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NÍVEL DOUTORADO

ESTUDOS SOBRE A RELAÇÃO ENTRE PERIODONTITE E CÂNCER BUCAL

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Linha de pesquisa: Epidemiologia, Etiopatogenia e Repercussão das Doenças da Cavidade Bucal e Estruturas Anexas

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Orientador:

Prof. Dr. Cassiano Kuchenbecker Rösing

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“O conhecimento amplia a vida. Conhecer é viver uma realidade que a ignorância impede desfrutar.”

“Tudo que o homem não conhece não existe para ele. Por isso o mundo tem, para cada um, o tamanho que abrange o seu conhecimento.”

Carlos Bernardo Gonzáles Pecotche

RESUMO

O câncer bucal representa um problema de saúde pública mundial classificado como o sexto tipo de câncer mais comum. Apresenta índices de mortalidade em cinco anos preocupantes nas últimas décadas. Estima-se que aproximadamente 15% dos canceres bucais tenham causa desconhecida. Estudos publicados recentemente têm sugerido que a doença periodontal pode representar um risco aumentado para o desenvolvimento de câncer bucal. Entretanto, o impacto da doença periodontal sobre a patogênese do câncer bucal *in vivo* é quase inexistente na literatura. Esta tese avaliou, através de dois artigos, a relação entre a doença periodontal e o câncer bucal. O primeiro artigo da presente tese trata de uma revisão de estudos epidemiológicos sobre a associação da doença periodontal e câncer bucal, assim como estudos atuais a respeito de mecanismos biológicos. Os estudos epidemiológicos na sua maioria apontam a periodontite como um fator de risco independente para o câncer bucal após ajustes estatísticos para diferentes fatores confudindores, como o fumo. Os estudos biológicos tem na sua grande maioria a *Porphyromonas gingivalis* como agente etiológico para a doença periodontal e estaria envolvida em várias etapas de carcinogênese bucal como a proliferação, migração e invasão celular, assim como a metástase e resistência a agente quimioterapêuticos em estudos *in vitro* e *in vivo* a periodontite. O segundo artigo da presente tese teve como objetivo avaliar o efeito da periodontite induzida por meio de ligadura sobre a carcinogênese bucal através da exposição ao carcinógeno 4-Nitroquinolina 1-Óxido (4NQO) em ratos Wistar. Os resultados mostram o aumento da incidência de carcinoma espinocelular na língua de ratos quando na presença de periodontite previa. Além disso, houve um maior tamanho tumoral e uma menor diferenciação celular através da expressão de citoqueratina 10 nas línguas dos animais. Os resultados desta tese vêm ao encontro do que se encontra na literatura reforçando que a presença da doença periodontal é um importante modificador no desenvolvimento de câncer bucal.

Palavras-chave: periodontite, câncer bucal, 4-nitroquinolina-1-óxido,ratos

ABSTRACT

Oral cancer is a worldwide public health problem rated as the sixth most common cancer. Its survival rates have remained below 50% for 5 years throughout the last decades. It is estimated that approximately 15% of oral cancer have an unknown etiology. Recently published studies have suggested that periodontal disease may represent an increased risk for oral cancer development. However, the impact of periodontal disease on oral carcinogenesis in vivo is almost non-existent. The current thesis evaluated, through two articles, the relationship between periodontal disease and oral cancer. The first article was a review of epidemiological studies about the association of periodontal disease and oral cancer, as well as current studies about biologic mechanisms. Periodontal disease and oral cancer share many common established risk factors, but after adjusting for different confounding factors, periodontal disease seems to be an independent risk for oral cancer in most of epidemiologies studies. Studies focusing in biological mechanisms of the relationship between both diseases mainly focus in *P.gingivalis. P. gingivalis* seems to be involved with various steps of oral carcinogenesis as proliferation, migration invasion, aggressiveness and also metastasis and resistance to anti-cancer agents *in vitro* and *in vivo* studies. The second article of the current thesis aimed to evaluate the effect of induced-periodontitis by ligature on the experimental oral carcinogenesis induced by the chemical 4-nitroquinoline 1-oxide (4NQO) in Wistar rats. The results showed an increased incidence of squamous cell carcinoma in the rats' tongues when in presence of previous periodontitis. The results of this thesis corroborate what we may find in the literature, reinforcing that the presence of periodontal disease act as an important modifier to oral cancer.

Keywords: periodontitis, oral cancer, 4-nitroquinoline-1-oxide, rats.

SUMÁRIO

1. APRESENTAÇÃO

A presente tese de doutorado é parte de uma trajetória acadêmica do Cirurgião-Dentista Tobias Rauber Spuldaro, graduado em Odontologia pela Universidade de Santa Cruz do Sul, Rio Grande do Sul, Brasil. Após a graduação, obteve os títulos de especialista em Periodontia, Implantodontia Saúde Coletiva em Odontologia. Atualmente atua como professor de Odontologia no Centro Universitário da Serra Gaúcha (FSG). Foi contemplado com uma bolsa do programa CAPES, nível mestrado, tendo defendido dissertação neste programa.

No período de pós-graduação produziu os seguintes artigos:

- Oballe HJ, Gaio EJ, Spuldaro T, Cavagni J, Gomez R, Rösing CK. Effects of alcohol and/or tobacco exposure on spontaneous alveolar bone loss in rat. Braz Dent J. 2014;25(3):197-202.

Artigos submetidos:

- Effects of low molecular weight heparin on alveolar bone loss in Wistar rats: morphometric and histological analyses.
  Submission ID: AJH-17-0800
  Revista: American Journal of Hematology
  Harry J Oballe; Marcelo L Lamers; Tobias R Spuldaro; Eduardo J Gaio; Cassiano K Rösing; Francisco WMG Muniz.

Artigos em processo de submissão:

- Effect of induced periodontitis on the oral carcinogenesis in Wistar rats.
  Tobias Rauber Spuldaro, Vivian Petersen Wagner, Felipe Nör, Eduardo José Gaio, Vinicius Coelho Carrard, Rogerio Castilho, Cassiano Kuchenbecker Rösing.
- The relationship between Periodontal Disease and Oral Cancer: a review of current epidemiological and biological studies.

Tobias Rauber Spuldaro, Rogério Castilho, Cassiano Kuchenbecker Rösing.

Atualmente, participa ativamente dos seguintes projetos de pesquisa:

- Propranolol em baixas doses sobre a carcinogênese de língua e a doença periodontal induzidos em ratos Wistar.
  Tobias Rauber Spuldaro, Vivian Petersen Wagner, Felipe Nör, Eduardo José Gaio, Vinicius Coelho Carrard, Rogerio Castilho, Cassiano Kuchenbecker Rösing.
- Efeito da exposição ao 4-Nitroquinolina 1-Óxido (4NQO) na perda óssea alveolar induzida e espontânea em ratos Wistar.
  Tobias Rauber Spuldaro, Harry Juan Rivera, Marcelo Ekman, Ribas Fernanda Visioli, Vinicius Coelho Carrard, Cassiano Kuchenbecker Rösing.
- Efeito da cerveja sobre a doença periodontal induzidas em ratos Wistar
  Rafael Lourenci, Tobias Rauber Spuldaro, Harry Juan Rivera Oballe, Vinicius Coelho Carrard, Eduardo José Gaio.
- Efeito de Enxaguatórios Bucais à base de Óleos Essenciais sobre a formação inicial do Biofilme Supra e Subgengival.
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2. ANTECEDENTES E JUSTIFICATIVA

2.1 Câncer bucal

Câncer é uma das quatro principais doenças crônicas responsáveis pelo maior número de mortes no mundo (PETERSEN e BAEHNI, 2012). Em 2012, a carga mundial de câncer aumentou para cerca de 14 milhões de novos casos por ano, um número que deverá subir para 22 milhões por ano nas próximas duas décadas. Para estes mesmos períodos, há previsão de incremento de estimativa de morte de 8,2 milhões por ano para 13 milhões por ano (WHO, 2014).

O câncer bucal representa um problema de saúde pública mundial classificado como o sexto tipo de câncer mais comum de todos os cânceres (LEEMANS *et al.*, 2011; PERERA *et al.*, 2016). O carcinoma espinocelular é considerado o tipo com a maior prevalência e representa cerca de 90% das malignidades bucais e uma incidência de mais de 300 mil casos de carcinoma espinocelular a cada ano no mundo (SILVERMAN, 2001; WARNAKULASURIYA, 2009). É uma doença que apresenta um mau prognóstico pelo fato do diagnóstico ser feito em um estágio muito avançado, pois muitas vezes se apresenta assintomático (LAMBERT *et al.*, 2011). Apesar dos avanços em tratamentos cirúrgicos, radioterapia e quimioterapia, os índices de mortalidade em cinco anos são de 40-50% e mantiveram-se inalterados nas últimas décadas, o que o torna como um dos piores dos principais tipos de cânceres (LEEMANS *et al.*, 2011).

No Brasil, a incidência do câncer bucal ocupa a quinta posição para homens (11.280 novos casos em 2014) e a sétima posição para mulheres (4.010 novos casos em 2014) sendo o carcinoma espinocelular a neoplasia maligna mais incidente (BRASIL, 2015). As localizações mais frequentes do carcinoma espinocelular bucal são a língua, seguido pelo assoalho bucal e o lábio inferior (BRENER, 2007).

2.2 A relação entre câncer bucal e doença periodontal

Os principais fatores de risco para o câncer são o fumo e álcool (PETTI, 2009). Estima-se que aproximadamente 15% das causas do câncer bucal tenham etiologia desconhecida e, que, cerca de 20% de todos casos de cânceres estejam

associados a infecção microbiana e a inflamação (KUPER *et al.*, 2000). Pode-se citar como exemplo clássico a associação entre a bactéria *Helicobacter pylori* como a primeira espécie bacteriana ser causadora direta do câncer gástrico (WHITMORE e LAMONT, 2014).

Em uma situação análoga ao envolvimento do câncer gástrico e a *Helicobacter pylori,* pode se citar uma possível relação entre microrganismos bucais e o desenvolvimento de câncer bucal (WHITMORE e LAMONT, 2014). A boca é composta por uma enorme diversidade de microrganismos, incluindo mais de 750 espécies distintas de bactérias, que estão em constante contato com o epitélio escamoso bucal desencadeando uma variedade de desafios microbianos do ponto de vista celular e molecular (KUMAR, 2013). Neste contexto, deve-se chamar a atenção para como isso poderia estar relacionado com o desenvolvimento de câncer bucal (RAJEEV *et al.*, 2012).

As doenças infecciosas bucais que estão mais associadas ao câncer bucal são as doenças periodontais (FITZPATRICK e KATZ, 2010; YAO, Q. W. *et al.*, 2014). Esta relação tem recebido especial atenção nos últimos anos e, segundo uma revisão da literatura com meta-análise, as doenças periodontais podem incrementar em até três vezes as chances de desenvolvimento câncer de boca (YAO, Q. W. *et al.*, 2014). Dentre as doenças periodontais, a periodontite é o estágio mais preocupante por estar em um estágio mais avançado. A periodontite é uma condição crônica caracterizada pela inflamação das estruturas de proteção e sustentação do dente (periodonto) e que a sua patogênese é causada pela combinação da resposta do hospedeiro frente a um desafio microbiano pelo biofilme dentário (READY *et al.*, 2008). Na sua forma mais avançada se estima que se 10,8% do mundo apresenta periodontite grave e que estes índices de prevalência podem passar de 20% em algumas regiões específicas (KASSEBAUM *et al.*, 2014). Ela contribui para o estabelecimento da condição inflamatória em pacientes geneticamente suscetíveis, o que estaria potencialmente envolvido no aumento de produção de mediadores inflamatórios e no colapso das vias responsáveis pela resolução imune (GIBSON *et al.*, 2006; MOUTSOPOULOS e MADIANOS, 2006).

Quando se relaciona o câncer bucal e a doença periodontal, duas teorias foram propostas: direta e indireta (Figura 1) (GONDIVKAR *et al.*, 2013).

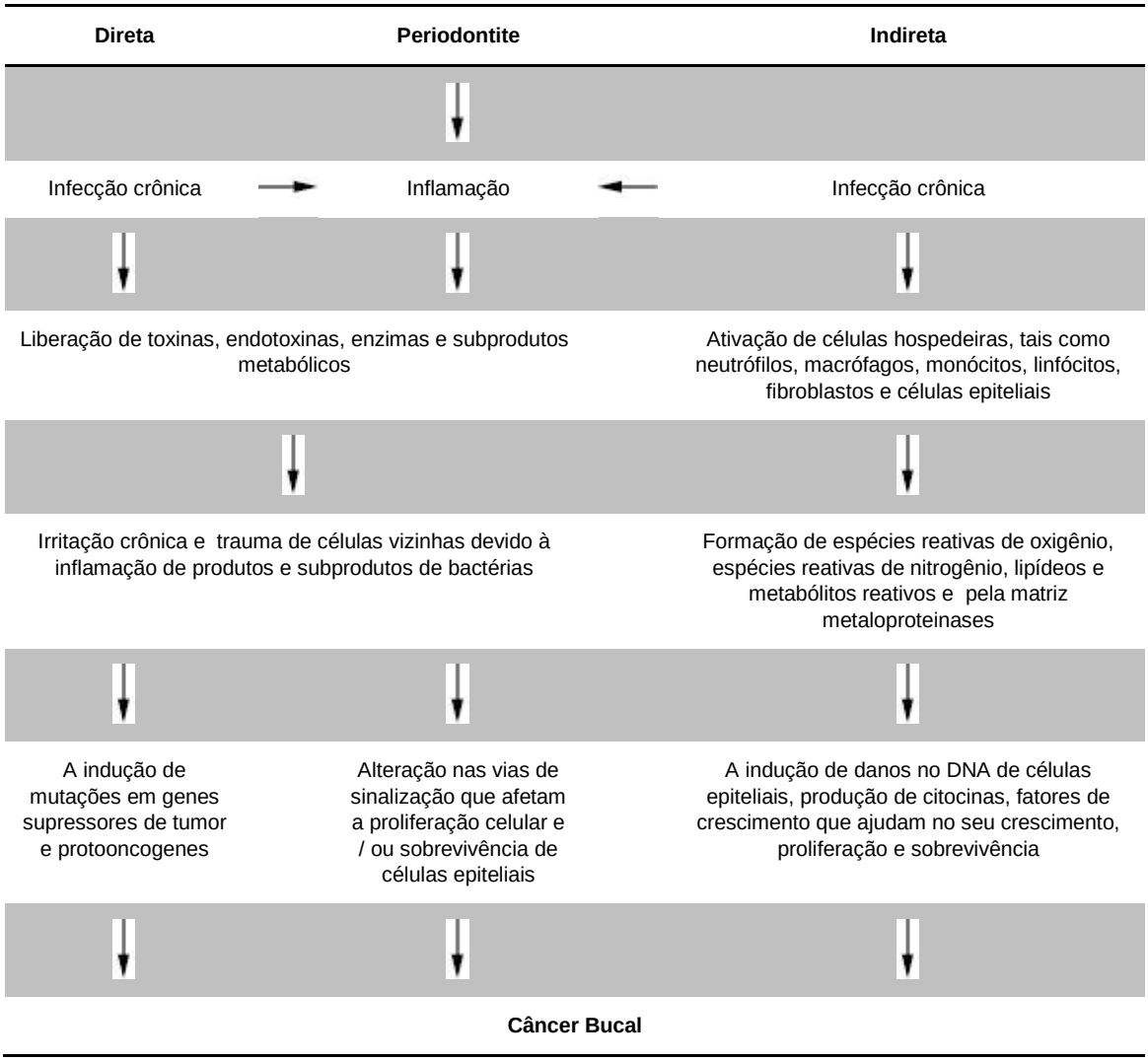

Figura 1 - Possíveis mecanismos sugerindo a correlação entre periodontite e câncer bucal. Adaptado de GONDIVKAR, S. M. et al*. Chronic periodontitis and the risk of head and neck squamous cell carcinoma: facts and figures.* Exp Oncol*,* v. 35, n. 3, p. 163-7, 2013.

A principal etiologia das doença periodontal é o biofilme dentário. A má higiene buccal permite que se tenha inicialmente uma inflamação dos tecidos gengivais marginais através do biofilme supragengival e, que se manter inalterad, haverá uma formação de bolsas periodontais e o estabelecimento da periodontite com a destruição irreversível dos tecidos de suporte periodontais através do biofilme subgengival (LARSEN e FIEHN, 2017).

A diversidade da microbiota presente nas doenças periodontais é bastante grande, sendo que já foram identificados como os principais responsáveis pela destruição dos tecidos periodontais os periodontopatógenos *Porphyromonas gingivalis, Treponema denticola* e *Tannerella forsythia* (SOCRANSKY *et al.*, 1998; READY *et al.*, 2008). Uma revisão da literatura recente descreveu a possível relação da carcinogênese com alguns periodontopatógenos, em especial *Porphyromonas gingivalis* e *Fusobacterium nucleatum*, ressaltando diferentes mecanismos como a inibição de apoptose, ativação de proliferação celular, promoção de invasão celular, indução de inflamação crônica e produção de carcinógenos (PERERA *et al.*, 2016). Em outra revisão sistemática com meta-análise mostrou-se que a presença de *P. gingivalis* pode aumentar em 1,36 vezes as chances de desenvolver câncer (SAYEHMIRI *et al.*, 2015).

A explicação pela qual as periodontite poderia estar indiretamente relacionada ao câncer bucal embasa-se na presença de um processo inflamatório onde as bactérias bucais e seus produtos ativam as células hospedeiras locais, tais como neutrófilos, monócitos, macrófagos, linfócitos, fibroblastos e células epiteliais (TEZAL *et al.*, 2005). A partir dessa ativação, uma série de eventos ocorre, o que pode induzir danos no DNA das células epiteliais (GONDIVKAR *et al.*, 2013). Também estariam relacionadas a isto a produção de citocinas, quimiocinas, fatores de crescimento e outros sinais que fornecem um ambiente para a sobrevivência celular, a proliferação, a migração, angiogênese e a inibição da apoptose (COUSSENS e WERB, 2002; MOUTSOPOULOS e MADIANOS, 2006). Este ambiente pode ajudar as células epiteliais a acumular mutações e conduzir essas células epiteliais mutantes a proliferar, migrar e dar-lhes uma vantagem de crescimento (GONDIVKAR *et al.*, 2013).

Estudos sobre os mecanismos biológicos para a relação das doenças periodontais e o câncer bucal em sua maioria são recentes (SAHINGUR e YEUDALL, 2015; PERERA *et al.*, 2016). A grande maioria destes estudos são *in* vitro e tem a *P. gingivalis* como o agente etiológico para simular as doenças periodontais em células epiteliais e/ou de câncer bucal (SAHINGUR e YEUDALL, 2015; PERERA *et al.*, 2016). Esse interesse emergiu de um estudo em que foram encontrados a presença de *P. gingivalis* em biópsias de carcinoma espinocelular de gengiva e se verificou a habilidade de penetrar em tecidos de câncer bucal (KATZ *et al.*, 2011). Por outro lado,

estudos *in vivo* relacionando as duas doenças são quase inexistentes. Um estudo recente mostrou pela primeira vez que tumores infectados por *P.gingivalis/F.nucleatum* se tornaram 2,5 vezes maiores e foram bem mais invasivos em camundongos (BINDER GALLIMIDI *et al.*, 2015). Resistência a agentes quimioterapêuticos e metástase também foram observadas em camundongos que tiveram a indução de tumores pela injeção de células de câncer bucal infectadas por *P. gingivalis* (WOO *et al.*, 2017).

2.3 Carcinogênese bucal e periodontite induzidos em ratos

O modelo de carcinogênese em modelos animais é de suma importância, pois representa mudanças celulares e moleculares de maneira muito semelhante a humanos (RIVERA *et al.*, 2011). É um consenso que o uso de animais é um passo inevitável no ententimento do processo de carcinogênese e a avaliação de intervenções terapêuticas (RIVERA MARTINEZ, 2012). Através de modelos em ratos, é possível induzir a carcinogênese de língua muito semelhante ao que acontece no desenvolvimento do carcinoma em humanos (KANOJIA e VAIDYA, 2006). O agente alquilante 4-nitroquinolina 1-óxido (4NQO) é o agente químico mais adotado para isso, pois constitui um método que reproduz a sequência dos estágios de hiperplasia, displasia e carcinoma de maneira semelhante àquela da cavidade bucal de seres humanos (KANOJIA e VAIDYA, 2006; RIBEIRO e SALVADORI, 2007). Outra vantagem que ele apresenta é o fato de que os danos causados pelo 4NQO são muito parecidos com outros carcinógenos presentes no tabaco, o principal fator de risco para o câncer bucal (KANOJIA e VAIDYA, 2006).

A diluição do agente 4NQO em água parece ser a melhor maneira para a indução de carcinoma espinocelular em ratos comparado com a aplicação tópica com pincel, devido à dificuldade de acesso à região anatômica onde irá ser aplicada, o risco de contaminação e ocorrência de acidentes durante a manipulação do animal (TANG *et al.*, 2004). A ingestão de 4NQO diluído em água por um período de 20 semanas faz com que se tenha 100% de alterações como a displasia e carcinoma espinocelular na língua de ratos Wistar muito semelhante ao de humanos do ponto de vista clínico e histopatológico (RIBEIRO e SALVADORI, 2007; CARVALHO *et al.*, 2012).

Ratos são modelos muito utilizados também para o estudo da patogênese da doença periodontal (STRUILLOU *et al.*, 2010). Isso se dá pelo fato de os tecidos periodontais e o processo de patogênese da perda óssea alveolar induzida serem muito parecidos com o que acontece em humanos(STRUILLOU *et al.*, 2010). Existem diferentes métodos para a indução da perda óssea alveolar como a partir da colocação de ligaduras, pela aplicação de lipopolissacarídeos e pela inoculação de periodonpatógenos específicos (DUMITRESCU *et al.*, 2004; VERZELETTI *et al.*, 2012; ZHANG *et al.*, 2014). Atualmente, a forma de indução de perda óssea alveolar através da colocação de ligadura (fio de sutura) ao redor dos dentes um dos métodos mais utilizados garantindo a indução de perda óssea alveolar, na medida em que permite o acúmulo do biofilme dentário, o principal fator etiológico das doenças periodontais (ROVIN *et al.*, 1966; VARGAS-SANCHEZ *et al.*, 2017). Além disso, a composição da microbiota do biofilme dentário adquirido pelo método da ligadura é muito semelhante àquelas em humanos (DUARTE *et al.*, 2010).

Existem diferentes métodos para avaliação da perda óssea alveolar em ratos. Dentre eles destacam as avaliações morfométrica, histológica e por microtomografia computadorizada (FERNANDES *et al.*, 2007; LI e AMAR, 2007; VARGAS-SANCHEZ *et al.*, 2017). O método morfométrico vale-se da quantificação da distância de medidas lineares ou da área entre a junção amelocementária e a crista óssea por meio de fotografias (SOUZA *et al.*, 2009; VERZELETTI *et al.*, 2012). O método histológico se mede de maneira muito parecida com os mesmos pontos de referências, porém requer o processamento histológico das peças e a mensuração através de microscópio (FERNANDES *et al.*, 2007). Todos estes métodos têm reconhecida capacidade de quantificar os efeitos do processo inflamatório na destruição periodontal (FERNANDES *et al.*, 2007; LI e AMAR, 2007; VARGAS-SANCHEZ *et al.*, 2017). Independente da metodologia utilizada, o tempo de indução de perda óssea alveolar é muito importante no que diz respeito a patogênese da periodontite. Um estudo recente comparou diferentes tempos (0, 3, 7, 15, 30 e 60 dias) de indução a periodontite em ratos e observou-se que o período de três dias já é o suficiente para se ter uma diferença significativa de perda óssea alveolar e que após 15 dias ela se mantém estabilizada(VARGAS-SANCHEZ *et al.*, 2017).

Com base no exposto até aqui, justifica-se que estudos procurando estabelecer efeitos e eventuais mecanismos biológicos da presença de doença

periodontal sobre o desenvolvimento de câncer bucal, especialmente pelos dados epidemiológicos que colocam ambas as doenças como altamente prevalente e a segunda como de altos índices de morbi-mortalidade.

3. OBJETIVOS

3.1 Objetivo Geral

Analisar através de uma revisão de estudos epidemiológicos e biológicos, assim como um estudo experimental sobre o efeito da periodontite induzida por meio de ligadura sobre a carcinogênese bucal através da exposição ao carcinógeno 4-Nitroquinolina 1-Oxido (4NQO) em ratos Wistar a relação entre a periodontite e o câncer bucal.

3.2 Objetivos específicos

- Revisar a literatura atual a respeito dos estudos epidemiológicos e biológicos associando a periodontite e o câncer bucal.
- Avaliar a incidência de carcinoma espinocelular bucal e o tamanho tumoral entre os grupos que foram induzidos à periodontite e expostos ao carcinógeno 4NQO em ratos Wistar.
- Avaliar o efeito da periodontite induzida associado à exposição ao carcinógeno 4NQO sobre perda óssea alveolar em ratos Wistar.

4. ARTIGOS CIENTÍFICOS

THE RELATIONSHIP BETWEEN PERIODONTAL DISEASE AND ORAL CANCER: A REVIEW OF CURRENT EPIDEMIOLOGICAL AND BIOLOGICAL STUDIES

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The relationship between Periodontal Disease and Oral Cancer: a review of current epidemiological and biological studies

ABSTRACT

Oral cancer is considered a major threat to public health with a low 5-year survival rate. It has a multifactorial origin with several risk factors that can act individually or in combinations. Infection and inflammation have been considered as a major risk to initiate carcinogenesis in about 20% of all cancers. Periodontitis, a chronic infecto-inflammatory disease and highly prevalent worldwide, has been considered to increase the oral cancer risk as an independent risk factor in epidemiological studies. The role of periodontal disease and its potential contribution as a risk factor/indicator in the development and/or progression of oral cancer *in vitro* and *in vivo* studies has been the subject of much interest and investigation in recent years. Published studies mainly focus on *P.gingivalis*, a major periodontopathic pathogen, and its involvement with various steps of oral carcinogenesis. The purpose of the present study is to review the current literature concerning epidemiological and biological evidences associating periodontal disease and oral cancer.

Keywords: Periodontal disease, periodontitis, oral cancer, *P. gingivalis*, risk factor

INTRODUCTION

Oral cancer is one of the highly prevalent cancers worldwide with an incidence of more than 500.000 cases per year posing as a major threat to public health worldwide(SIEGEL *et al.*, 2013). It is considered as one of worst type of cancer because its low 5-year survival rate of 40-50%(LEEMANS *et al.*, 2011). There are several risk factors identified for the development of oral cancer like smoking, alcohol consumption, viral infections, nutritional factors, genetic factors and infections(SCULLY, 2011). Interestingly, infection and inflammation have been considered as a major risk to initiate carcinogenesis in approximately 20% of all cancers (ELINAV *et al.*, 2013). Besides that, it is estimated that 15% of oral cancers still present an unknown etiology, which raises the need to explore other potential risk factors (CHOCOLATEWALA *et al.*, 2010; PERERA *et al.*, 2016).

Periodontal disease is characterized as a chronic infection caused by oral bacteria which results in inflammation of the gums and can continue to a gradual destruction of the periodontal tissues around the teeth leading to alveolar bone loss and consequently tooth loss(MICHAUD *et al.*, 2007). Whereas periodontitis is a chronic infecto-inflammatory and highly prevalent worldwide, it is plausible to further study its involvement in oral cancer pathogenesis. In fact, recent systematic reviews with meta-analyses have shown that individuals with history of periodontitis may have a nearly 3-fold increased risk to oral cancer (YAO, Q. W. *et al.*, 2014; YE *et al.*, 2016). The interest of associating both diseases emerged from two studies which found an increased incidence of oral cancer in patients with clinical and radiographic periodontal destruction (TEZAL *et al.*, 2005; TEZAL *et al.*, 2007).

The biological mechanism by which periodontitis could be related to oral cancer is hypothesized by two theories: the direct involvement of the periodontopathogens and indirectly by the periodontal inflammatory response (TEZAL *et al.*, 2005; GONDIVKAR *et al.*, 2013). Katz *et al*. (2011) found for the first time higher amounts of *Porphyromonas gingivalis* in gingival squamous cell carcinoma compared to healthy gingival tissue samples, indicating the ability of periodontopathogens to penetrate oral cancerous tissues (KATZ *et al.*, 2011). Periodontal disease release inflammatory mediators to aid in elimination of periodontopathogens, but once the

individual's immune system is unable to clear periodontal infection, the persistent chronic inflammation could be related to the breakdown of normal cell growth control and have a possible repercussion on tumor growth and progression (AHN *et al.*, 2012; GONDIVKAR *et al.*, 2013).

Despite the advances in the understanding of the relationship between periodontal disease and oral cancer, the existing evidence still does not support periodontitis as a true risk factor (SAHINGUR e YEUDALL, 2015). However, emerging studies have been exploring the topic. The purpose of the present article is to review the current literature concerning epidemiological and biological evidences associating periodontal disease and oral cancer.

METHODS

Epidemiological Studies

The evidence in the literature supporting an association between periodontal disease and oral cancer is increasing (FITZPATRICK e KATZ, 2010; SAHINGUR e YEUDALL, 2015). The meta-analyses studies that pointed the increased chances (YAO, Q. W. *et al.*, 2014; YE *et al.*, 2016) included studies in which tooth loss was used as a proxy of periodontal disease. This brings a strong limitation, since tooth loss can be caused by caries, trauma and other factors (Fitzpatrick & Katz, 2010).

In the present study, a literature search using PubMed (National Library of Medicine, Washington, DC) including studies published in the last fifteen years with different combinations of the following key words was performed: "oral cancer", "head and neck cancer", "oropharyngeal cancer", "oral squamous cell carcinoma", "periodontal disease", squamous cell carcinoma", "periodontitis", "clinical attachment". In order to be included in this review, inclusion criteria comprised: (a) study type: case–control study or cohort study; (b) the odds ratio (OR) or RR value and its 95% CI should be provided; (c) paper published in English. The main studies associating periodontal disease and oral cancer are summarized in Table 1.

Table 1 Summary of studies associating periodontal disease and oral cancer according to the chronological order of publication

	Sample	Study design	Criteria for Oral Cancer	Criteria for Periodontal Disease	Main Results	Factors adjustment
Tezal et al. 2005 USA	131 oral tumor 323 pre-cancerous	Cohort	Tumor (non-specific) Pre-cancerous lesions Oral soft tissue lesions	Severity Dichotomization CAL ≥ or ≤ 1.5 mm	CAL was related with an increased oral tumor (OR 4.57) and pre-cancerous lesions (OR 1.55) in nonsmokers.	Age, gender, race, oral conditions, education, smoke and alcohol
Rosenquist et al. 2005 Sweden	132 cases 320 controls	Case-control	Oral carcinoma Stage grouping (I-IV) systems	Assessment of dental panoramic and periapical radiographs	There was an increased risk for bone loss (OR 3.0), but not when adjusted. Average (OR 2.0) and poor (OR 5.3) PI were significant risk factors after adjustment for tobacco smoking and alcohol consumption	Age, gender, oral conditions, education, smoke and alcohol.
Guha et al. 2007 **Central Europe Latin America**	274 cases in Europe; 414 cases in Latin America	Comparative Cohort	Oral Cancer (International Classification of Diseases for Oncology)	Self reported bleeding gums frequency Oral health/hygiene (good, average, poor) assessed by the presence of tartar, gingival bleeding, mucosal irritation, and decaying teeth.	Poor oral health/hygiene showed an increased risk for oral cancer (OR 4.51 in Europe and OR 2.91 in Latin America). Gingival bleeding – Sometimes (OR 1.25) always (OR 1.94) in Latin America	Age, gender, country, education, tobacco pack-years, cumulative alcohol consumption, and all other oral health variables.
Tezal et al. 2007 USA	51 cases 54 controls	Case-control	Squamous cell carcinoma of the tongue (*International Classification of Diseases for Oncology)*	ABL from panoramic radiographs (mm)	Each mm of ABL was associated with an increased risk of tongue cancer (OR 5.23)	Age, smoke, ethnicity/race, oral conditions

Rezende et al. 2008 Brazil	50 cases 50 controls	Case-control	Oral and oropharyngeal squamous cell carcinoma	CPTTN	76% of the subjects evaluated had the presence of periodontal pockets with depths of 6mm or greater in, while only 10% of the subjects in the control group showed the same level of disease.	-
Michaud et al. 2008 USA	118 cases	Cohort	Oropharyngeal cancers	Self reported history of periodontal disease with bone loss and validated by radiographs	The presence of periodontal disease was not significant to increase risk oropharyngeal cancers (OR 1,15)	Age, ethnic, physical activity, diabetes, alcohol, geographical location, diet, smoke
Tezal et al. 2009 USA	266 cases 100 (Oral Cancer) 207 Controls	Case-control	Head and neck Cancer and Oral Cancer Stage grouping (I-IV) and differentiation International Classification of Diseases for Oncology, third edition	ABL from panoramic radiographs (mm)	Each mm of ABL was associated with an increased risk of HNSCC (OR 4.36) and oral cancer (OR 4.52). Periodontitis history was associated with poorly differentiated tumors in the oral cavity.	Age, gender, race/ethnicity, marital status, smoke, alcohol
Sharma et al. 2011 India	20 cases periodontitis and leukoplakia 20 cases periodontitis 20 controls	Case-control	Precancerous lesions (leukoplakia), level of dysplasia and level of IL-6 in saliva	CPTTN	Significant higher levels of IL-6 in individuals in both groups with periodontitis. Levels of IL-6 increased in leukoplakia according to its severity.	Men, smoke
Ahn et al. 2012 USA	12605 cases 7852 IgG P.g.	Cohort	Orodigestive Cancers	AAP Severe periodontitis (2 teeth had interproximal CAL ≥ 6 mm and at 1 tooth had interproximal PD ≥ 5mm) moderate periodontitis (2 teeth interproximal CAL ≥ 4 mm and 2 teeth PD ≥ 5mm) Serum IgG antibody to P.g	Periodontitis (moderate and severe) was associated to Orodigestive cancer mortality (OR 2.28) and was increased according to the severity of periodotitis. Orodigestive cancer mortality is related P. g., independent of periodontal disease	Smoking, education, race/ethnicity and BMI

Meisel et al. 2012 Germany	123 leukoplakia 246 controls	Case-control	Precancerous lesions (leukoplakia)	PD, CAL, PI, BOP	Periodontitis-related dose-dependent increase in the probability of having oral leukoplakia (second, third and fourth quartiles of CAL were OR = 1.7, 3.3 and 5.3; BOP were OR 2.0, 2.9 and 3.8, respectively).	Smoke, socioeconomic, oral hygiene
Eliot et al. 2013 USA	513 cases 148 (oral cancer) 567 controls	Case-control	Head and neck cell carcinoma Oral Cancer International Classification of Disease	Self reported	Periodontal disease was associated with a slightly elevated risk of HNSCC (OR 1.09) and oral cancer (OR 1.09)	Smoke, alcohol, gender, age, education
Moergel et al. 2013 Germany	178 cases 127 controls	Case-control	oral squamous cell carcinoma limited to the area of periodontitis	Panoramic radiographs Self reported	ABL was higher in case group (mean 4.3 mm compared to 2.9 mm in control group). Periodontitis represents an increased risk for oral cancer (OR 2.4). A history of periodontal treatment was associated with significantly reduced oral cancer risk.	Oral conditions, smoke, age, gender, alcohol, history of periodontal treatment
Wen et al. 2013 China	141 cases	Cohort	Oral Cancer International Classification of Diseases for Oncology	Gingivitis and periodontitis ICD-9-CM codes 523.3 and 523.4	Periodontitis increased the chance of oral cancer (HR 1.8). Periodontitis exhibited a higher risk of developing oral cancer than those in the gingivitis. Women with gingivitis and or periodontitis had more chances to develop oral cancer.	Age, gender, comorbidities
Narayan et al. 2014 India	242 cases 254 controls	Case-control	Oral Squamous Cell Carcinoma	CPITN	Individuals with oral cancer had worse periodontal parameters with a significant presence of PD > 6mm. Periodontal disease experience is directly proportional to oral cancer.	-
Laprise et al 2016 India	350 cases 371 controls	Case-control	Oral squamous cell carcinoma International Classification of Diseases	Inflammation and gingival recession evaluated visually (color, size, contour, consistency, surface texture, position and spontaneous bleeding)	Individuals with generalized gingival recession and gingival inflammation showed a higher chance to develop oral cancer (OR 1,8 and 2,02), but not for all factors adjusted	Paan chewing smoke, alcohol, age, gender, number of educational years

De Moraes et al. 2016 Brazil	35 cases 40 controls	Case-control	Oral/Oropharyngeal cancer	AAP PI, GI, PD, CAL Severe periodontitis (2 teeth had interproximal CAL ≥ 6 mm and PD ≥ 7mm) generalized chronic periodontitis (>30% with CAL ≥4 mm).	80% and 89% of the patientes with Oral/Oropharyngeal cancer had generalized chronic periodontitis and severe periodontitis, respectively. They showed higher BOP and CAL levels. The extent (OR 12.5) and severity (OR 10.9) of chronic periodontitis remained as risk indicators for oral cavity and/or oropharyngeal cancer	Smoke, alcohol.

Legend: OR - Odds Ratio; CAL – Clinical attachment loss; ABL – Alveolar bone loss; PD – Probing depth; BoP – Bleeding on probing; Pg – Phorphyromonas gingivalis; PI – Plaque index; GI – Gingival index; mm - milimeters; CPTTN - community periodontal index of treatment needs; ICD –International classification of diseases; AAP - American Academy of Periodontology; IL - interleucin; Ig -Imunmoglobulin

The existing evidence presents several limitations that require attention. In terms of methodological design, all studies are observational. Observational studies have limitations such as establishing a clear chronology and determining temporal relationship between exposure and outcome. There is no randomized clinical trial, the gold standard for epidemiological studies, which is understandable, due to the nature of the outcome (oral cancer, which presents high mortality rate). Ethical considerations upon diagnosis prevent the follow-up of premalignant lesions without treatment. Furthermore, in a pilot study, patients could not endure a complete dental exam because most of them were frail and had large lesions in their mouth (LAPRISE *et al.*, 2016).

The criteria for the diagnosis of periodontal disease is another point that deserves attention. Four studies considered periodontal disease as self-reported and different questionnaires were applied. For instance, individuals were asked if their gums bleed (frequency) or if they had any history of periodontal disease (yes or no) (GUHA *et al.*, 2007; MICHAUD *et al.*, 2008; ELIOT *et al.*, 2013; MOERGEL *et al.*, 2013). One study considered periodontal disease as gum recession and gingival inflammation when they were assessed visually by calibrated examiners (LAPRISE *et al.*, 2016). Five studies used panoramic and/or periapical radiographs to quantify bone loss to infer the history of periodontitis, in which is a complementary diagnostic procedure (ROSENQUIST *et al.*, 2005; TEZAL *et al.*, 2007; TEZAL *et al.*, 2009; MOERGEL *et al.*, 2013). Eight studies used clinical periodontal measurement with different indexes (plaque index, gingival index, pocket depth, clinical attachment loss) alone or combined and also different methods to diagnose periodontal disease such as community periodontal index of treatment needs (CPTTN), international classification of diseases (ICD) and American Academy of Periodontology (AAP) (ROSENQUIST *et al.*, 2005; TEZAL *et al.*, 2005; REZENDE *et al.*, 2008; SHARMA *et al.*, 2011; AHN *et al.*, 2012; MEISEL *et al.*, 2012; NARAYAN *et al.*, 2014; MORAES *et al.*, 2016). Only four studies used clinical attachment loss (gold standard) as a parameter to measure periodontitis (REZENDE *et al.*, 2008; AHN *et al.*, 2012; NARAYAN *et al.*, 2014; MORAES *et al.*, 2016). Extension and severity of periodontitis were assessed in only one study using a full mouth examination (MORAES *et al.*, 2016). Most of the studies have biases such as partial indexes, self-assessment

questionnaires and radiographic parameters that underestimate the prevalence of periodontitis (MORAES *et al.*, 2016).

We included in this review all studies that were related to the diagnosis of oral cancer. There are two studies that associate leukoplakia/precancerous and periodontitis (TEZAL *et al.*, 2005; MEISEL *et al.*, 2012). However, the malignization of leukoplakia is low stipulated in only six to 29 per 100,000 (PETTI, 2003; MEISEL *et al.*, 2012). One study associated nonspecific oral tumors and periodontitis(TEZAL *et al.*, 2005). Three studies, one study and two studies considered oropharyngeal, orodigestive and head and neck cancer to associate with periodontitis, respectively. Reviewing the literature on oral and oropharyngeal cancers is difficult because these tumors often are reported in aggregate with other pharyngeal or head and neck malignancies, and anatomic subsite definitions are at times unclear or may not allow for distinction between the oral cavity and the oropharynx oral and oropharyngeal cancers (CHI *et al.*, 2015). Despite this heterogeneity, the majority of the studies were considered oral cancer/oral carcinoma (62%) and found a positive association with periodontal disease.

There are many confounding factors that can possibly affect the association between periodontal disease with various cancer risks (JAVED e WARNAKULASURIYA, 2016). Smoking, socioeconomic status, age, gender, alcohol consumption and ethnicity are the most common potential confounding factors affecting cancer risk. For this, statistical methods are necessary to adjust for potentially confounding effects (FITZPATRICK e KATZ, 2010; JAVED e WARNAKULASURIYA, 2016). For instance, smoking, the main risk factor for cancer, was adjusted in 87.5% of the studies listed. Two studies did not adjust for confounding variables, which might affected the results (REZENDE *et al.*, 2008; NARAYAN *et al.*, 2014). After adjusting for different confounding factors, periodontal disease seems to be an independent risk for oral cancer in most of the studies(YAO, Q. W. *et al.*, 2014).

Although poor oral hygiene and increased accumulation of dental plaque are associated with a high prevalence and severity of periodontal disease, host susceptibility still a key factor to the development of periodontitis (ALBANDAR, 2002). Similarly, poor oral hygiene has been associated with an increased prevalence of HNSCC (ROSENQUIST *et*

al., 2005; GUHA *et al.*, 2007; BERTL *et al.*, 2016). A recent cohort with a large population-based of HNSCC showed a significant association between poor oral health indicator and survival and this was more pronounced for sites closer to the dentition (FARQUHAR *et al.*, 2017). Another study showed that 50% of patients did not visit a dentist after cancer diagnosis and two thirds of them suffered of severe periodontitis (BERTL *et al.*, 2016). Since the association of oral cancer and periodontal diseases are complex and share common established risk factors, prevention of oral diseases seems of utmost importance since the recent evidence have shown history of periodontal treatment significantly reduced oral cancer risk (MOERGEL *et al.*, 2013; HWANG *et al.*, 2014).

The plausible biological mechanism linking periodontitis and oral cancer: *the role of P.gingivalis*

Periodontitis is a chronic disease characterized by inflammation of the supporting structures (periodontium) and its pathogenesis involves the combination of host response and microbial challenge by dental biofilms. The role of periodontitis and its potential contribution as a risk factor/indicator in the development and/or progression of oral cancer by the direct and indirect mechanisms have been the subject of much interest and investigation in recent years (TEZAL *et al.*, 2005; GONDIVKAR *et al.*, 2013; SAHINGUR e YEUDALL, 2015).

In the last two decades, a significant number of studies have associated the microbiota as one of the etiologic factors of various cancers. *Helicobacter pylori* was the first bacterial species officially recognized as the direct cause of gastric cancer (WHITMORE e LAMONT, 2014). The oral cavity is prone to a number of bacterial infectious diseases, such as periodontitis, and it is possible that oral bacteria may initiate or promote tumor development, analogous to the association of gastric cancer with *Helicobacter pylori* infection (SAHINGUR e YEUDALL, 2015). A recent meta-analysis showed that *P. gingivalis* increased the chance of cancer development and periodontal disease as much as 1.36 times (SAYEHMIRI *et al.*, 2015).

Studies focusing in biological mechanisms of the relationship between periodontal disease and oral cancer mainly focus in *P.gingivalis. P.gingivalis* is a major

etiological agent in the development of chronic periodontitis that expresses a plethora of virulence factors and modulates the immune response of the host cells (HOW *et al.*, 2016). *P. gingivalis* is as a keystone pathogen based on its ability to orchestrate inflammatory disease by remodeling the oral microbiome and locally invading periodontal tissues avoiding the immune surveillance while maintaining its viability (HAJISHENGALLIS *et al.*, 2012; HOW *et al.*, 2016).

P.gingivalis has been associated with accelerating cell cycle progression and it represses chemically induced apoptosis in primary cultures of gingival epithelial cells (YAO, L. *et al.*, 2010; HAJISHENGALLIS e LAMONT, 2014; PAN *et al.*, 2014). Epithelial cells constitute the physical barrier to infections and the first mediator of innate immunity (YAO, L. *et al.*, 2010; HAJISHENGALLIS e LAMONT, 2014; PAN *et al.*, 2014). Promotion of cell invasion, induction of chronic inflammation and production of carcinogens are other mechanisms that could be associated with oral carcinogenesis (PERERA *et al.*, 2016). The main *in vitro* and *in vivo* studies associating P. *gingivalis* and oral cancer are summarized in Table 2.

Table 2 – Summary of the main *in vitro* and *in vivo* studies associating *P.gingivalis* and oral cancer

Author	Microorganism	Study design	Sample	Main Results
2011 Katz	P *gingivalis* *S. gordonii*	Case-Control	Human normal gingival tissues Human gingival carcinoma	P. *g.* was found in higher levels (>33%) in the epithelium of gingival carcinoma. The staining intensity was also significantly enhanced in the malignant tissue by 2 folds compared to non-invasive *S.g*
2011 Groeger	*P gingivalis* W83 ATCC 33277	*In Vitro*	OSCC cells (SCC-25) HGK	P.*g.* was able to induce the expression of B7-H1 and B7-DC receptors in OSCC cells and HGK.
2014 Yee	*P gingivalis* ATCC 33277 e W38 (live or heat-killed)	*In Vitro*	OSCC cells (HSC-3, H413) GMSM-K cells	GMSM-K cells produced less IL-8 than HSC-3 and H413 cells. Live *P.g.* induced significant IL-6 and IL-8 secretion in GMSM-K and HSC-3 cells, and heat-inactivation of bacteria enhanced greatly IL-6 and IL-8 stimulation in these cells. Uninfected H413 cells produced high levels of IL-6 and IL-8, but were not responsive to live *P.g.*; heat-inactivated *P.g.* upregulated IL-6 and IL-8 secretion in these cells.
2014 Cho	*P gingivalis* *381*	*In Vitro*	OSCC cells (SCC25 and Ca9-22)	*P.g.* inhibited proliferation of oscc by inducing G1 cell cycle arrest. The expression of cyclin D1 and Cdk4 was decreased in OSCC, whereas p21, a Cdk inhibitor, was upregulated. Autophagy was prominently enhanced in infected cells contributing to the suppressed proliferation.
2014 Inaba	*P gingivalis* ATCC 33277 *F nucleatum* ATCC 25586	*In Vitro*	OSCC cells (SAS e Ca9-22)	*P. g.* activates the ERK1/2-Ets1, p38/HSP27, and PAR2/NFκB pathways to induce proMMP9 expression, after which the proenzyme is activated by gingipains to promote cellular invasion of OSCC cell lines.
2015 Inaba	*P gingivalis* ATCC 33277	*In Vitro*	OSCC cells (SAS)	Following *P. g.* infection, *PAR4* mRNA expression was increased and proMMP9 production was enhanced, leading to acceleration of SAS cell invasion. The phosphorylation of p38 and ERK1/2 was reduced in *PAR4* gene knockdown cells infected with *P. g.*, whereas nuclear translocation of NF-kB was not inhibited.
2015 Ha	*P gingivalis* 381	*In Vitro*	OSCC cells (Ca9-22)	Repeated infection of OSCC by *P. g.* resulted in morphological changes of OSCC, suggesting acquisition of an EMT phenotype. Promoted migratory and invasive properties of OSCC cells and provided resistance against a chemotherapeutic agent. Increased invasiveness of *P. g.* was correlated with enhanced production of MMP-1 and MMP-10 that was stimulated by IL-8 release.

2015 Binder	*P gingivalis* 381 *F nucleatum* PK1594	*In Vivo* *In Vitro*	4NQO induced OSCC OSCC cells (SCC-25 and CAL 27)	*P.g./F.n.* chronic infection promoted the growth and severity of 4NQO induced tongue tumors. *P.g./F.n.* triggered TLR signaling, resulting in IL-6 production that activates STAT3 which in turn induces important effectors driving OSCC growth and invasiveness.
2016 Ha	P *gingivalis* *381* E. *coli*	*In Vitro*	OSCC cells (SCC-25, OSC-20 and SAS)	Exposures to *P.g.* promoted the invasive ability of OSC-20 and SAS cells via the upregulation of MMP-1 and MMP-2. IL-8 secretion was substantially increased in the OSC-20 and SAS cells. The downregulation of IL-8 in *P.g.*-infected OSC-20 and SAS cells attenuated their invasive potentials and MMP levels.
2016 Hoppe	*P gingivalis* ATCC 33277 *A. actinomycetemcomitans* Y4	*In Vitro*	Tissue biopsies of healthy gingiva and head and neck tumors OSCC cells (BHY)	Incubation of oral tumor cells with *P.g.* and human α-defensins led to an increase in cell proliferation. *A.a.* enhanced cell death. These two oral pathogens exhibited opposite primary effects on the proliferation behavior of oral tumor cells.
2016 Sztukowska	*P gingivalis* ATCC 33277 ΔfimA ATCC 4941 W83 11029 and 10512 *F nucleatum* *S gordonii*	*In Vitro* *In Vivo*	Mice gingival epithelial cells TIGKs OSCC biopsies	*P.g.* induced expression and nuclear localization of the ZEB1, which controls EMT. ZEB1 levels was correlated with an increase in expression of vimentin and MMP-9, and with enhanced migration of epithelial cells into matrigel. Infection of mice by *P.g.* increased ZEB1 levels in gingival tissues, and intracellular *P.g.* were detected by antibody staining in biopsy samples from OSCC.
2017 Groeger	P. *Gingivalis* W83	*In Vitro*	PHGK and OSCC cells (SCC-25)	*P.g.* membrane up-regulates the expression of genes involved in downstream TLR, NFκB and MAPK signaling pathways involved in the pro-inflammatory immune response in primary and OSCC.
2017 Geng	P *gingivalis* ATCC 33277	*In Vitro*	HIOECs cells	Persistent exposure to *P.g.* caused cell morphological changes, increased proliferation ability with higher S phase fraction in the cell cycle, and promoted cell migratory and invasive properties. Key regulators genes TLR were found in response to long-time exposure of *P.g..*

2017 Woo	*P gingivalis* 381	*In Vitro* *In Vivo*	OSCC cells (OSC-20) Tumor xenografts and Injection of OSCC cells	Tumor xenografts composed of *P.g.* – infected OSCC cells demonstrated a higher resistance to Taxol through Notch1 activation (EMT). *P.g.* – infected OSCC cells formed more metastatic foci in the lung than uninfected cells. Infected mice showed higher serum levels of MMP-1, MMP-2, MMP-9, and MMP-10.

Legend: *P.g.- Porphyromonas gingivalis; S.g. –Streptococcus gordonii; F. n. - Fusobacterium nucleatum; E. c. – Escherichia coli;* OSCC - oral squamous cell carcinoma; GMSM-K - immortalized human oral epithelial; IL – interleucin; HGK - human gingival keratinocytes; Cdk - Cyclin-dependent kinase; PAR - proteinase-activated receptor ; NF-κB - factor nuclear kappa B; EMT - epithelial–mesenchymal transition; CD - cluster of differentiation molecules; 4NQO - 4-Nitroquinoline 1-oxide; STAT - *signal transducers and activators of transcription;* ERK - extracellular signal–regulated kinases; MMP - Matrix metalloproteinase; TLR- Toll like receptor; ZEB - Zinc finger E-box-binding homeobox; TIGKs - Human telomerase immortalized keratinocytes; PHGK - Primary human gingival keratinocytes; HIOECs - human immortalized oral epithelial cells; fim – Fimbrae

Bacterial infection and inflammation in the tumor microenvironment can manipulate host cells and facilitate their own survival and persistence and, as a result, the host cells usually undergo either apoptosis or proliferation following bacterial invasion (CHO *et al.*, 2014). The host response to bacterial infection is coordinated by a sophisticated molecular regulatory pathways and their deregulation is recognized as a key promoter of tumor progression and metastasis through potentiation of chronic inflammation at tumor sites (SAHINGUR e YEUDALL, 2015).

Human defensins, which are antimicrobial peptide members of the innate immune system, were expressed at tissue biopsies of healthy gingiva, head and neck tumors, and after oral cancer cells had been treated with P. gingivalis, they modulated the human defensin gene expression promoting proliferation properties of oral tumor cells (HOPPE et al., 2016). P.gingivalis strains were able to induce the expression of the B7-H1 and B7-DC receptors, types of transmembrane protein normally expressed on immunocytes, in squamous carcinoma cells and human gingival keratinocytes, which might have facilitated immune evasion by oral cancers (GROEGER et al., 2011). P.gingivalis can up-regulates the expression of genes involved in downstream TLR, NFκB and MAPK signaling pathways involved in the pro-inflammatory immune response in primary and malignant oral epithelial cells (GROEGER et al., 2017).

Cell proliferation was increased when OSCC cells were infected by P. gingivalis when compared to other bacteria or uninfected OSCC cells (BINDER GALLIMIDI et al., 2015; HOPPE et al., 2016). On the other hand, some studies found that P.gingivalis suppressed host cell proliferation in OSCC cells by the modulation of cell cycle-related molecules and reduced tumor growth in BALB/c xenograft mice harboring OSCC cells that were chronically infected with P.gingivalis (CHO et al., 2014; HA et al., 2015; WOO et al., 2017).

The epithelial–mesenchymal transition (EMT), a process by which epithelial cells change shape and acquire an invasive phenotype, has been associated with initiating the malignant transformation or oncogenic progression of epithelial cells (LAMOUILLE et al., 2014). Human gingival epithelial infected with P. gingivalis showed increased EMT marker ZEB1 responsible for the switch in cell differentiation and behavior, in gingival tissue of mice (SZTUKOWSKA et al., 2016). Similarly, the same bacterial

species (AT 33277) was found in human biopsy samples from OSCC (SZTUKOWSKA et al., 2016). In order to see tumor-promoting effect, human oral epithelial cells infected with P.gingivalis for 23 weeks result in morphological changes, increased proliferation and promotion of cell migratory and invasion properties (GENG et al., 2017). Repeated infection of oral cancer cells by P. gingivalis resulted in morphological changes of host cancer cells into an elongated shape, along with decreased expression of epithelial cell markers, suggesting the acquisition of an EMT phenotype (HA et al., 2015).

Increased cellular invasion of OSCC cell was observed upon P.gingivalis exposure resulting in the activation of the ERK1/2-Ets1, p38/HSP27, and the PAR2/NF-κB pathways to induce proMMP9 expression (INABA et al., 2014). Similarly, elevated levels of PAR4 mRNA were followed by p38 and ERK1/2 phosphorylation and led to increased proMMP9 production with resulting enhanced cellular invasion (INABA et al., 2015). An in vivo study showed, for the first time, that tumors from chronic infected mice with P.gingivalis/F.nucleatum were markedly 2.5 times larger, they were significantly more invasive and the expression of cyclin D1, a pivotal oncogene of oral tumorigenesis, was significantly enhanced in comparison to non-infected mice, both in cancerous and non-cancerous tongue epithelia (BINDER GALLIMIDI et al., 2015). Higher migratory and invasive properties of OSCC cells when infected with P. gingivalis induced an increase in the expression level of CD44 and CD133, well-known cancer stem cell markers, and promoted the tumorigenic properties of infected cancer cells compared to non-infected controls (HA et al., 2015). Exposures to P.gingivalis promoted the invasive ability of two OSCC cells via the upregulation of MMP-1 and MMP-2 (HA et al., 2016).

Ha et al. (2015) were pioneers to report resistance against chemotherapeutic (Taxol) when OSCC cells were infected with P.gingivalis (HA et al., 2015). Recently, OSCC cells were infected with P.gingivalis and implanted into the flanks of nude mice, showing a higher resistance to Taxol through Notch1 activation (EMT marker), as compared to uninfected cells. Furthermore, when P.gingivalis–infected OSCC cells were injected into their tail vein, they formed more metastatic foci in the lung than uninfected cells did. (WOO et al., 2017).

CONCLUSION

The current evidence indicates that periodontal disease seems to contribute as a modifier in the oral cancer development. Periodontal disease and oral cancer share many common established risk factors, but after adjusting for different confounding factors, periodontal disease seems to be an independent risk for oral cancer in most of epidemiologies studies. Studies focusing in biological mechanisms of the relationship between both diseases mainly focus in *P.gingivalis. P. gingivalis* seems to be involved with various steps of oral carcinogenesis as proliferation, migration invasion, aggressiveness and also metastasis and resistance to anti-cancer agents *in vitro* and *in vivo* studies. The mechanisms are still very incipient and future studies are needed in order to contribute to the etiopathogenesis and molecular pathways involved. While the plausible biological mechanism is under construction, prevention is strongly recommended in order to prevent the appearance of both diseases.

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INDUCTION OF PERIODONTITIS IN AN ANIMAL MODEL OF CHEMICAL CARCINOGENESIS

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Induction of Periodontitis in an Animal Model of Chemical Carcinogenesis

ABSTRACT

Emerging evidence is suggestive of the involvement of chronic inflammatory diseases with cancer development. Although the correlation between bacterial-induced chronic inflammation and cancer have been recently demonstrated in gastric cancer, the impact of chronic periodontitis lesions on oral cancer still missing. Here we decided to tackle the effects of periodontitis in cancer development using 42 Wistar rats distributed throughout four experimental groups comprised of controls (n=8); Periodontitis (n=8); chemical carcinogen 4-Nitroquinoline 1-oxide (4NQO) (n=13); and Periodontitis + 4NQO (n=13). Ligature placement drove the induction of periodontitis on the 2nd molars of rats, while administration of the tobacco surrogate 4NQO (25ppm for 20 weeks) was used to induce tumor formation. Bone loss was measured through the morphometrical evaluation of the alveolar maxillae, Perio, 4NQO, and Perio + 4NQO groups presented significant higher alveolar bone loss compared to Control littermates. Interestingly, the incidence and tumor size was higher in the Perio + 4NQO group (69%) compared to 4NQO alone (45%). Overall, our results suggest that the presence of the chronic inflammatory disease periodontitis accelerated/intensified the process of oral carcinogenesis in rats.

Keywords: periodontitis, 4NQO, rats, oral cancer.

INTRODUCTION

Oral cancer is a major public health problem with more than 500.000 new cases per year worldwide (SIEGEL *et al.*, 2013). Oral squamous cell carcinoma (OSCC) is the most common oral cancer and represents 90% of all cases (SAHINGUR e YEUDALL, 2015). The survival rates remain below 50% in 5 years and have not changed in the last three decades (WARNAKULASURIYA, 2009; LEEMANS *et al.*, 2011). It has a multifactorial origin with several risk factors that can act individually or in combinations (PERERA *et al.*, 2016). The predominant etiological factors involved in oral cancer are tobacco and alcohol use, but around 15% of oral cancers still present an unknown etiology (CHOCOLATEWALA *et al.*, 2010; SAHINGUR e YEUDALL, 2015).

Periodontitis is an infection-driven chronic inflammatory disease that leads to irreversible bone loss of supporting structures and tooth loss. Furthermore, periodontitis has been recently associated with OSCC in several studies (TEZAL *et al.*, 2005; GONDIVKAR *et al.*, 2013; SAHINGUR e YEUDALL, 2015; PERERA *et al.*, 2016). The prospective involvement of periodontitis and oral cancer have been proposed by two distinct theories: the direct involvement of the periodontopathogens in cancer formation and development; and by the indirect accumulation of chronic inflammation (TEZAL *et al.*, 2005; GONDIVKAR *et al.*, 2013). Indeed, it is plausible that a long-term presence of a polymicrobial infection associated with a continuous production and accumulation of inflammatory mediators within the periodontal disease may lead to continuous DNA damage and consequently the accumulation of mutations as one of the hallmarks of oral cancer. Such alterations are likely to modify the oral microenvironment and increase susceptibility to cancer (SAHINGUR e YEUDALL, 2015).

Epidemiological studies present several confounding factors that can affect the real association of periodontitis and oral cancer, such as smoking. The current biological studies of both diseases are mainly *in vitro studies* with a special focus in the carcinogenic potential of *Porphyromonas gingivalis,* a major pathogen of periodontal disease, on OSCC cell lines (PERERA *et al.*, 2016). However, *in vivo* studies demonstrating the direct involvement of periodontitis and oral cancer are still missing. The first *in vivo* study looking for a causal role of periodontal pathogens in chemically induced OSCC was performed quite recently (BINDER GALLIMIDI *et al.*, 2015).

The objective of this study was to explore the effects of periodontitis during the carcinogenesis process of Wistar rats receiving the carcinogen 4-Nitroquinoline 1-Oxide (4NQO).

MATERIAL AND METHODS

Animals and Experimental Procedures

Forty-two 60-day-old male Wistar rats, weighting approximately 250g, provided by the Center for Reproduction of Animals of Laboratory Experimentation (CREAL-UFRGS) (Porto Alegre, RS, Brazil) and acclimated for 2 weeks at the the Animal Experimentation Unit from the Hospital de Clínicas de Porto Alegre (Porto Alegre, RS, Brazil) were used in this study. Three animals were allocated per cage (25x25x15 cm) and had a light/dark cycle of 12h and room temperature (22 ± 2ºC), relative humidity of 50–70% and with free access to Sterilized rodent laboratory chow (Nuvilab CR1; Quimtia, Colombo, PR, Brazil). This study was carried out in accordance with the Brazilian Federal Law N°11.794/2008 for the scientific use of animals. The study protocol was submitted and approved by the Animal Use Ethics Committee, Hospital de Clínicas de Porto Alegre (protocol number 150475) according to the ARRIVE guidelines.

Periodontitis was induced by placing 4-0 silk ligature (Ethicon, Johnson & Johnson, São Paulo, Brazil) subgingivally around the right upper second molar under isoflurane anesthesia in the first 2 weeks. The ligatures remained in place during the entire experimental period and served as a way to accumulate periodontal microorganisms similar to humans (VERZELETTI *et al.*, 2012; CAVAGNI *et al.*, 2016). In order to induce the carcinogenesis of the tongue, fresh solutions were prepared every two days and rats were exposed to 25 ppm of the 4NQO carcinogen solution (Sigma, St. Louis, MO, USA; #N8141) for 20 weeks after the induced periodontitis (RIBEIRO e SALVADORI, 2007). Figure 1 shows the study flowchart. All animals were submitted to anesthesia and the body weight was checked every week.

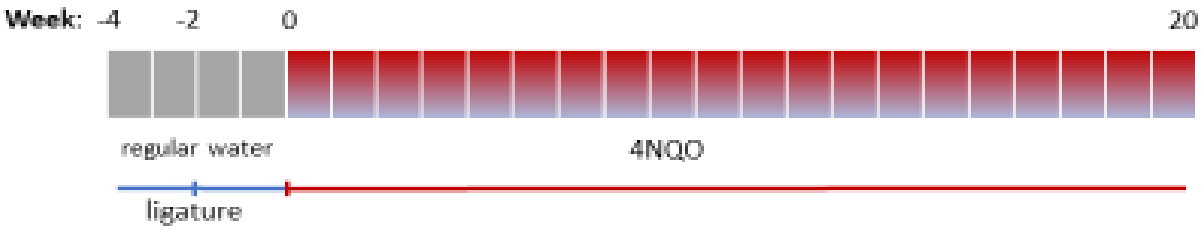

Figure 1 - Study flowchart. All groups were acclimated for 2 weeks. Periodontitis was induced by ligature in groups Perio and Perio + 4NQO 2 weeks before the exposure to 4NQO.

Animals were randomly distributed into four groups stratified by weight: Control (n=8); Periodontitis (n=8); 4NQO (n=13); Periodontitis + 4NQO (n=13). They were euthanized by overdose with isoflurane and tongues and maxillae were removed and stored in pots containing 10% formalin for 24-72 hours. Pictures of the tongues were taken in order to measure the tumor surface.

Morphometrical analysis

Maxillae were defleshed with 9% sodium hypochlorite for 4 hours and soft tissue removed carefully. They were stained for 1 min in methylene blue 1% to outline the cementoenamel junction (CEJ). Standardized pictures were taken of the buccal and palatal sides of the maxillae and measured using ImageJ 1.46r software (National Institutes of Health, MD, USA) (Figure 2A). Five linear points were recorded and the mean of the values considering the distance between the CEJ and the alveolar bone crest as bone loss. To warrant the blindness of the examiner, pictures were randomized by a computer program and coded and renamed by an external examiner (EG). About 10% of the maxillae were randomly selected and twice measured in terms of alveolar bone loss, and compared using the intraclass correlation coefficient, which showed excellent reproducibility (0.96).

Histological and immunostaining analysis

Tongues were embedded in paraffin blocks and longitudinally thin-sectioned (4 μm). They were stained with hematoxylin and eosin (H.E.; Merck) and graded as normal, dysplasia and carcinoma (Figure 3A) by two blinded expertal oral pathologists (R.C. and V.C.). Differences in grading were discussed and agreement was achieved. Tumor surface was measured using ImageJ 1.46r software (National Institutes of Health, MD, USA) in areas that tumor was delineated.

Blindness of the examiners

All analyses in the present study were performed by examiners blinded to group distribution.

Statistical Analysis

The variables of this study were tested for normality. Data were analyzed using the statistical package GraphPad Prism 7 (GraphPad Software, San Diego, CA). Mean ± SEM was used to report findings. One-way and Two-way ANOVA test were used for weight gain, solution consumption and alveolar bone loss and Student's t test for tumor size.

RESULTS

During the experiment, two animals of the 4NQO group died due to anesthetic complications.

All animals gained weight during the study (Figure 2A). In groups that were exposed to 4NQO, animals maintained their weight in the first 4 weeks and they started to lose weight in the last 4 weeks. Animals in Perio group increased the their weight during the whole experiment. When groups exposed to 4NQO are compared to Perio group, statistically significant differences are observed in all weeks for group 4NQO and in the sixteenth and twentieth weeks for group Perio + 4NQO. When they were compared to Control group, 4NQO and Perio + 4NQO showed a significant difference only in the last week (data not shown). A parallel may be drawn in regard to solution consumption. Groups exposed to 4NQO drank less

than control groups (Figure 2C). A significant lower solution consumption was observed during the 20 weeks exposure to the carcinogen in group 4NQO and Perio + 4NQO in the first and last weeks compared to Control group.

Statistically significant differences for alveolar bone loss were detected between groups. The intergroup analysis for alveolar bone loss in the right side, in which periodontitis was induced, animals in groups Perio, 4NQO and Perio + 4NQO presented statistically significant differences when compared to Control group. Also, statistically significant difference was observed between Perio compared to Perio + 4NQO group (Figure 3B). Surprisingly, group 4NQO presented an alveolar bone loss similar to Perio group even when periodontitis was not induced. When The left side and the right side (ligature) were compared for the intragroup analysis in groups that periodontitis was induced, a higher alveolar bone loss was observed in the Perio + 4NQO group (Figure 3C).

When the histopathological characteristics of the tongues were analyzed between groups, all animals exposed to 4NQO presented epithelial changes. Perio + 4NQO group showed a higher incidence of OSCC in 69% of cases compared to 45% in 4NQO group, and dysplasia was more incident in the 4NQO group compared to Perio + 4NQO (Figure 4B). Likely, tumor surface size in group Perio + 4NQO (62.75 ± 9.72) was statistically larger when compared to 4NQO group (44.25 ± 6.69) (Figure 4C).

DISCUSSION

The role of periodontitis as a risk factor for cancer have become of interest during recent years (GONDIVKAR *et al.*, 2013; PERERA *et al.*, 2016). Most of studies focusing on the biological mechanisms between periodontal disease and oral cancer are focused on the role of *Porphyromonas gingivalis (PG) (PERERA et al., 2016).* PG is considered a major etiological agent associated with the development of chronic periodontitis, yet little is known about its implications in cancer development and progression. Similar to the bacteriocentric vision on cancer initiation, little is known on the role of chronic inflammation driven by long-standing bacterial colonies as a risk factor for oral cancer. In order to explore the prospective association

of periodontitis with tumor development, we decided to use a in vivo strategy that closely resembles the development of periodontitis in humans. The use of ligatures in rat molars result in the formation and accumulation of bacterial biofilm, followed by the establishment of a localized chronic inflammatory disease that progresses to alveolar bone loss (GRAVES *et al.*, 2008; DUARTE *et al.*, 2010). In order to explore the potential contribution of chronic inflammation on cancer initiation, we decided to use low doses of the tobacco surrogate 4NQO in combination with dental ligatures. To the best of our knowledge this is the first study that associates the induction of periodontitis with the chemical oral carcinogenesis protocol in rodents.

The animals' body weight was used as a proxy to systemic health. All animals increased their weight during the experiment comparing to the baseline. Interestingly, Perio group increased the most their average weight during the whole experiment, but they had no differences compared to Control group, which is accordance with other studies(VERZELETTI *et al.*, 2012; CAVAGNI *et al.*, 2016). Regarding to groups exposed to 4NQO, they gained approximately 10% less weight from the baseline to the final week compared to control groups with a particular slightly drop in the final four weeks, which is in line with other study (RIBEIRO e SALVADORI, 2007). In fact, statistically significant differences could be observed in the last weeks between Perio group and groups exposed to 4NQO. A parallel may be drawn in regard to solution consumption, when groups exposed to 4NQO had a significant lower solution consumption in various time points compared to control groups. We supposed those differences of body weight and solution consumption behavior between control and 4NQO groups might be because of the bad taste in the first weeks and carcinogenesis development at the final weeks of OSCC as is proposed in the literature(RIBEIRO e SALVADORI, 2007).

The challenge of the periodontal etiopathogenesis induced by ligature in producing an alveolar bone loss in this study was achieved and corroborate with other studies (VERZELETTI *et al.*, 2012; CAVAGNI *et al.*, 2016). This could be observed in the Perio group when periodontitis was induced on the right side and a greater alveolar bone loss was statistically significant different to the left side (non induced periodontitis). On the other hand, despite having a greater alveolar bone loss in the right side, Perio + 4NQO did not show statistical significant difference. Surprisingly, for the first time we could see that the exposure to 4NQO

alone was able to induce alveolar bone loss very similar to the ligature when comparing 4NQO and Perio group. This emerge further studies to evaluate if the administration of 4NQO locally induced the alveolar bone loss by itself or its carcinogenesis effect was systemically responsible for that.

The main outcome of the present study was to establish a direct correlation between the presence of periodontal disease with high incidence of oral cancer. Towards this goal, the choice of 4NQO was essential to oral cancer once that

The selection of the 4NQO as the carcinogen is of importance to this study due to its probably the best chemical carcinogen to specifically induce oral squamous cell carcinoma in rats via drinking water because it reproduces the sequential carcinogenesis stages of hyperplasia, dysplasia and carcinoma, which is very similar to human carcinogenesis (KANOJIA e VAIDYA, 2006; RIBEIRO e SALVADORI, 2007; EL-ROUBY, 2011). In this study all animals exposed to 25 ppm of 4NQO had changes in the tongue epithelium during the 20-week period. Our hypothesis that the presence of periodontitis increases the chances of oral cancer risk is in agreement with epidemiological and biological studies (GONDIVKAR *et al.*, 2013; YAO, Q. W. *et al.*, 2014; BINDER GALLIMIDI *et al.*, 2015; PERERA *et al.*, 2016). We found a higher incidence of OSCC in 69% of the rats with the presence of periodontitis before the exposure to 4NQO (Perio+4NQO) compared to 45% exposed only to 4NQO. Furthermore, we found macroscopically that tumor surface size in group Perio + 4NQO was statistically larger than 4NQO group. This could be seen also in a recent *in vivo* study that tumors from chronic infected mice with *P.gingivalis/F.nucleatum* topically on the tongue and exposed to 4NQO in drinking water were histologically 2.5 times larger (BINDER GALLIMIDI *et al.*, 2015).

Taken all together, we can assume that the presence of periodontitis modulated the oral carcinogenesis in rats based on the alveolar bone loss, a higher incidence of OSCC and tumor size.

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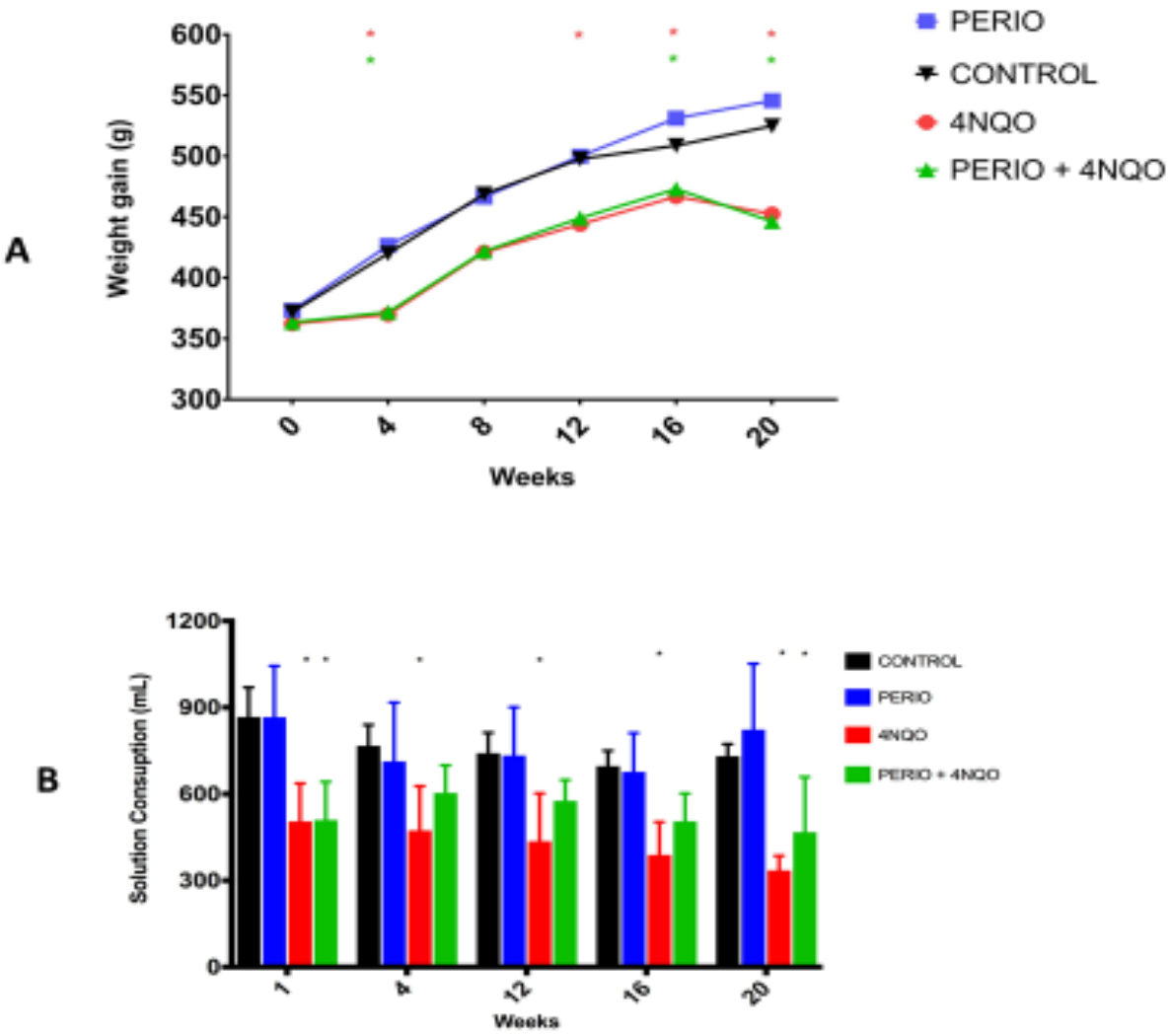

Figure 2 Body weight and solution consumption for different groups. (A) Weight of rats in different groups (Control, Perio, 4NQO and Perio + 4NQO) every 4 weeks during the 20 weeks of 4NQO exposure. Each point represents mean. *Significantly different from Perio group ($p<0.05$) (B) Solution consumption of rats in different groups every 4 weeks during the 20 weeks of 4NQO exposure Each point represents mean ± SEM. *Significantly different from Control group ($p<0.05$).

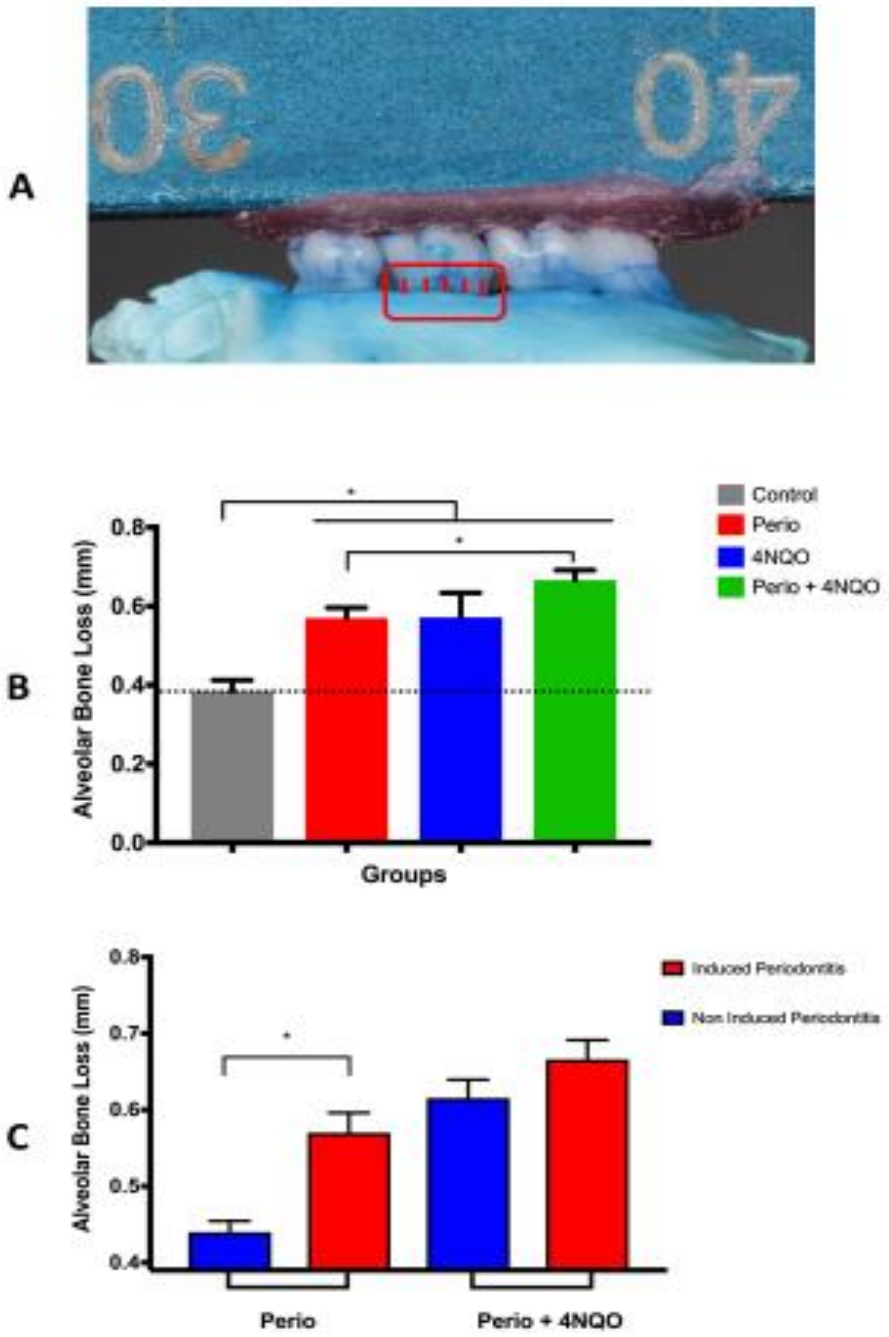

Figure 3 Morphometrical alveolar bone loss evaluation in different groups. (A) Photograph of the second upper molar with five linear measurements between the cementoenamel junction and alveolar bone crest (B) Mean alveolar bone loss in the right sides in different groups (Control, Perio, 4NQO and Perio + 4NQO). Each point represents mean ± SEM. *Significantly different in different groups ($p<0.05$). (C) Mean alveolar bone loss in the right side (ligature – induced periodontitis) and left side in groups (non induced periodontitis). Each point represents mean ± SEM. *Significantly different in different sides ($p<0.05$).

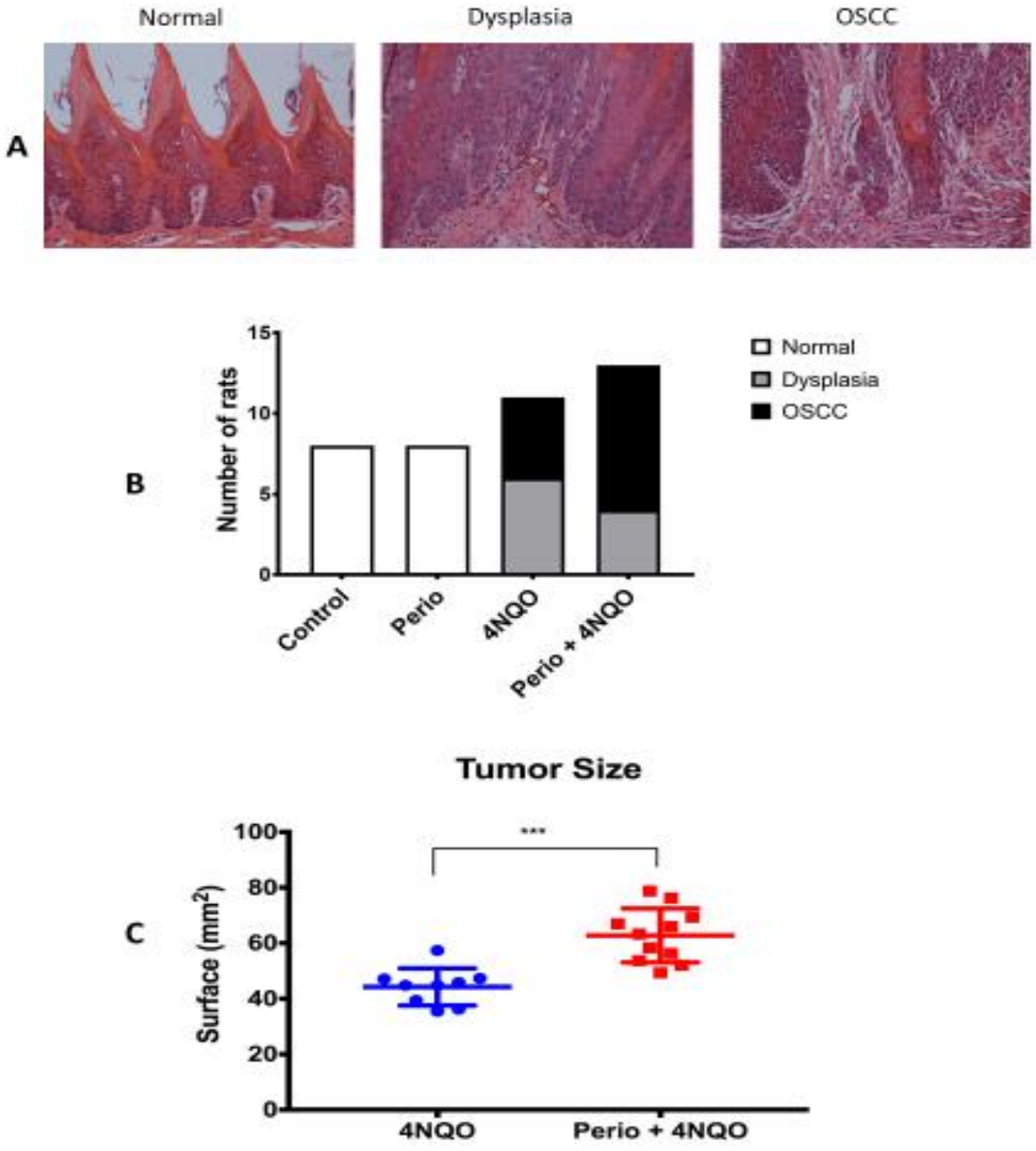

Figure 4 Histopathological diagnosis in different groups and tumor size of rat tongue exposed to 4NQO. (A) Photomicrographies showing different histopathological diagnosis: normal, dysplasia and oral squamous cell carcinoma (OSCC) (Hematoxylin & Eosin stain; ×200 magnification) (B) Incidence of different histopathological diagnosis according to the experimental group (C) The surface of the tumor areas of each tongue were digitally quantified. Each point represents mean ± SEM. *Significantly different in different groups ($p<0.05$).

5. CONSIDERAÇÕES FINAIS

As taxas de incidência para o câncer bucal têm cada vez aumentado mais e os índices de mortalidade continuam de 50% em 5 anos(LEEMANS *et al.*, 2011). Estes índices têm se mantido inalterados em várias regiões do mundo, o tornando como um dos piores tipos de cânceres. Estima-se que aproximadamente 15% das causas do câncer bucal tenham etiologia desconhecida e, que, cerca de 20% de todos casos de cânceres estejam associados a infecção microbiana e a inflamação(KUPER *et al.*, 2000; CHOCOLATEWALA *et al.*, 2010). Neste caso, vale chamar a atenção, que, a doenças periodontais, doenças infectoinflamatória altamente prevalente na cavidade bucal, tem dado fortes indicadores de estar associado ao câncer bucal.

As doenças periodontais e o câncer bucal apresentam vários fatores de risco em comum. Para isso, o presente trabalho verificou através de uma crítica revisão de literatura os estudos epidemiológicos e biológicos existentes entre as duas doenças. As evidências epidemiológicas, em sua grande maioria, têm mostrado que de fato a periodontite pode ser um fator de risco independente para o câncer bucal após ajustes estatísticos para fatores de confusão (YAO, Q. W. *et al.*, 2014). Da mesma forma, nos estudos de natureza biológica apontam que periodontopatógenos estão relacionados à diferentes estágios da carcinogênese bucal como a proliferação, migração, invasão e agressividade celular, assim como em metástase e resistência à agentes quimioterápicos (PERERA *et al.*, 2016; WOO *et al.*, 2017).

A grande maioria dos estudos biológicos presentes na literatura no que diz respeito ao envolvimento das doenças periodontais e câncer bucal são *in* vitro e têm a *P. gingivalis* como o agente etiológico para simular as doenças periodontais em células epiteliais e/ou de câncer bucal. Estudos *in vivo* relacionando as duas doenças são escassos e envolvem periodontopatógenos como método para induzir a doença periodontal (BINDER GALLIMIDI *et al.*, 2015; WOO *et al.*, 2017). A literatura aponta que a melhor forma de indução de perda óssea alveolar através da colocação de ligadura

pelo fato de permitir o acúmulo do biofilme dentário, o principal fator etiológico das doenças periodontais (READY *et al.*, 2008; VARGAS-SANCHEZ *et al.*, 2017).

O objetivo da presente tese foi avaliar o efeito da periodontite induzida por meio de ligadura sobre a carcinogênese bucal através da exposição ao carcinógeno 4-Nitroquinolina 1-Oxido (4NQO) em ratos Wistar. Os resultados mostraram, pela primeira vez, um aumento na incidência de carcinoma espinocelular na língua de ratos quando na presença de periodontite prévia pelo meio de ligadura. Além disso, houve um maior tamanho tumoral nas línguas dos animais. Os resultados desta tese vêm ao encontro do que se encontra na literatura reforçando que a presença da doença periodontal é um importante modificador no desenvolvimento de câncer bucal.

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ANEXO

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão de Ética no Uso de Animais (CEUA/HCPA) analisou o projeto:

Projeto: 150475
Data da Versão do Projeto: 08/10/2015

Pesquisadores:
VINICIUS COELHO CARRARD
TOBIAS RAUBER SPULDARO

Título: EFICÁCIA DE PROPRANOLOL EM BAIXAS DOSES SOBRE A CARCINOGÊNESE DE LÍNGUA E A DOENÇA PERIODONTAL INDUZIDOS EM RATOS WISTAR.

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais.

- Os membros da CEUA/HCPA não participaram do processo de avaliação de projetos onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Porto Alegre, 16 de janeiro de 2016.

Biol. Michael Everton Andrades
Coordenador CEUA/HCPA