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Avaliação da expressão imuno-histoquímica das enzimas DNA Metiltransferases 1 e 3b e infiltrado inflamatório em língua de Camundongos *Swiss* submetidos à fumaça de narguilé

Florianópolis

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O presente trabalho em nível de Mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de mestre em Odontologia.

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Florianópolis, 2020.

À minha avó Maria do Carmo (in memoriam).

"A vida é um sopro."

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APRESENTAÇÃO

Esta dissertação foi originalmente escrita como dois artigos na língua inglesa, com o objetivo de serem submetidos nas revistas *Critical Reviews in Oncology/Hematology* e *Oral Oncology*. Essa pesquisa foi realizada em parceria com a pesquisadora Ma. Sarah Freygang Mendes Pilati da Universidade do Vale do Itajaí/Universidade Federal de Santa Catarina.

RESUMO

A metilação do DNA é uma das alterações epigenéticas mais estudadas atualmente, especialmente com relação às DNA metiltransferases (DNMTs). A sua expressão aumentada ou reduzida pode levar à uma modificação no padrão da metilação. A desregulação das DNMTs está fortemente correlacionada com a exposição à agentes externos tóxicos e carcinogênicos, principalmente através do fumo com tabaco. O uso do aparelho de narguilé se tornou um hábito comum em todo o mundo, no entanto, seus efeitos na cavidade oral ainda não são bem elucidados. Este estudo teve como objetivo avaliar a expressão das enzimas DNMT1 e DNMT3b, assim como a inflamação, na superfície dorsal, ventral e margem lateral de língua de camundongos *Swiss* expostos à fumaça de narguilé. Para o experimento, os animais foram divididos em 6 grupos (n=60): controle, 7, 15, 30, 60 e 90 dias de exposição consecutiva à fumaça por um sistema de exposição de corpo-todo. Após cada período, as línguas foram analisadas através de coloração com hematoxilina/eosina para análise de inflamação e de imuno-histoquímica para análise de DNMT1 e DNMT3b. Os resultados mostraram que a DNMT3b apresentou diferença estatística ($p<0,05$) entre tempos de exposição e diferentes localizações anatômicas, observando-se uma expressão reduzida nos grupos de 7 a 60 dias; e aos 90 dias uma expressão semelhante ao grupo controle ou até mesmo o ultrapassando como visto em ventre lingual. A DNMT1 não apresentou diferença estatística, no entanto, demonstrou uma reduzida expressão em todos os tempos de exposição, com a superfície ventral apresentando uma expressão similar ao grupo controle aos 90 dias. A fumaça do narguilé não foi capaz de induzir inflamação aguda ou crônica em língua de camundongos. Este estudo mostrou que a fumaça de narguilé pode resultar em uma hipometilação do DNA em períodos iniciais de exposição, causando a ativação de proto-oncogenes e/ou uma instabilidade genômica; e aos 90 dias de exposição, a fumaça pode contribuir para um padrão de metilação similar ao grupo controle ou até mesmo a uma futura hipermetilação do DNA, silenciando genes supressores tumorais. Essas alterações que ocorrem no genoma devido à hipo ou hipermetilação contribuem em grande parte para o desenvolvimento de doenças como o câncer.

Palavras-chave: Narguilé. Epigenética. Metilação de DNA.

ABSTRACT

DNA methylation is one of the most studied epigenetic changes nowadays, especially regarding DNA methyltransferases (DNMTs). Its up or downregulation may lead to a different DNA methylation status. DNMT deregulation is strongly correlated to exposure to toxic and carcinogenic external compounds, especially through tobacco smoking. Narghile smoking has become a common habit worldwide, and its effects in the oral cavity are poorly understood. This study aimed to evaluate DNMT1 and 3b expression, as well as inflammation, in the dorsal, ventral surface and lateral border of Swiss mice's tongues exposed to narghile smoke. For the experiment, animals were divided into 6 groups (n=60): control, 7, 15, 30, 60, and 90 days of consecutive exposure to smoke in a whole-body exposure system. After each period, their tongues were analyzed through hematoxylin/eosin staining for inflammation status and immunohistochemistry for DNMT1 and DNMT3b. Results showed that DNMT3b presented statistical differences ($p<0,05$) between exposure periods and different tongue sites; it showed lower immunoexpression from 7 to 60 days; at 90 days there was an expression similar to control group or even an upregulation in the ventral surface. DNMT1 did not present any statistical differences; however, there was a lower expression in all exposed times, with ventral surface showing an expression similar to control group at 90 days. Narghile smoke was not able to induce acute or chronic inflammation in the mice's tongues. The study showed that narghile smoke may result in a DNA hypomethylation pattern at initial exposure periods, favoring the activation of proto-oncogenes and/or genomic instability; and at 90 days, the smoke may contribute to a methylation pattern similar to that of the control or even to a future hypermethylation of DNA, inactivating tumor suppressor genes. These alterations that occur in the genome due to hypo or hypermethylation contribute largely for the development of diseases like cancer.

Keywords: Smoking Water Pipes. Epigenomics. DNA Methylation.

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LISTA DE ABREVIATURAS E SIGLAS

CEC: Carcinoma Espinocelular

CpG: Dinucleotídeo Citosina e Guanina (*dinucleotide cytosine and guanine*)

DNA: Ácido Desoxirribonucléico (*deoxyribonucleic acid*)

DNMTs: DNA metiltransferases (*DNA methyltransferases*)

HDAC: Histona Desacetilases

HE: Hematoxilina e Eosina

HPV: Papilomavírus Humano (*human papillomavirus*)

HRP: Peroxidase de Rábano (*horseradish peroxidase*)

LPB: Laboratório de Patologia Bucal

mRNA: RNA mensageiro (*Messenger RNA)*

PBS: Tampão Fosfato (*phosphate buffered saline*)

RNA: Ácido Ribonucleico (*ribonucleic acid*)

RNA^{ASP}: Ácido Ribonucleico de Proteína Antisense

SAM: S-adenosilmetionina (*S-Adenosylmethionine*)

UFSC: Universidade Federal de Santa Catarina

UNIVALI-CEUA: Comissão de Ética na Utilização de Animais da Universidade do Vale do Itajaí (*Ethics Committee on use of Animals at University of Vale do Itajaí*)

UNIVALI: Universidade do Vale do Itajaí

LISTA DE SÍMBOLOS

Símbolo	Significado
CH_3	Metil
%	Por cento
Mg	Miligramas
N	Tamanho da Amostra
®	Marca Registrada
Ml	Mililitros
µL	Microlitros
g/ml	Gramas por Mililitros
g	Gramas
pH	Potencial Hidrogeniônico
µm	Micrômetros
ºC	Graus Celsius
M	Molar
H_2O_2	Peróxido de Hidrogênio
$<$	Menor

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1 INTRODUÇÃO

Com a difusão da cultura árabe para os países ocidentais, ocorreu concomitantemente a difusão de alguns hábitos desta população, dentre eles, o consumo do tabaco através do narguilé (também conhecido como arguilé, shisha, narguile, hookah e water pipe smoke) (MAMTANI *et al.*, 2017). O uso deste dispositivo entre os jovens aumentou vertiginosamente devido à falta de conhecimento sobre os possíveis efeitos causados pela sua fumaça (MAZIAK *et al.*, 2015; AKRAM *et al.*, 2018).

O aparelho de narguilé, proveniente dos países de origem árabe, é composto em sua estrutura por cabeça, corpo de metal, uma espécie de garrafa de vidro, mangueira e uma peça de boca (CHAOUACHI, 2009; ABOAZIZA e EISSENBERG, 2015). O processo do consumo da fumaça se dá através da queima da essência pelo carvão, localizada na cabeça do aparelho, passando pela água e indo em direção à peça de boca (MAZIAK, 2014).

Com o aumento do uso deste dispositivo associado à escassa literatura existente e ao pouco conhecimento público sobre os efeitos gerados pela fumaça proveniente do narguilé, levanta-se a questão de quais seriam os efeitos epigenéticos causados nos tecidos epiteliais da cavidade oral pela fumaça deste aparelho (CHAOUACHI, 2011). Surge então a preocupação com possíveis malefícios visto que se observa a presença de tabaco, toxinas e carcinógenos na sua fumaça (VIEGAS, 2008; WALTERS *et al.*, 2017).

A forma mais comum de ocorrer a regulação epigenética em mamíferos é através da metilação do DNA. Ela é essencial durante a morfogênese para um desenvolvimento normal do organismo (ROBERTSON, 2001; CHENG e BLUMENTHAL, 2008; CHAOUACHI, 2009; USHIJIMA e ASADA, 2010). O processo de metilação tem um importante papel na modulação da estrutura da cromatina, controlando a expressão gênica e outros processos que dependem da cromatina para se realizarem (CHENG e BLUMENTHAL, 2008). As DNA Metiltransferases (DNMTs) são enzimas que estão presentes neste processo, sendo elas: DNMT1, DNMT3a e DNMT3b (ROBERTSON *et al.*, 1999).

A função das DNMTs é transferir um grupo metil da S-adenosilmetionina (SAM) para o quinto carbono do nucleotídeo citosina (SUBRAMANIAM *et al.*, 2014). A enzima DNMT1 está presente na furca de replicação do DNA e tem a função de copiar o padrão de metilação do DNA da fita mãe para a fita filha recém-sintetizada (DANIEL *et al.*, 2011). A DNMT3a e a DNMT3b atuam no DNA não modificado previamente, estabelecendo um novo padrão de metilação conhecido como Metilação *de novo* (OKANO *et al.*, 1999). Quando as ilhas CpG da

região promotora de genes se encontram metiladas, os genes ali presentes não são expressos (LI e ZHANG, 2014). Quando a metilação é desregulada, ocorrem modificações no controle da expressão gênica, dentre as quais se inclui o silenciamento de genes supressores de tumor, contribuindo para o desenvolvimento de doenças como o câncer (ROBERTSON, 2005; GOPALAKRISHNAN, VAN EMBURGH e ROBERTSON, 2008; BAYLIN e JONES, 2014; HATTORI e USHIJIMA, 2014).

Como uma forma de aprofundar o conhecimento dos efeitos da fumaça do narguilé em cavidade oral, este trabalho teve como foco um tema ainda pouco abordado em pesquisas científicas e de grande preocupação em relação a saúde pública, principalmente pela falta de conhecimento da população em relação ao uso do dispositivo (ASLAM *et al.*, 2014). Tem-se como crença popular que o narguilé é inofensivo quando comparado com outras formas de fumo, como o cigarro convencional. No entanto, estudos relatam que esse método de fumo pode apresentar efeitos nocivos à saúde do usuário (KATURJI *et al.*, 2010). Com isso, devido à alta popularidade que o consumo de narguilé vem ganhando mundialmente e à escassa literatura pertinente, o objetivo deste estudo foi avaliar em língua de camundongos Swiss o infiltrado inflamatório encontrado nas amostras e a expressão imuno-histoquímica de proteínas da família DNA metiltransferases (DNMT1 e DNMT3b) relacionadas com a carcinogênese bucal após a exposição da fumaça do narguilé.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 EPIGENÉTICA

A epigenética se refere ao estudo das modificações herdáveis ou adquiridas e que não modificam a sequência de base do DNA (FOLEY *et al.*, 2009). Essas alterações podem ocorrer no momento em que está ocorrendo a divisão celular e podem acarretar importantes alterações na biologia do organismo (PORTELA e ESTELLER, 2010). O DNA de um indivíduo sofre diversas agressões de agentes ambientais, como alimentação e consumo de álcool e cigarro (BAYLIN e JONES, 2014). Assim, as alterações epigenéticas têm sido consideradas, em associação com as modificações genéticas, um importante fator na carcinogênese devido ao seu comportamento aberrante no genoma, causando alterações que levam ao desenvolvimento de

diversas neoplasias malignas como já relatado pela literatura (KAMIYA *et al.*, 1995; MITHANI *et al.*, 2007; POSTEL-VINAY *et al.*, 2012; DU e CHE, 2017).

Existem três tipos principais de alterações epigenéticas: RNA não codificante, envolvido nos processos celulares fundamentais e que são de grande importância nas transcrições (CHRUN, MODOLO e DANIEL, 2017), alterações de histonas, geralmente com seus estados de acetilação alterados que podem permitir ou restringir o acesso de fatores de transcrição ao DNA, podendo ocorrer também sua metilação ou fosforilação (EGGER *et al.*, 2004; SZYF, 2007; MAURANO *et al.*, 2015; DANIEL *et al.*, 2016) e a metilação do DNA (realizada pelas DNA metiltransferases) que é uma modificação onde ocorre uma adição de um grupo metil à uma citosina que faz parte de um dinucletídeo CpG (ROBERTSON, 2001; JONES e BAYLIN, 2002; CHENG e BLUMENTHAL, 2008; SCHÜBELER, 2015; SHIN *et al.*, 2016). Além disso, a metilação do DNA ocorre em associação com a desacetilação das histonas através de interação com o complexo de co-repressores da expressão gênica (FEINBERG e TYCKO, 2004; CHRUN, MODOLO e DANIEL, 2017; CHRUN *et al.*, 2017). Essas alterações podem persistir por toda a vida útil da célula e serem herdadas pelas gerações subsequentes (MASCOLO *et al.*, 2012).

As consequências das modificações epigenéticas incluem o aumento da expressão de genes ou silenciamento completo dos mesmos, dependendo do tipo de alteração que ocorre nos ativadores e supressores de regiões promotoras localizadas na cromatina (DAWSON e KOUZARIDES, 2012; IRIMIE *et al.*, 2018). Assim, as alterações epigenéticas influenciam na indução à superexpressão de oncogenes ou ao silenciamento de genes supressores de tumores (KANEDA e TSUKADA, 2017; RUSSO *et al.*, 2018).

2.2 METILAÇÃO DO DNA

A forma mais comum de ocorrer a regulação epigenética em mamíferos é através da metilação do DNA. Ela é essencial durante a morfogênese para um desenvolvimento normal do organismo (ROBERTSON, 2001; CHENG e BLUMENTHAL, 2008; USHIJIMA e ASADA, 2010). O processo de metilação, juntamente com a modificação de histonas, tem um importante papel na modulação da estrutura da cromatina, controlando a expressão gênica e outros processos que dependem da cromatina para se realizarem (CHENG e BLUMENTHAL, 2008). A metilação do DNA se dá por uma ligação covalente na qual um grupo metil (CH_3) acaba por ser transferido da SAM para o carbono 5 de uma citosina que normalmente precede a uma guanina (dinucleotídeo CpG) (LARSEN *et al.*, 1992). Para manter o padrão de metilação

no DNA, a mesma pode ser transferida para as células filhas durante as mitoses. Quando essa metilação sofre alguma alteração, sendo conhecida também como metilação aberrante do DNA, ela contribui para uma possível inativação de alguns genes, ação essa que tem uma grande influência no processo de carcinogênese (BAYLIN e JONES, 2014). A depender dos genes afetados, essas funções podem ser: controle da divisão celular, estabilização e manutenção da expressão gênica, regulação da diferenciação celular, dentre outros mecanismos importantes para o desenvolvimento normal do indivíduo (USHIJIMA *et al.*, 2003; PORTELA e ESTELLER, 2010; USHIJIMA e ASADA, 2010; JIN *et al.*, 2011). Esse processo é realizado basicamente por uma família de enzimas que recebe o nome de DNA metiltransferases que é constituída por DNMT1, DNMT2, DNMT3a, DNMT3b e DNMT3L (JIN *et al.*, 2008; SMITH e MEISSNER, 2013).

As DNA metiltransferases se dividem em classes de representantes: as metiltransferases de manutenção (DNMT1); as DNMT2 que na verdade são RNA transferases que catalisam a metilação do RNA^{ASP} (JELTSCH *et al.*, 2017); o grupo responsável pelo processo de metilação *de novo* (DNMT3a e DNMT3b), que ocorre em sítios previamente não metilados (KLUTSTEIN *et al.*, 2016); e DNM3L que é um importante regulador sem atividade catalítica, operando na forma de heterotetrâmeros com a DNMT3a facilitando a metilação de resíduos de citosina (ZHANG e XU, 2017).

Quando esse processo de metilação é desregulado por algum motivo, como por exemplo, agentes externos, ocorrem modificações no controle da expressão gênica. Dentre essas modificações, pode ocorrer uma perda global do padrão normal da metilação, o que consequentemente causa uma instabilidade no genoma, processo esse conhecido como hipometilação global do DNA. Quando há o acúmulo de metilação em áreas específicas do DNA, principalmente na região promotora de genes, é estabelecido um novo padrão conhecido por hipermetilação. Essas alterações que ocorrem no genoma devido a hipo ou hipermetilação contribuem em grande parte para o desenvolvimento de doenças como o câncer (ROBERTSON, 2005; GOPALAKRISHNAN, VAN EMBURGH e ROBERTSON, 2008; BAYLIN e JONES, 2014; HATTORI e USHIJIMA, 2014).

A hipometilação foi o primeiro processo estudado, reportado por Gama-sosa *et al.* (1983). Ainda que necessitem de mais definições de como ocorre esse processo, a hipometilação global contribui potencialmente para a instabilidade do genoma, principalmente nas regiões codificadores de genes, e para o acúmulo de alterações no material genético,

podendo ter como consequências a recombinação mitótica, reorganização cromossômica e aniquilação do *imprinting* genômico, sendo essas alterações clássicas no câncer (EHRLICH e LACEY, 2013; BAYLIN e JONES, 2014). Além disso, a ativação de proto-oncogenes silenciados é possível devido a remoção da metilação na região promotora de genes (ZHANG e XU, 2017). No entanto, a hipometilação não recebe tanta atenção se tratando de câncer quando comparado à hipermetilação devido à frequência e a quantidade de estudos indicando maior incidência de câncer relacionado à hipermetilação em certas áreas do genoma (SHAW, 2006).

A hipermetilação das ilhas CpG localizadas na região promotora dos genes induz o silenciamento de diversos genes supressores de tumor. Ela ocorre nas regiões promotoras ricas em dinucleotídeos CpG e por isso exerce uma importante função sobre a expressão gênica, levando à perda da expressão dos genes, com importante papel nos cânceres (LUCZAK e JAGODZINSKI, 2006; RUSSO *et al.*, 2018). Esse silenciamento ocorre devido à existência de sítios CpG metilados na região promotora do gene que impedem a ligação dos fatores de transcrição aos seus respectivos domínios específicos, inibindo assim o processo de transcrição (SAWADA *et al.*, 2007; ESTELLER, 2008; MASCOLO *et al.*, 2012).

Ocorre no processo de hipermetilação, uma metilação excessiva das ilhas de CpG, podendo afetar genes envolvidos no ciclo celular, genes que realizam o reparo do DNA, interação entre células, apoptose e angiogênese. Todos esses processos estão envolvidos no desenvolvimento do câncer (ESTELLER, 2008; CHANG *et al.*, 2016; SHRIDHAR *et al.*, 2016; ZHANG e XU, 2017). No câncer de boca, ainda não há uma correlação exata de como ocorre o processo de desregulação da expressão gênica (MASCOLO *et al.*, 2012; IRIMIE *et al.*, 2018), no entanto o estudo de JONES e BAYLIN (2002) alega que mais de 40 grupos de genes hipermetilados foram identificados no carcinoma oral de células escamosas.

As células tumorais sofrem alterações progressivas em seu DNA pela metilação desde o estágio potencialmente cancerizável até o desenvolvimento do câncer (RODRÍGUEZ-PAREDES e ESTELLER, 2011; BAYLIN e JONES, 2014). Esse fenômeno pode ocorrer por diversos motivos, mas o principal fator causal são as alterações nas famílias de genes das DNMTs que promovem a desregulação na metilação do genoma (DAWSON e KOUZARIDES, 2012). Em um estudo realizado por ZHANG e XU (2017) foram avaliadas amostras de genes que codificam as DNMTs e analisados quais foram os padrões de alterações encontrados e se os mesmos influenciavam no desenvolvimento do câncer. Com isso, essas alterações foram classificadas em expressão aumentada, mutação e deleção (ZHANG e XU, 2017). Vale lembrar que por mais que a família das DNMTs esteja envolvida no processo de carcinogênese, ela é

importante para os indivíduos e está altamente expressa no processo de desenvolvimento na embriogênese. Assim que as células começam a se diferenciar uma das outras nesse processo, a expressão das DNMTs é reduzida e estabilizada (MOORE, LE e FAN, 2013).

2.3 DNMT1

As DNMT1 estão envolvidas na metilação de fitas de DNA em processo de replicação, ou seja, nas fitas hemi-metiladas. Essas fitas são caracterizadas quando somente uma das duas fitas de DNA é metilada, sendo encontrada no DNA recém duplicado. As DNMT1 localizam a região da furca de replicação (local onde uma nova fita hemimetilada sintetizada é formada) se ligam à fita de DNA recém sintetizada e realizam a metilação para reproduzir o padrão de metilação original (MOORE, LE e FAN, 2013). As DNMT1 também podem interagir com proteínas associadas a ela, como as histonas desacetilases (HDAC1 e HDAC2), contribuindo com a inibição da transcrição gênica (SAITO *et al.*, 2003). Quando sobre-expressa, a DNMT1 contribui para a hipermetilação nas ilhas CpG, sendo responsável, em partes, pelo desenvolvimento anormal da metilação observado na carcinogênese (SAWADA *et al.*, 2007; SUBRAMANIAM *et al.*, 2014).

Um estudo realizado por ISSA *et al.* (1993) confirmou a sobre-expressão de DNMT1 em células primárias do câncer. Sua expressão aberrante também está relacionada com a regulação do ciclo celular, contribuindo assim para o desenvolvimento e progressão da carcinogênese (ROBERTSON *et al.*, 2000). Associações entre a metilação e agentes carcinogênicos como vírus (SHEN *et al.*, 2002), cigarro (KIM *et al.*, 2001) e radiação (ISSA *et al.*, 1996) foram observadas, porém, ainda não levaram à explicação do mecanismo definitivo que ocorre no processo (ISSA, 2004; SAWADA *et al.*, 2007).

Dentre os tumores estudados que apresentam sobre-expressão da DNMT1, se encontram tumores sólidos que resultam em metástases nos linfonodos e pior prognóstico (RAHMAN *et al.*, 2015), de bexiga (NAKAGAWA *et al.*, 2003), fígado (SAITO *et al.*, 2003), estômago (SUBRAMANIAM *et al.*, 2014), dentre outros, além de serem encontradas em grandes quantidades em cânceres de cólon (VAIOPOULOS, ATHANASOULA e PAPAVASSILIOU, 2014), próstata (LEE *et al.*, 2016), mama (MIRZA *et al.*, 2013), leucemia (BENETATOS e VARTHOLOMATOS, 2016), laringe e colo uterino (RAHMAN *et al.*, 2015).

Com relação ao câncer de boca, um estudo realizado por SUPIC *et al.* (2016) avaliou a expressão das DNMTs 1, 3a e 3b em amostras de carcinoma espinocelular oral, chegando ao resultado de que a sobre-expressão da DNMT com pior desfecho para o paciente com carcinoma de células escamosas foi a DNMT1, destacando uma lesão mais agressiva e com menor índice de sobrevivência dos pacientes (SHIAH *et al.*, 2009; SUPIC *et al.*, 2016). Em tumores de pacientes não tabagistas, a imunoexpressão da DNMT1 apresenta um resultado significantemente maior frente às outras DNMTs (DANIEL *et al.*, 2010).

2.4 DNMT3a E DNMT3b

As DNMT3a e DNMT3b são o grupo responsável pelo processo de metilação *de novo,* que estabelece a adição do radical metil na fita de DNA que não foi metilada durante a sua duplicação (JIN *et al.*, 2008; LI e ZHANG, 2014; ZHANG e XU, 2017). As DNMT3a e DNMT3b não conseguem diferenciar entre ilhas de CpG já metiladas e não metiladas e assim não copiam um padrão específico na metilação. Por este motivo atuam com a função *de novo* das metiltransferases e ficam distribuídas no núcleo de forma dispersa, sem associação com locais de replicação mesmo durante a fase de síntese do DNA (SUBRAMANIAM *et al.*, 2014). Embora sejam altamente expressas durante a embriogênese, as mesmas decaem após a diferenciação celular (SUBRAMANIAM *et al.*, 2014). A DNMT3a primeiramente metila uma sequência de genes no último estágio da embriogênese e especialmente após o nascimento, enquanto que a DNMT3B modifica uma região mais ampla da sequência do DNA nas fases iniciais da embriogênese (SMITH e MEISSNER, 2013; LI e ZHANG, 2014). O estudo de OKANO *et al.* (1999) demonstrou que, ao inativar as DNMT3a e DNMT3b, a deleção do processo *de novo* das metiltransferases levou a um fenótipo letal (OKANO *et al.*, 1999).

Os níveis de presença das DNMT3a e DNMT3b estão aumentados em diversos tecidos afetados pelo câncer e em linhagens celulares dos mesmos, contribuindo parcialmente para a hipermetilação das ilhas CpG de uma grande quantidade de genes supressores tumorais e uma variedade de malignidades (SUBRAMANIAM *et al.*, 2014). Estudos de ZHANG e XU (2017) demonstram que mutações nas DNMT3a em neoplasias hematológicas têm sido observadas no genoma do câncer. Foi encontrado também que a DNMT3a está frequentemente mutada na leucemia mielóide, leucemia linfoblástica e na síndrome mielodisplásica, associadas também à intensidade da doença e resistência ao tratamento (LEY *et al.*, 2010; ZHANG e XU, 2017). A deleção das DNMT3a leva à progressão de linfoma, tumores pulmonares e proliferação de

progenitores hematopoiéticos (CHALLEN *et al.*, 2013). No câncer de boca as DNMT3a apresentam na análise imuno-histoquímica uma expressão significativamente maior frente às outras DNMTs, assim como uma maior incidência em pacientes que apresentam a lesão e fazem uso de bebida alcóolica (DANIEL *et al.*, 2010). Outro estudo realizado por ADHIKARI *et al.* (2017) verificou a presença aumentada de DNMT3a na hipermetilação do câncer de boca, fato esse que necessita de maiores estudos (ADHIKARI *et al.*, 2017).

Quando ocorre a hipermetilação de regiões promotoras de genes, desencadeado pela ação da DNMT3a, pode haver alterações no crescimento e progressão dos tumores, com modificação tanto na velocidade de crescimento quanto na agressividade. Por outro lado, alguns autores sugerem que a DNMT3a não esteja associada com o processo de iniciação da neoplasia (ROBERTSON *et al.*, 1999; GAO *et al.*, 2011; KANEDA e TSUKADA, 2017; ZHANG e XU, 2017).

A DNMT3b participa da carcinogênese de diversos tipos de neoplasias, sendo elas o câncer de esôfago, gástrico e de pulmão (SU *et al.*, 2010; CHEN *et al.*, 2013). No câncer de boca, o estudo de Chen, Chen e Lin (2014) relatou que foi verificada uma grande expressão imuno-histoquímica das DNMT3b. Para investigar se a DNMT3b era responsável pela agressividade do tumor, os pesquisadores suprimiram a ação da enzima, o que resultou numa progressão mais lenta do câncer comparado quando a DNMT3b estava ativa, além de uma significante ligação com o risco de envolvimento de linfonodos, recorrência da doença e baixa chance de sobrevivência em pacientes com câncer de boca nos estágios III e IV (CHEN, CHEN e LIN, 2014). O estudo de Supic *et al.* (2016) também confirmou a sobre-expressão da DNMT3b no estágio III do carcinoma oral (SUPIC *et al.*, 2016).

2.5 EPIGENÉTICA E CÂNCER DE BOCA

O câncer de boca é uma neoplasia associada ao consumo de substâncias nocivas ao organismo como álcool e tabaco, presença de HPV e predisposição genética (LINGEN *et al.*, 2011; MASCOLO *et al.*, 2012). Além das diversas alterações genéticas que estão presentes no genoma do câncer descritas na literatura, como mutações somáticas e translocações cromossômicas, algumas alterações epigenéticas no genoma também foram observadas (KORF e MIKHAIL, 2017). Dentre as alterações, a metilação aberrante do DNA e a modificação de histonas desempenham um importante papel no desenvolvimento, progressão e prognóstico do

câncer bucal (OGI *et al.*, 2002). Um estudo desenvolvido por Piyathilake *et al.* (2005) avaliou o DNA de amostras de tecidos extraídos de carcinomas orais e amostras de tecidos da mucosa saudável, encontrando uma hipermetilação do DNA nas amostras das neoplasias malignas orais. Ao avaliarem amostras de carcinomas orais em pacientes que fazem uso de tabaco, Baba *et al.* (2009) e Guerrero-Preston *et al.* (2009) encontraram uma hipometilação global no genoma, porém, ao serem avaliadas amostras de carcinomas orais em pacientes que fazem uso de álcool, Supic *et al.* (2011) verificaram uma hipermetilação de genes relacionados ao câncer de boca. Além desses dois fatores estudados, outro estudo observou que quando há a presença de inflamação crônica na mucosa oral, a expressão de genes relacionados ao desenvolvimento do câncer de boca pode ser modificada pela metilação dos mesmos (GASCHE *et al.*, 2011). Além de todos esses fatores, a quantidade de metilação presente nos genes supressores de tumores podem estar associados ao estágio em que se encontra o câncer e à maior possibilidade de metástases (JHA *et al.*, 2015).

2.6 NARGUILÉ

O Narguilé é um aparelho para consumo de tabaco muito comumente utilizado no oriente, sendo composto por diversas estruturas, como cabeça, corpo de metal, uma espécie de garrafa de vidro, mangueira e uma peça de boca (ABOAZIZA e EISSENBERG, 2015). O dispositivo funciona a partir de um sistema de queima de carvão, no qual pode ser a base de pólvora ou outro material, aquecendo a essência localizada na cabeça do aparelho, a qual pode ser composta por diversos componentes incluindo ou não o tabaco, criando assim uma fumaça que passa por um recipiente composto de água, sendo finalmente aspirada pelo usuário (AKL *et al.*, 2010; COBB *et al.*, 2010; JAWAD *et al.*, 2013). Diversos usuários de narguilé sugerem que por passar através da água, a mesma funciona como uma espécie de filtro para as substâncias presentes na fumaça, além de acreditarem que a fumaça é produzida em temperaturas menores que a de outros tipos de fumo, como o cigarro convencional. No entanto, estudos comprovaram que os produtos tóxicos encontrados na fumaça são semelhantes ao cigarro convencional e que a água basicamente tem a função de resfriar a fumaça (SMITH-SIMONE *et al.*, 2008; AKL *et al.*, 2010; AZAB *et al.*, 2010; ASLAM *et al.*, 2014; EL-ZAATARI, CHAMI e ZAATALI, 2015; ALANAZI *et al.*, 2017; LIPKUS e MAYS, 2018).

Esta forma de consumo de tabaco está se espalhando ao redor do mundo, principalmente entre adolescentes e adultos jovens, mesmo entre jovens ditos “saudáveis” ou atletas (ASLAM *et al.*, 2014; NEMMAR *et al.*, 2015; PATEL, KHANGOORA E MARIK, 2019; SALLOUM *et al.*, 2019). Este grupo tem atração por esse tipo de cigarro pelo mesmo ser saborizado com frutas, mel e umectantes conhecido como “massel”, “moassel” ou apenas como essência de narguilé, e também por ser uma forma de uso social de reuniões para conversas e “passar o tempo”. Esse tipo de fumo se encontra em segundo lugar em frequência de uso entre jovens americanos, com frequência entre 20-40% dos jovens (KNISHKOWY e AMITAI, 2005; JACKSON e AVEYARD, 2008; SMITH-SIMONE *et al.*, 2008; ALJARRAH, ABABNEH e AL-DELAIMY, 2009; PRIMACK *et al.*, 2009; AKL *et al.*, 2010; COBB *et al.*, 2010; PRIMACK *et al.*, 2010; NAKKASH, KHALIL e AFIFI, 2011; JAWAD *et al.*, 2013; PRIMACK *et al.*, 2013; ASLAM *et al.*, 2014; MINAKER *et al.*, 2015; ALANAZI *et al.*, 2017; LIPKUS e MAYS, 2018). Além do “moassel”, mais utilizado por ter 30% de tabaco e 70% de mel, umectantes e outros, existem outras formas da essência como o “tumbak” ou “ajami” que é composto por uma pasta negra e pura de tabaco e o “jurak” que é originário da Índia e tem um composto intermediário dos anteriormente citados (KNISHKOWY e AMITAI, 2005).

Entende-se que é muito difícil padronizar estudos sobre a ação do narguilé em seres humanos, pois há muitas variedades no uso incluindo: quantidade variada de tabaco, tipo de tabaco usado, frequência de sessões, duração de sessões, anos de uso e uso concomitante de outras substâncias (EISSENBERG e SHIHADEH, 2009; AKL *et al.*, 2010; KATURJI *et al.*, 2010; TOUKAN *et al.*, 2020), apesar de que a literatura em sua maioria, quando se refere ao narguilé, fazer referência ao uso do “moassel” (JAWAD *et al.*, 2013). Dessa forma, ainda há a necessidade de mais estudos padronizados sobre riscos e efeitos desse tipo de fumo (AKL *et al.*, 2010; ASLAM *et al.*, 2014; PEPPER e EISSENBERG, 2014; EL-ZAATARI, CHAMI e ZAATARI, 2015; EISSENBERG, 2019).

Os estudos de SHIHADEH e SALEH (2005) demonstram que o tabaco do narguilé apresenta cerca de 2-4% de nicotina contra 1-3% do cigarro tradicional. O monóxido de carbono também se apresenta em maior quantidade e podem ser encontradas as seguintes substâncias na fumaça: alcatrão, metais pesados, arsênio, benzopireno, níquel, cobalto, berílio, cromo e chumbo, inclusive naquelas essências e carvões ditos “naturais” (SHIHADEH e SALEH, 2005). Em estudos realizados para dosar a quantidade desses componentes, verificou-se que uma sessão de narguilé libera maiores quantidades de formaldeído, acetaldeído, acroleína

propionaldeído e metacroleína na fumaça principal ao se comparar com o cigarro tradicional (SHIHADEH e SALEH, 2005; AL RASHIDI, SHIHADEH e SALIBA, 2008; VIEGAS, 2008; EISSENBERG e SHIHADEH, 2009; JAWAD *et al.*, 2013). Além disso, apesar da concentração de nicotina ser semelhante, o tempo de uso de uma sessão de narguilé é muito mais longo, causando uma exposição maior à nicotina (EISSENBERG e SHIHADEH, 2009; ASLAM *et al.*, 2014; TOUKAN *et al.*, 2020). As sessões duram em média de 45 a 60 minutos, no entanto essas reuniões podem durar horas (KNISHKOWY e AMITAI, 2005). Foi observado que uma sessão de narguilé com duração de 45 minutos tem três vezes mais exposição de monóxido de carbono que o uso de um cigarro convencional e em nível plasmático os pacientes apresentaram alto nível de nicotina e monóxido de carbono podendo gerar doenças semelhantes àquelas causadas pelo cigarro convencional (EISSENBERG e SHIHADEH, 2009). Em estudo realizado por KATURJI *et al.* (2010) com humanos na qual foi efetuada a avaliação da fumaça tragada pelos mesmos, foram aspirados 119 litros de fumaça contendo 150mg de monóxido de carbono e 602mg de resíduos da essência, resultando em duas vezes mais nicotina que no cigarro convencional (4mg) (KATURJI *et al.*, 2010).

O narguilé apresenta riscos, pois além de alguns estudos associarem o mesmo a doenças como câncer de pulmão, foi demonstrado que o uso de narguilé está associado a uma tendência dos usuários de iniciarem o uso de cigarros, seja comportamental (mudando para o cigarro por ser mais conveniente e ter mobilidade) ou seja pelo fato da nicotina causar dependência independente da sua forma de consumo (VIEGAS, 2008; COBB *et al.*, 2010; MAZIAK, 2014; ALANAZI *et al.*, 2017; ALZYOUD, VEERANKI e PBERT, 2020). Observou-se que a água presente no dispositivo filtra apenas 0,5% do total de nicotina que compõe a essência, podendo assim causar uma dependência dessa forma de fumo. Estudos demonstraram que cerca de um terço dos usuários de narguilé apresentam sintomas frente a ausência do uso da nicotina, onde após o fumo através do aparelho os sintomas de abstinência, que são observados também por exemplo no uso do cigarro convencional, se tornam presentes (NEERGAARD *et al.*, 2007; JACKSON e AVEYARD, 2008; VIEGAS, 2008; RASTAM *et al.*, 2011).

Diversos estudos demonstraram a presença de agentes tóxicos e carcinogênicos na fumaça deste tipo de fumo, associando o mesmo com doenças cardiovasculares e pulmonares (câncer, asma, bronquites, etc.), carcinomas gástricos, esofágicos, de bexiga e de laringe, bem como afetando células epiteliais de traqueia e pulmão. As alterações como leucoplasias em cavidade oral ainda necessitam de mais estudos para poderem ser relacionadas ao uso do

narguilé (MOHAMMAD, KAKAH e MOHAMMAD, 2008; MIRSADRAEE *et al.*, 2010; EL-ZAATARI *et al.*, 2015; STRULOVICI-BAREL *et al.*, 2016; WALTERS *et al.*, 2017; AKRAM *et al.*, 2018).

2.7 NARGUILÉ E ALTERAÇÕES BUCAIS

Se tratando de alterações bucais, o tabaco é considerado um dos principais fatores que provocam riscos à saúde do ser humano, especialmente em relação ao cigarro convencional (AL-AMAD, AWAD e NIMRI, 2014). Poucos são os estudos que associam o consumo de tabaco através do narguilé com alterações bucais, no entanto, há relatos de que seu uso influencia no desenvolvimento de doenças periodontais, em uma resposta inflamatória prejudicada, maior susceptibilidade à infecção por *Candida albicans*, mucosa ressecada, no desenvolvimento de lesões potencialmente cancerizáveis e no câncer bucal (AL-BELASY, 2004; WARNAKULASURIYA, 2011; BIBARS *et al.*, 2015; MUNSHI, HECKMAN e DARLOW, 2015; NOCITI, CASATI e DUARTE, 2015; RAMOA, EISSENBERG e SAHINGUR, 2017).

ZAID *et al.* (2018) sugeriram que mutações no gene *p53* estão associadas ao uso de narguilé. Para o estudo foram incluídos pacientes não-fumantes e fumantes com mucosa saudável, com lesões potencialmente cancerizáveis e com câncer de boca. Como resultados todas as amostras apresentaram mutação da proteína, no entanto, a maior porcentagem de mutações ocorreu nos pacientes fumantes e com câncer de boca, onde de 52 amostras de carcinoma proveniente de pacientes fumantes, 83,1% apresentaram mutações. Em outro estudo realizado por PATIL *et al,*. (2019a) o DNA genômico e o RNA de queratinócitos da mucosa oral expostos ao extrato da fumaça do narguilé foram extraídos para análise, verificando-se que 247 genes apresentaram expressão alterada quando comparados com células saudáveis, incluindo genes que desempenham um papel importante no desenvolvimento do câncer de boca.

Por haver grandes quantidades de substâncias tóxicas na fumaça, como os compostos aldeídos, o alcatrão e seus constituintes, radicais como metais pesados e monóxido de carbono em quantidades maiores do que as encontradas na fumaça do cigarro convencional, não se pode descartar que a fumaça influencia no desenvolvimento de lesões potencialmente cancerizáveis e malignas (SHIHADEH e SALEH, 2005; AL RASHIDI *et al.*, 2008; KHABOUR *et al.*, 2012). Além disso, sabe-se que tais alterações quando em boca, como por exemplo leucoplasias ou

carcinoma epidermóide, são frequentemente encontradas em pacientes fumantes (WARNAKULASURIYA, 2011; JAVED *et al.*, 2017; RAMOA *et al.*, 2017; WAZIRY *et al.*, 2017). Sendo assim, por mais que hajam diversos artigos relatando essa possível associação, os efeitos biológicos completos na cavidade oral ainda não são totalmente elucidados, sendo necessários maiores estudos frente às consequências do uso do narguilé em boca (JAVED *et al.*, 2017; PATIL *et al.*, 2019b).

3 PERGUNTA NORTEADORA

A exposição da mucosa lingual de ratos à fumaça de narguilé, em diferentes períodos de tempo, é capaz de induzir alterações inflamatórias bem como a alteração da expressão imuno-histoquímica das DNMT1 e DNMT3b em diferentes regiões da língua?

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar as alterações inflamatórias e a expressão imuno-histoquímica das enzimas DNMTs 1 e DNMT3b na mucosa lingual de camundongos expostos à fumaça de narguilé durante 7, 15, 30, 60 e 90 dias.

4.2 OBJETIVOS ESPECÍFICOS

- Avaliar microscopicamente a presença de células inflamatórias em língua de camundongos não-expostos e expostos à fumaça de narguilé.
- Avaliar e comparar os níveis de expressão imuno-histoquímica da DNMT1 e DNMT3b dos grupos estudados nas diferentes localizações da língua.
- Correlacionar a presença de células inflamatórias com a expressão imuno-histoquímica das DNMTs 1 e 3b e com o tempo de exposição à fumaça nos grupos estudados.

5 METODOLOGIA EXPANDIDA

5.1 DELINEAMENTO DO ESTUDO

O estudo realizado foi do tipo experimental descritivo.

5.2 ASPECTOS ÉTICOS E LEGAIS

O projeto foi submetido à Comissão de Ética na Utilização de Animais da Universidade do Vale do Itajaí (Univali-CEUA) com parecer 063/17 (Anexo A).

5.3 LOCAL DE REALIZAÇÃO DO ESTUDO

A parte experimental do estudo com animais foi realizada no Biotério do Curso de Odontologia da Universidade do Vale do Itajaí e o processamento/análise das amostras no Laboratório de Patologia Bucal (LPB) da Universidade Federal de Santa Catarina (UFSC).

5.4 PROCEDIMENTOS LABORATORIAIS

5.4.1 Procedimentos com animais

Para o procedimento experimental com animais utilizou-se do sistema de exposição de corpo-inteiro (SHRAIDEH e NAJJAR, 2011; KHABOUR *et al.*, 2012; SEMENZATI *et al.*, 2012).

Neste estudo, camundongos fêmeas da linhagem *Swiss* (n= 60), com idade de 02 meses e em média 25g de peso, foram alojados em gaiolas convencionais sendo mantidos num ciclo de luz-escuro de 12 horas com um período de troca diário de comida e água *ad libitum* juntamente com a climatização e umidade do ar controladas. Os animais passaram por um período de ambientação no biotério uma semana antes da realização do experimento. Em sequência, realizou-se a divisão dos animais aleatoriamente em seis grupos com dez animais cada, sendo eles: controle (sem exposição à fumaça), 7, 15, 30, 60 e 90 dias de exposição consecutivos. O número de animais para cada grupo foi decidido baseando-se na literatura

experimental em que Garcia Martins *et al.* (2012) usou grupos com 10 animais para experimentos com fumaça de cigarros e posterior avaliação histopatológica (GARCIA MARTINS *et al.*, 2012). Além disso, um estudo prévio realizado por nosso grupo de pesquisa como trabalho de conclusão de curso no curso de Odontologia no ano de 2017 na Universidade do Vale do Itajaí serviu como base para aprimoramento, refinamento e redução do número de animais e do tempo da pesquisa levando em conta o Princípio dos 3 R's (Redução, Refinamento e Substituição/*Replacement, Reduction and Refinement*) (PRESCOTT e LIDSTER, 2017).

Os animais do grupo experimental foram submetidos à uma exposição de corpo-todo (MIRSADRAEE *et al.*, 2010; SHRAIDEH e NAJJAR, 2011; KHABOUR *et al.*, 2012; SEMENZATI *et al.*, 2012; MINAKER *et al.*, 2015) através de confinamento em uma caixa vedada medindo 175x170x270 mm e ligada ao aparelho de narguilé comum por uma bomba de ar elétrica que realiza a sucção da fumaça e a transfere para dentro dessa caixa. Para a exposição à fumaça de narguilé utilizou-se a essência sabor maçã da marca Mizo (**Al Nakhla Tobacco Company – Free Zone S.A.E®**, Shibin El Kom, Egito), altamente consumida pelos usuários de narguilé e popularmente conhecida como "moassel", com porcentagem de 0,5% de tabaco não lavado e carvão de pólvora Bamboo Brasil Carvão de Narguilé (Egitape Importação e Exportação LTDA®, São José, Santa Catarina) com dimensão de 2x2 cm. A duração da sessão se deu por 30 minutos/dia durante 7, 15, 30, 60 e 90 dias contínuos (SHIHADEH *et al.*, 2004). A água presente no recipiente do dispositivo foi trocada a cada nova sessão. Os animais foram expostos à um padrão de um "sopro" de fumaça durante dois segundos, intervalados com 58 segundos de ar ambiente (BENTUR *et al.*, 2014; NEMMAR *et al.*, 2015), totalizando uma sessão de 30 minutos baseada no estudo de HAKIM *et al.* (2011). Um "sopro" de fumaça segundo o Método Beirute, descrito por KATURJI *et al.* (2010) equivale a 530 ml de fumaça. Sendo assim, ao longo do experimento obteve-se um total acumulado de 15.900 ml por grupo em cada sessão (SHIHADEH *et al.*, 2004; MAZIAK *et al.*, 2009). Assume-se que dentro deste período de experimento a diferença na quantidade de exposição à fumaça por camundongo dentro do aquário foi desprezível. A bomba de ar elétrica foi ajustada de forma a resultar um volume total de exposição de 530 ml, protocolo também definido pelo Método Beirute (SHIHADEH e SALEH, 2005). Este protocolo foi escolhido por se aproximar, em média, da topografia do sopro humano durante o uso do aparelho de narguilé (SHIHADEH *et al.*, 2004; KHABOUR *et al.*, 2012). Os animais do grupo controle foram expostos somente ao ar com as

mesmas condições dos grupos experimentais, mantidos em biotério controlado e sacrificados somente ao final do experimento com o grupo de 90 dias.

Para a obtenção das amostras, após a última sessão de exposição de cada grupo em seus respectivos dias realizou-se primeiramente anestesia nos animais com uma dose contendo 10,5µL de Xilazina (0,23 g/ml) e 42µL de Quetamina (0,1 g/ml) para cada 10 gramas de peso do animal e posterior retirada das línguas em monobloco. Logo em seguida, a eutanásia se sucedeu através de sobredose anestésica com as mesmas soluções utilizadas durante a anestesia. A sobredose foi composta por 50 µL de Xilazina (0,23 g/ml) e 210 µL de Quetamina (0,1 g/ml) para 10 g de peso do animal, ou seja, em um animal pesando 20 g utilizou-se 100 µL de xilazina com 420 µL de Quetamina.

5.4.2 Procedimentos com as amostras

Os tecidos a serem estudados foram fixados em paraformaldeído 4% tamponado – pH 7,4 e devidamente processados e incluídos em parafina. A confecção das lâminas para estudo sucedeu-se no LPB da UFSC onde realizou-se cortes de 3 µm e coloração com Hematoxilina e Eosina (H.E.) para posterior análise microscópica referente à presença de inflamação.

5.4.3 Processamento imuno-histoquímico

As amostras também foram submetidas à reação de imuno-histoquímica pelo método do polímero marcado com HRP, para avaliação das DNMT1 e DNMT3b, utilizando-se anticorpos primários específicos para esses antígenos. Controles positivos para todos os anticorpos (placenta humana) foram incluídos nas reações. O controle negativo de todas as reações se deu pela omissão do anticorpo primário.

Das amostras previamente fixadas em formol e emblocadas em parafina, realizou-se cortes teciduais de 3 µm de espessura estendidos em lâminas de vidro previamente tratadas com solução de *3-aminopropyltriethoxylene* (Zymed Laboratories, Inc. San Francisco, CA, USA) e levados à estufa a 60 ºC por 15 minutos.

Em seguida os cortes passaram por desparafinização em xilol e reidratação em uma sequência decrescente de etanol constituída por três passagens de cinco minutos cada, começando pelo etanol absoluto (I, II e III), seguida pelo etanol 95% e etanol 85%. As lâminas foram posteriormente lavadas em água destilada por dez minutos. A peroxidase endógena foi

bloqueada com peróxido de hidrogênio a 6% em metanol, por 30 minutos. Seguiu-se com a lavagem em água corrente por dez minutos e depois a passagem em água destilada.

Para a recuperação dos sítios antigênicos realizou-se o tratamento dos cortes teciduais com tampão citrato 0,01M pH 6,0 em banho-maria a 96 ºC por 40 minutos. O bloqueio de ligações inespecíficas foi feito por meio da incubação com leite em pó desnatado (5% em solução tampão Phosphate-buffered saline - PBS), à temperatura ambiente, por 40 minutos, seguido de lavagem com água destilada até a remoção total do leite e duas incubações de cinco minutos cada, com PBS.

As secções foram então incubadas com anticorpos monoclonais de camundongos contra DNMT1 (60B1220.1, diluição 1:1500, Novus Biologicals, Centennial, EUA) e DNMT3b (NB300-516, diluição 1:500, Novus Biologicals, Centennial, EUA) a 4ºC durante a noite, seguido de incubação com conjugado de EnVision™ (DAKO North America Inc., Carpinteria, EUA) e Dako líquido DAB + Sistema de Substrato de Cromogenio™ (3,3' - diaminobenzidina) (DAKO North America Inc., Carpinteria, EUA) para a visualização de complexos antígeno-anticorpo. Todas as secções foram contra-coradas com Hematoxilina de Harris. Como controle negativo, os anticorpos primários foram omitidos da sequência reacional. Utilizou-se espécimes de tecido de placenta em cada reação, os quais sabe-se da detecção de imunorreatividade positiva para DNMT1 e DNMT3b (DANIEL *et al.*, 2016). Posteriormente montou-se as lâminas com Entellan (Merck, Alemanha).

5.5 ANÁLISE MICROSCÓPICA E IMUNO-HISTOQUÍMICA

Para análise de células inflamatórias e imuno-histoquímica, capturou-se quatro imagens de maneira equidistante em dorso e ventre de língua e duas em cada uma das margens laterais (Figura 1), método adaptado de estudos prévios (DANIEL *et al.*, 2016; CHRUN *et al.*, 2017; WU *et al.*, 2018), a uma ampliação de 400x em um microscópio de luz (Axiostar Plus, Carl Zeiss, Oberkochen, Alemanha) acoplado a um sistema de aquisição de imagem digital (A620, Cannon, Lake Success, NY, USA), e a um microcomputador (HP Compaq 6005, São Paulo, Brasil), onde foram armazenadas as imagens.

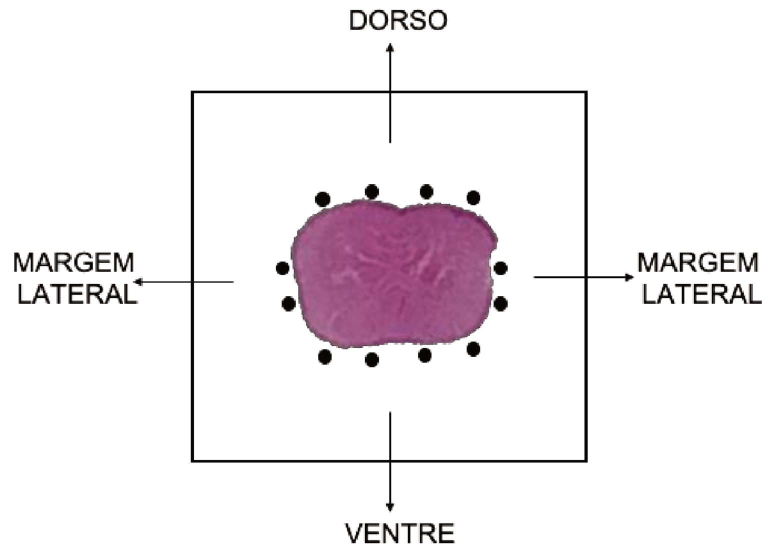

Figura 1 - Simulação da marcação dos pontos equidistantes (4 em dorso e ventre e 2 em cada bordo) para avaliação de infiltrado inflamatório. A mesma marcação foi realizada nas lâminas de imuno-histoquímica.

Para avaliar e quantificar as células inflamatórias selecionou-se como base o estudo de Bósio *et al.* (2014) na qual a classificação da intensidade de inflamação é definida como: (0) inflamação ausente, 0-10 células/área; (1) inflamação leve, 11-25 células/área; (2) inflamação moderada, 26-65 células/área; e (3) inflamação intensa com mais de 65 células/área. As células foram contadas através do programa de domínio público *ImageJ* versão 1.51p (Instituto Nacional de Saúde, Bethesda, Maryland, EUA).

Foi realizada a avaliação nuclear da imunorreatividade à DNMT1 e DNMT3b nas células epiteliais, com a utilização do mesmo programa descrito anteriormente, através da contagem das células com núcleos positivos e negativos e cálculo da porcentagem de imunopositividade para cada área analisada em cada caso (ventre, dorso e bordos de língua). Estes valores foram submetidos a uma análise estatística para comparação entre as regiões e os grupos estudados.

As avaliações para ambas as análises descritas se deram com o avaliador cegado quanto ao grupo a que cada caso pertencia. Os valores foram registrados em uma planilha previamente elaborada no Microsoft Excel® (Microsoft Office Corporation).

5.6 CALIBRAÇÃO INTRA-EXAMINADOR

Na análise da imunorreatividade e do infiltrado inflamatório, a calibração intra-examinador ocorreu através da contagem de núcleos com marcações positivas e negativas e da contagem de células inflamatórias baseadas em seu aspecto morfológico, respectivamente, em 10 imagens de bordo de língua, 10 imagens de dorso e 10 imagens de ventre. A mesma análise foi realizada novamente após uma semana ($ICC>0,8$). Todas as imagens foram selecionadas aleatoriamente dentro dos grupos estudados. Analisou-se os resultados através do cálculo do Coeficiente de Correlação Intraclasse obtendo-se para inflamação 0,802 em margens laterais, 0,831 em dorso e 0,811 em ventre; e para a análise imuno-histoquímica 0,839 nas margens laterais, 0,994 em dorso e 0,884 em ventre.

5.7 ANÁLISE ESTATÍSTICA

Os dados coletados foram armazenados em planilha eletrônica e analisados no software SPSS® versão 11 (SPSS Inc., Headquarters, EUA). Realizou-se teste de normalidade Shapiro-Wilk para avaliação da distribuição dos dados coletados. Para as expressões de DNMTs aplicou-se o teste de Kruskal-Wallis ($p<0,05$) e *post-hoc* de Dunn-Bonferroni ($p<0,05$) em todos os grupos. Para avaliação da classificação do infiltrado inflamatório realizou-se teste Exato de Fisher ($p<0,05$).

6 ARTIGOS

6.1 ARTIGO DE REVISÃO

Artigo formatado conforme as normas da revista *Critical Reviews in Oncology/Hematology* (acessadas em 20/06/2020) conforme Anexo B.

DNA Methylation in Oral Squamous Cell Carcinoma and Its Inhibitors

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Resumo

A metilação do DNA é uma das alterações epigenéticas mais frequentemente estudadas nos dias atuais, juntamente com sua associação à carcinogênese oral. Um grupo de enzimas é responsável pelo processo de metilação, conhecidas como DNA metiltransferases (DNMTs). Embora a metilação do DNA seja essencial durante a embriogênese, alterações no padrão de metilação, incluindo hipometilação global ou hipermetilação da região promotora de genes, podem ser respectivamente associadas com instabilidade cromossômica e com o silenciamento de genes supressores tumorais. Expressões aumentadas de DNMTs são um achado comum no câncer bucal e podem contribuir para a inativação de importantes genes supressores tumorais, influenciando no desenvolvimento, progressão, metástases e prognóstico do tumor. Para controlar essas alterações, drogas inibidoras vêm sendo desenvolvidas como um modo de regular a super-expressão de DNMTs, devendo ser associadas à quimio e radioterapia em andamento nos pacientes que realizam o tratamento do câncer oral. Neste artigo, nós objetivamos destacar o conhecimento atual sobre a metilação do DNA no câncer oral, incluindo a hiper/hipometilação de genes, expressão de DNMTs e seus tratamentos com inibidores.

Abstract

DNA methylation is one of epigenetic changes most frequently studied nowadays, together with its relationship with oral carcinogenesis. A group of enzymes is responsible for methylation process, known as DNA methyltransferases (DNMT). Although essential during embryogenesis, DNA methylation pattern alterations, including global hypomethylation or gene promoter hypermethylation, can be respectively associated with chromosomal instability and tumor suppressor gene silencing. Higher expression of DNA methyltransferases is a common finding in oral cancer and may contribute to inactivation of important tumor suppressor genes, influencing development, progression, metastasis, and prognosis of the tumor. To control these alterations, inhibitor drugs have been developed as a way to regulate DNMT overexpression, and they are intended to be associated with ongoing chemo- and radiotherapy in oral cancer treatments. In this article, we aimed to highlight the current knowledge about DNA methylation in oral cancer, including main hyper/hypomethylated genes, DNMT expression and its inhibitor treatments.

Keywords: Epigenomics; DNA Methylation; Methyltransferases; Mouth Neoplasms; Oral Cancer; Squamous Cell Carcinoma

1 Introduction

The word “epigenetics” was stablished in the early 1940s by a scientist named Conrad Waddington, who used the term to describe how genes interact with the environment to produce a phenotype and to explain why phenotypic variations may not be led by genetic variations.[1] The heritability of a phenotype is passed on through either mitosis or meiosis, and epigenetic research is particularly focused on providing further insights into all mechanisms that are involved in its initiation, maintenance, and heritability.[2]

Any heritable alterations in gene expression with no changes in DNA sequence are considered to be epigenetic modifications.[3, 4] These changes can persist for a particular period of life, and are directly related to harmful substances that we are exposed to in the environment, or they may persist for the entire cell life and be passed down through generations.[5] This can be explained during gene transcription, when a specific gene is expressed at one time and is then completely silenced after an external stimulus.[6]

Epigenetic events are necessary in our life as of the embryogenic period. The gene expression pattern is dependent on epigenetic modifications[7]; this is why all cells in our organism present the same DNA but do not perform the same function. There is a complex epigenetic network that controls gene expression [8]; however, when the environment interferes in this network, modifications may arise in gene expression and cause cell transformation, resulting in a malignant neoplasm.[9]

There are three known epigenetic mechanisms: DNA methylation, histone modification, and RNA-mediated silencing.[3] Although any mechanisms can lead to a malignant neoplasia and other diseases [10], the most studied one is DNA methylation.[11] After the 1980s, studies started to demonstrate that this epigenetic alteration was involved in gene regulation and cell differentiation.[12] Nowadays, it is established that DNA methylation is a major epigenetic factor influencing gene activity in association with other regulators. The majority of DNA methylation that can be found in our body is located in cytosines that precede a guanine nucleotide, and DNA regions rich in this dinucleotides sequence are called CpG islands.[5, 6]

A family of enzymes is responsible for DNA methylation, named DNA methyltransferases (DNMT). There are three important enzymes present in this process: DNMT3a, DNMT3b, and DNMT1.[13] Their function is to transfer a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine.[14] The enzyme DNMT1 is present in the DNA replication fork and copies the DNA methylation pattern from the DNA mother strand to the newly synthetized daughter strand.[12] DNMT3a and DNMT3b act on the previously

unmodified DNA, establishing a new pattern of methylation, which is called *de novo* methylation.[15] Nevertheless, when there is a malignant transformation, the DNA methylation pattern changes, and global hypomethylation and gene promoter hypermethylation occur.[5]

During cancer development, DNA methylation presents an aberrant pattern which can be classified as hypomethylation or hypermethylation.[16, 17] As a consequence of hypomethylation throughout the genome, chromosomal instability as well as activation of some proto-oncogenes may occur as a result of the removal of previous methylation. However, few reports in literature have related hypomethylation to cancer development when compared to hypermethylation.[18, 19] Hypermethylation is known to reduce or completely silence gene expression, especially tumor suppressor genes, by addition of a methyl group to CpG islands located at the promoter regions of these genes.[20] When silenced, these genes can influence neoplasm progression, prognosis and others factors that interfere with the course of carcinogenesis.[16, 21] DNMT overexpression occurs in a variety of malignant neoplasms, suggesting that they are involved in stablishing aberrant DNA methylation patterns in cancer.[22] Several studies reported DNMT overexpression in breast, hepatocellular, gastric, lung, pancreatic and head and neck neoplasms.[17, 23-28]

Recently, inhibitors have been studied for cancer therapy as therapeutic agents (natural compounds or laboratory drugs) that may be used to act on a specific malignant neoplasm.[29-31] DNMT overexpression and hypermethylation of tumor suppressor genes are directly related to carcinogenesis; thus, new anticancer drugs have been used as demethylating agents to restore re-expression and normal function of tumoral suppressor genes.[30, 32]

Few studies have investigated DNMT expression in oral squamous cell carcinomas (OSCC) and the use of inhibitors; therefore, the objective of this paper is to review literature data about DNMT overexpression, methylated and hypomethylated genes in OSCC and the use of DNMT inhibitors in oral carcinogenesis.

2 Methylation, Cancer and Inhibitors

DNA methylation is one of the aberrant epigenetic changes that occur in cancer [33-35], with global hypomethylation and CpG island hypermethylation in gene promoter regions.[19] The hypomethylation process increases chromosome instability by reducing 5-mC, especially in gene-coding regions. Some of the consequences can be mitotic recombination, chromosomal rearrangement and annihilation of genomic imprinting.[16] Moreover, activation of

silenced oncogenes may be possible because of the removal of methylation in the gene promoter region.[31] On the other hand, cancer-specific promoter CpG island hypermethylation causes a barrier for transcription factors and, consequently, inactivation of tumor suppressor genes, which are related to tumor progression.[18, 36] In this process, the methyl group is added to the fifth carbon of cytosine, which is catalyzed by DNMTs in CpG islands. These mechanisms have been seen in many different neoplasms, including head and neck cancer.[31] It should be noted that cancers induced by hypermethylation were more commonly found when compared to cancers induced by hypomethylation.[37] Furthermore, some studies showed that the aberrant pattern of DNMTs can be associated with faster tumor progression and poor prognosis.[38-40]

As DNMT overexpression is related to several malignant neoplasms [23-26], the literature shows some strategies to control this aberrant process, e.g., deletion of a specific DNMT, which leads to an important reduction in genomic methylation (and, consequently, slower cellular proliferation). However, previous studies reported that some DNMTs retained in CpG islands (such as DNMT3a and DNMT3b) can maintain the aberrant process.[41] The inhibition of those DNMTs is an important strategy that is currently studied for cancer treatment. On the other hand, different researchers have shown problems with inhibition of pan-DNMT (DNMT1, 3a and 3b) because it may activate pro-metastatic genes.[42] The solution found to date is to inhibit selected DNMTs, for example only DNMT1, 3a or 3b, to slow down progression of the neoplasm and avoid metastasis. [37]

3 DNA methylation in Oral Squamous Cell Carcinoma

OSCC is associated with harmful substances to the body (e.g., alcohol, tobacco, and others) human papillomavirus (HPV), and genetic predisposition.[3, 8] In addition to the well-known genetic changes that have been extensively described in the literature, including somatic mutations and chromosome translocations, several epigenetic alterations have been described in cancer genomes.[43] Aberrant DNA methylation and histone modifications play an important role in development, progression and prognosis of OSCC.[44] However, aberrant methylation in OSCC has not been fully demonstrated yet.[45] Although there are studies trying to understand these processes, there are limited data about the specific changes that occur in oral premalignancy and malignant lesions.[8, 20, 46] One of such studies analyzed DNA extracted from OSSC and normal tissue cells and found a higher frequency of DNA methylation in oral cancer.[36] Among external factors associated with OSCC development, global hypomethylation was found to occur in patients that use tobacco.[47, 48] In contrast, alcohol users seem to show

increased CpG hypermethylation of genes related to oral cancer.[49] Expression of OSCC-related genes can also be modified by methylation when there is chronic inflammation in the oral mucosa.[50] Also, the number of genes silenced by hypermethylation can be associated with OSCC stage and metastasis.[31] Some studies have also correlated development, growth, and poor prognosis of OSCC and higher chances of metastasis with DNMT expression status. As an example, Baba et al. (2009) performed a study in mice tongue exposed to 4NQO, a carcinogenic substrate. They found that DNMT1 overexpression was associated with increased OSCC development when compared to mice tongue also exposed to the substrate but with inhibited DNMT1, which decreased OSCC development.[47] Chen, Chen and Lin (2014) showed that DNMT3b inhibition in SCC4/SCC25 cell lines was able to prevent tumor cell proliferation when compared to cells with DNMT3b acting normally.[51] Table 1 shows other studies that report DNMT expressions in OSCC tissues or cell lines.

Table 1 - DNMT expression in OSCC

DNMT	Substrate	Expression	Reference
DNMT1, 3a, 3b	SAS / HSC-2/3/4[a] Ca9-22 / Ho-l-u-1[a] Ho-l-N-1 / OK-92[a]	Overexpression	26
DNMT1	OSCC tissues	Overexpression	52
DNMT3b	OSCC tissues	Overexpression	51
DNMT1, 3a, 3b	DOK / OC2 / Ca9-22 HSC3 / TW2.6[a]	DNMT3a/3b overexpression	53
DNMT1, 3a, 3b	OSSC tissues	Overexpression	27, 28, 54
DNMT3a	OSCC tissues	Overexpression	55

[a]Cell lines

4 Hypermethylated genes in Oral Squamous Cell Carcinoma

More than 40 tumor suppressor genes silenced by hypermethylation and related to OSCC have been described in the literature. These genes are associated with important cellular process such as cellular cycle, apoptosis, Wnt signaling pathway, cell-to-cell adhesion and DNA repair.[3] However, the exact pattern of gene methylation in OSCC is not completely understood yet.[19] Some genes and their clinical consequences in OSCC are frequently described by the literature. Some of them are detailed below:

Adenomatous polyposis coli (*APC*) gene, i.e., is a tumor suppressor gene, which is involved in early OSCC development when hypermethylated.[18] *APC* is usually translated into a multi-domain protein that binds to different molecules. One of these molecules is *ß-catenin*, which is related to adherence junctions and Wnt signaling.[19, 56] When *APC* is silenced by hypermethylation, *ß-catenin* cannot be degraded.[57] As a consequence, the increased levels of *ß-catenin* may lead to the activation of growth-promoting oncogenes that trigger canonical Wnt signaling, which is an essential pathway for cell proliferation and differentiation.[58]

E-cadherin is a glycoprotein specially involved in cell-to-cell adhesion, cell polarity, intracellular signaling and tissue architecture.[45] This glycoprotein is synthetized by the *CDH1 (cadherin 1 type 1)* gene, which shows a dysregulated pattern of expression when hypermethylated.[19] *CDH1* silencing and, consequently, absence of *E-cadherin* has been related to a more aggressive pattern of OSCC, poor prognosis and higher chances of metastasis.[59] However, in a literature review, Gasche and Goel (2012) reported that the rate of *CDH1* methylation may range between 17% and 85%.[18] The variability of results suggests that methylation of this gene may not be a good marker for OSCC detection.[35]

PTEN (phosphatase and tensin homolog) is a tumor suppressor gene that plays an important role in cell survival, proliferation, differentiation, apoptosis and invasion. Because of all roles played by this gene, it has been called "the new guardian of the genome".[19, 45] Its hypermethylation has been reported in patients with OSCC; however, the exact clinical consequence in these cases is still uncertain.[60] Some studies reported that patients whose PTEN has been silenced have aggressive tumors, metastasis and a poor prognosis.[3, 61]

MGMT (O-6-methylguanine-DNA methyltransferase) is a DNA repair gene [45] that removes guanine DNA adducts, keeping cell physiology normal and maintaining genomic stability. In addition, *MGMT* protects normal cells against carcinogens and spontaneous mutations.[62] When silenced, it plays a key role in early development of OSCC [18] and is associated with a poor prognosis [49, 63]. For this reason, it needs to be investigated further as a possible diagnostic tool for OSSC.[64] Moreover, this is one of the most studied genes as an inhibitory target for a better understanding of the mechanisms of demethylating drugs used in anticancer treatments.[62]

MLH1 (mutL homolog 1) is a DNA mismatch repair gene that prevents accumulation of DNA mutations. Given its importance , the methylation pattern of this gene has been widely studied.[18] Hypermethylation of *MLH1* has been associated with initial

development of OSCC [65]; consequently, it is another gene that may be used as a diagnostic tool for early detection of OSCC in patients.[66]

$p14^{ARF}$ is an important tumor suppressor gene involved in cell proliferation, division and angiogenesis regulation.[18] Hypermethylation of $p14^{ARF}$ leads to a loss of *p53* function and inactivation of cell proliferation induced by the *p21* gene.[67] However, clinicopathologic correlation of epigenetic changes in $p14^{ARF}$ is still controversial. Some studies showed that $p14^{ARF}$ hypermethylation is correlated with a greater tumor size, tumor stage and nodal metastasis [56, 68, 69], while other studies reported an association with lower recurrence of the disease and a better clinical result.[44, 70]

$p15^{INK4B}$ is a tumor suppressor gene that inhibits cell growth and, consequently, stops cell cycle progression during the G1 phase, which is originated from the extracellular stimuli of transforming factors *BETA* and *IFN-ALPHA*.[18] Its hypermethylation may desensitize cells so that they can receive these extracellular signs and, thus, influence OSCC development.[69] Normal tissues do not show $p15^{INK4B}$ methylation and that´s why its aberrant methylation may be used as a OSCC marker.[71]

$p16^{INK4A}$ is one of the most studied methylated genes in OSCC. This gene is an inhibitor of the cell cycle and, when hypermethylated, it is associated with larger tumors, tumor stage (specially III and IV), nodal metastasis, higher recurrence ratio and poor prognosis.[18, 70] The $p16^{INK4A}$ gene could be used as a prognostic biomarker of aggressiveness when hypermethylated.[19, 35, 72]

Table 2 shows these and other hypermethylated genes described in the literature about OSCC in carcinogenesis.

Table 2 - Hypermethylated genes in OSCC

Gene	Function	Clinical Implication	Reference
ABO	Blood group antigen	Tumor progression	3, 73, 74
APC	Cell proliferation	Tumor development / progression	3, 18, 19, 31, 49, 57, 58
ATM	Cell proliferation, DNA repair	Poor prognosis	3, 75
C/EBPα	Cell cycle regulation, body weight homeostasis	Poor prognosis	3, 76
CALCA	Calcium regulation, phosphorus metabolism	Poor prognosis	77

CCNA1	Cell cycle regulation	Poor prognosis	[20, 78, 79]
CD44	Cell-to-cell interaction, adhesion and migration	Tumor aggressiveness	[50, 80]
CDH1	Cell-to-cell adhesion	Tumor progression, invasion	[3, 19, 38, 56, 78]
CDKN2A	Cell cycle regulation, senescence	Tumor initiation, progression	[3, 19, 31, 38, 50, 56]
CDKN2B	Cell cycle regulation	Cancer recurrence	[38, 81]
CHFR	Cell cycle regulation	Tumor progression	[50, 82]
CRABP2	Transcriptional regulation	Poor prognosis	[3, 83]
CYGB	Encodes a globin protein	Unknown	[78, 84]
DAP-kinase	Apoptosis	Poor prognosis	[3, 19, 20, 31, 36, 38, 44, 49, 56, 57, 67, 85]
DBC1	SIRT1 downregulation, cellular stress response, *p53* regulation	Tumor development	[38, 86]
DCC	Neural development, apoptosis	Tumor development	[3, 20, 35, 36, 38, 44, 87]
DKK3	Transcriptional regulation	Metastasis, poor prognosis	[3, 88]
E-cadherin	Signal transduction	Metastasis	[3, 18, 31, 35, 36, 49, 51, 56, 57, 67, 85, 89]
EDNRB	Signal transduction	Cancer-induced pain	[3, 20, 90]
EPHA7	Neural development	Tumor progression	[91]
ERCC1	DNA repair	Poor prognosis	[20, 92]
EYA4	Transcriptional regulation	Tumor progression	[77, 93]
FHIT	Cell cycle regulation, apoptosis	Tumor development	[38, 94]
GATA5	Transcriptional regulator	Poor prognosis	[50, 95]
GSTP1	Detoxification of carcinogens	Tumor progression	[3, 45]
H3K4	Histone 3	Poor prognosis	[3, 19]
HIN1	Tumor suppressor	Poor prognosis	[3, 96]
hMLH1	DNA repair	Tumor development	[3, 18, 19, 49, 65, 67, 85, 89]
hMSH2	DNA repair	Tumor development	[65, 67]
HOXA11	Transcriptional regulation	Tumor progression	[77, 97]
HOXA9	Gene expression, morphogenesis, differentiation	Tumor growth, metastasis	[77, 98]
HS3ST2	Circadian rythm control, glycosaminoglycan metabolism	Tumor development	[77]

HTR1B	Thermoregulation, respiration, appetite control, sexual behavior	Poor prognosis	77, 99
KIF1A	Membranous transportation along axonal microtubules	Tumor pathogenesis	20, 100
LHX6	Transcriptional regulation	Tumor development	3, 101
MED15	Transcriptional regulation	Poor prognosis	20, 102
MGMT	DNA repair	Tumor development	3, 18, 19, 31, 36, 38, 49, 56, 57, 64, 67, 78, 85, 89
MINT Family	Encodes a hormone inducible transcriptional repressor	Poor prognosis	3, 20, 35, 36, 44
miR137	Tumor suppresor	Tumor progression	3, 103
miR193a	Tumor suppresor	Tumor development	3, 104
MME	Opioid peptides destruction	Tumor development	77, 105
MX1	Cellular antiviral response	Tumor size, vascular invasion	3, 83
NID2	Cell adhesion	Tumor invasion, metastasis	20, 106
NPY	Circadian rhythm modulation	Tumor progression	77, 91
$p14^{ARF}$	Cell proliferation, angiogenesis	Tumor development, size and metastasis; good prognosis	3, 18, 19, 31, 35, 36, 44, 56, 67
$p15^{INK4B}$	Cell cycle regulation	Tumor development	3, 18, 19, 31, 35, 36, 44, 67, 69, 71, 89
$p16^{INK4a}$	Cell cycle regulation, senescence	Tumor development	3, 18-20, 26, 31, 35, 36, 44, 49, 56, 57, 64, 67, 69, 71, 78, 84, 85, 89, 107
p53	DNA repair, cell division	Tumor development	3, 31, 38, 71, 107-109
p73	Apoptosis	Tumor development	3, 109
PAX6	Transcriptional regulation	Poor prognosis	50, 110
PAX1	Cell adhesion	Tumor development	20, 50, 111
PI3K	Cell division	Tumor growth and metastasis	8, 51
PRTFDC1	Protein homodimerization, magnesium ion binding	Tumor growth	31, 67, 112
PTEN	Differentiation, survival, proliferation, invasion, apoptosis	Tumor invasion, poor prognosis	3, 19, 31, 61, 67

RARβ	Cell growth, differentiation	Tumor development	[19, 31, 72, 78]
RARB2	Cell proliferation	Poor prognosis	[3, 19, 38, 50]
RASSF	Cell cycle regulation, apoptosis	Poor prognosis	[3, 18-20, 31, 38, 49, 85]
Rb	Tumour suppressor	Poor prognostis	[3, 8]
RUNX3	Transcriptional regulation	Tumor stage, metastasis	[3, 31, 49, 56, 85]
SFRP1-2-4-5	Transcriptional regulation	Tumor development	[3, 113]
SOX17	Transcriptional regulation	Poor prognosis	[46, 77]
STAT3	Transcriptional regulation	Tumor agressiveness, poor prognosis	[51]
TAL1	Transcriptional regulation	Poor prognosis	[77]
TCF21	Epithelial-mesenchymal interactions	Tumor progression, metastasis	[3, 114]
THBS1	Cell-to-cell and cell-to-matrix interactions	Tumor invasion	[3, 115]
TIMP3	Epithelial-mesenchymal interactions	Tumor growth	[3, 20, 78, 116]
TP73	Stress cellular response and cell development	Poor prognosis	[38, 110]
WIF1	Transcriptional regulation	Tumor invasion	[3, 31, 49, 56, 85]
WRN	DNA repair, replication	Tumor aggressiveness	[57, 117]
WT1	Transcriptional regulation	Better prognosis	[50, 77, 95]
σ-14-3-3	Signal transduction	Tumor development	[3, 118]

5 Hypomethylated genes in Oral Squamous Cell Carcinoma

While hypermethylation in the gene promoter region is frequently associated with OSCC development, there are few studies, to date, on non-specific genes or on the role of (global) hypomethylation in oral carcinogenesis.[19, 119]

Global hypomethylation may contribute to carcinogenesis by reducing methylated CpG dinucleotides through the whole genome, as seen in the *long-interspersed nuclear element-1 (LINE-1)*, whose demethylation increases genome instability. Also, by demethylating some previously methylated promoter regions of multiple oncogenes, global hypomethylation may contribute for carcinogenesis development by damaging oncogene expression.[18] These

characteristics are related to the development of malignant neoplasms because they can promote genome instability.[45, 47, 56, 120]

Table 3 shows the hypomethylated genes previously described in the literature about OSCC.

Table 3 - Hypomethylated genes in OSCC

Gene	Function	Clinical implication	Reference
AIM2	Cell proliferation	Poor prognosis	77, 121
CEACAM1	Tumor cell growth	Poor prognosis	77, 122
EMR3	Protein-coding gene	Unknown	77
IFNG	Immunoregulator	Poor prognosis	77, 123
LINE-1	Global methylation level	Tumor development	18, 50, 119
PI3	Elastase-specific inhibitor	Poor prognosis	77, 124
PTHLH	Cell growth, development, migration, differentiation, survival	Tumor progression	77, 125
SPP1	Cell-matrix interaction	Tumor progression, metastasis	77
Survivin	Cell proliferation, apoptosis	Tumor aggressiveness, invasion	18, 19

6 Inhibitors and Oral Squamous Cell Carcinoma

All OSCC-related hypermethylated genes are frequently studied for epigenetic treatment or, at least, for the purpose of providing a better prognosis for patients on the basis of methylation inhibition.[31]. There are some DNMT inhibitors that have been described in the literature for patients with OSCC.[37] Some of these inhibitors are chemotherapy drugs currently in use in cancer treatment that stop tumor progression, promoting a decrease in cell growth and reduction in the number of cells found in the G2/M phase of the cell cycle.[126] However, research on the use of these inhibitors in OSCC is still incipient, especially in cell culture experiments. Some studies showed that the association of DNMT inhibitors with other chemotherapy drugs may decrease the treatment efficacy, reinforcing that the use of DNMT inhibitors in OSCC treatment needs to be cautious.[127, 128]

Zebularine (4-Deoxyuridine) demethylates DNA previously methylated by DNMTs *in vitro* by stabilizing and blocking the enzymes, preventing methylation on other sites.[54] *Zebularine* sensitizes tumor cells for chemo and radiotherapy, as well as prevents metastasis

and angiogenesis.[129, 130] In summary, after each cell cycle, DNA becomes progressively hypomethylated.[131] An important point highlighted by Suzuki et al. (2009) is that *Zebulaine* has the capacity to activate genes transcriptionally, even if the promoter region was not methylated previously; this event is known as the pleiotropic effect.[128] This epigenetic change suggests that the drug may have the potential to remodel the chromatin in an independent manner, disregarding its effect in cytosine methylation.[132] The complete detection of all the pleiotropic effects produced by *Zebularine* would be really important for new strategies in the treatment of OSCC, eliciting a better cell response to chemo- and radiotherapy.

Decitabine (5-aza-2'-deoxycytidine; 5-aza-Cdr) is an antimetabolite analogue to deoxycytidine as well as an inhibitor of DNMTs, including DNMT1/3a/3b. *Decitabine* irreversibly replaces a cytosine with a covalent attachment of the DNMTs to DNA, inhibiting the enzymes and leading to genomic demethylation.[12, 32, 133] *Decitabine* has high activity in tumor cells, as demonstrated by cell cycle withdrawal and apoptosis induction.[38]

EGCG (green tea polyphenol epigallocatechin-3-gallate) and green tea extract [14, 31] are natural compounds that play a role in cell cycle withdrawal in the G1 phase, as well as induce cell apoptosis.[134] In addition, green tea extract inhibits CAL-27, SCC-25, and KB, human squamous carcinoma cell lines, throughout the S and G2/M phases.[135] There are two main cascades, EGFR and Notch, influenced by *EGCG* and *green tea extract*, which are also related to cell cycle control.[134, 135] In OSCC cell lines, it has been demonstrated that *EGCG* may inhibit hypermethylation of new synthesized DNA strands, thereby re-expressing silenced genes, acting especially in *RECK*, *MMP-2* and *MMP-9* genes.[136]

Aloe-emodin (derived from Rheum undulatum L.) is found in the root and rhizome of Chinese plants, and it is commonly used for treatment of several diseases.[137] In cancer, it has been reported that *Aloe-emodin* plays an apoptotic effect in cells of multiple types of cancer.[138] There are few studies about its effect on OSCC cells, but the results also showed apoptosis induction and higher levels of *caspase-9* and *caspase-3*.[139] Furthermore, one study reported that *Aloe-emodin* has inhibited DNA methylation of all DNMTs in an OSCC cell line, suggesting that this natural compound modulates the DNMT pathway that is related to tumor formation and progression in oral carcinogenesis.[137] More studies correlating *Aloe-emodin* with OSCC treatment are needed to offer more in-depth data about the effects of this natural compound.

Nevertheless, preliminary studies showed promising results in this new option of oral cancer management.

Table 4 shows the main DNMT inhibitors drugs studied in OSCC.

Table 4 - DNMT inhibitors used in the treatment of OSCC

Inhibitor	Subtrate	Associated drug	Effects	Reference
Zebularine	HSC-3[a]	None	34% of DNTM inhibition; Decreases cellular growth and G_2/M cell cycle accumulation; reduces stability of HIF-1α and its targets activities	[126, 140]
	HSC-3[a]	5-FU	Suppresses apoptotic potential of 5-FU[b]	[126, 128]
	HSC-3[a]	CDDP	Chemosensitivity efficacy; apoptosis induced effects	[126, 128]
5-aza-2’ -deoxycytidine	SAS[a]	None	None	[58]
	HSC-2[a]		None	[58]
	HSC-3[a]		Restoration of APC mRNA expression	[58]
	HSC-4[a]			
	Ca-9-22[a]			
	HO-lu-1[a]			
	HO-1-NI[a]			
	OK92[a]		None	[58]
	SCC15[a]		Demethylation of p16[INK4A] after 35 cycles	[141]
	SCC40[a]		Demethylation of p16[INK4A] after 60 cycles	[141]
Green tea polyphenol epigallocatechin-3-gallate (EGCG)	OSC2[a]	None	Upregulation of p21[WAF1] promoting stop on tumor growth and cellular apoptosis	[142]
	HSC-3[a]		Upregulation of RECK gene; suppression of MMP-2 and MMP-9; Inhibition of cancer-invasive ability in a three-dimensional collagen model	[136]
	HSC-4[a]		Inhibition of cancer-invasive ability in collagen model	[136]
	SCC9[a]		Upregulation of RECK gene; suppression of MMP-2 and MMP-9; repressed significantly	[136]

			tumor invasion in a three-dimensional collagen model	
	SCC25[a]		Repressed tumor invasion significantly in a three-dimensional collagen model	136
Aloe-emodin	Buccal mucosa tissue	None	Non tumor formation; reduced severity of oral dysplasia; upregulation of Akt, MAPK, ERK and DNMT1/3a/3b	137
	SCC15[a]		Inhibits cell viability and tumor growth; induces apoptosis	139

[a]Cell line; [b]5-FU: 5-fluorouracil; CDDP: Cisplatin.

7 Conclusion

In this review, we were able to identify that hypermethylation is one of the most studied epigenetic process occurring in OSCC genes. The development of other types of cancer (e.g., colon cancer, esophageal and hematological malignancies) have been widely associated with hypermethylation promoted by DNMTs. For this reason, it is clear why hypermethylation has become an important target for cancer therapies, including the DNMT inhibitors described above. Although evidence is still incipient, the use of DNMT inhibitors has been gaining more and more attention, especially when associated with ongoing typical chemotherapy in oral cancer. However, the consequences of using DNMTs over the global methylation pattern are not known yet and may cause chromosomal instability, favoring the expression of oncogenes and metastatic genes. For all these reasons, further studies are needed on methylation in oral carcinogenesis, as they may contribute to the development of safe epigenetic therapies.

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6.2 ARTIGO DE PESQUISA

Artigo formatado conforme as normas da revista *Oral Oncology* (acessada em 22/09/2020) conforme Anexo C.

Title: DNA methyltransferases expressions in mice tongue exposed to waterpipe smoke

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Resumo

Objetivo: Esse estudo objetivou avaliar a expressão de DNMT1 e 3b, assim como inflamação, na superfície de dorso, ventre e margem lateral de língua de camundongos *Swiss* expostos à fumaça de narguilé. **Materiais e Métodos:** Os animais foram divididos em 6 grupos (n=60): controle, 7, 15, 30, 60 e 90 dias consecutivos de exposição à fumaça através de um sistema de corpo-todo. Após cada período, as línguas foram analisadas através de coloração com hematoxilina/eosina para classificação da inflamação e imuno-histoquímica para DNMT1 e DNMT3b. **Resultados:** A DNMT3b demonstrou uma redução na expressão a partir de 7 até 60 dias; aos 90 dias, a expressão se deu similar ao grupo controle, havendo até mesmo uma sobre-expressão na região central quando comparado com o grupo controle. DNMT1 demonstrou redução na expressão em todos os períodos de exposição, com a superfície ventral mostrando uma expressão similar ao grupo controle aos 90 dias. A fumaça do narguilé não foi capaz de induzir a inflamação aguda ou crônica na língua de camundongos. **Conclusão:** O estudo mostrou que a fumaça do narguilé pode resultar na hipometilação do DNA em períodos iniciais de exposição, contribuindo para a ativação de proto-oncogenes e/ou instabilidade genômica; após longos períodos, pode levar à um padrão de metilação similar ao grupo controle ou até mesmo à uma hipermetilação, silenciando genes supressores tumorais. Essas alterações que ocorrem no genoma devido a hipo/hipermetilação contribuem em grande parte para o desenvolvimento de doenças como o câncer bucal.

Abstract

Objective: This study aimed to evaluate DNMT1 and 3b expression, as well as inflammation, on the dorsal surface, ventral surface and lateral border of Swiss mice's tongues exposed to waterpipe smoke. **Materials and Methods:** Animals were divided into 6 groups (n=60): control, 7, 15, 30, 60, and 90 days of consecutive exposure to smoke in a whole-body exposure system. After each period, tongues were analyzed through hematoxylin/eosin staining for inflammation status and immunohistochemistry for DNMT1 and DNMT3b. **Results:** DNMT3b showed lower immunoexpression from 7 to 60 days; at 90 days, expression was similar to that of the control group or there was upregulation on the ventral surface when compared to the control group. DNMT1 showed lower expression at all exposure times, with the ventral surface showing similar expression to that of the control group at 90 days. Waterpipe smoke was not able to induce acute or chronic inflammation in the mice's tongues. **Conclusion:**

The study showed that waterpipe smoke may result in a DNA hypomethylation pattern in initial exposure periods, contributing for activation of proto-oncogenes and/or genomic instability; and over long periods, it may lead to a methylation pattern similar to that of the control or even to hypermethylation, silencing tumor suppressor genes. These alterations that occurs in the genome due to hypo/hypermethylation contributes largely for the development of diseases as oral cancer.

Introduction

The Middle East culture has been widespread globally and is disseminating the habit of tobacco consumption through waterpipes [1]. A waterpipe is a tobacco smoking apparatus (also known as narghile, arghile, hookah, shisha) which is becoming increasingly popular, especially among young adults, and taking the place of conventional cigarette smoking among youth [2-4]. It is already known that waterpipe smoke contains toxic and carcinogenic substances originated from the heated charcoal and the flavored tobacco, such as nicotine, tar, polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, volatile aldehydes, benzene, nitric oxide, carbon monoxide, phenols, and heavy metals [5, 6]. These substances may vary according to the design and the structure of the waterpipe, time of use, volume of water inside the bowl and even hose porosity [5, 7]. There is a wrong belief that waterpipe smoke is less harmful when compared to conventional cigarettes [8]; for this reason, its use has become frequent, even in public places and especially between groups, as a social inclusion tool among young people [9, 10]. The lack of specific legislation, different research designs and the absence of the same social stigma carried by conventional cigarettes have also reinforced the widespread use of it [7, 11].

Tobacco use has been described as one of the major risk factors to influence genetic and epigenetic changes relative to the development of oral squamous cell carcinoma (OSCC) [12]. However, it is not known whether there is a relationship between oral cancer and waterpipe use. Epigenetic changes (modifications in DNA expression without alteration in DNA sequence) have been recently studied in OSCC development. The major one is DNA methylation [13], which is a heritable epigenetic change performed by DNA methyltransferases (DNMTs), a group of enzymes responsible for catalyzing a covalent addition of a methyl group to the fifth carbon position of a cytosine located at a guanine base in a CpG dinucleotide [12]. DNA methylation is essential for normal development and survival; however, when this process is deregulated, it may cause several diseases, including malignant neoplasms [14].

DNMT1 is responsible for maintenance of DNA replication by copying the methylation pattern of the DNA mother strand to the daughter after each duplication [15]. DNMT3b is part of *de novo* methylation, a process that adds a methyl group to non-previously methylated DNA [16]. Overexpression of DNMTs has been related to OSCC development [17]. DNMT1 overexpression is correlated with DNA aberrant methylation in solid tumors, lymph nodes metastasis, and poor prognosis for patients [18]. DNMT3b overexpression is associated with lymph node metastasis, higher recurrence rate and poor prognosis [16].

Given the fact that the use of waterpipes has spread worldwide and favors exposure to carcinogen substances, the aim of this study was to evaluate DNMT1 and DNMT3b immunoexpression and inflammation in the tongue of Swiss mice exposed to waterpipe smoke in different periods of time.

Material and Methods

Animals and Treatments

This study was approved by the Ethics Committee on use of Animals at University of Vale do Itajaí (approval number 063/17). Based on the method of Garcia Martins et al. (2012), sixty female three-week old Swiss mice were selected and maintained in conventional animal cages in an animal care unit at 24± 1ºC with a 12:12 light/dark cycle [19]. Water and pelleted food were available ad libitum.

Waterpipe Smoke Exposure

After one week of acclimatization, the mice were randomly divided (n=10) into six groups: control (no exposure), 7, 15, 30, 60, and 90 days of consecutive waterpipe smoke exposure. A whole-body exposure system was used [6], with animals placed inside a closed glass chamber with a 4mm diameter orifice for inserting a silicon hose connected to an electric air machine and attached to the waterpipe device. A commercially available apple-flavored *moassel* tobacco (Al Nakhla Tobacco Company – Free Zone S.A.E®, Cairo, Egypt) was used together with 0.5% of non-washed tobacco and an instant lightening charcoal by Bamboo Brasil (Egitape Importação e Exportação. LTDA®, Florianópolis, Santa Catarina). The animals were exposed to one 2-second puff, interspersed with 58 seconds of fresh air, in a total of 30 minutes/day, which is the average length of one human smoking event, according to Hakim et al. (2011) [20]. This protocol was selected because it is similar to human breath topography

during waterpipe use [21]. The control group was exposed only to air with the same conditions of the experimental groups and euthanatized at 90 days. The other groups were euthanatized at 7, 15, 30, 60, and 90 days.

Tongue microscopic analysis

The animal tongues were surgically removed after euthanasia, fixed with 10% formaldehyde, and included in paraffin. For the immunohistochemical study, three micrometers sections were dewaxed, rehydrated and treated in 6% H_2O_2 methanol solution (1:1) for 30 minutes to quench endogenous peroxidase activity. For antigen retrieval, the sections were treated with citrate buffer pH 6.0 in a water bath at 96°C for 40 minutes. The non-specific binding sites were blocked with 5% skim powdered milk with phosphate-buffered saline (PBS) solution for 40 minutes. The sections were then incubated with mouse monoclonal antibody against DNMT1 (60B1220.1, 1:1500 dilution, Novus Biologicals, Centennial, EUA) and DNMT3b (NB300-516, 1:500 dilution, Novus Biologicals, Centennial, EUA) at 4°C overnight. Incubation with EnVision^{TM} (DAKO North America Inc., Carpinteria, EUA) and DAKO Liquid DAB + Substrate Chromogen System^{TM} (3,3'-dyaminobenzyne) (DAKO North America Inc., Carpinteria, EUA) for antigen-antibody complex visualization was performed, followed by counterstaining with Harris hematoxylin. As negative control, the primary antibodies were omitted from the reaction sequence. As positive control, we used placental tissue specimens [15].

Nuclear immunopositivity of DNMT1 and DNMT3b in epithelial cells was evaluated by one calibrated blinded observer (ICC>0,8). In each sample, immunopositive cells were counted using *ImageJ* version 1.51p (Health National Institute, Bethesda, Maryland, EUA) in four fields of each tongue site (dorsal surface, ventral surface, and lateral borders), in an adaptation of Daniel et al. (2016), Chrun et al. (2017) and Wu et al. (2018) studies, at a 400x magnification, equidistantly captured with a light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany) [15, 22, 23]. The ratio immunopositive cells/total number of cells was determined for each tongue site.

For inflammation analysis, three-micrometers sections were obtained and stained with hematoxylin and eosin. Inflammatory cells were quantified using the software *ImageJ* 1.51p version (Health National Institute, Bethesda, Maryland, EUA), according to Bósio et al. (2014) and classified as absent (0-10 cells/area), mild (11-25 cells/area), moderate (26-65 cells/area)

and severe inflammation (more than 65 cells/area) [24]. Evaluation was conducted by one blinded and calibrated evaluator (ICC>0.8).

Statistical analysis

The data were analyzed in SPSS® software version 11 (SPSS Inc., Headquarters, EUA). Fisher's exact test was used for classification of inflammatory infiltrate. The Kruskal-Wallis test was used to analyze the incidence of nuclear immunopositivity for each DNMT. Pairwise comparisons were made using the Dunn-Bonferroni test. For all tests, differences were considered significant when $p<0.05$.

Results

The immunohistochemical expression of DNMT1 and DNMT3b was localized in the nuclei of epithelial cells [Figures 1 and 2]. The percentage of positive cells for DNMT1, as shown in Table 1 and Figure 3, did not show statistically significant differences when compared to time of exposure or tongue locations. DNMT3b immunopositivity showed statistical differences ($p<0,05$) according to exposure time and tongue locations, as shown in Table 2 and Figure 4. Both enzymes showed a lower expression pattern at 7, 15, 30, and 60 days when compared to the control group. At 90 days DNMT1 maintained the lower expression in the dorsal surface and lateral border, however, the ventral surface showed an increase with a tendency to expression similar to control group. DNMT3b at 90 days in each tongue site showed a expression similar to the non-exposed group, with exception of the ventral surface that exceeded control group expression.

The inflammatory cell quantification did not show statistically significant differences between the groups. Most cases were classified as absent inflammation. Mild inflammation was found in only 10% of the control group (when analyzing dorsal surfaces) and in the 90-day group (when analyzing dorsal and lateral border surfaces), while 15/30-day groups presented up to 22% of cases with mild inflammation on dorsal surfaces. Ventral surfaces showed absent inflammation in all study groups.

Discussion

The lack of information about harmful effects of waterpipe smoke has contributed to its worldwide use. Some studies have associated the habit of waterpipe smoking with a higher risk

for lung and head and neck cancer development [7, 25]. However, there are very few microscopic studies analyzing waterpipe-related cell and DNA alterations [1]. To the best of our knowledge, this is the first standardized study to evaluate the expression pattern of DNMT1 and DNMT3b, and inflammatory response, on different tongue sites of mice exposed to waterpipe smoke in different periods.

Although there were no DNMT3b statistical differences among all the groups, there was a similar lower expression pattern at 7, 15, 30, 60 days of exposure to waterpipe when compared to the control group. While it is well established that DNMT3b is responsible for de novo methylation, Gagliardi, Strazzullo and Matarazzo (2018) [26] reported that DNMT3b also works in conjunction with DNMT1 in DNA methylation maintenance during cell division. Also, there are few proto-oncogenes whose inactivation depends exclusively on DNMT3b activity, such as CREB, FOS, SP1, SP4, C/EBPα, and NF-κB p65 [30]. When the two enzymes, i.e., DNMT1 and 3b show lower expression (as found in this study), DNA hypomethylation (global hypomethylation) [27] may occur, and it is strongly linked to chromosomal instability and proto-oncogenes activation, both frequently reported as a common occurrence in early carcinogenesis [28, 29]. Therefore, DNMT3b lower expression at 7, 15, 30, and 60 days suggests that waterpipe smoke may promote proto-oncogenes activation, favoring early phases of carcinogenesis. Based on all these possibilities, it is plausible to expect a possible DNA hypomethylation pattern (both global and proto-oncogenes promoter regions) caused by initial periods of exposure to waterpipe smoke.

In contrast to these results, the 90-day group showed increased DNMT3b expression, in a similar level to that of the control group. Although the mice were not exposed beyond 90 days, if there had been more time of exposure, higher expressions of DNMT3b could have occurred. If so, we could hypothesize a tendency for DNA hypermethylation. Although there is no evidence in the literature relating to DNMT3b down-regulation followed by up-regulation in oral cancer, this pattern has been described in lymphomas, with initial hypomethylation leading to proto-oncogenes activation and chromosomal instability, followed by secondary hypermethylation, which may silence important tumor suppressor genes [29]. When this possible occurrence is associated with OSCC, and DNMT3b is found to be overexpressed, the latter is commonly associated with lymph node involvement, tumor recurrence and a poor prognosis [16]. Furthermore, other clinical and histological consequences of the tumor may differ according to the tumor suppressor genes that are silenced by the hypermethylation process [28, 31].

In the 90-day group, the ventral surface showed a higher expression when compared to the control, and although there was no statistical significance with other surfaces, this expression may show a higher tendency for oral cancer development on the ventral surface, as already reported for OSCC in cigarette smokers [5, 32]. This higher frequency of oral cancer on the ventral surface may be associated with its non-keratinized epithelium, which is less protective and more vulnerable to several products originated from the tobacco burning process [33, 34] that are also found in waterpipe smoke, such as nicotine, tar, polycyclic aromatic hydrocarbons, volatile aldehydes, phenols, carbon monoxide and heavy metals [5]. Even though the same compounds are found in tobacco and waterpipes, the latter present a higher concentration of them, with levels corresponding up to 10 cigarettes per waterpipe smoking session [35]. However, the amounts of toxicants are still uncertain owing to variations in time and days of use [36, 37].

Regarding DNMT1, although there was no statistical difference for any group exposed to waterpipe smoke, all groups showed a lower expression on the dorsal surface and the lateral border when compared to control. On the ventral surface, as in DNMT3b, there was low expression from 7 to 60 days and an increase that was similar to control at 90 days. In this study, lower expression of both DNMT1 and DNMT3b may lead to DNA hypomethylation, thus favoring initial carcinogenesis. Furthermore, in all periods of exposure, waterpipe smoke was not able to induce acute or chronic inflammation in the mice’s tongues.

Although this research did not evaluate enzyme activity or DNA methylation pattern, waterpipe smoke was able to decrease DNMT3b and DNMT1 expression in shorts periods of exposure in mice, contributing to a possible hypomethylation status at all tongue sites. As a consequence, it may lead proto-oncogenes activation and/or genome instability. Due to DNMT3b normal regulation or even upregulation at ventral surface after 90 days’ exposure, we cannot discard a possible DNA hypermethylation event caused by longer periods of waterpipe smoke exposure, silencing important tumor suppressor genes. All these DNA methylation pattern modifications by waterpipe smoking may contribute to initial oral carcinogenesis. We reinforce that further studies are needed to improve the knowledge about the effects of waterpipe smoke on the overt epigenetic status.

Conflict of Interest

None Declared.

Role of the Funding Source

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Figures

Figure 1 – Epithelial immunohistochemical expression of DNMT1 in each group of exposure. (400x)

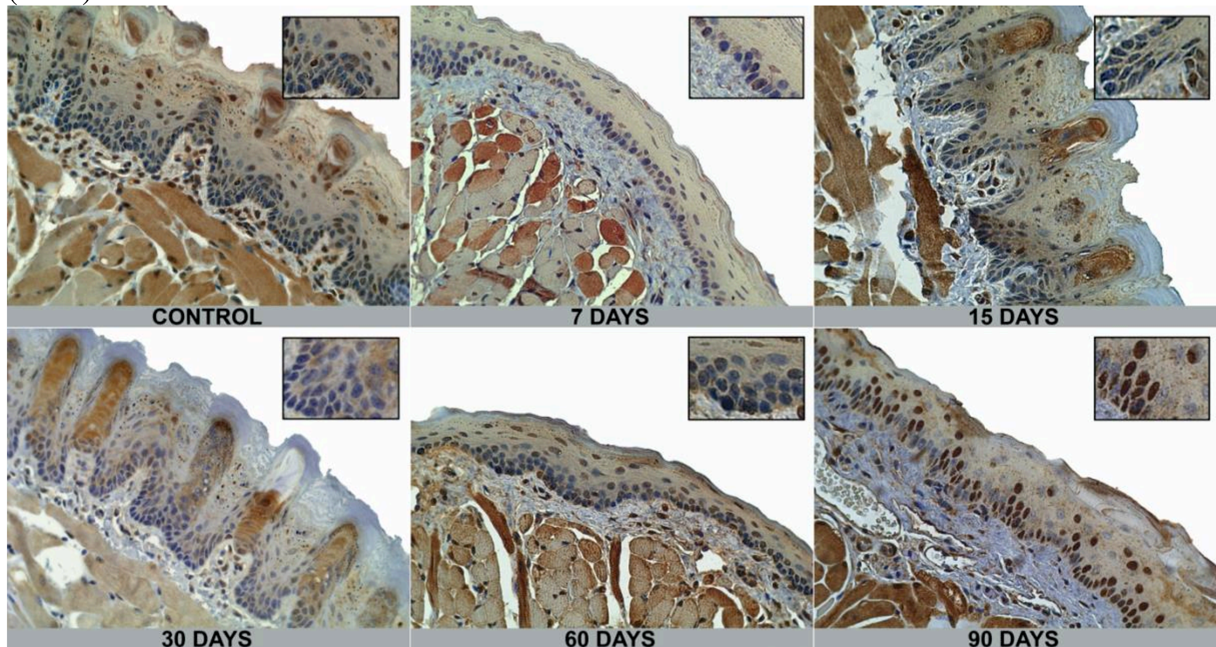


Figure 2 – Epithelial immunohistochemical expression of DNMT3b in each group of exposure. (400x)

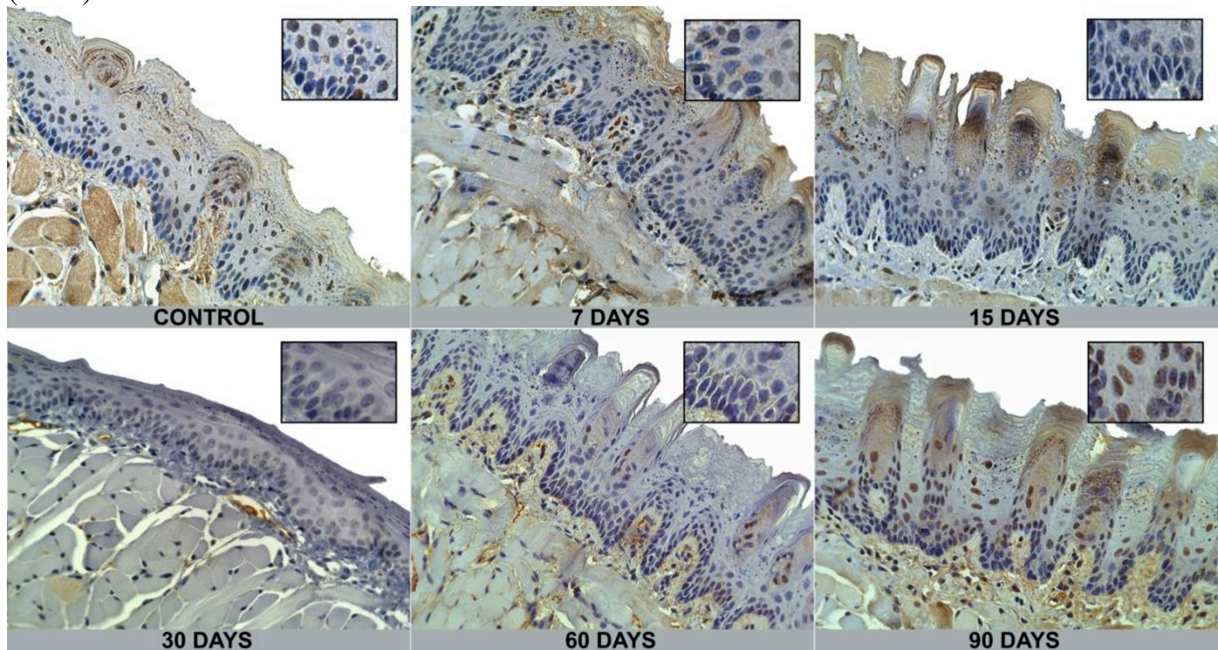


Figure 3 – DNMT1 immunohistochemical expression pattern according to exposure times on each tongue site.

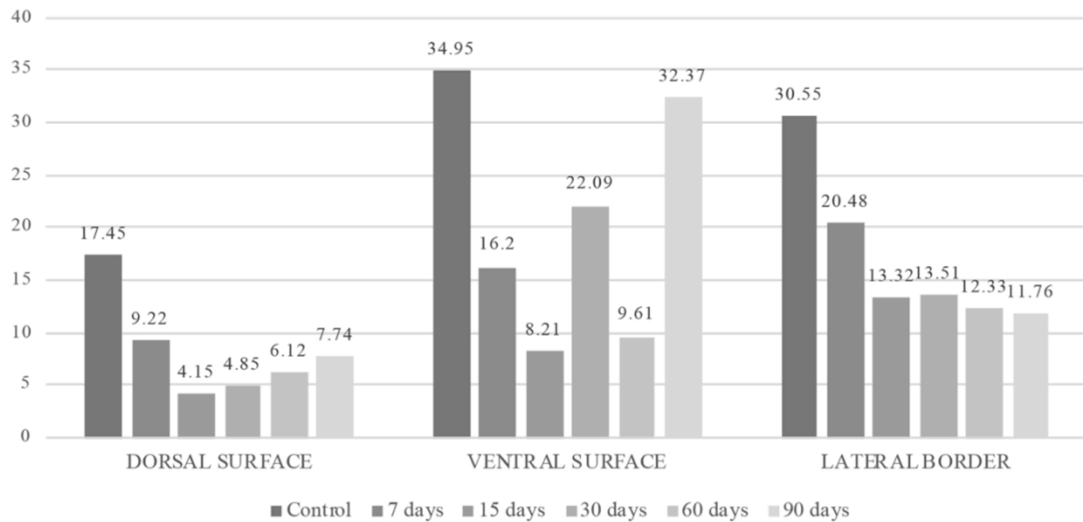
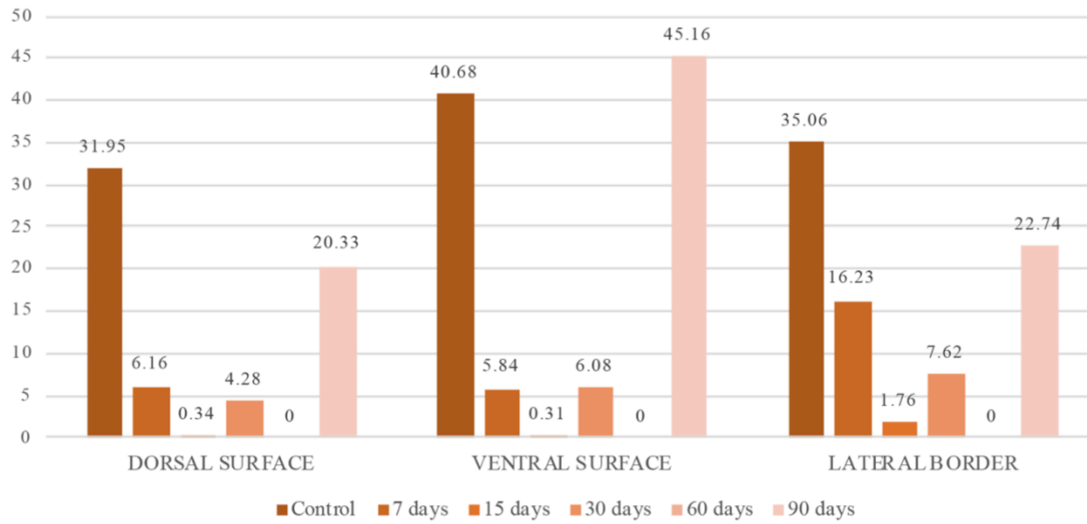


Figure 4 – DNMT3b immunohistochemical expression pattern according to exposure times in each tongue site.



Tables

Table 1 – DNMT1 immunopositivity percentage according to narghile exposure times on tongue sites.

		DORSAL SURFACE	**VENTRAL SURFACE**	**LATERAL BORDER**	**P value**
Exposure Times	Control	17.45 (36.25)	34.95 (25.52)	30.55 (23.25)	0.671
	7 days	9.22 (12.49)	16.20 (23.79)	20.48 (22.37)	0.264
	15 days	4.15 (19.31)	8.21 (20.46)	13.32 (25.31)	0.774
	30 days	4.85 (20.90)	22.09 (42.25)	13.51 (30.15)	0.197
	60 days	6.12 (10.07)	9.61 (37.69)	12.33 (19.04)	0.217
	90 days	7.74 (15.24)	32.37 (41.60)	11.76 (22.83)	0.255
P-value		0.337	0.385	0.169	

Data shown as median (interquartile range).

Table 2 – DNMT3b immunopositivity percentage according to narghile exposure times on tongue sites.

		DORSAL SURFACE	VENTRAL SURFACE	LATERAL BORDER	P value
Exposure Times	Control	31.95 (31.89)^{AB}*	40.68 (31.41)^{AB}	35.03 (28.03)^{AB}*	0.005
	7 days	6.16 (13.57)*#	5.84 (13.47)^{C}*	16.23 (30.53)#	0.004
	15 days	0.34 (4.80)^{AC}*	0.31 (11.53)^{A}	1.76 (5.97)^{AC}*	0.009
	30 days	4.28 (4.03)*	6.08 (5.95)	7.62 (2.90)*	0.024
	60 days	0.00 (4.83)^{B}*	0.00 (7.98)^{BD}	0.00 (7.92)^{BD}*	0.008
	90 days	20.33 (27.96)^{C}*	45.16 (22.68)^{CD}	22.74 (35.12)^{CD}*	0.006
P-value		0.000	0.000	0.000	

Data shown as median (interquartile range).

Same letters indicate statistical differences between rows in the same column.

Same symbols indicate statistical differences between columns in the same row.

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7 CONSIDERAÇÕES FINAIS

O uso do narguilé atualmente adquiriu uma proporção a nível mundial, principalmente entre adolescentes e jovens adultos. Um dos motivos dessa rápida ascensão se dá pela falsa crença popular de que esse método de fumo é menos nocivo quando comparado ao uso do cigarro convencional. No entanto, através da literatura sabe-se que o narguilé é prejudicial à saúde em diversos aspectos, porém, em relação às alterações causadas na cavidade oral dos usuários os estudos ainda são incipientes.

A partir dos dados apresentados, este trabalho analisou a expressão das enzimas DNMT1 e DNMT3b em língua de camundongos expostos à fumaça de narguilé em diferentes períodos de exposição. Visto que a metilação do DNA é uma das alterações epigenéticas relacionadas ao desenvolvimento do CEC mais estudadas atualmente, optou-se por analisar a língua de camundongos por ser uma região anatômica onde a frequência de desenvolvimento do câncer de boca é alta quando comparado a outros locais da cavidade oral. Por se tratar de uma pesquisa com a técnica de imuno-histoquímica e essa não avaliar a capacidade enzimática das DNMTs, pode-se sugerir que novos estudos com metodologias mais específicas sejam realizados, como RT-PCR para avaliação da expressão gênica, Western Blotting para quantificação das enzimas e por fim o método baseado no ensaio ELISA específico para avaliação da atividade das DNMTS (BOTIA *et al.,* 2012), assim obtendo uma melhor e mais completa análise do comportamento enzimático frente à exposição à fumaça de narguilé. A complementação do trabalho com a análise da enzima DNMT3a, que exerce importante função no processo de metilação *de novo*, assim como de manutenção (JEONG *et al.,* 2009), culminaria em um estudo ideal para a avaliação completa das três principais enzimas envolvidas no processo de metilação do DNA. Entretanto, não foi possível a inclusão da enzima DNMT3a neste estudo devido a dificuldade para aquisição do anticorpo.

Frente à impossibilidade da avaliação do estado de metilação do DNA neste trabalho, foram levantadas hipóteses sobre a influência das DNMTs neste processo. Mesmo com esta limitação, ao analisar a expressão das enzimas nos diferentes períodos de tempo foi possível sugerir, com o amparo da literatura disponível, que a reduzida expressão das enzimas em períodos iniciais pode contribuir para a hipometilação do DNA, o que leva à ativação de proto-oncogenes e/ou instabilidade genômica. Em períodos maiores, o retorno da expressão similar ao grupo controle e até mesmo o ultrapassando, como no caso da DNMT3b em ventre lingual

(grupo 90 dias), levanta a hipótese de uma possível subsequente hipermetilação do DNA caso maiores tempos de exposição ocorressem, assim causando a inativação de genes supressores tumorais. Tanto as alterações visualizadas na hipo e na hipermetilação do DNA contribuem para o desenvolvimento da carcinogênese oral.

Pela falta de maiores elucidações dos processos celulares envolvidos, a interpretação dos dados se faz de certa forma limitada. Identificamos que a fumaça do narguilé não apresentou capacidade de induzir um processo inflamatório significante na lâmina própria. Com a presença muito baixa de infiltrado inflamatório, não foi possível estabelecer uma correlação com a expressão imuno-histoquímica das enzimas DNMT1 e DNMT3b identificadas neste trabalho. Além disso, uma análise do epitélio lingual para identificação de alterações displásicas, principalmente em ventre lingual, poderia complementar a hipótese de que a fumaça do narguilé favorece alterações fenotípicas epiteliais no desenvolvimento de lesões potencialmente-cancerizáveis e malignas.

A identificação de alterações epigenéticas no DNA que contribuem para o desenvolvimento do câncer bucal vêm como um modo de incentivar pesquisas futuras utilizando as DNMTs como enzimas importantes a serem analisadas no desenvolvimento inicial da carcinogênese causada pela fumaça de narguilé. Além disso, a comprovação do efeito carcinogênico do narguilé pode destacar a importância de um efetivo controle e regulamentação governamental frente ao uso e venda indiscriminada dos diversos produtos relacionados ao dispositivo.

8 CONCLUSÕES

Com base nos resultados dessa pesquisa verificou-se que:

- A fumaça do narguilé não foi capaz de induzir alterações inflamatórias significantes em nenhum período de exposição, sendo assim, não foi possível realizar uma correlação com a expressão imuno-histoquímica das DNMTs.
- Houve uma redução da imunoexpressão da enzima DNMT3b nos grupos de 7, 15, 30 e 60 dias de exposição à fumaça de narguilé, com elevação no grupo de 90 dias se aproximando dos níveis do grupo controle nas amostras de dorso e margem lateral e até mesmo o ultrapassando nas amostras de ventre lingual.
- Houve uma redução da enzima DNMT1 em dorso e margem lateral de língua em todos os tempos de exposição em comparação ao grupo controle. Em ventre lingual, a redução da imunoexpressão se deu até 60 dias, sendo que no grupo de 90 dias houve uma aproximação da expressão a níveis semelhantes ao grupo controle.
- Essas alterações podem sugerir um estado de hipometilação do DNA logo nos períodos iniciais de exposição à fumaça, favorecendo a ativação de proto-oncogenes e/ou instabilidade genômica. Em períodos prolongados (a partir de 90 dias), a DNMT3b tende à uma normalização e possível hipermetilação, como observado em ventre lingual, favorecendo a inativação de proto-oncogenes. Ambas quando ocorrem favorecem o desenvolvimento inicial da carcinogênese oral.

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ANEXO A – PARECER COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA/UNIVALI

PARECER COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA/UNIVALI

Protocolo: 063/17	Data: 01/12/2017
Titulo: Alterações em boca, traqueia e pulmão de camundongos expostos a fumaça do narguilé	
Coordenador do Projeto: Sarah Freygang Mendes Pilati	
Executores: Mayara de Arruda Tomaz, Morgana de Souza, Fernando Galli, Laura Sagaz	
Colaboradores: David R. Tames, Maria de Lourdes Correa, Claudia Fernanda da Silva, Filipe Modolo Siqueira,	
Objetivos Objetivos Geral: Determinar os efeitos e as alterações celulares na cavidade oral, traqueia e pulmão de roedores, decorrentes do emprego da fumaça produzida pelo narguilé. Objetivos Especificos: Avaliar as caracteristicas clinicas e histológicas de roedores submetidos à fumaça do narguilé durante um periodo de 7,15,30,60 e 90 dias, identificando, após o periodo de exposição, alterações presentes na cavidade oral, traqueia e pulmão, bem como, reunir histopatológicos para estabelecer uma comparação entre os tecidos sadios e expostos à fumaça. Também realizar análise de presença de proteinas associadas a inflamação, proliferação celular e carcinogênese através de imunohistoquimica.	

I. RESUMO:

II. ANÁLISE:

- Data de inicio e término: 03/2018 – 06/2018, adequadas;
- Qualificação da equipe e treinamento: Pesquisador responsável e colaboradores possuem experiência nos procedimentos a serem aplicados, executores necessitam de treinamento (Previamente ao inicio do projeto o aluno será treinado pela orientadora e co-orientador);
- Justificativa do projeto: adequada;
- Detalhamento dos procedimentos experimentais: **descritos, porém solicita-se maior detalhamento sobre volume e fluxo de "fumaça" a que os animais serão expostos (vide item III. PARECER)**
- Condições de manutenção: adequados;
- Número de animais solicitados e planejamento estatistico: adequados;
- Espécie, linhagem e sexo: **Swiss, fêmeas, 25g.**

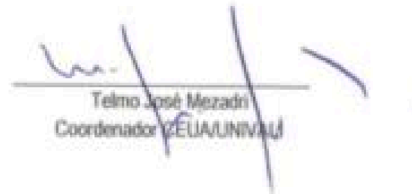
III. PARECER: APROVADO

- Listar pendências ou critérios de aprovação ou reprovação.

"A duração da sessão será de 30 minutos/dia durante 7,15,30,60 e 90 dias dias. O volume de fumaça de "tabaco" a que os animais dos grupos testes serão expostos é de 35 ml por **dois segundos** enquanto os outros 58 segundos serão de ar puro (NEMMAR et al., 2015; HOFFMEISTER et al, 2017). A taxa de fluxo da bomba será ajustada manualmente para manter o volume de **530mL/2seg** (sopro especificado pelo método Beirute), este regime foi escolhido por que ele se aproxima, em média, da topografia do sopro humano durante o uso do aparelho de narguilé (KHABOUR et al., 2012)."

IV. REANÁLISE:

V. PARECER FINAL: APROVADO



Telmo José Mezadri
Coordenador CEUA/UNIVALI

ANEXO B – NORMAS DA REVISTA *CRITICAL REVIEWS IN ONCOLOGY/HEMATOLOGY*

Preparation of the Manuscript - Specific:

Sections of the Article
The first pages of the manuscript should contain: (1) title; (2) the name(s) and complete affiliation(s) of the author(s); (3) table of contents; (4) abstract; (5) keywords; and (6) the name and full contact details of the corresponding author.

Title
Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

Corresponding Author
Clearly indicate who is willing to handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.

Abstract
A concise and factual abstract is required (maximum length 150 words). The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separate from the article, so it must be able to stand alone.

Keywords
Immediately after the abstract, provide a maximum of 8 keywords, avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Subdivision of the article
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ?), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to "the text". Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Abbreviations
Define abbreviations that are not standard in this field at their first occurrence in the article, in the abstract but also in the main text after it. Ensure consistency of abbreviations throughout the article.

Acknowledgements
All contributors who do not meet the criteria for authorship as defined above should be listed in an acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Authors should disclose whether they had any writing assistance and identify the entity that paid for this assistance.

Conflict of interest
At the end of the text, under a subheading "Conflict of interest statement" all authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/ registrations, and grants or other funding. If the author(s) has no conflict of interest this should be stated.

Role of the funding source
All sources of funding should be declared as an acknowledgement at the end of the text. Authors should declare the role of study sponsors, if any, in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. If the study sponsors had no such involvement, the authors should so state.

Vitae
Include in the title page a short biography of each author.

Tables
Number tables consecutively in Arabic in accordance with their appearance in the text. All tables must have a title, and may be accompanied by a brief description of the data contained within the table. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Standard abbreviations of units of measurements should be added between parentheses. Numerical data should be aligned using decimal points; in numbers less than one, a zero should precede the decimal point. Ditto marks must not be used. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References
Responsibility for the accuracy of bibliographic citations lies entirely with the Authors.

Citations in the text: Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the

text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either "Unpublished results" or "Personal communication" Citation of a reference as "in press" implies that the item has been accepted for publication.

Citing and listing of Web references. As a minimum, the full URL should be given. Any further information, if known (Author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Text: Indicate references by number(s) in square brackets in line with the text. The actual Authors can be referred to, but the reference number(s) must always be given.

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:
[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2000;163:51-9.

Reference to a book:
[2] Strunk Jr W, White EB. The elements of style. 3rd ed. New York: Macmillan; 1979.

Reference to a chapter in an edited book:
3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 1999, p. 281-304

Note shortened form for last page number. e.g., 51-9, and that for more than 6 Authors the first 6 should be listed followed by "et al." For further details you are referred to "Uniform Requirements for Manuscripts submitted to Biomedical Journals" (J Am Med Assoc 1997;277:927-934) (see also http://www.nlm.nih.gov/tsd/serials/terms_cond.html). Journal names should be abbreviated according to Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/ serials/lji.html. Recheck references in the text against the reference list after your manuscript has been revised. Incomplete references can result in publication delay.

The digital object identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. The correct format for citing a DOI is shown as follows (example taken from a document in the journal Physics Letters B):https://doi.org/10.1016/j.physletb.2003.10.071. When you use the DOI to create URL hyperlinks to documents on the web, they are guaranteed never to change.

Equations
Equations should be typed at the appropriate position in the text, and should be numbered consecutively using Arabic numbers. Symbols, e.g., Greek letters, should be clearly identified in cases where confusion could arise. Please check that the spacing before and after each symbol is correct and that superscript or subscript symbols are clearly evident.

Equipment and Drugs
When quoting specific equipment or drugs, authors must state in parentheses the name and address of the manufacturer. Generic names should be used wherever possible.

Units and Abbreviations

Measurements of length, height, weight and volume should be given in metric units (metre, kilogram, litre) or their decimal multiples in terms of the International System of Units http://www.bipm.fr/en/si/. Temperatures should be given in degrees Celsius and blood pressure in mmHg. Define abbreviations that are not standard in the field at their

first occurrence in the article, in the abstract but also in the main text after it. Ensure consistency of abbreviations throughout the text.

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower- case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Twitter handle

We can now include your Twitter handle on your published paper. If you would like your Twitter handle to be included, please provide it on your Title page.

Highlights

Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork

Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files.

A detailed guide on electronic artwork is available.
You are urged to visit this site; some excerpts from the detailed information are given here. *Formats*
Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the

resolution is too low. • Supply files that are too low in resolution.
• Submit graphics that are disproportionately large for the content.

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Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

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This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

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http://open.mendeley.com/use-citation-style/critical-reviews-in-oncology-hematology When preparing your manuscript, you will then be able to select this style using the Mendeley plug- ins for Microsoft Word or LibreOffice.

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There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style
Reference style
Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication. Citations may be made directly (or parenthetically). Groups of references can be listed either first alphabetically, then chronologically, or vice versa.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999).... Or, as demonstrated (Jones, 1999; Allan, 2000)... Kramer et al. (2010) have recently shown ...'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.
Examples:
Reference to a journal publication:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
Reference to a chapter in an edited book:
Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304. Reference to a website:
Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).
Reference to a dataset:
[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T., 2015. Mortality data for Japanese oak wilt disease and surrounding forest compositions. Mendeley Data, v1. https://doi.org/10.17632/ xwj98nb39r.1.

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Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

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Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.

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AFTER ACCEPTANCE

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ANEXO C – NORMAS DA REVISTA *ORAL ONCOLOGY*

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower- case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

• ***Word Count.***Please include a word count on your Title Page. Your word count should exclude the abstract, keywords, references, tables and figures.

Highlights

Highlights are optional yet highly encouraged for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

A concise and factual abstract of no more than 250 words is required. The abstract must be

structured for original research articles and articles reporting the results of clinical trials.

The abstract should be divided by subheadings as follows: Objectives, Materials and Methods, Results and Conclusion.

The abstract **should not be structured for review articles.** The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separate from the article, so it must be able to stand alone.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.

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Highlights

Highlights are mandatory for Original Research Articles, Review Articles, and Perspectives. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

See: https://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract provide a maximum of ten keywords, to be chosen from the Medical Subject Headings from Index Medicus. These keywords will be used for indexing purposes. It is usually necessary to include keywords such as Oral Cancer, or Head and Neck cancer.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Artwork

Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
• Ensure that color images are accessible to all, including those with impaired color vision.

A detailed guide on electronic artwork is available.
You are urged to visit this site; some excerpts from the detailed information are given here. *Formats*
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi. TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.
Please do not:
• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
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