Anaíra Ribeiro Guedes Fonseca Costa

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Absence of inducible nitric oxide synthase in 4-nitroquinoline-N-oxide murine model of oral carcinogenesis: alterations of histopathological, systemic inflammatory, and epigenetic markers

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Orientador: Prof. Dr. Adriano Mota Loyola
Co-orientador: Prof. Dr. Paulo Rogério de Faria

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Reuniu-se em Web Conferência pela plataforma Zoom, em conformidade com a PORTARIA Nº 36, DE 19 DE MARÇO DE 2020 da COORDENAÇÃO DE APERFEIÇOAMENTO DE PESSOAL DE NÍVEL SUPERIOR - CAPES, pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Odontologia, assim composta: Professores Doutores: Ana Cristina Araújo Lemos da Silva (UFU); Maria Luiza Gonçalves dos Reis Monteiro (UFU); Carla Silva Siqueira Miranda (UFJ); Eneida Franco Vêncio (UFG); Adriano Mota Loyola (UFU) orientador da candidata.

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Aos meus pais, Edilamar Ribeiro Guedes e Juarez Fonseca Costa.

„Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis macht, daß ein Ding kein Gift sei.“

Philippus Aureolus Theophrastus Bombastus von Hohenheim

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

•NO - óxido nítrico

4NQO - N-óxido de 4-nitroquinolina

8-nitro-dG - 8-nitro-2'desoxiguanosina

8-oxo-dG - 8-oxo-2'desoxiguanosina

A - adenina

AP-1 - proteína ativadora 1

atm - atmosfera de pressão

BH4 - tetraidrobiopterina

C - citosina

CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CCEB - carcinoma de células escamosas bucal

CEUA-UFU - Comissão de Ética na Utilização de Animais da Universidade Federal de Uberlândia

cGMP - monofosfato cíclico de guanosina

c-Jun - fator de transcrição Jun

CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico

COX2 - enzima ciclo-oxigenase 2

c-Src - proteína tirosina quinase sarcoma celular

Cys - cisteína

DMBA - 7,12 dimetilbenzantraceno (DMBA)

DNA - ácido desoxirribonucleico

DOI - *depth of invasion,* profundidade de invasão

ED - *epithelial dysplasia*, displasia epitelial

EMT - transição epitélio-mesênquima

eNOS - óxido nítrico sintase endotelial

ERN - espécie reativa de nitrogênio

FAD - dinucleotídeo de adenina e flavina

FAPEMIG - Fundação de Amparo à Pesquisa de Minas Gerais

FMN - mononucleotídeo de flavina

G - guanina

$g.mol^{-1}$ - massa molar equivalente a g/mol

GAPDH - gliceraldeído-3-fosfato desidrogenase

gNO - *gaseou nitric oxide*, óxido nítrico gasoso

GTP - guanina trifosfato

H3 - histona H3

H3K27 - lisina 27 da histona H3

H3K27ac - acetilação da lisina 27 da histona H3

H3K27me3 - trimetilação de lisina 27 da histona H3

H3K36 - lisina 36 da histona H3

H3K9 - lisina 9 da histona H3

H3K9ac - acetilação da lisina 9 da histona H3

H3K9me2 - dimetilação da lisina 9 da histona H3

H3K9me3 - trimetilação da lisina 9 da histona H3

H4K12ac - acetilação da lisina 12 da histona H4

H4K20 - lisina 20 da histona H4

HAT - histona acetiltransferase

Hct - *hematocrit,* hematócrito

HDAC - histona desacetilases

Hgb - *hemoglobin*, hemoglobina

HIF-1 - fator induzido por hipóxia-1

ICB-USP - Instituto de Ciências Biomédicas da Universidade de São Paulo

IFN-γ - interferon-gama

IL-1β - interleucina 1-beta

IL-10 - interleucina-10

iNOS - óxido nítrico sintase induzível

JAK - Janus quinase

JNK - c-Jun N-terminal quinase

KDM - *lysine demethylase*, enzima demetilase de lisina

L-NAME - L-NG-Nitro arginine metil ester

LPS - lipopolissacarídeos

MCHC - *mean corpuscular hemoglobin concentration,* concentração de hemoglobina corpuscular média

MCV - *mean corpuscular volume*, volume corpuscular médio

MD - *moderate dysplasia*, displasia moderada

MDLC - *manual differential lymphocyte count*, contagem manual de linfócitos

MDNC - *manual differential neutrophil count,* contagem manual de neutrófilos

mg/mL - miligrama por mililitro

MHC - *mean hemoglobin concentration,* concentração média de hemoglobina

ML - *mild dyplasia*, displasia leve

mM/L - milimolar por litro

MMP - metaloproteinase de matriz

M-NO - complexo metal nitrosil

MPT - modificações pós-traducionais de proteínas

MPTH - modificações pós-traducionais de histonas

mRNA - RNA mensageiro

N_2O_3 - anidrido nitroso

NADPH - fosfato de dinucleotídeo de adenina e nicotinamida

NF-ƙB - fator nuclear kappa-B

NHA - N-hidroxi-L-arginina

NLR - *neutrophil-to-lymphocyte ratio*, razão neutrófilo-linfócito

NM - *normal mucosa*, mucosa normal

nNOS - óxido nítrico sintase neuronal

NO^+ - nitrosônio

NO_2 - dióxido de nitrogênio

NO_2^- - nitrito

NOS1 - gene da óxido nítrico sintase neuronal

NOS2 - gene da óxido nítrico sintase induzível

$Nos2^{-/-}$ - animais geneticamente modificados que não expressam o gene da iNOS

$Nos2^{+/+}$ - animais selvagens que expressam o gene da iNOS

NOS3 - gene da óxido nítrico sintase endotelial

NPM1 - nucleofosfomina-1

O_2^- - íon superóxido

ºC – graus celsius

OH^- - hidroxila

$ONOO^-$ - peroxinitrito

ONOOH - ácido peroxinitroso

OPMD - *oral potentially malignant disorders*, desordens orais potencialmente malignas

OSCC - *oral squamous cell carcinoma*, carcinoma de células escamosas bucal

PCC *platelet cell count,* contagem de plaquetas

PI3K - fosfatidilinositol 3-quinase

PKG - proteína quinase G

PLR - *platelet-to-lymphocyte ratio*, razão plaqueta-linfócitos

PPG - *propylene glycol*, propilenoglicol

PTM - *post-translational modifications*, modificações pós-traducionais

PTHM – *post-translational histone modifications*, modificações pós-traducionais de histonas

QS - quickscore

RBCC - *red blood cell count*, contagem de células vermelhas

RDW - *red blood cell width*

REBIR-UFU - Rede de Biotérios de Roedores da Universidade Federal de Uberlândia

RNS – *reactive nitrogen species,* espécies reativas de nitrogênio

SCC - *squamous cell carcinoma*, carcinoma de células escamosas

SD - severe dysplasia, displasia intensa

sGC - guanilato ciclase solúvel

SII - *systemic immune-inflammation index*, índice imunidade-inflamação sistêmico

SNO - nitrosilação

SP - *squamous papilloma*, papiloma escamoso

STAT - proteína transdutora de sinal e ativadora de transcrição

T - timina

TGF-β - fator de crescimento tumoral-beta

TNF-α - fator de necrose tumoral-alfa

Tyr - tirosina

VE-caderina - caderina vascular endotelial

VEGF - fator de crescimento vascular endotelial

WBCC - *white blood cell count,* contagem de leucócitos

WHO - *World Health Organization*, Organização Mundial da Saúde

μM – micromolar

RESUMO

O óxido nítrico atua em vários processos fisiológicos e patológicos, inclusive no câncer. Durante a carcinogênese, exerce um papel dicotômico, atuando como agente ora pró, ora antitumoral. Seus efeitos no desenvolvimento do câncer de boca, entretanto, permanecem pouco explorados. O objetivo deste trabalho foi analisar a influência da óxido nítrico sintase induzível (iNOS) sobre biomarcadores histopatológicos, inflamatórios sistêmicos e epigenéticos de camundongos selvagens ($Nos2^{+/+}$) e desprovidos de iNOS ($Nos2^{-/-}$) tratados por 16 semanas com N-óxido de 4-nitroquinolina (4NQO) a 50µg/mL. Após 8 semanas de observação, as línguas foram avaliadas quanto à presença de hipotrofia, hiperplasia, papiloma escamoso, displasia epitelial e carcinoma de células escamosas, sendo mensurada a profundidade de invasão (DOI) neste último. Parâmetros hematológicos e marcadores inflamatórios sistêmicos foram analisados em amostras de sangue periférico. Imunomarcação para H3K9ac e H3K27ac foi analisada nas lesões epiteliais através do método de *quickscore* (QS). A incidência das lesões foi similar entre as linhagens, porém a DOI foi menor no grupo $Nos2^{-/-}$ (p=0,018). Correlações foram identificadas entre DOI e contagem de neutrófilos (ρ=-0,68, p=0,017), linfócitos (ρ=0,72, p=0,011), razão neutrófilo-linfócito (ρ=-0,65, p=0,025), razão plaqueta-linfócito (ρ=-0,73, p=0,013) e índice imunidade-inflamação sistêmico (ρ=-0,67, p=0,037) em animais $Nos2^{-/-}$. Para H3K9ac, o QS foi maior na displasia moderada do grupo $Nos2^{-/-}$ (p=0,025) quando comparado ao $Nos2^{+/+}$. A displasia leve apresentou menor QS para H3K9ac quando comparada às displasias moderada e intensa no grupo $Nos2^{-/-}$ (p=0,015). A expressão de H3K27ac aumentou da mucosa normal para a displasia leve em camundongos $Nos2^{+/+}$ (p=0,007), sendo observado maior número de displasias positivas para H3K27ac nesses animais (p=0,023). Estes resultados sugerem que a iNOS exerce um papel importante no processo de progressão do carcinoma de células escamosas bucal, que pode estar associado a alterações na dinâmica das células da resposta imunológica e inflamatória evidenciadas por marcadores de sangue periférico. Além disso, alterações epigenéticas nos estágios iniciais da carcinogênese, mais especificamente do padrão de acetilação de histonas na displasia epitelial, podem ser mediadas por esta enzima.

Palavras-chave: carcinogênese, óxido nítrico, câncer bucal, invasão tumoral, histonas

ABSTRACT

Nitric oxide is a signaling molecule involved in several physiological and pathological conditions, including cancer. During carcinogenesis, it plays a dichotomous role, acting both as a tumorigenic and antitumor agent. Its effects in oral cancer development are, however, not sufficiently explored. This study purposed to analyze the influence of inducible nitric oxide synthase (iNOS) on histopathological, systemic inflammatory, and epigenetic biomarkers in $Nos2^{+/+}$ (wild-type) and $Nos2^{-/-}$ (knockout) mice treated for 16 weeks with a 50ug/mL solution of 4-nitroquinoline N-oxide (4NQO). After an 8-week observation period, tongues were collected and evaluated for the presence of epithelial hypotrophy, hyperplasia, squamous papilloma, epithelial dysplasia and oral squamous cell carcinoma, in which the depth of invasion (DOI) was measured. Red blood cell, white blood cell, and platelet cell parameters were analyzed in blood samples. Immunostaining for H3K9ac and H3K27ac expression was evaluated with the quickscore method (QS). $Nos2^{+/+}$ and $Nos2^{-/-}$ mice had similar incidence of tongue lesions, but depth of invasion was lower in $Nos2^{-/-}$ (p=0.018). Strong correlations were found between DOI and neutrophil count (ρ=-0.68, p=0.017), lymphocyte count (ρ=0.72, p=0.011), neutrophil-to-lymphocyte ratio (ρ=-0.65, p=0.025), platelet-to-lymphocyte ratio (ρ=-0.73, p=0.013), and systemic immune-inflammation index (ρ=-0.67, p=0.037) in $Nos2^{-/-}$ mice. For H3K9ac, QS values were higher in moderate dysplasia of $Nos2^{-/-}$ (p=0.025) when compared to $Nos2^{+/+}$. Mild dysplasias had lower values of H3K9ac when compared to moderate and severe in $Nos2^{-/-}$ group (p=0.015). H3K27ac increased from normal mucosa to mild dysplasia in $Nos2^{+/+}$ mice (p=0.007). $Nos2^{+/+}$ mice also had a higher number of H3K27ac-positive dysplasias when compared to $Nos2^{-/-}$ (p=0.023). These results suggest that iNOS has an important role in OSCC invasion and progression, which might be associated to alterations in immune-inflammatory cell dynamics evidenced by peripheral blood biomarkers. Additionally, epigenetic alteration in early stages of oral carcinogenesis, mainly changes in histone acetylation pattern of epithelial dysplasia, might be iNOS-mediated.

Keywords: carcinogenesis, nitric oxide, oral cancer, neoplasm invasiveness, histones

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

1.1. O carcinoma de células escamosas bucal

O carcinoma de células escamosas bucal (CCEB) é uma neoplasia maligna que se origina do epitélio de revestimento da mucosa oral e possui etiologia multifatorial, tendo como principais fatores de risco o tabagismo e o etilismo crônicos (Irimie *et al*., 2018). Representa 90% de todas as malignidades que podem acometer a boca e é o sexto tumor mais frequente no mundo, sendo responsável por uma incidência de 560.000 novos casos e aproximadamente 300.000 mortes anuais (Van Monsjou *et al*., 2013; Irimie *et al*., 2018).

No Brasil, o CCEB foi o quinto tumor mais comum no sexo masculino e o décimo terceiro no sexo feminino na Estimativa 2020 do Instituto Nacional do Câncer (INCA), no qual foi registrada uma previsão para o triênio 2020-2022 de 11.200 novos casos em homens e 4.010 em mulheres (INCA, 2020). Para cada ano do triênio 2023-2025, está prevista a incidência de 15.200 novos casos de câncer de boca, ocupando assim o quarto lugar no *ranking* das neoplasias malignas afetando homens na região sudeste do Brasil (INCA, 2022). Este tipo de câncer apresenta, em geral, elevada taxa de morbidade e mortalidade, sendo que 70% dos pacientes diagnosticados no país já se encontra em estágio avançado (Nóbrega *et al*., 2018) e menos de 60% sobrevive por mais de cinco anos (Amit *et al*., 2013).

A história natural do CCEB se inicia com a exposição das células epiteliais a vários estímulos que causam um desequilíbrio na homeostase tecidual, promovendo alterações genéticas e epigenéticas responsáveis pela modulação do ciclo celular, reparo de DNA, diferenciação celular e apoptose, as quais levam à transformação neoplásica progressiva dos queratinócitos (Rivera, 2015). Ao longo deste processo, o surgimento do CCEB pode ser precedido por alterações pré-neoplásicas da mucosa, caracterizadas pela presença de hipotrofia, hiperplasia e/ou displasia epitelial em diferentes graus de severidade, alterações que podem ser percebidas clinicamente na forma de desordens orais potencialmente malignas (DOPM) (Rivera, 2015; Woo, 2019).

As DOPM são definidas como qualquer anormalidade da mucosa oral associada a um risco elevado para o surgimento do câncer de boca (Warnakulasuriya *et al*., 2020), embora tal risco varie de acordo com uma variedade de fatores relacionados ao paciente

e à lesão propriamente dita, sendo difícil sua predição (Speight, Khurram & Kujan, 2018). As principais DOPM listadas pela Organização Mundial de Saúde são a eritroplasia, eritroleucoplasia, leucoplasia bucal, fibrose submucosa oral, disqueratose congênita, entre outras (Reibel *et al*., 2017). A prevenção e o diagnóstico precoce do CCEB envolvem, portanto, a detecção e o tratamento adequado das DOPM, bem como a educação em saúde quanto aos riscos do uso crônico de produtos do tabaco e álcool (Abati *et al*., 2020).

A cirurgia permanece sendo a principal modalidade de tratamento do CCEB, podendo incluir radioterapia adjuvante (Belcher *et al*., 2014). Há poucos estudos investigando possíveis agentes quimioterápicos para a eliminação de tumores primários, enquanto a proposta terapêutica básica permanece caracteristicamente mutiladora (Cohen *et al*., 2008). Neste contexto, a identificação de novos alvos moleculares pode auxiliar na problemática da prevenção, diagnóstico, prognóstico e tratamento desta doença, de modo a reduzir a morbidade e mortalidade dos pacientes. Além disso, pesquisas recentes têm almejado a quimioprevenção aplicada às DOPM que precedem o CCEB, como a leucoplasia bucal, eritroplasia e eritroleucoplasia (Foy *et al*., 2013).

Para tanto, o estudo dos mecanismos moleculares envolvidos nas várias etapas do desenvolvimento do câncer se faz necessário, de modo a eleger uma ou mais moléculas-alvo com potencial para aplicação diagnóstica, prognóstica e terapêutica, tendo em vista que, apesar do avanço crescente na área de oncobiologia molecular, biomarcadores visando o diagnóstico e tratamento do CCEB continuam sendo pouco explorados (Sasahira & Kirita, 2018).

1.2. O processo de carcinogênese

O desenvolvimento do câncer, fenômeno denominado de carcinogênese, é um processo que se desenvolve em múltiplas etapas, as quais refletem a presença de alterações nos mecanismos regulatórios da homeostase que direcionam a transformação progressiva das células normais em células malignas (Hanahan & Weinberg, 2000). Tais alterações resultam de sucessivas modificações no genoma e epigenoma, conferindo à célula o fenótipo neoplásico e favorecendo a seleção e expansão de clones que apresentam as características mais vantajosas de crescimento e longevidade (Chaffer & Weinberg, 2015).

Determinadas capacidades funcionais, responsáveis pela sobressalência dos clones neoplásicos em detrimento das células vizinhas, são compartilhadas por todos os tipos tumorais, ainda que adquiridas em momentos diferentes e com variável impacto no seu desenvolvimento e progressão: autossuficiência de sinais de crescimento, insensibilidade ao sinais inibitórios de crescimento, evasão à apoptose, angiogênese sustentada, potencial de replicação ilimitado, invasão tecidual e metástase, metabolismo celular desregulado e evasão à destruição imunológica, cuja aquisição é possibilitada pela instabilidade genômica e inflamação pró-tumoral (Hanahan & Weinberg, 2011).

Cada uma destas características, denominadas *hallmarks* do câncer, é regulada por uma ou mais vias de sinalização intracelular, ao que se atribui a ocorrência de recidivas e a resistência de determinadas neoplasias aos quimioterápicos (Hanahan & Weinberg, 2011). Consequentemente, a identificação de moléculas-chave que participem das vias de sinalização envolvidas nas etapas da tumorigênese constitui um importante objetivo de estudo, visando elucidar novos mecanismos cruzados e propor métodos terapêuticos que atuem de maneira eficaz sobre um ou mais *hallmarks*.

Uma molécula candidata a este tipo de estudo é o monóxido de nitrogênio, mais comumente mencionado na literatura como óxido nítrico (•NO). Desde o reconhecimento de sua função como fator de relaxamento derivado do endotélio (Ignarro *et al*., 1987; Palmer *et al*., 1987), o •NO tem sido identificado nos mais variados processos biológicos, atuando como vasodilatador, inibidor da agregação plaquetária, modulador da resposta inflamatória e imunológica, neurotransmissor, analgésico, entre outros (Moncada & Higgs, 1991). Suas propriedades físico-químicas o qualificam como um segundo mensageiro peculiar, em se tratando de uma pequena molécula de gás inorgânico volátil com toxicidade dose-dependente e elevada reatividade química (Koshland, 1992).

1.3. Propriedades físico-químicas e biossíntese do óxido nítrico

O •NO é um composto químico inorgânico na forma de molécula heteronuclear diatômica linear que contém um átomo de oxigênio e outro de nitrogênio, unidos por ligação covalente dupla. A presença de um elétron não pareado na nuvem eletrônica do nitrogênio lhe confere a classificação de radical livre (Lancaster, 2015). Em temperatura ambiente, o •NO é um gás incolor, inodoro e insípido, possuindo uma massa molar de 30,01 $g.mol^{-1}$, meia-vida biológica de dois segundos e aproximadamente 2 mM/L de

solubilidade em água sob 1 atm de pressão atmosférica (Ignarro, 2000). Sua natureza lipofílica, no entanto, proporciona uma solubilidade até oito vezes maior em solventes apolares, ao que se atribui seu elevado potencial de difusão entre células sem o intermédio de receptores proteicos de membrana (Koshland, 1992).

A síntese de •NO depende de três enzimas de uma mesma família, denominadas óxido nítrico sintases (NOS): neuronal (nNOS), induzível (iNOS) e endotelial (eNOS) (Michel & Feron, 1997). A nNOS foi a primeira isoforma purificada e clonada do tecido neural, cujo gene NOS1 se localiza no cromossomo 12q24.2-24.316. A eNOS, por sua vez, é codificada pelo gene NOS3 localizado no cromossomo 7q35-36 e produzida em endoteliócitos (Kishimoto *et al.*, 1992; Marsden *et al*., 1993; Robinson *et al.,* 1994). Ambas são classificadas como enzimas constitutivas em função de sua atividade contínua e dependente de cálcio e calmodulina, sintetizando concentrações baixas e transitórias de •NO (Michel & Feron,1997). Em contrapartida, a iNOS é uma isoforma induzível e independente de cálcio, codificada pelo gene NOS2 localizado no cromossomo 17q35-17q1122 (Nathan & Xie, 1994). A síntese de •NO catalisada pela iNOS é contínua e caracterizada por concentrações elevadas que podem durar horas ou dias; (Nathan, 1997).

A iNOS está presente principalmente em fagócitos, como macrófagos e neutrófilos, e constitui um importante componente da resposta imunológica do hospedeiro para a eliminação de microrganismos (Nathan, 1997). O •NO, nestas células, reage com íon superóxido (O_2^-) para formar peroxinitrito ($ONOO^-$), responsável por danificar lipídios, proteínas e ácidos nucleicos de microrganismos no interior dos fagolisossomos (Nathan & Xie, 1994). Tal efeito ocorre mediante estímulo por IFN-γ, IL-1β, TNF-α, LPS e depende da ativação de fatores de transcrição, como NF-ƙb, JAK/STAT e JNK, enquanto sua inibição é induzida por esteroides, TGF-β, IL-10, p53 e pelo próprio •NO (Nathan & Xie, 1994; Nathan, 1997; Alderton *et al.,* 2001).

Todas as NOS catalisam a síntese de •NO a partir da L-arginina e do oxigênio molecular com o auxílio de cofatores, tais como o fosfato de dinucleotídeo de adenina e nicotinamida (NADPH), dinucleotídeo de adenina e flavina (FAD), mononucleotídeo de flavina (FMN), tetraidrobiopterina (BH4) e protoporfirina férrica (Marletta, 1994; Alderton *et al*., 2001). De modo geral, todas as isoformas são hemoproteínas sintetizadas inicialmente como monômeros inativos que possuem dois domínios catalíticos: um domínio redutase C-terminal, no qual ocorre a ligação com NADPH, FAD e FMN; e um

domínio oxigenase N-terminal, com o qual interagem BH4, oxigênio e L-arginina (Marletta, 1994; Alderton *et* al., 2001). A formação dos dímeros funcionalmente ativos depende da ligação dos monômeros a duas moléculas de calmodulina após elevação da concentração de cálcio intracelular (com exceção da iNOS), constituindo, ao final, uma estrutura tetramérica (Marletta, 1994; Alderton *et* al., 2001).

A síntese de •NO endógeno ocorre em duas etapas. Na primeira, dois elétrons são doados pelo NADPH para os carreadores FAD e FMN no domínio redutase de um monômero (Marletta, 1994; Alderton *et* al., 2001). Na segunda etapa, os elétrons são direcionados pelas flavinas para o grupo heme ligado à pterina no domínio oxigenase do segundo monômero. O grupo heme catalisa a oxidação dos substratos em dois ciclos, um envolvendo L-arginina para formar NHA (monoxigenase I) e outro para converter NHA em citrulina e •NO (monoxigenase II) (Marletta, 1994; Alderton *et al*., 2001).

As funções biológicas do •NO variam segundo a isoforma da enzima que o sintetizou e a localização da mesma em diferentes tecidos e compartimentos celulares (Alderton *et al.*, 2001). Isto se relaciona a sua interação com alvos subcelulares específicos em determinadas organelas (Oess *et al*., 2006). Uma vez sintetizado, o •NO busca o pareamento de seu elétron de valência para formação de um orbital ligante reagindo com dois tipos de moléculas: metais de transição e outros radicais livres (Lancaster, 2015). A partir disso, os efeitos do •NO nos sistemas biológicos podem ser categorizados como diretos, quando mediados pela interação do •NO propriamente dito com diferentes moléculas, ou indiretos, quando mediados por produtos da reação do •NO com radicais livres (Oess *et al*., 2006). No contexto da carcinogênese, ambos os efeitos são relevantes e contribuem diferencialmente para as propriedades pró e antitumorais do •NO.

1.4. Reações diretas e indiretas do óxido nítrico

Em pequenas concentrações (<1μM), o •NO reage diretamente com metais de transição presentes em proteínas, notadamente aquelas constituídas por grupamentos heme (hemoglobina e guanilato ciclase) (Mocellin *et al.,* 2007). Tal reação forma complexos metal-nitrosil (M-NO), processo denominado nitrosilação (SNO) (Mancardi *et al.,* 2004). Em células não eritróides, o •NO interage principalmente com a guanilato

ciclase solúvel (sGC), proteína responsável pela conversão de GTP em cGMP, aumentando em 100 vezes sua atividade catalítica (Derbyshire & Marletta, 2012).

Em suma, o •NO participa de vias de sinalização dependentes ou não de cGMP. Na presença deste, o óxido nítrico interage com o grupamento heme da guanilato ciclase, elevando os níveis de cGMP, que se acopla à proteína quinase G (PKG), fosfodiesterases e canais de nucleotídeos cíclicos (Burke *et al*., 2013). A ativação da PKG pela via •NO-sGC-cGMP desencadeia a fosforilação de substratos variados (Friebe & Koesling, 2003), sendo o relaxamento muscular, a vasodilatação e diminuição da agregação plaquetária os efeitos mais conhecidos (Davies *et al*., 1995).

Em baixas concentrações de cGMP, o óxido nítrico reage com outras moléculas contendo metais de transição ou com oxigênio e ânion superóxido, formando espécies reativas de nitrogênio (ERN) responsáveis por seus efeitos indiretos (Mancardi *et al.*, 2004). Ambas as vias dependente e independente de cGMP são de relevância para o estudo do efeito bimodal do óxido nítrico na carcinogênese.

Na literatura, é possível observar a aplicação do termo nitrosilação como um sinônimo de nitrosação, uma vez que o primeiro implica em modificações pós-traducionais de proteínas (MPTs), que geralmente recebem o sufixo "-ilação" (Wink *et al.*, 2013). Contudo, tendo em vista que a nitrosilação constitui um efeito direto (adição de •NO a diferentes biomoléculas) e a nitrosação um efeito indireto (adição de NO^+), empregaremos esses termos segundo os diferentes contextos bioquímicos, assumindo significados biológicos distintos, portanto.

Em concentrações elevadas (>1μM), o •NO reage rapidamente com superóxido (O^-_2) e oxigênio (O_2) para formar ERN. A reação com superóxido produz peroxinitrito ($ONOO^-$), enquanto a auto-oxidação do •NO forma dióxido de nitrogênio (NO_2), anidrido nitroso (N_2O_3) e nitrito (NO_2^-). O N_2O_3, por sua vez, decompõe-se rapidamente em nitrosônio (NO^+) e NO_2^- (Mocellin *et al*., 2007).

As ERN, normalmente formadas via ativação inflamatória da iNOS (Mancardi *et al*., 2004), são responsáveis pelos efeitos indiretos do •NO através de duas reações principais: nitração e *S*-nitrosação (Jadeski *et al*., 2002). Ambas as reações produze MPT de proteínas, uma vez que a adição de ERN a grupamentos específicos de resíduos de aminoácidos altera suas propriedades físico-químicas, e, consequentemente, sua função (Mocellin *et al*., 2007).

Na *S*-nitrosação, o NO^+, formado a partir da decomposição do N_2O_3, interage principalmente com o grupamento tiol de resíduos de cisteína de proteínas-alvo, formando *S*-nitrosotiois (SNO) (Shahani & Sawa, 2012). A nitração, alternativamente, ocorre a partir da ligação de NO_2^-, derivado do $ONOO^-$, ao anel aromático de resíduos de tirosina, formando de 3-nitrotirosina (Romero *et al*., 2018).

1.5. Propriedades pró e antitumorais do óxido nítrico

A ampla atuação do •NO em diferentes processos fisiológicos implica, invariavelmente, na sua contribuição para a patogênese de várias doenças, entre elas o câncer. Curiosamente, o •NO exerce um papel dicotômico na tumorigênese, atuando alternativamente como agente pró e antitumoral (Jadeski et al., 2002; Xu et al., 2002; Mocellin et al., 2007; Muntané & de La Mata, 2010; Burke et al., 2013). Neste contexto, fica evidente a necessidade de identificar os mecanismos e vias de sinalização da carcinogênese nos quais o •NO está envolvido. Este conhecimento apresenta alto potencial de interesse na formulação de abordagens antitumorais, considerando sua participação nas vias de sinalização de diferentes *hallmarks* do câncer.

O desenvolvimento do fenótipo neoplásico durante a carcinogênese depende da aquisição destes *hallmarks*, que é possibilitada por fenômenos considerados "habilitadores" (Hanahan & Weinberg, 2011). Alterações genéticas e epigenéticas envolvendo componentes de vias que sinalizam a proliferação celular se enquadram em um dos fenômenos habilitadores, denominado instabilidade genômica. Outro fenômeno capaz de habilitar *hallmarks* é a inflamação, considerando a produção de biomoléculas e espécies reativas que, respectivamente, estimulam o crescimento e promovem danos celulares (Hanahan & Weinberg, 2011).

A participação do •NO na carcinogênese envolve ambos os fenômenos habilitadores. A princípio, a síntese contínua e elevada de •NO pela iNOS, ultrapassando os limites fisiológicos e exercendo efeitos celulares deletérios, é estimulada por citocinas inflamatórias. O •NO e as RNS produzidas durante a resposta inflamatória são altamente mutagênicos, reagindo com moléculas de DNA ou promovendo MPTs de proteínas supressoras de tumor, contribuindo, assim, para a instabilidade genômica (Lala & Chakraborty, 2001).

As bases nitrogenadas do DNA são particularmente susceptíveis à nitração e oxidação mediada por $ONOO^-$ e intermediários. A nitração da guanina, por exemplo, forma o aduto 8-nitro-2'desoxiguanosina (8-nitro-dG), que posteriormente sofre depurinação seguida de clivagem do DNA (Burney et al., 1999). Alternativamente, sua oxidação forma 8-oxo-2'desoxiguanosina (8-oxo-dG), responsável pela ocorrência de mutações por transversão G:C→T:A (Wink et al., 1998). Nitrito e OH-, formados após clivagem da forma protonada do peroxinitrito (ONOOH), removem hidrogênios da desoxirribose, resultando em quebra da fita de DNA (Calmels et al., 1997).

Em síntese, o •NO e as espécies reativas dele derivadas, ao interagir com o DNA, produzem mutações que constituem um dos mecanismos responsáveis pela instabilidade genômica (Burney et al., 1999; Kilinc & Kilinc, 2005). Contribuem para isto, também, a inativação de proteínas chave de reparação do DNA, como a DNA-alquil-transferase, proteína A do *xeroderma pigmentosum* e 8-oxoguanosina-glicosilase-1, que ocorre mediante *S*-nitrosação de resíduos de cisteína e de domínios *zinc-finger* nas regiões de ligação ao DNA (Morita et al., 1996; Wink et al., 1998; Jaiswal et al., 2001).

Além de contribuir para a inflamação e instabilidade genômica, o •NO também está associado a diferentes *hallmarks* do câncer, ora promovendo, ora impedindo a sua aquisição. Um dos principais mecanismos através do qual o •NO regula vias de sinalização oncogênicas envolve fatores da transcrição. Alterações em fatores de transcrição podem ocorrer via *S*-nitrosação ou via sGC-cGMP-PKG, que ativam a cascata de quinases ativadas por mitógenos (MAPK) (Thum & Bauersachs, 2007). Os efeitos destes dois mecanismos são, no entanto, específicos para determinados tipos celulares e dependem da concentração de •NO, podendo, inclusive, atuar de forma oposta. Um exemplo disso é a atividade do dímero AP-1, cuja transcrição das subunidades c-Jun, c-Fos e sua posterior ligação ao DNA são estimuladas pelo •NO via sGC-cGMP (Tsuchiya et al., 2002; Nikitovic et al., 1998; Marshall & Stamler, 2002). Por outro lado, a *S*-nitrosação dos resíduos de cisteína nas regiões de ligação do DNA à c-Jun e c-Fos inibe a atividade da AP-1 (Boss et al., 2018; Marshall et al., 2002). A AP-1 é uma família de fatores de transcrição responsável por regular a proliferação e diferenciação celular, e sua elevada expressão tem sido identificada em neoplasias malignas como o melanoma (Kappelmann et al., 2002), no entanto, sua associação com o óxido nítrico no contexto da carcinogênese ainda não foi explorada.

Em contrapartida, os efeitos da *S*-nitrosação no NF-ƙB foram bem descritos na literatura: o •NO interage com o grupamento tiol do resíduo Cys62 do monômero p50, impedindo sua ligação ao DNA. Além disso, o monômero p65 também é alvo da *S*-nitrosação, desta vez inibindo a ligação do NF-ƙB ao promoter do gene NOS2. Assim, a expressão de iNOS é reduzida, tendo em vista que um dos reguladores da ativação da enzima é o próprio NF-ƙB, e que sua inativação via *S*-nitrosação constitui um mecanismo de autorregulação (Marshall & Stamler, 2002; Sha & Marshall, 2012). A *S*-nitrosação do NF-ƙB, consequentemente, promove a inibição à jusante de várias proteínas anti-apoptóticas, constituindo um dos principais mecanismos de apoptose em resposta ao estresse nitrosativo (Marshall & Stamler, 2001). Este, portanto, se enquadra no rol de efeitos antitumorais do •NO.

Mecanismos pró-apoptóticos relacionados ao receptor Fas (CD95) também podem ser regulados via *S*-nitrosação de um resíduo de cisteína em seu domínio citoplasmático (Leon-Bollote et al., 2011; Aranda et al., 2011). Mediante ativação do receptor Fas, a tioredoxina-2 promove desnitrosação e ativação da caspase-3 mitocondrial, reforçando o sinal apoptótico (Aranda et al., 2011; Benhar et al., 2008).

Tal atividade de desnitrosação, por sua vez, faz-se necessária para o estímulo apoptótico, pois as caspases estão sujeitas a um efeito inibitório da *S*-nitrosação em seu sítio ativo. Este fenômeno envolvendo a caspase-3 foi identificado no câncer epitelial de ovário mediante coexpressão de mieloperoxidase, promovendo resistência das células tumorais à apoptose (Saed et al., 2010). Outro mecanismo de inibição apoptótica e resistência à quimioterapia envolve a proteína Bcl-2, cuja ubiquitinação e degradação proteossomal são impedidas pela *S*-nitrosação dos resíduos de cisteína 158 e 259 (Azad et al., 2006; Nimmannit et al., 2006).

Além disso, em baixas concentrações (de 0,1 a 10 μM), o •NO promove a *S*-nitrosação da p21 ras, ativando a via de sinalização NF-ƙB e possibilitando a transcrição de proteínas anti-apoptóticas (Lander et al., 1995). A MPT induzida na proteína ras pelo •NO também é capaz de estimular a via de sinalização anti-apoptótica PI3K-Akt (Mocellin et al., 2007), contribuindo, assim, para a aquisição do *hallmark* evasão à apoptose.

Um dos mecanismos mais estudados de evasão à apoptose mediada pelo •NO consiste na nitração dos resíduos de tirosina da proteína p53, responsável pela perda de

sua atividade de ligação ao DNA (Forrester et al., 1996; Calmels et al., 1997; Chazotte-Aubert et al., 2000). O ambiente rico em •NO contribui para a perpetuação deste fenótipo, uma vez que a atividade da iNOS está associada a mutações G:C→A:T em dinucleotídeos CpG contendo 5-metilcitosina no gene TP53 em amostras de câncer colorretal (Felley-Bosco et al., 2002).

Além da evasão à apoptose, o •NO está envolvido na sinalização de outros *hallmarks* do câncer, como a angiogênese sustentada. Deste cenário participam principalmente o fator induzível por hipóxia 1 (HIF-1) e o fator de crescimento vascular endotelial (VEGF). No primeiro, o •NO pode atuar estabilizando a subunidade HIF-1α através da *S*-nitrosação de Cys533, bloqueando sua ubiquitinação e degradação proteossomal (Li et al., 2007). A neoangiogênese é promovida, dentre outros mecanismos, através da liberação de VEGF via •NO-sGC-cGMP (Ziche & Morbidelli, 2003). Além disso, a COX-2, responsável pela produção de prostaglandinas e outros fatores pró-angiogênicos, também é ativada em células tumorais via *S*-nitrosação (Morbidelli et al., 2003).

Quanto ao *hallmark* invasão e metástase, a *S*-nitrosação induzida pela eNOS em Cys619 da β-catenina contribui para a permeabilidade vascular. Sua liberação da VE-caderina facilita a dissociação das junções aderentes estimulada pelo VEGF, permitindo a penetração de células em vasos sanguíneos e linfáticos (Thibeault et al., 2010). O rompimento das junções aderentes contendo E-caderina também depende da produção de •NO e da consequente *S*-nitrosação em Cys498 da tirosina quinase c-Src (Rahman et al., 2010). Esse mecanismo ativa vias de sinalização oncogênicas envolvendo c-Myc, Akt e β-catenina, aumentando a transição epitélio mesênquima (EMT) e a expressão de COX-2 em células basaloides de câncer de mama (Wink et al., 2013). Alternativamente, a c-Src fosforila o resíduo Tyr1055 da iNOS, estabilizando sua meia-vida e aumentando a produção de •NO em células tumorais do pulmão (Zeng et al., 2009).

Proteínas contendo zinco em sua constituição também são alvo de PTM induzidas pelo •NO, por exemplo, as metaloproteinases de matriz (MMP). Em condições de inflamação prolongada e atividade contínua da iNOS, a MMP-9 pode ser ativada mediante *S*-nitrosação (O'Sullivan et al., 2014). Em carcinomas de nasofaringe, o •NO ativa indiretamente a MMP-9, inibindo, porém, a conversão de pro-MMP-2 em MMP-2 (Zergoun et al., 2016). Em células de câncer de próstata, a maior invasividade das células

tumorais pode ser associada à hiperativação da MMP-9 mediada por iNOS/•NO, sendo necessária a utilização de inibidores de iNOS de forma complementar à terapia fotodinâmica, que induz a expressão da enzima (Fahey & Girotti, 2015).

As ligações entre •NO e os *hallmarks* do câncer são as mais variadas e têm sido fruto de pesquisa nas últimas décadas (Rizi *et al*., 2017), inclusive com propostas para terapia antineoplásica (Shang *et al*., 2006; Steele *et al.*, 2010). Há, na literatura, vários trabalhos abordando o papel dicotômico do •NO e das NOSs, em especial a iNOS, na tumorigênese (Lala & Chakraborty, 2001; Mocellin *et al.*, 2007; Burke *et al.*, 2013; Vanini *et al*., 2015). No entanto, a atividade das NOSs e do •NO parece variar na dependência do tipo tumoral estudado, não havendo um consenso quanto ao seu efeito definitivo. Ainda, grande parte das pesquisas envolvendo •NO é realizada *in vitro* com cultura de células normais e neoplásicas, aplicando-se doadores de •NO em concentrações variadas ou estimulando a expressão de iNOS com LPS e IFN-γ, o que dificulta a extrapolação de seus efeitos nas vias de sinalização oncogênicas em tumores *in vivo*.

1.6. O óxido nítrico na modulação da paisagem epigenética

Nos últimos anos, a literatura tem evidenciado o papel do •NO na manutenção direta da "paisagem" epigenética, atuando através de vários mecanismos, incluindo modificações pós-traducionais de histonas (MPTHs), metilação de DNA e regulação de microRNAs (Vasudevan *et al.*, 2016; Socco *et al*., 2017). Neste contexto, a regulação da atividade de enzimas modificadoras de histonas é um dos efeitos mais recentemente identificados, envolvendo principalmente as histonas acetiltransferases (HATs) e as histonas desacetilases (HDACs). Em neurônios, a HDAC2 se apresentou como um alvo da *S*-nitrosação nos resíduos Cys262 e Cys274, que, no entanto, não resulta na inativação da enzima, mas sim em sua dissociação da cromatina, aumentando a acetilação de histonas próximas de promotores de genes dependentes de neurotrofina e promovendo, assim, a transcrição de genes importantes para o desenvolvimento neuronal (Nott *et al*., 2008).

Em linhagens de células de carcinoma de células escamosas bucal, o •NO induz a expressão de NPM1 e GAPDH, que ativa p300 HAT e promove a hiperacetilação da histona H3 (Arif *et al*., 2010). Embora através de mecanismos ainda pouco esclarecidos, a iNOS participa da carcinogênese experimental induzida em camundongos, sendo

associada a alterações no gene *Kras* e ao estímulo da expressão de microRNA-21, promovendo maior crescimento e inflamação tumorais no carcinoma de pulmão (Hussain *et al*., 2012).

1.7. A iNOS em lesões cancerizáveis e malignas da cavidade bucal

Vários estudos realizados nas últimas duas décadas demonstraram a presença da iNOS em diferentes lesões da cavidade bucal, ao mesmo tempo em que evidenciam a ausência desta enzima na mucosa sadia (Brennan, Conroy & Spedding, 2000; Brennan et al., 2001; Chen, Hsuen & Lin, 2002; Franchi et al., 2006; Mozet et al., 2009; Sappayatosok et al., 2009; Varghese, Sunil & Medhavan, 2010; Morelatto et al., 2013; Mastrangelo et al., 2014). Quando presente, a expressão imuno-histoquímica da iNOS nas células epiteliais pode ser detectada tanto no núcleo quanto no citoplasma, com intensidade e distribuição variadas (Brennan, Conroy & Spedding, 2000; Brennan et al., 2001). Em geral, a imunopositividade não é ubíqua.

Na displasia epitelial, por exemplo, a expressão de iNOS pode ser encontrada em até 80% das amostras (Chen, Hsuen & Lin, 2002), sendo que a intensidade e a distribuição da marcação aumentam da displasia leve para as displasias moderada e severa (Brennan, Conroy & Spedding, 2000). Outras lesões bucais, como fibrose submucosa oral e a hiperplasia verrucosa, também apresentam uma incidência de imunorreatividade para iNOS acima de 65% (Chen, Hsuen & Lin, 2002).

O percentual de carcinomas de células escamosas bucal (CCEB) positivos para iNOS, no entanto, variam entre 54 e 87% na literatura (Mozet et al., 2009; Sappayatosok et al., 2009; Varghese, Sunil & Medhavan, 2010; Morelatto et al., 2013). De fato, menores níveis de iNOS foram detectados em amostras de CCEB quando comparados à leucoplasia e hiperqueratose (Mastrangelo et al., 2014). Embora não esteja presente em todos os casos, a expressão proteica e gênica de iNOS no CCEB parece ocorrer principalmente nas margens tumorais, *fronts* de invasão e apresenta correlação significativa com a presença de metástase linfonodal (Brennan et al., 2001; Chen, Hsuen & Lin, 2002; Franchi et al., 2006; Mozet et al., 2009; Sappayatosok et al., 2009).

Mais recentemente, Servato *et al*. (2019) identificaram um aumento na expressão proteica e gênica de iNOS, bem como da imunomarcação para 3-nitrotirosina, em amostras de leucoplasia bucal e CCEB. Além disso, os autores relataram associações

significativas entre a expressão destes dois marcadores e a presença de metástase, o estadiamento tumoral, a ocorrência de recidivas e taxa de sobrevida, sugerindo que a via de sinalização do •NO exerce um papel importante no desenvolvimento e progressão de lesões pré-neoplásicas e neoplásticas da cavidade bucal, e que marcadores associados ao •NO possuem potencial para aplicação no prognóstico do CCEB.

A carcinogênese experimental em modelos animais surge, portanto, como um método que permite a investigação da participação do •NO e das NOSs em diferentes etapas do desenvolvimento tumoral dentro de condições controladas, considerando também as variadas interações celulares existentes no organismo e possibilitando a proposição de vias de sinalização relacionadas a esse fenômeno. Poucos estudos desta natureza foram desenvolvidos (Jadeski *et al*., 2002; Hussain *et al*., 2012).

Neste contexto, o objetivo deste trabalho foi verificar a influência da iNOS no processo de carcinogênese bucal experimental induzido por 4NQO em modelo murino, verificando-se diferenças na incidência e características morfológicas das lesões observadas, bem como em marcadores histopatológicos, inflamatórios sistêmicos e epigenéticos entre camundongos selvagens e desprovidos de iNOS.

2. CAPÍTULO 1

Journal: Experimental and Molecular Pathology.

Title: Absence of inducible nitric oxide synthase is associated to reduced depth of invasion in 4NQO-induced murine oral squamous cell carcinoma

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Abstract

Nitric oxide has been identified in a variety of physiological and pathological conditions, including cancer, where it seems to act alternatively as a tumor promoter and inhibitor. During multistep oral carcinogenesis, the expression of nitric oxide-related markers seems to increase. In this context, the purpose of this study was evaluate the influence of inducible nitric oxide synthase (iNOS) in oral carcinogenesis by analyzing the incidence and clinicopathological features of epithelial tongue lesions in the $Nos2^{+/+}$ (wild-type) and $Nos2^{-/-}$ (knockout) mice treated with 4-nitroquinoline N-oxide (4NQO). A 50ug/mL solution of 4NQO was administered in the drinking water of 31 mice, 15 $Nos2^{+/+}$ and 16 $Nos2^{-/-}$, for 16 weeks, and results were compared to control groups (n=15) treated with propylene glycol after 8-week observation period. Tongues were evaluated for the incidence of macroscopic and microscopic lesions. Data collected included the number, location, and histological features of the observed lesions, including grade of dysplasia, growth pattern, tumor differentiation, pattern of invasion and depth of invasion. There were no significant differences in the incidence and clinicopathological features of 4NQO-induced lesions between $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. Median (range) for depth of invasion in $Nos2^{+/+}$ and $Nos2^{-/-}$ cancers were 233.5 μm (933.3) and 142.6 μm (458.7), a significant difference (p=0.0184). We concluded that the absence of iNOS does not affect the incidence of carcinogen-induced epithelial lesions but reduces tumor invasiveness in oral cancer.

Keywords: carcinogenesis, nitric oxide, oral cancer, neoplasm invasiveness

Introduction

Carcinogenesis is a multistep process in which alterations of the regulatory mechanisms of homeostasis promote the transformation of normal cells and tissues into benign and malignant tumors (Hanahan and Weinberg, 2011; Chaffer and Weinberg, 2015). Among such are the changes in the genome and epigenome that progressively grant the cell a neoplastic phenotype with advantageous growth and longevity traits, known as the hallmarks of cancer (Hanahan and Weinberg, 2011).

These hallmarks are usually regulated by redundant signaling pathways within the cellular circuitry, which hinders the proposal of broad-spectrum antineoplastic agents for cancer treatment (Hanahan and Weinberg, 2011). The identification of key molecules in cancer development is therefore a relevant topic for research, aiming to elucidate core mechanisms that could be targeted for antineoplastic therapy. A candidate to this type of study is the nitric oxide (•NO).

Nitric oxide has been identified in a variety of biological processes, mainly vasodilation, inflammatory response, and neurotransmission (Moncada and Higgs, 1991). It is also involved in the pathogenesis of several diseases, including neoplasms. In oncogenesis, however, •NO seems to play a dichotomous role, acting alternatively as a tumor promoter and inhibitor (Muntané and De La Mata, 2010; Burke et al., 2013).

Nitric oxide act as a carcinogenic molecule by contributing to the acquisition of hallmarks through genomic instability and tumour-induced inflammation (Hanahan and Weinberg 2011). Reactive nitrogen species (RNS) resulting from the elevated synthesis of •NO by

inducible nitric oxide synthase (iNOS) might either interact directly with the DNA or promote post-translations modifications (PTMs) of tumor suppressor and oncoproteins (Lala and Chakraborty, 2001). Nitration of tyrosine residues in p53, for example, interferes with its DNA-binding activity, which contributes to resisting cell death (Chazotte-Aubert et al., 2000). *S*-nitrosation of NF-κB, on the other hand, promotes the downstream inhibition of several anti-apoptotic proteins, which accounts for a tumor inhibition effect (Marshall and Stamler, 2001). This •NO-mediated protein PTM is also responsible for activating matrix metalloproteinases (MMP) (O'Sullivan et al., 2014), such as MMP-9, which in prostate cancer cells causes increased invasiveness (Fahey et al., 2015). The connections between •NO and the hallmarks of cancer are many and have been subject for cancer-therapy research in the last decades (Li et al., 2015).

In oral cancer and precursor lesions, however, the effect of •NO is not sufficiently explored. A few studies have shown that normal mucosa (NM) does not express iNOS, whereas epithelial dysplasia (ED) and oral squamous cell carcinoma (OSCC) lesions show iNOS reactivity, which correlate to grade of dysplasia (Brennan et al., 2000), and lymph node metastasis (Brennan et al., 2000). Recently, our research group demonstrated that iNOS and 3-nitrotyrosine expression increases during oral carcinogenesis and were associated to tumor staging, metastasis, relapse, and survival (Servato et al., 2019).

In this context, the aim of this study was to investigate the influence of iNOS in oral carcinogenesis by analyzing the incidence and clinicopathological features of epithelial lesions in $Nos2^{+/+}$ (wild-type) and $Nos2^{-/-}$ (knockout) mice treated with the chemical carcinogen 4-nitroquinoline-N-oxide (4NQO).

Materials and methods

Study design and ethical issues

The experimental procedures were approved by the Ethical Commission in Animal Experimentation (CEUA-UFU, registration n. 100/18). Our experimental unit consisted of single *Mus musculus* male mice of two different strains: C57BL/6J (wild-type, $Nos2^{+/+}$) and B6.129P2-$Nos2^{tm1Lau}$/J (knockout, $Nos2^{-/-}$). Treatment groups received the carcinogen 4NQO, and controls were treated with vehicle propylene glycol (PPG). To verify the effect of iNOS in epithelial carcinogenesis, the genetically modified $Nos2^{-/-}$ mice were used as negative control for the wild-type, $Nos2^{+/+}$ strain. Sample size was determined based on Tang et al. (2004), with 61 animals randomly assigned to four groups: $Nos2^{+/+}$/4NQO (n=15), $Nos2^{+/+}$/PPG (n=15), $Nos2^{-/-}$/4NQO (n=16), $Nos2^{-/-}$/PPG (n=15).

Animals

Animals included were 8-12 weeks old and weighed 24.2 ± 2.6g at the beginning of the treatment. Thirty $Nos2^{+/+}$ mice were provided by the Rodents Animal Facilities Complex of the Federal University of Uberlândia (REBIR-UFU), and 31 $Nos2^{-/-}$ mice (JAX stock #002609) were provided by the Institute of Biomedical Sciences of the University of São Paulo (ICB-USP). The $Nos2^{-/-}$ genetic modified strain was developed in the University of Virginia Health Sciences Center and provided internationally by the Jackson Laboratory (Bar Harbor, USA). Briefly, Laubach et al. (1995) used a targeting vector with neomycin resistance and herpes simplex virus thymidine kinase genes to disrupt exons 12 and 13 of the calmodulin binding domain. The animals obtained with this method were crossed to

C57BL/6 and backcrossed to C57BL/6J for 11 generations and have no serum nitric oxide response.

Experimental protocol

$Nos2^{+/+}$ and $Nos2^{-/-}$ mice were treated with 4NQO diluted in propylene glycol and water at 50µg/mL (N8141, Sigma-Aldrich, San Louis, USA) for 16 weeks, as proposed by Tang et al. (2004). Control groups received a solution of 5mg/mL of PPG solution, used in the dilution of 4NQO. Animals were followed for 8 weeks after the treatment period, when the following humane endpoints were verified at every eight hours: ≤ 20% of weight loss, inability to ambulate, labored respiration, dehydration, hunched posture, ocular or nasal discharge, and inability to access food and/or water. Animals were promptly euthanized upon detection of these signs. The total period of experimentation was 24 weeks. All experimental procedures were conducted in REBIR-UFU. Animals were kept in 32 x 20 x 21cm micro-isolator cages, at 22ºC, light-dark cycles of 12h, free access to commercial mouse chow, and sterilized water.

Macroscopy and histopathological analysis

Tongues were dissected and submitted to gross examination. We considered the tongue largest lesion as reference for the longitudinal section. In the absence of visually detectable lesions, the section was performed in the midline. Tissues were fixed with paraformaldehyde 7% for 24h and embedded in paraffin before microtomy. Histological sections of 3µm were stained with hematoxylin and eosin and scanned using Aperio AT2 (Leica Biossystems, Nussloch, Germany) with 400x magnification.

Alterations in the tongue epithelial lining were analysed by two pathologists and classified into five categories according to Tang et al. (2004), with modifications, as follows: hypotrophy, hyperplasia without dysplasia, squamous papilloma (SP), epithelial dysplasia (ED), and oral squamous cell carcinoma (OSCC). The entire length of the epithelium was analysed in all samples. Number of affected animals and lesions per animal and experimental group were considered outcome measures for the histopathological analysis.

Tongue cancers were further classified according to growth pattern (endophytic, exophytic), tumor differentiation (Sloan et al., 2017) and pattern of invasion (Bryne et al., 1991). For tumor differentiation, lesions were considered well, moderately or poorly differentiated based on the degree of keratinization of the neoplastic cells. Pattern of invasion was analyzed with the identification of one among four types: (I) pushing, well delineated infiltrating borders, (II) infiltrating solid cords, bands or strands; (III) small groups or cords of infiltrating cells, usually with >15 cells; (IV) and cellular dissociation in small groups (<15) or single cells.

Depth of invasion (DOI) was measured as recommended by Müller et al., (2019) in 400x magnification scanned slides using the ruler tool of ImageScope (Leica Biossystems, Nussloch, Germany). Briefly, the basement membrane was identified, and an imaginary line was drawn across the tumor, from one margin to the other. A perpendicular line was drawn in sequence, extending to the deepest part of the tumor, whose lenght represented the DOI.

Histopathological parameters for classification of the epithelial lesions

Lesions were classified according to Tang et al. (2004), with mild modifications, such as the inclusion of epithelial hypotrophy and the classification of epithelial dysplasia according to WHO criteria (Reibel et al., 2017). Hypotrophy was defined as a reduction of the epithelial thickness based on the aspects of the tongue mucosa in control animals. Epithelial hyperplasia without dysplasia was defined as areas of thickened epithelium with or without hyperkeratosis, usually with loss of the "serrated" aspect of the mouse epithelium. Epithelial dysplasia was identified in areas of thinned or thickened epithelium where alterations of the tissue architecture and cell differentiation were observed. It was further classified in mild (ML), moderate (MD) or severe (SD) based on the affected epithelial layers (Reibel et al., 2017). Squamous papillomas were considered as exophytic, papillary and hyperkeratotic growths of benign cells. OSCC were considered as microinvasive or frankly invasive lesions. In the first case, we observed either a broad border of pushing infiltration to the subepithelial tissues, or the presence of incipient neoplastic growth where the basement membrane was ill-defined.

Statistical analysis

Inferential analyses were carried out in GraphPad Prism 8.2.1 (GraphPad, San Diego, California, USA) and IBM® SPSS Statistics software 28.0.0 (SPSS Inc., Chicago, Illinois, USA), with α=5%. Differences in food and water consumption were verified with Kruskal-Wallis and Dunn's test. Contingency analyses with Fisher's exact test were performed to verify differences in the incidence of tongue lesions between the groups. Unpaired *t* test was performed to investigate differences in the number of altered areas in the tongue between the strains. Differences in the distribution of these areas according to

animal strain were verified with Z-test. Differences in the DOI of $Nos2^{+/+}$ and $Nos2^{-/-}$ OSCCs were tested with Mann-Whitney's. Survival analysis was performed with log-rank test.

Results

All $Nos2^{+/+}$ and $Nos2^{-/-}$ mice survived the 16-week treatment with 4NQO. The percentage of animals that reached the final 8-week observation period (24 weeks of experiment) was 66.7% and 87.5%, respectively. Overall survival curves were not significantly different (Log-rank test, p=0.169, Figure 1).

From 61 treated animals, fifty-five were eligible for histopathological analysis: 26 $Nos2^{+/+}$ and 29 $Nos2^{-/-}$. Two $Nos2^{-/-}$/PPG mice died during acclimation period and 1 $Nos2^{+/+}$/PPG died at week 6 due to emaciation secondary to malocclusion. Five $Nos2^{+/+}$/4NQO died before week 24, 2 at week 20, 1 at week 21, and 2 at week 23. Only two $Nos2^{-/-}$/4NQO mice died by week 21. The tongue sections of the animals that died at week 20 and of a $Nos2^{+/+}$/PPG mouse were excluded from histopathological analyses due to autolysis. The others remained in the study, for they had already developed OSCC and were euthanized after the observation of humane endpoints, mainly weight loss <20%, obvious alterations according to the mouse grimace scale, and hunched posture. The final number of samples per group was: $Nos2^{+/+}$/4NQO (n=13), $Nos2^{+/+}$/PPG (n=13), $Nos2^{-/-}$/4NQO (n=16), $Nos2^{-/-}$/ PPG (n=13).

Macroscopic lesions were observed in the tongue of 69.2% (n=9/13) $Nos2^{+/+}$ and 75% (n=12/16) $Nos2^{-/-}$ mice. They occurred mainly in the posterior tongue of $Nos2^{-/-}$ mice

(55.6%) and in the anterior dorsum of $Nos2^{+/+}$ (45.5%), consisting of OSCC (44.4% in $Nos2^{-/-}$ and 41.7 in $Nos2^{+/+}$) and SP (38.9% and 41.7%, respectively). All treated animals who did not exhibit alterations at gross-examination had microscopic lesions in the epithelium (Figure 2).

There were no significant differences in the incidence of 4NQO-induced microscopic lesions between $Nos2^{+/+}$ and $Nos2^{-/-}$ mice (Table 1). Similarly, no difference was found regarding the number of altered areas observed in the tongue sections (Table 2). The average number of lesions in $Nos2^{+/+}$ and $Nos2^{-/-}$ animals was 7.7 ± 2.3 (5-12) and 7.5 ± 3.2 (1-12), respectively. All treated animals developed epithelial alterations, and those who did not show OSCC had ED. No alterations were observed in the control groups. With regard to histopathological features of OSCC (Figure 3), most lesions were endophytic, well-differentiated and had pattern of invasion type II in both strains, although no difference related to tumor growth and invasion patterns was found (Table 3). Only DOI was significantly higher in $Nos2^{+/+}$ OSCC than in $Nos2^{-/-}$ tumors (Mann-Whitney U test, p=0.0184).

Discussion

Based on the current evidence for the involvement of nitric oxide in OSCC development and progression, we aimed to evaluate the influence of iNOS in oral carcinogenesis analyzing the incidence and clinicopathological features of epithelial tongue lesions in $Nos2^{+/+}$ (wild-type) and $Nos2^{-/-}$ (knockout) mice treated with 4-nitroquinoline N-oxide (4NQO).

The carcinogen 4NQO is a DNA-reactive genotoxin widely used in chemically induced models for squamous cell carcinoma (Bouaoud et al., 2021). It mimics the carcinogenic effects of chronic tobacco use, the main risk factor for oral cancer, which includes intracellular oxidative stress and DNA adduct formation (Ishida et al., 2017). With this model, different stages of oral carcinogenesis can be detected in the field of cancerization, from mild dysplasia to frankly invasive tumors (Ishida et al., 2017; Bouaoud et al., 2021). To investigate the influence of different molecules in this process, several mouse models combining genetic modifications with 4NQO treatment have also been tested, including $XPA^{-/-}$;$p53^{+/-}$, $L2D1^{+}$, $Dusp1^{-/-,}$ PIK3CA-GEMM, K14-GFP-miR-211, and $Gal3^{-/-}$ (de Faria et al., 2011; Ishida et al., 2017). To the author's knowledge, this is the first investigating the effect of 4NQO treatment in Nos2-knockout mice.

Nitric-oxide has long been associated to the pathogenesis of tobacco and alcohol-related diseases (Cooper and Magwere, 2008), thus raising the possibility of its involvement in oral tumorigenesis. In fact, it is an important signaling molecule in the initiation and progression of oral cancer (Choudhari et al., 2012), as a few studies have already reported the differential expression of NOS and other •NO-related markers in normal mucosa, oral potentially malignant disorders (OPMD) and OSCC (Brennan et al., 2000; Chen et al., 2002; Connelly et al., 2004; Varghese et al., 2010; Morelatto et al., 2014; Mastrangelo et al., 2014).

Inducible nitric oxide synthase (iNOS) expression, for example, does not normally occur in the epithelium lining of the oral mucosa (Connely et al., 2005; Mastrangelo et al., 2014; Ambe et al., 2015), although chronic tobacco use seems to induce it (Meric et al., 2012).

When it comes to OPMD, Brennan et al. (2000) reported positive iNOS immunostaining in samples of ED, which correlated to the degree of dysplasia. Chen et al. (2002) also identified high iNOS expression in samples of ED, submucous fibrosis, and verrucous leukoplakia. Enhanced iNOS mRNA and protein expression was also observed in OSCC when compared to normal mucosa (Brennan et al., 2000; Chen et al., 2002; Connelly et al., 2004; Varghese et al., 2010; Morelatto et al., 2014), although it might be at lower levels when compared to leukoplakia (Mastrangelo et al., 2014).

Besides its increasing expression throughout oral carcinogenesis, iNOS has been related to lymph node metastasis (Brennan et al., 2001; Chen et al., 2002; Mozet et al., 2009; Sappayatosok et al., 2009; Servato et al., 2019), lymphangiogenesis (Sappayatosok et al., 2009), tumor stage (Sappayatosok et al., 2009; Yang et al., 2015; Servato et al., 2019), tumor grade (Yang et al., 2015), relapse and survival (Servato et al., 2019), therefore being of prognostic value for OSCC.

Considering the dichotomous role of •NO in cancer development, and the high expression of •NO-related markers in oral potentially malignant and malignant lesions, we conducted an *in vivo* study using the standard murine 4NQO model of carcinogenesis with a negative control consisting of a Nos2 gene knockout mouse. With this mixed model, it was possible to analyze the influence of iNOS absence on the clinicopathological features of 4NQO-induced epithelial lesions. Differences observed were, however, restricted to the depth of invasion of OSCC, which was significantly lower in $Nos2^{-/-}$ mice.

Despite the lack of experiments on oral cancer with this genetic model, other *in vivo* studies have explored iNOS expression in animals treated with carcinogens. Chen et al. (2003) found elevated mRNA and protein expression in DMBA-induced hamster buccal-pouch carcinomas. Similarly, Tanaka et al. (2003) observed that iNOS was not present in normal rat tongue, but in dysplasias, papillomas and carcinomas induced by 4NQO, with strong reactivity in advanced lesions and invasion fronts. The same authors also reported that tumor invasion reduced following EGMP (antioxidant) treatment, as well as iNOS expression. This is an interesting finding, since our experiment demonstrated that animals lacking iNOS had OSCC with lower depths of invasion. Based on these results, we suggest that the absence of iNOS does not affect the incidence of epithelial but is associated to the reduction in tumor invasiveness and therefore to OSCC progression.

Similar findings were identified in human cells and tissues. In fact, Brennan et al. (2008) observed that iNOS immunostaining was significantly higher in nodal OSCC, where peripheral cells of the tumor nests and invading fronts were consistently positive. The authors carried out Transwell invasion assays using H357 tongue carcinoma cells treated with 1400 W, an iNOS inhibitor, and observed a reduction in cell invasion. In prostate cancer cells, iNOS inhibition and •NO scavenging also suppresses growth, migration, and invasion (Fahey and Girotti, 2015). Similarly, •NO inhibition reduced tumor growth in iNOS-expressing mouse bladder samples (Belgorosky et al., 2014).

This •NO-related tumor invasion seems to occur upon iNOS-dependent MMP activation, as demonstrated by Chen et al. (2008) with a lung cancer mouse model. These authors reported an increase in MMP-2 activity following gaseous nitric oxide (gNO) treatment

to A549 cells, which in turn diminished if iNOS expression was blocked with L-NAME. When injected to SCID mice, gNO treated A549 cells formed more metastatic nodules, whilst animals pre-treated with L-NAME had a reduced number of metastases. Activation of some MMP by •NO seems to occur by *S*-nitrosation of the zinc-finger domains in pro-MMP (O'Sullivan et al., 2014). In head and neck squamous cell carcinoma, Franchi et al. (2002) identified correlations among MMP-9 expression, iNOS activity, p53 status and angiogenesis, suggesting that p53 mutation may result in increased invasiveness related to •NO production and MMP activation. Further research is therefore warranted to explore the associations between iNOS, •NO and MMP in oral cancer.

This study is limited to a single observation period of 8 weeks after 4NQO treatment, considering that other studies followed the animals for up to 16 weeks (Tang et al., 2004; de Faria et al., 2011). A higher number of OSCC would be expected by the end of 32 weeks of experimentation in comparison to 24 weeks, and perhaps more invasive tumors. It would be interesting to analyze how iNOS absence would influence the features of more advanced lesions, especially when many tumors were well-differentiated, exophytic and had invasion pattern Type I, which are sometimes difficult to classify as malignant and to obtain the DOI. Morphological parameters for the classification of murine tongue lesions are not clear in the literature, what hinders some level of subjectivity to the analyses. Some of these parameters were established for human potentially malignant and malignant lesions and might not always be applicable to mice.

Based on these results, we concluded that the absence of iNOS has no apparent effect on the incidence and clinicopathological features of 4NQO-induced epithelial lesions in mice but is associated to a significant reduction in oral squamous cell carcinoma invasiveness.

Tables

Table 1. Incidence of 4NQO-induced lesions in Nos$2^{+/+}$ and Nos$2^{-/-}$ mice

Tongue lesions	Experimental groups, n[1] (%)				
	Nos$2^{+/+}$ (n=13)		Nos$2^{-/-}$ (n=16)		
	Absent	Present	Absent	Present	*p*-value[2]
Hypotrophy	11 (84.6)	2 (15.4)	13 (81.2)	3 (18.8)	ns
Epithelial hyperplasia	4 (30.8)	9 (69.2)	5 (32.2)	11 (68.8)	ns
Squamous papilloma	6 (46.2)	7 (53.8)	9 (56.2)	7 (43.8)	ns
Epithelial dysplasia	0 (0)	13 (100)	1 (6.2)	15 (93.8)	ns
Mild	1 (7.7)	12 (92.3)	3 (18.8)	13 (81.3)	ns
Moderate	6 (46.2)	7 (53.8)	9 (56.2)	7 (43.8)	ns
Severe	3 (23.1)	10 (76.9)	4 (25)	12 (75)	ns
Squamous cell carcinoma	4 (30.8)	9 (69.2)	3 (18.8)	13 (81.2)	ns

[1]Number of affected animals; [2]Fisher's exact test, $p<.05$; ns: not significant.

Table 2. Number of 4NQO-induced lesions observed in $Nos2^{+/+}$ and $Nos2^{-/-}$ mice.

Tongue lesion	Experimental groups, n[1] (%)		
	$Nos2^{+/+}$	$Nos2^{-/-}$	*p*-value[2]
Hypotrophy	2 (2.0)	5 (4.2)	ns
Epithelial hyperplasia	17 (16.7)	23 (19.2)	ns
Squamous papilloma	8 (7.8)	8 (6.7)	ns
Epithelial dysplasia	61 (59.8)	70 (58.3)	ns
Mild	30 (29.4)	28 (23.3)	ns
Moderate	10 (9.8)	21 (17.5)	ns
Severe	22 (21.6)	21 (17.5)	ns
Squamous cell carcinoma	13 (12.7)	15 (12.5)	ns
Total	102 (100)	121 (100)	ns

[1]Number of lesions; [2]Z-test with Bonferroni adjustment, $p<.05$; ns: not significant.

Table 3. Histopathological features of OSCC in Nos$2^{+/+}$ and Nos$2^{-/-}$ mice

Histopathological features	n	Experimental groups, n[1] (%)		*p*-value[4]
		Nos2$^{+/+}$	Nos$2^{-/-}$	
Growth pattern				
Endophytic	18	9 (69.1)	9 (60.0)	ns
Exophytic	10	4 (30.8)	6 (40.0)	ns
Tumor differentiation[2]				
WD	22	11 (84.6)	11 (73.3)	ns
MD	5	1 (7.7)	4 (26.7)	ns
PD	1	1 (7.7)	0 (0.0)	ns
Pattern of invasion				
Type I	6	3 (23.1)	3 (20.0)	ns
Type II	14	5 (38.5)	9 (60.0)	ns
Type III	5	3 (23.1)	2 (13.3)	ns
Type IV	3	2 (15.4)	1 (6.7)	ns
DOI median (range)[3]		233.5 (933.3)	142.6 (458.7)	0.0184*

[1]Number of lesions; [2]WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated; [3]DOI: depth of invasion; [4]Z-test with Bonferroni adjustment, $p<.05$; ns: not significant, *statistically significant.

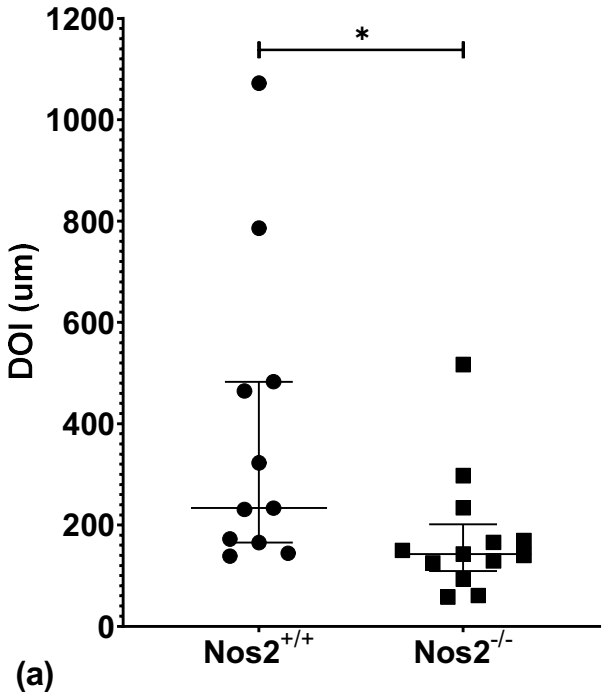
Figure Captions

Figure 1. (a) Depth of invasion (DOI) of oral squamous cell carcinoma (OSCC) in $Nos2^{+/+}$ and $Nos2^{-/-}$ mice, Mann-Whitey U test, p=0.0148. (b) Overall survival of 4NQO treated mice, Log-rank test, p=0.169.

Figure 2. Macroscopic and microscopic features of 4NQO-induced epithelial lesions in the tongue of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. (A) Exophytic lesion in the tongue mid-dorsum of a $Nos2^{+/+}$ mouse; (B) Exophytic lesions in the lateral surface of a $Nos2^{+/+}$ mouse tongue; (C) Exophytic lesion in the anterior dorsum of a $Nos2^{-/-}$ mouse; (D) normal epithelial lining of the tongue mucosa; (E) epithelial hyperplasia without dysplasia; (F) squamous papilloma; (G) mild epithelial dysplasia; (H) moderate epithelial dysplasia; (I) severe epithelial dysplasia. Hematoxylin-and-eosin, 100x original magnification.

Figure 3. Microscopic features of 4NQO-induced oral squamous cell carcinoma (OSCC) in the tongue of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. (A) Pattern of invasion type I; (B) Pattern of invasion type II; (C) Pattern of invasion type III; (D) Pattern of invasion type IV; (E) Moderately differentiated oral squamous cell carcinoma (OSCC); (F) Poorly differentiated OSCC. Hematoxylin-and-eosin, 100x original magnification.

Figure 1


1200
1000
800
600
400
200
0
DOI (um)
*
Nos2+/+
Nos2-/-
(a)

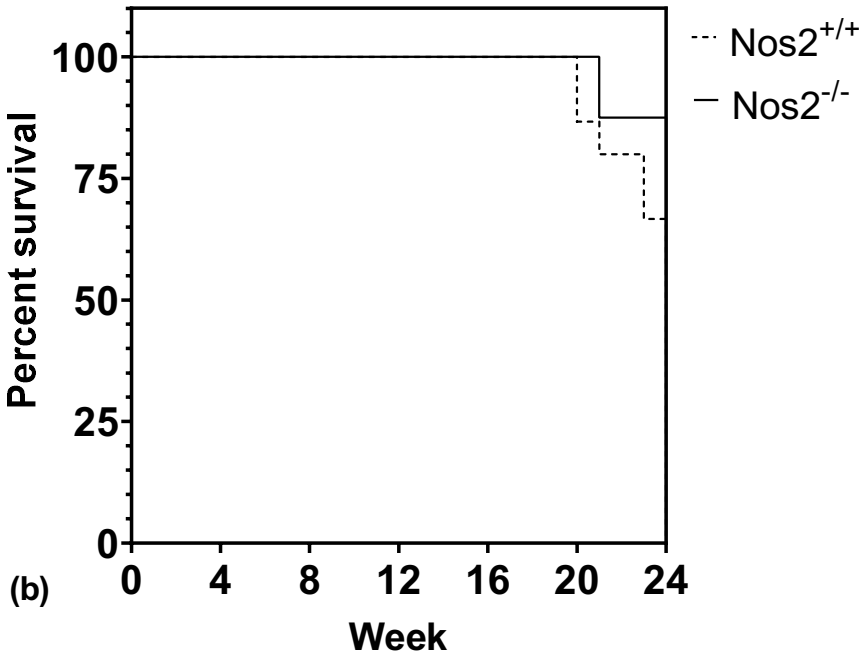

100
75
50
25
0
Percent survival
0
4
8
12
16
20
24
Week
(b)
Nos2+/+
Nos2-/-

Figure 2

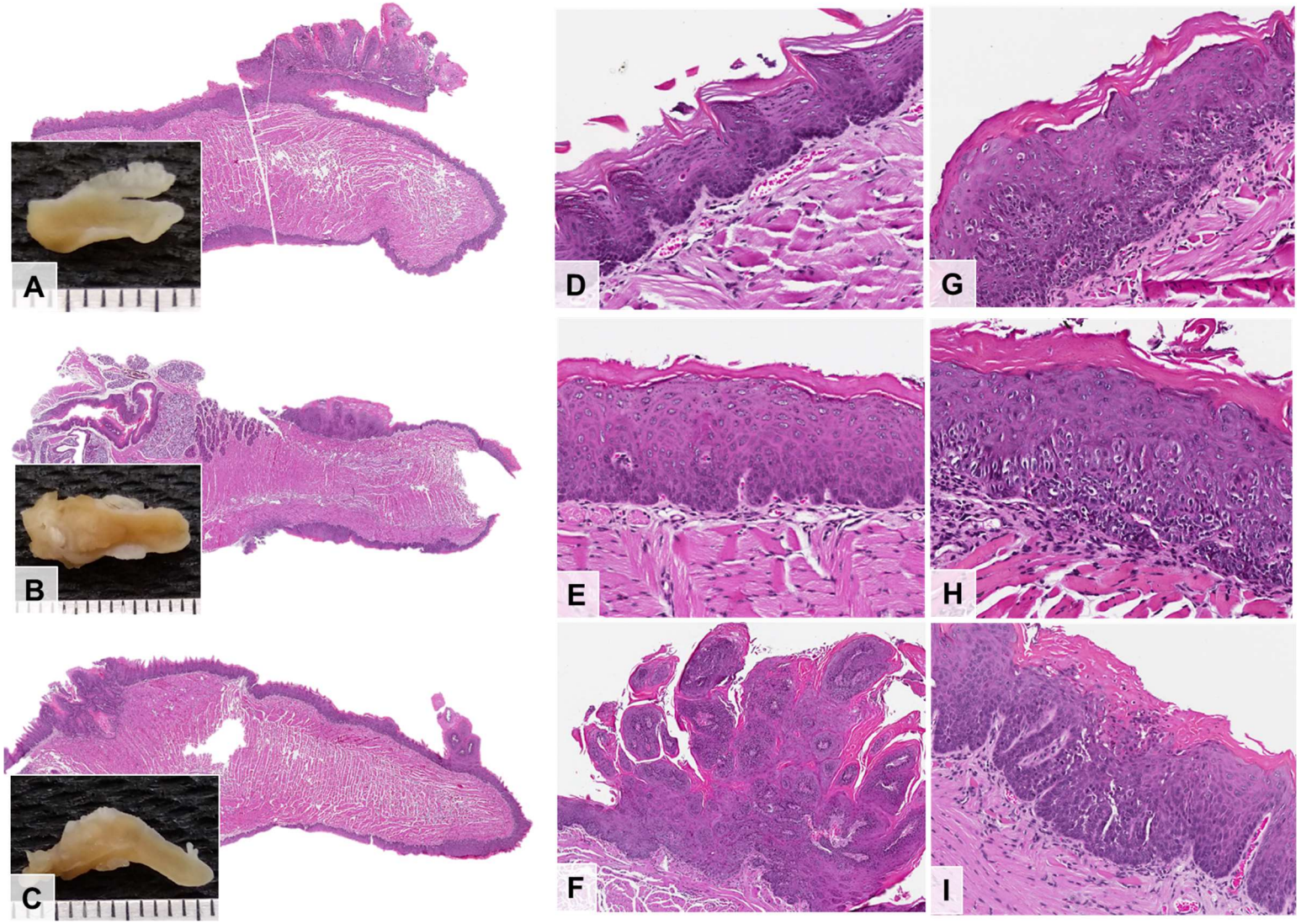
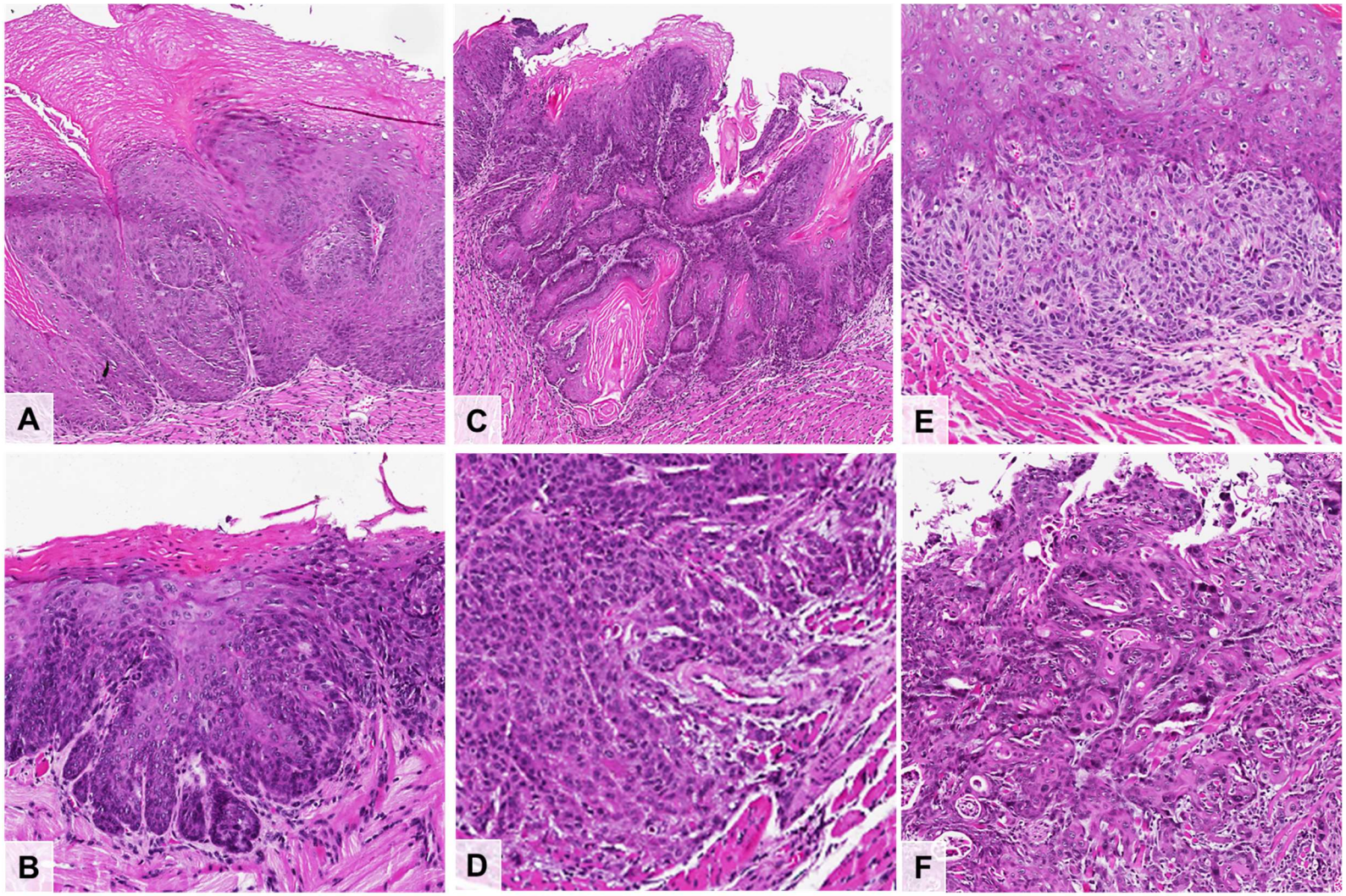


Figure 3



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3. CAPÍTULO 2

Journal: Experimental and Molecular Pathology.

Title: Depth of invasion of squamous cell carcinoma in Nos2-knockout mice correlated to alterations in systemic inflammatory markers following 4NQO treatment

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Abstract

Peripheral blood analysis is a non-invasive and low-cost technique of prognostic value for several diseases, including oral cancer. Considering the role of inducible nitric oxide synthase (iNOS) in tumor-associated inflammation, this study purposed to evaluate the influence of this enzyme on peripheral blood parameters and systemic inflammatory biomarkers during murine oral carcinogenesis. A 50ug/mL solution of 4-nitroquinoleine-N-oxide (4NQO) was provided to 15 C57BL/6J ($Nos2^{+/+}$) and 16 B6.129P2-$Nos2^{tm1Lau}$/J ($Nos2^{-/-}$) for 16 weeks. Animals were followed for 8 weeks after treatment, then blood samples and tongues were collected for hematological and histopathological analyses. Red blood cells, white blood cells, and platelet cell parameters were analyzed. The neutrophil-to-lymphocyte (NLR), platelet-to-lymphocyte ratio (PLR), and the immune-inflammation index (SII) were also calculated. The depth of invasion (DOI) of all carcinomas was measured. Differences were found in several blood parameters. The DOI in $Nos2^{-/-}$ was lower than in $Nos2^{+/+}$ (p=0.009), and strong correlations were found between DOI and neutrophil count (ρ=-0.68, p=0.017), lymphocyte count (ρ=0.72, p=0.011), NLR (ρ=-0.65, p=0.025), PLR (ρ=-0.73, p=0.013), and SII (ρ=-0.67, p=0.037) in $Nos2^{-/-}$ mice. In conclusion, iNOS seems to have an important role in OSCC invasion and progression, which might be associated to alterations in immune-inflammatory cell dynamics evidenced by peripheral blood and systemic inflammatory biomarkers.

Keywords: carcinogenesis, nitric oxide, oral cancer, tumor biomarkers, hematological tests.

Introduction

Neoplasms of the oral cavity and pharynx represent the 7th more frequent type of cancer worldwide and the 9th cause of death, with an annual incidence of 710,000 new cases and 359,000 deaths (Borsetti et al., 2020). Only in 2020, there were 377,713 new cases of oral cancer in 185 countries and 177,757 related deaths (Sung et al., 2021). The increasing incidence of oral cancer has long been a healthcare concern in several countries, along with low survival rates related to delayed diagnosis (Chakraborty et al., 2019). In general, up to 50% of the patients are diagnosed at advanced stages, with overall 5-year survival rates lower than 60% (Abati et al., 2020).

In this context, several biomarkers have been studied to improve its prognosis. The analysis of peripheral blood and systemic inflammatory biomarkers was recently shown to a be non-invasive and low-cost technique providing significant prognostic predictors for oral cancer (Tangthongkum et al., 2017; Wu et al., 2017; Diao et al., 2018; Diao et al., 2019; Kao et al., 2018; Yang et al., 2018; Spanier et al., 2020; Salzano et al., 2022). These hematological parameters are of clinical interest because they reflect the dynamics between anti-tumor and tumor-promoting functions of the immune system in cancer patients (Li et al., 2018).

Considering that neutrophilia and lymphocytopenia are common features of the cancer-associated chronic inflammation, the neutrophil-to-lymphocyte ratio (NLR), for example, can be applied to measure the balance between the detrimental effects of neutrophils and the beneficial effects of lymphocyte-mediated adaptive immunity (Howard et al., 2019).

In cancer-related inflammation, platelets might participate by increasing angiogenesis and releasing growth factors (Kim et al., 2015). Tumor cells also activate platelets to shield them from blood shear forces and natural killers (Palacios-Acedo et al., 2019), hence platelet-related markers, such as the platelet to lymphocyte ration (PLR) and the systemic immune-inflammation index (SII), might be useful in determining cancer diagnosis and prognosis (Tangthongkum et al., 2017; Diao et al., 2019).

High PLR was associated, for example, to recurrence and poor survival in oral cancer patients (Tangthongkum et al., 2017). High NLR, in turn, was associated to nodal metastasis and poor overall survival rates (Yang et al., 2018). It also correlated to tumor invasion and was used with depth of invasion (DOI) to predict neck metastasis (Ventura et al., 2021; Salzano et al., 2022). In fact, patients with pretreatment NLR values above 2.93-2.96 have a higher risk for occult metastasis and are therefore candidates for elective neck dissection (Ventura et al., 2021; Salzano et al., 2022). These reports briefly illustrate how peripheral blood parameters might aid in oral cancer prognosis as well as in treatment planning.

In this matter, investigating peripheral blood and systemic inflammatory biomarkers in experimental models for oral squamous cell carcinoma (OSCC) can help elucidate how changes in these parameters are related to tumor development and progression, and to antineoplastic therapy. Nitric oxide (•NO) inhibition has been considered as an ancillary treatment for certain cancer cells (Girotti, 2020), and its role in OSCC progression is evidenced in the literature (Servato et al., 2019). Considering the role of iNOS in tumor-associated inflammation, the aim of this study was to verify whether its absence would

alter peripheral blood biomarkers and their association with tumor invasion in experimental OSCC.

Materials and methods

Study design and ethical issues

This *in vivo* study was approved by the institutional Ethics Commission on Animal Use (#100/18). Experiments were conducted as previously described (Tang et al., 2004; de Faria et al, 2011). Briefly, 15 C57BL/6J ($Nos2^{+/+}$) and 16 B6.129P2-$Nos2^{tm1Lau}$/J ($Nos2^{-/-}$) male mice were treated with the chemical carcinogen 4-nitroquinoleine-1-oxide (4NQO) in the drinking water at 50µg/mL for 16 weeks, whilst controls for each strain (n=15) were treated with vehicle propylene glycol (PPG). Animals were then observed for 8 weeks and submitted to euthanasia and sample collection for histopathological analyses and hematological profiling. Weight and water intake of the animals were measured weekly.

Blood sample collection

Peripheral blood samples were collected at the end of the 24-week experiment period from the following groups: $Nos2^{+/+}$/4NQO (n=11), $Nos2^{+/+}$/PPG (n=9), $Nos2^{-/-}$/4NQO (n=15), and $Nos2^{-/-}$/PPG (n=9). After anesthesia with intraperitoneal administration of ketamine (100mg/kg) and xylazine (10mg/kg), approximately 1mL was collected into ethylenediaminetetraacetic acid (EDTA) tubes from the retro-orbital plexus, as proposed by Parasuraman et al. (2010). Animals were then submitted to cervical dislocation and necropsy.

Hematological profiling

Samples were processed in auto-hematology analyzer Hematoclin 2.8 VET (Quibasa-Bioclin, Belo Horizonte, Brazil). Red blood cell count (RBCC), hemoglobin (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBCC), platelet cell count (PCC) and RDW (red blood cell distribution) values were obtained. Manual differential leukocyte counts were performed on blood smears stained with panoptic fast stain kit (LaborClin, Santa Bárbara, Brazil).

Systemic inflammation markers based on the blood cell counts were also calculated, including NLR, PLR, and SII. The NLR was calculated dividing the manual differential neutrophil count (MDNC) by the manual differential lymphocyte count (MDLC) (Kao et al., 2018; Li et al., 2021). We calculated the PLR dividing the PCC by the MDLC (Li et al., 2021). SII was obtained multiplying the MDNC by the PCC, and then dividing the result by the MDNC (Wang and Zhu, 2019; Li et al., 2021).

Histopathological analyses

Tongues were collected at necropsy, sectioned in the midline, fixed in paraformaldehyde 7% for 24 hours, and embedded in paraffin. Hematoxylin and eosin-stained slides were then analyzed by two pathologists to identify areas of epithelial dysplasia (ED) and oral squamous cell carcinoma (OSCC) in 4NQO-treated mice, as proposed by Tang et al. (2004). Regions of the tongue surface lined with either atrophic or hyperplastic epithelium showing alterations in the tissue architecture and cell differentiation were

considered dysplastic. OSCC was identified in areas of malignant neoplastic invasion manifested in the form of broad pushing borders, microinvasion spots with ill-defined basement membrane, or more frankly invasive epithelial nests. We considered the worst lesion in the mouse tongue as the outcome measure. The depth of invasion (DOI) of all OSCC was measured as proposed by Müller et al. (2018) in ImageScope software (Leica Biossystems, Nussloch, Germany) after H&E-stained slides were scanned in 400x magnification with Aperio AT2 (Leica Biossystems, Nussloch, Germany). In summary, a line was drawn in parallel to the basement membrane from one healthy margin to the other, and the distance between this line and the deepest part of the tumor was considered the DOI.

Statistical analyses

All analyses were carried out in GraphPad Prism 8.2.1 (GraphPad, San Diego, California, USA), with α=5%. Differences in the weight, water intake and hematological parameters between experimental and control groups were verified with Kruskal-Wallis and Dunn's *post hoc* test. Comparisons between $Nos2^{+/+}$ and $Nos2^{-/-}$ animals with ED and OSCC were tested with unpaired *t* test and Mann-Whitney's. Correlations between DOI and the hematological parameters were verified with Spearman's ρ (rho).

Results

Among the experimental animals from which blood samples were collected and analyzed, 72% (n=8/11) of the $Nos2^{+/+}$ and 80% (n=12/15) of the $Nos2^{-/-}$ mice had OSCC. The remaining animals had only epithelial dysplasia. A total of 11 OSCC were identified in

$Nos2^{+/+}$ mice, for three of them had two distinct malignant lesions. This was observed in only one $Nos2^{-/-}$ mouse. Median (range) of the DOI was 322.7 μm (927.6) in $Nos2^{+/+}$ carcinomas and 141.3 μm (458.7) in $Nos2^{-/-}$. This difference was statistically significant (Mann-Whitney U test, p=0.009). The median (range) water intake of the animals treated with 4NQO, 204.3mL (83.3mL) in $Nos2^{+/+}$ and 194.3mL (126.3 mL) in $Nos2^{-/-}$, was significantly lower when compared to controls, 235.3mL (60.2mL) and 214.7mL (58.3mL), respectively (Kruskal-Wallis with Dunn's test, p<0.001).

Table 1 describes the red series and hematimetric indices obtained from blood samples of $Nos2^{+/+}$ and $Nos2^{-/-}$ animals. The RBCC was significantly higher in $Nos2^{+/+}$ mice treated with 4NQO when compared to control, and a similar finding was observed in $Nos2^{-/-}$ for MCHC. The inverse occurred with MCV and MCH in both strains. The PCC was higher in $Nos2^{-/-}$ treated mice than in $Nos2^{+/+}$, although no difference was found in comparison to controls. Additionally, MDNC, NLR, PLR and SII were significantly higher in the experimental groups, whilst only MDLC reduced (Table 2).

There were significant differences in the hematological parameters when control, ED and OSCC groups of both strains were analyzed (Figures 1 and 2). Comparisons for OSCC between the strains showed that MCV was higher (Mann-Whitney U test, p=0.04) and the PCC lower (Unpaired t test, p<0.01) in $Nos2^{+/+}$ mice. Regarding ED, however, there were no statistical differences. Correlations were found between DOI and the following hematological parameters for $Nos2^{-/-}$ mice: MDNC (ρ=-0.68, p=0.017), MDLC (ρ=0.72, p=0.011), NLR (=-0.65, p=0.025), PLR (ρ=-0.73, p=0.013), and SII (ρ=-0.67, p=0.037).

Discussion

In this study, we analyzed the hematological profile of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice treated with 4NQO to investigate whether the absence of iNOS would alter blood parameters and systemic inflammatory markers during oral carcinogenesis. We observed that red blood cell count was significantly higher in $Nos2^{+/+}$ treated mice when compared to controls, as well as in animals with dysplasia and OSCC. Considering that nearly half of the patients with OSCC have anemia (Spanier et al., 2020) and that cancer can directly cause anemia by suppressing hematopoiesis and erythropoiesis (Dicato et al., 2010), this paradoxical increase in erythrogram values might occur due to animal dehydration rather than from erythrocytosis, which can sometimes manifest in the form of a paraneoplastic syndrome. The latter occurs in certain types of cancer, including renal cell carcinoma, hepatocellular carcinoma, cerebellar hemangioblastoma, pheochromocytomas, ovarian tumors, and Wilm's tumors (Hodges et al., 2007), but not in OSCC.

In fact, animals of the experimental groups had lower water intake in comparison to untreated controls. Other exams, however, are necessary to confirm dehydration, usually serum albumin test (Barcessat et al., 2014). In this line, Barcessat et al. (2014) detected increased serum albumin levels following the topical application of 4NQO on the ventral tongue of rats for 16 weeks, which were associated to animal dehydration and hemoconcentration in bloods exams.

Regarding hematimetric indices, lower MCV and MCH were observed in experimental animals of both strains when compared to controls, as well as in OSCC. The opposite was observed in MCHC, but only for $Nos2^{-/-}$ mice. Although our main hypothesis is that

alterations in the red series might be associated to animal dehydration, we cannot reject the possibility that 4NQO directly affects hematopoiesis, for immature red blood cells, mainly micronucleated polychromatic erythrocytes, were found in the bone marrow of female ICR mice following 4NQO treatment (Lu et al., 1990).

In human OSCC, Battathiri et al. (2001) reported that most patients had normal or higher MCH and MCV, but slightly lower MCHC values. According to this author, high MCH and MCV could be related to erythropoietin and folate deficiencies, and that associations between MCH and MCHC with tumor size resulted from compensatory regeneration attempts of the bone marrow in response to tumor-induced hemolysis and anemia. In this case, immature red blood cells (reticulocytes) would also be found in peripheral blood smears, but it did not occur in mice. Further research on the effect of 4NQO treatment in hematopoiesis and erythropoiesis should be performed in this model to better comprehend the alterations of the hematological profile herein reported.

For white series parameters, our data revealed that the number of neutrophils increased from normal mucosa to OSCC, whilst lymphocyte counts decreased. In fact, neutrophilia and lymphocytopenia are common features of the cancer-associated inflammation, in which neutrophil recruitment enhances tumorigenesis and the lower number of lymphocytes reflects a reduced adaptive immune response against malignant cells (Howard et al., 2019). The interplay between the damage caused by neutrophilia and the protective effect of the lymphocytes can be measured by the NLR, which was also higher in carcinomas than in normal mucosa in both strains.

We have also found that tumor invasion was lower in $Nos2^{-/-}$, which showed strong but inverse correlations with neutrophil count and NLR. In general, tumor invasion has been associated to neutrophil infiltration and high NLR in OSCC (Glogauer et al., 2015; Goertzen et al., 2018), where neutrophils might increase tumor invasiveness by activating invadopodia and matrix degradation through a paracrine loop stimulation between inflammatory and tumor cells (Glogauer et al., 2015). According to Goertzen et al. (2018), neutrophils secrete TNFα and induce PI3K and Src-kinase activity, which upregulates several invasion genes, such as MMP-9 and IL-8. In this context, we hypothesize that neutrophil-stimulated tumor invasion might be iNOS-dependent, for in the absence of this enzyme epithelial invasion was less prominent and not directly associated to neutrophil counts. Since •NO synthesis by iNOS is upregulated by TNFα and •NO-induced PTMs have been shown to participate in MMP-9 activation, it is possible to consider that iNOS is involved in the TNFα-signaled OSCC invasion.

Despite the significantly lower MDLC that we found in 4NQO-treated and OSCC-affected mice, this systemic inflammatory marker positively correlated to DOI in $Nos2^{-/-}$ mice. This might be interpreted either as a lymphocyte-mediated adaptive response to tumor growth, or to a pro-tumorigenic role of lymphocytes in oral carcinogenesis. This event has been described by Roberts et al. (2007) in MBA/PMA-induced skin cancers, who identified a tumor-infiltrating T lymphocyte population with activated cell receptor αβ+CD8+CD44+CD62L. These cells express pro-inflammatory cytokines IFN-γ, TNF-α, and COX-2, but not perforin, and hence have a more cytokine-expressing, non-cytolytic function that could promote tumor growth and invasion instead of the expected cytotoxicity. Also, •NO inhibits T-cell proliferation (Liew et al., 1995), what might

explain in part the positive correlation between MDLC and DOI in $Nos2^{-/-}$ mice. Since our study lacks further evidence pointing towards the anti-tumor or tumor-promoting effect of lymphocytes after iNOS inhibition, the role of these inflammatory cells in induced carcinogenesis remains unclear.

In terms of platelet cell parameters, we have found a higher PCC in $Nos2^{-/-}$ carcinomas when compared to normal mucosa, which is in line with the findings of human OSCC (Tangthongkum et al., 2017; Diao et al., 2019). Our higher PCC in $Nos2^{-/-}$ treated when compared to $Nos2^{+/+}$ mice might be explained by an increase in platelet aggregation due to the lack of •NO, although platelet function tests should be performed to confirm this hypothesis. Furthermore, PLR and SII also inversely correlated to DOI in $Nos2^{-/-}$ mice. The relationship between these cells and tumor invasion is well established, for it also depends on platelet activation and consequent release of growth factors (Kim et al., 2015). In similarity to what was observed in MDNC and NLR, platelet-mediated tumor invasion might be impaired in $Nos2^{-/-}$ mice. We suggest that high platelet aggregation might not suffice to promote invasion in the lack of •NO, who is responsible for activating other invasion-related molecules. There are no other studies in this regard, warranting further investigation on the relationship between •NO, platelet cell parameters and tumor invasion in OSCC.

In conclusion, our results suggest that iNOS has an important role in oral squamous cell carcinoma invasion, which could be associated to systemic alterations in immune-inflammatory cell dynamics evidenced by peripheral blood biomarkers.

Tables

Table 1. Red series and hematimetric indices of *Nos2*$^{+/+}$ and *Nos2*$^{-/-}$ mice.

Parameters	Experimental groups, median (range)				*p*-value[8]
	Nos2$^{+/+}$/4NQO[6]	Nos2$^{+/+}$/PPG[7]	Nos2$^{-/-}$/4NQO[6]	Nos2$^{-/-}$/PPG[7]	
RBCC[1] x (10^6/uL)	10.8 (2.1)^{a}	9.2 (3.6)^{b}	10.5 (7.0)^{ab}	9.0 (4.4)^{b}	<.01
Hemoglobin (g/dL)	15.3 (4.0)^{a}	13.8 (4.6)^{ab}	14.3 (10.0)^{ab}	13.2 (3.9)^{b}	.03
Hematocrit (%)	45.6 (9.2)^{a}	42.7 (13.4)^{ab}	43.6 (26.6)^{ab}	42.4 (10.2)^{b}	.02
MCV[2](fL)	43.3 (4.0)^{a}	45.8 (4.7)^{b}	42.1 (10.2)^{a}	45.9 (14.4)^{b}	<.01
MCH[3](pg)	14.0 (2.0)^{a}	14.9 (1.0)^{b}	13.8 (2.2)^{a}	14.4 (3.4)^{b}	<.01
MCHC[4](g/dL)	32.4 (7.2)^{ab}	32.0 (2.7)^{ab}	32.8 (3.2)^{a}	31.5 (3.7)^{b}	<.01
RDW[5](%)	14.9 (2.3)^{a}	15.4 (1.3)^{a}	15.4 (9.9)^{a}	15.8 (2.2)^{b}	.02

[1]RBCC: red blood cell count; [2]MCV: mean corpuscular volume; [3]MCH: mean corpuscular hemoglobin; [4]MCHC: mean corpuscular hemoglobin concentration; [5]RDW: red cell distribution width; [6]4NQO: 4-nitroquinoline N-oxide; [7]PPG: propylene glycol; [8]Kruskal-Wallis with Dunn's *post hoc* test, $p<0.05$.

Table 2. White series and platelet cell indices of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice.

Parameters	Experimental groups, median (range)				*p*-value[8]
	$Nos2^{+/+}$/4NQO[6]	$Nos2^{+/+}$/PPG[7]	$Nos2^{-/-}$/4NQO[6]	$Nos2^{-/-}$/PPG[7]	
WBCC[1] x (10^3/uL)	6.0 (9.6)[a]	8.0 (9.0)[a]	7.4 (19.4)[a]	9.7 (9.7)[a]	ns
Neutrophils (%)	50 (43)[a]	17 (32)[b]	50 (47)[a]	20 (18)[b]	<.01
Lymphocytes (%)	47 (39)[a]	80 (36)[b]	47 (46)[a]	79 (24)[b]	<.01
PCC[3] X (10^3/ uL)	1731 (1483)[a]	1367 (1518)[a]	2804 (1801)[b]	2034 (846)[ab]	<.01
NLR[2]	1.1 (2.1)[a]	0.2 (0.8)[b]	1.0 (4.5)[a]	0.3 (0.3)[b]	<.01
PLR[4]	56.5 (50.6)[ab]	18.6 (36.6)[a]	54 (144.4)[b]	25.5 (12.3)[a]	<.01
SII[5]	1768 (3462)[a]	282 (1802)[b]	2422 (8256)[a]	432 (643)[b]	<.01

[1]WBCC: white blood cell count; [2]NLR: neutrophil-to-lymphocyte ratio; [3]PCC: platelet cell count; [4]PLR: platelet-to-lymphocyte ratio; [5]SII: systemic inflammatory-immune index; [6]4NQO: 4-nitroquinoline N-oxide; [7]PPG: propylene glycol; [8]Kruskal-Wallis with Dunn's *post hoc* test, $p<0.05$; ns: not significant.

Figure captions

Figure 1. Comparative analyses for red blood cell and platelet cell parameters in normal mucosa (NM), epithelial dysplasia (ED) and squamous cell carcinoma (SCC) samples from $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. Graphs (a) to (e) are scatter plots for red blood cell count (RBCC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet cell count (PCC) of $Nos2^{+/+}$ mice. Graphs (f) to (j) represent the same data for $Nos2^{-/-}$ mice. Kruskal-Wallis with uncorrected Dunn's *post hoc* test, $p<0.05$.

Figure 2. Comparative analyses for white blood cell parameters in normal mucosa (NM), epithelial dysplasia (ED) and squamous cell carcinoma (SCC) samples from $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. Graphs (a) to (e) are scatter plots for manual differential neutrophil count (MDNC), manual differential lymphocyte count (MDLC), neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) of $Nos2^{+/+}$ mice. Graphs (f) to (j) represent the same data for $Nos2^{-/-}$ mice. Kruskal-Wallis with uncorrected Dunn's *post hoc* test, $p<0.05$.

Figure 3. Correlations between depth of invasion (DOI) and systemic inflammatory markers in $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. Graphs (a) to (e) are scatter plots for manual differential neutrophil count (MDNC), manual differential lymphocyte count (MDLC), neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) of $Nos2^{+/+}$ mice. Graphs (f) to (j) represent the same data for $Nos2^{-/-}$ mice. Correlations were found between DOI and MDNC (ρ=-0.68,

p=0.017), MDLC (ρ=0.72, p=0.011), NLR (=-0.65, p=0.025), PLR (ρ=-0.73, p=0.013), and SII (ρ=-0.67, p=0.037). Spearman's correlation test, $p<0.05$.

Figure 1

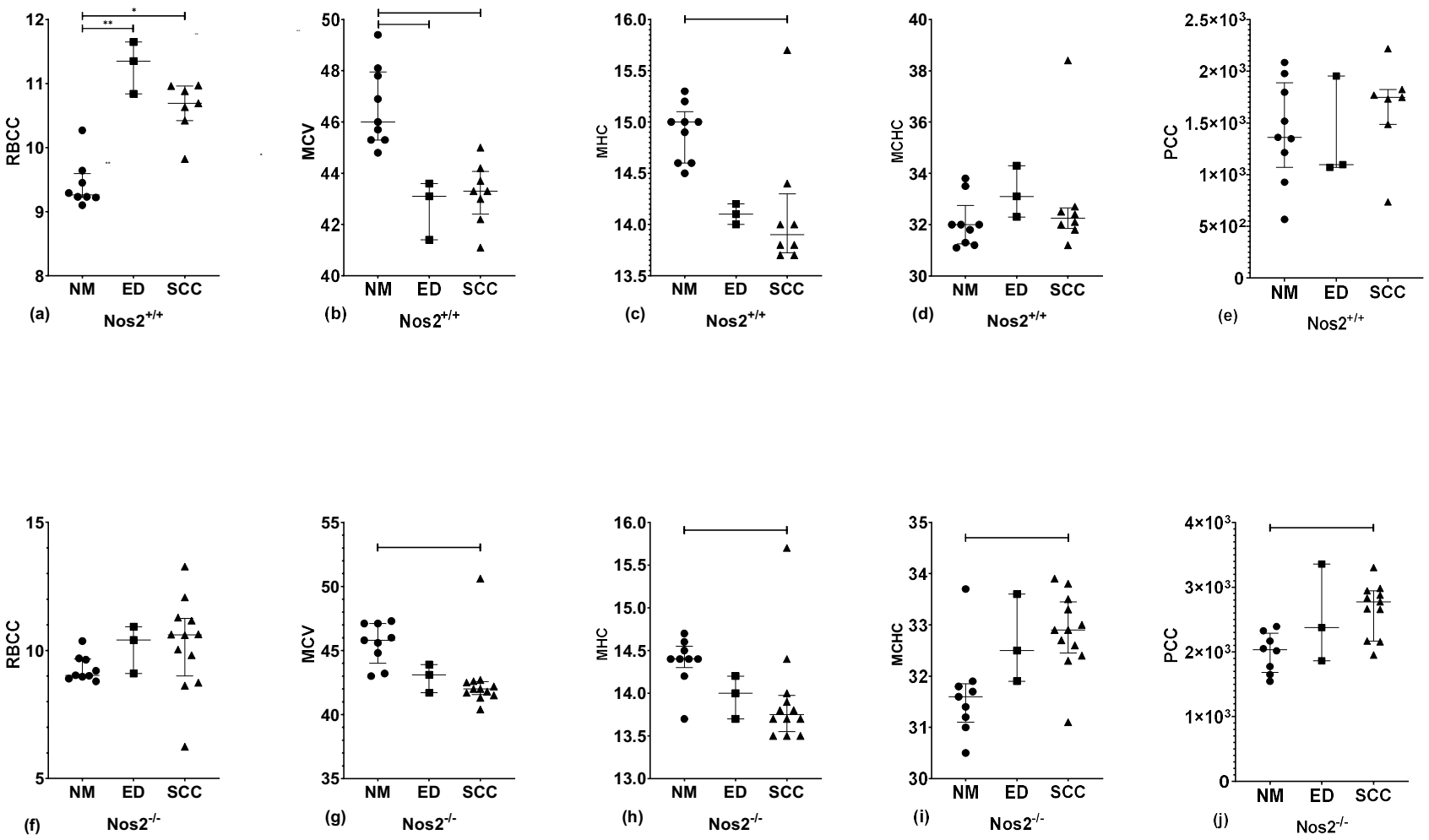
RBCC
MCV
MHC
MCHC
PCC
NM
ED
SCC
(a) Nos2+/+
(b) Nos2+/+
(c) Nos2+/+
(d) Nos2+/+
(e) Nos2+/+
(f) Nos2-/-
(g) Nos2-/-
(h) Nos2-/-
(i) Nos2-/-
(j) Nos2-/-

Figure 2

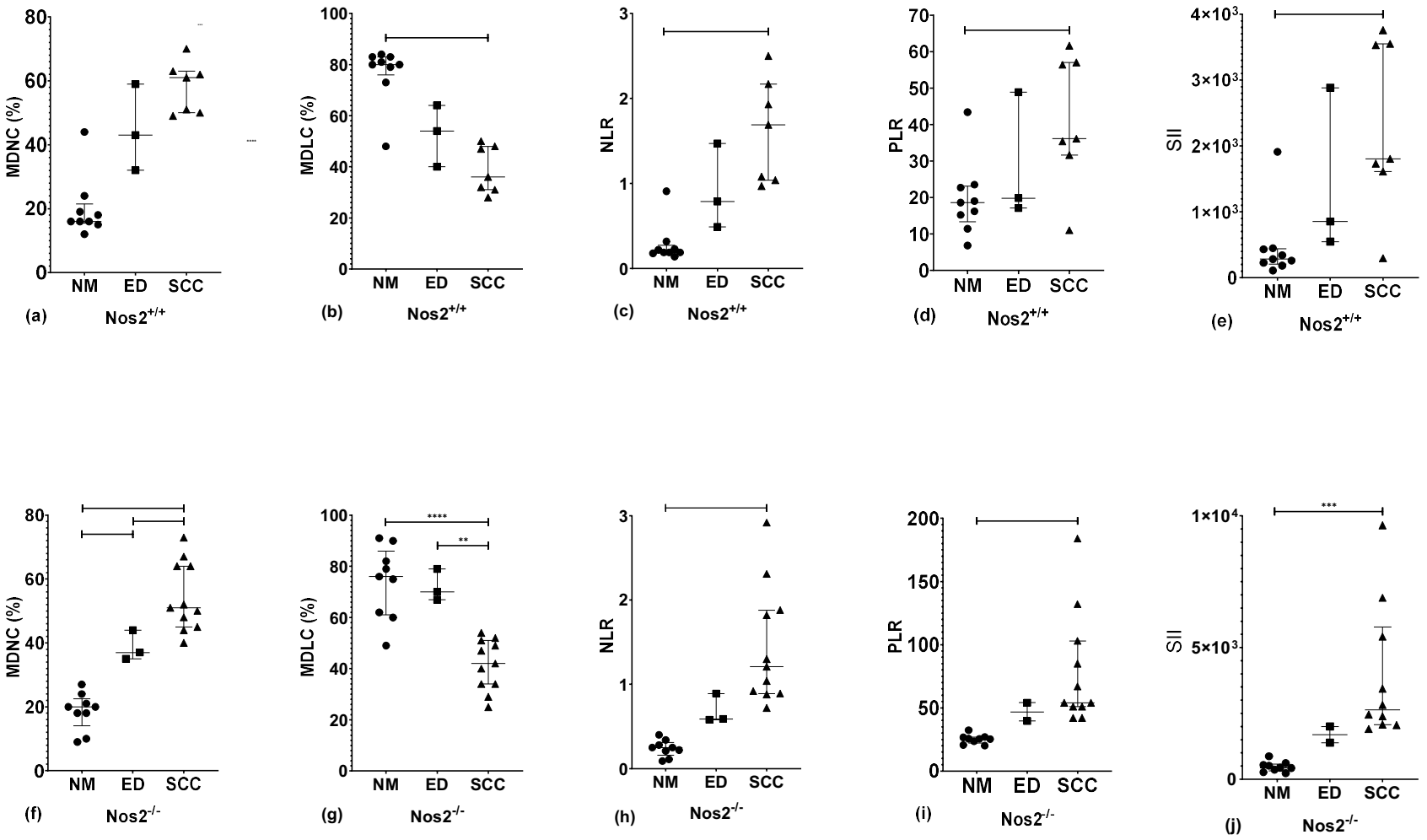
MDNC (%)
MDLC (%)
NLR
PLR
SII
NM
ED
SCC
(a) Nos2+/+
(b) Nos2+/+
(c) Nos2+/+
(d) Nos2+/+
(e) Nos2+/+
(f) Nos2-/-
(g) Nos2-/-
(h) Nos2-/-
(i) Nos2-/-
(j) Nos2-/-
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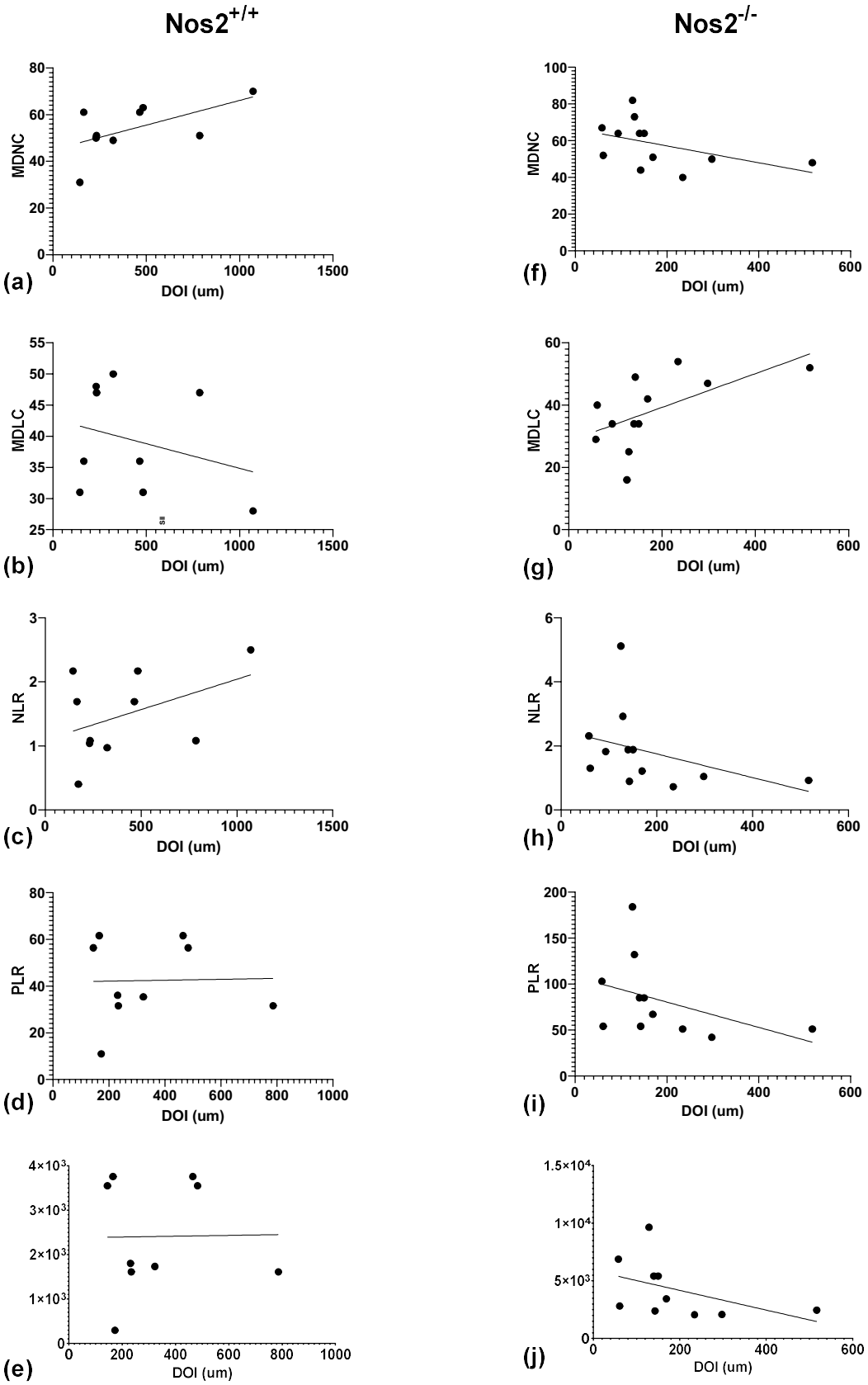
Figure 3



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4. CAPÍTULO 3

Journal: Applied Immunohistochemistry and Molecular Morphology

Title: Immunohistochemical expression of H3K9ac and H3K27ac in premalignant and malignant tongue lesions of wild-type and Nos2-knockout mice treated with 4NQO.

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ABSTRACT

Nitric oxide is an important regulator of the epigenetic landscape of cellular homeostatic and pathological states, in which post-translational modifications of the epigenome-modulating enzymes are the most well described mechanism. Considering that alterations of the histone acetylation pattern were associated to oral cancer development and progression, the purpose of this study was to analyze the immunohistochemical expression of H3K9ac and H3K27ac at different stages of oral carcinogenesis induced by 4-nitroquinoline-N-oxide (4NQO) in $Nos2^{+/+}$ (wild-type) and $Nos2^{-/-}$ (knockout) mice. C57BL/6J and B6.129P2-$Nos2^{tm1Lau}$/J mice were treated with 4NQO in the drinking water at 50μg/mL for 16 weeks and observed for 8 weeks. Tongues were submitted to histopathological analysis and immunohistochemistry for H3K9ac and H3K27ac expression. The antigen-antibody reaction was analyzed with quickscore (QS). Both histone acetylation marks were expressed in the normal epithelium. QS values were higher in moderate dysplasia of $Nos2^{-/-}$ mice (p=0.025) when compared to $Nos2^{+/+}$, and mild dysplasia had lower values for H3K9ac when compared to moderate and severe dysplasia in $Nos2^{-/-}$ group (p=0.015). H3K27ac significantly increased from normal mucosa to mild dysplasia in $Nos2^{+/+}$ mice (p=0.007). Additionally, $Nos2^{+/+}$ mice had a higher number of H3K27ac-positive mild dysplasias when compared to $Nos2^{-/-}$ (p=0.023). We concluded that the pattern of histone acetylation changes in murine oral carcinogenesis, mainly when the epithelial lining of the tongue becomes dysplastic, and that such epigenetic modifications might be iNOS-mediated.

Keywords: precancerous conditions, carcinogenesis, epigenetic process, histones, immunohistochemistry.

INTRODUCTION

In the past decade, nitric oxide (•NO) has emerged as an important regulator of the epigenetic landscape in both cellular homeostatic and pathological states[1-5]. The already multifaceted role of •NO in cell signaling also comprehends epigenetic alterations, i.e. DNA methylation, histone post-translational modifications (HPTM), and non-coding RNA expression[1-5]. Although •NO was shown to up and/or downregulate several well-known epigenetic markers, such as 5-methylcytosine, H3K9me2, H3K9ac and miRNA-21, most of the related mechanisms are still elusive[3].

Post-translational modifications of the epigenome-modulating enzymes are currently the most well described mechanism for epigenetic regulation by •NO, which might result from both direct and indirect effects, such as nitrosylation of lysine demethylases (KDM)[6] and *S*-nitrosation of histone deacetylases (HDAC), respectively[7], resulting in activity inhibition. Histone acetyltransferases (HAT) are also stimulated by nitric oxide, as shown in oral cancer cell lines and tissue, where histone hyperacetylation occurs following •NO-stimulated nucleophosmin (NPM1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, which activate histone acetyltransferase p300 (HAT p300)[8].

Taken together, these findings suggest an important role of •NO in the interplay between HAT and HDAC[3,?]. In fact, *in vitro* studies demonstrated that •NO exposure significantly modified the acetylation patterns of several lysine residues, such as H3K9, H3K27, H3K36 and H4K20, which have critical gene regulatory functions and were detected at the promoter regions of many genes involved in carcinogenesis[2,6].

Since the expression of inducible nitric oxide synthase (iNOS) increases from normal mucosa to oral pre-malignant and malignant lesions[9-13], changes in their histone acetylation patterns might therefore be •NO-regulated. H3K9ac expression, for example, reduces from oral leukoplakia (OL) to oral squamous cell carcinoma (OSCC)[14], and high H3K9ac-expression was associated to lymph node metastasis and relapse[15]. The number of H4K12ac-positive cells, in turn, was higher in OL when compared to normal mucosa[16]. In lip carcinogenesis, H4K12ac and H3K9ac significantly reduced from normal lip tissue to actinic cheilitis[17]. Despite the current evidence supporting the prognostic value of •NO-related markers[12] and histone modifications in oral cancer[14], the literature is still in lack for studies analyzing the associations between them.

In this context, the aim of this study was to analyze the immunohistochemical expression of histone acetylation marks H3K9ac and H3K27ac in 4NQO-induced pre-malignant and malignant oral lesions in wild-type and Nos2-knockout mice.

MATERIALS AND METHODS

Study design

This study was approved by the Ethics Commission on Animal Use of the Federal University of Uberlândia (#100/18). Experimental units consisted of *Mus musculus* male mice from two strains: 30 C57BL/6J (wild-type, $Nos2^{+/+}$) and 31 B6.129P2-$Nos2^{tm1Lau}$/J (Nos2-knockout, $Nos2^{-/-}$). Animals were treated with 4-nitroquinoline N-oxide (4NQO, N8141, Sigma-Aldrich, San Louis, USA) in the drinking water at 50µg/mL for 16 weeks, as previously described (Capítulo 1). Control groups for each strain (n=15) were treated

with propylene glycol at 5mg/mL (Labsynth, São Paulo, Brazil) used in 4NQO dilution. Tongues were collected after 8 weeks of observation (24 weeks after commencing the 4NQO treatment), fixed for 24h in paraformaldehyde 7%, and then embedded in paraffin. Tissue sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) for histopathological analyses.

Histopathological classification

Two pathologists classified the lesions in digital slides stained with hematoxylin and eosin (HE) as proposed by Tang et al. (2004). Regions of epithelial hypotrophy, hyperplasia and hyperkeratosis with alterations of the tissue architecture and cell differentiation were regarded as epithelial dysplasia (ED), which was further graded into mild (ML), moderate (MD), or severe (SD) based on the affected epithelial layers[18]. Microinvasive malignant lesions with small infiltrating nests, exophytic tumors with broad pushing borders, along with more frankly invasive tumor cells penetrating the tongue musculature were classified as oral squamous cell carcinoma (OSCC).

Immunohistochemistry

Sections of 3μm were mounted on Starfrost slides (Waldemar Knittel Glasbearbeitungs GmbH, Bielefeld, Germany) before immunohistochemistry (IHC). The reactions were manually performed using Starr Trek Universal HRP Detection System (Biocare Medical, Concord, CA, USA). Tongue sections of 15 $Nos2^{+/+}$ and 16 $Nos2^{-/-}$ mice treated with 4NQO were included in the assay, along with 10 animals of each control, so IHC reactions could be performed in a single day to avoid variations in the assay. After deparaffinization and rehydration, we conducted a heat-mediated antigen retrieval with Tris/EDTA pH 9.0

buffer with Tween 20 in three cycles of five minutes in the microwave oven. Intrinsic avidin and biotin activity were blocked as proposed by Miller *et al.*[19]. Blocking of endogenous peroxidase was performed with three baths of 15 minutes in a solution of hydrogen peroxide 5V and methanol (1:1). Slides were incubated for 30 minutes at room temperature with the following rabbit monoclonal primary antibodies: H3K9ac (Abcam, ab32129, 1:200), and H3K27ac (Abcam, ab177178, 1:10,000). Positive controls consisted of human normal colon (H3K9ac) and prostate cancer tissues (H3K27ac). Negative controls slides were incubated with Tris-HCl buffer only.

Immunohistochemical analysis

Slides were scanned with Aperio AT2 (Leica Biossystems Imaging, Nussloch, Germany) at 400x magnification. For each histological type, one to five areas of 1366 x 670 pixels were selected in QuPath (Quantitative Pathology & Bioimage Analysis, University of Edinburg). In control samples, five areas of the tongue dorsum were analyzed in each animal. Semiquantitation of the nuclear immunostaining was performed with the quickscore (QS) method proposed by Detre *et al.*[20]. Intensity was scored as negative (0), weak (1), intermediate (2) or strong (3), and the proportion of positive cells as 1-4% (1), 5-19% (2), 20-39% (3), 40-59% (4), 60-79% (5), or 80-100% (6). Intensity and proportion scores for each area were multiplied, and the median QS was considered the outcome measure, ranging from 1 to 18.

Samples with tissue processing, sectioning, histochemical, and slide scanning artefacts were excluded from IHC analyses. For H3K9ac expression, areas from 17 $Nos2^{+/+}$ and 22 $Nos2^{-/-}$ treated mice were evaluated. For H3K27ac expression, samples from 20 $Nos2^{+/+}$

and 21 $Nos2^{-/-}$ treated animals were included in the analysis. Table 1 shows the number of areas analyzed in each mouse strain, for each antibody.

Data analyses

All analyses were performed in GraphPad Prism software version 5.01 (GraphPad, San Diego, California, USA) with alpha value set at 5%. Quickscores were compared within each mouse strain using Kruskal-Wallis with Dunn's post-hoc test. Intragroup differences for each histological class were verified with Mann Whitney's U test. Chi-square test verified differences in the frequency distribution of H3K27ac-negative and positive cases.

RESULTS

Immunohistochemical expression of H3K9ac

Figure 1 shows the pattern of immunohistochemical reactivity for both PTHM in normal mucosa, epithelial dysplasia, and squamous cell carcinoma of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. In the normal mucosa of the mouse tongue, immunoreactivity for H3K9ac varied from weak to moderate intensity in the nuclei of keratinocytes. Positive cytoplasmic staining was observed in a few samples, usually with weak to moderate intensity. The entire epithelial lining exhibited H3K9ac-positive cells, although the basal cell layer usually had more negative cells. Areas of filiform and fungiform papillae were frequently positive.

In epithelial dysplasia and squamous cell carcinoma of mice, intensity of staining varied from weak to strong in the nuclei, and weak to moderate in the cytoplasm. Positive cells were diffusely distributed within the lesions. Negative cells were more frequently

observed in the basal and parakeratin layers of dysplasia, whilst some tumors showed immunoreactivity in some areas and no reactivity in others.

Immunohistochemical expression of H3K27ac

In the normal epithelial lining of the tongue, intensity of staining was weak in the nuclei of keratinocytes, mainly of those located in filiform papillae of the posterior dorsum. In contrast to control samples, which have all shown some level of positivity, there were cases of epithelial dysplasia and squamous cell carcinomas negative for H3K27ac. Once again, the basal layer had the lowest proportion of positive cells, and diffuse reactivity was observed in the upper layers.

Table 2 shows the QS values obtained for each histological class in $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. A significant difference was observed for H3K9ac only in moderate dysplasia, whose QS values were higher in the $Nos2^{-/-}$ group (Mann-Whitney U test, p=0.025). Additionally, intragroup comparisons showed that mild dysplasias had significant lower QS values when compared to moderate and severe dysplasias in $Nos2^{-/-}$ animals (Kruskal-Wallis with Dunn's post-hoc test, p=0.015). Regarding H3K27ac quickscores, there were no significant differences between the strains. Intragroup comparisons showed that mild dysplasia had significant higher QS values when compared to normal mucosa in $Nos2^{+/+}$ group (Kruskal-Wallis with Dunn's post-hoc test, p=0.042). Additionally, contingency analysis showed that $Nos2^{+/+}$ mice had a higher number of H3K27ac-positive mild epithelial dysplasias when compared to $Nos2^{-/-}$ (Fisher's exact test, p=0.023, Table 3).

DISCUSSION

Acetylation is the main histone post-translational modification and overall epigenetic mechanism responsible for the regulation of chromatin-templated processes, such as nucleosome assembly, chromatin folding, and gene transcription[21]. Lysine residues in core histones are susceptible for acetylation and deacetylation mediated by HAT and HDAC[22], and the impact of these modifications on the cell phenotype may result from either the combined acetylation of certain lysine residues[23], or by the modification of a single residue with site-specific effect[21].

Differences in the biological effect of this HPTM depends not only on the type and number of the amino acid residue, but also on the histone involved. Acetylation of lysine residues 9 and 27 of the histone H3, for example, are well-known marks of promoter and open/active chromatin regions[24]. During carcinogenesis, changes in histone acetylation contribute to the activation of oncogenes or deactivation of tumor suppressors[24]. In fact, several acetylated marks were found to be enriched at the promoter region of cancer-associated genes, especially following •NO administration to cell lines[2].

Since inducible nitric oxide synthase and 3-nitrotyrosine are highly expressed in OSCC[9-12], and that alterations of histone acetylation represent an epigenetic mechanism involved in oral cancer development[14-17], our study aimed to investigate the impact of Nos2 gene knockout in the H3 acetylation pattern of 4NQO-treated mice. We found that H3K9ac and H3K27ac are normally expressed in the mouse tongue, and that differences between the strains were found only for epithelial dysplasia. $Nos2^{+/+}$ mice had more H3K27ac-positive mild dysplasias, and $Nos2^{-/-}$ mice showed higher QS values for H3K9ac in

moderate dysplasia. Regarding the progression of normal mucosa to pre-malignant and malignant lesions, we identified an increase in H3K9ac expression from mild to moderate/severe dysplasia in $Nos2^{-/-}$ mice, and in H3K27ac from normal mucosa to mild dysplasia in $Nos2^{+/+}$ animals.

Increased H3K27ac and H3K9/K14ac expression in 4NQO and ethanol-treated mice was previously described by Urvalek *et al*[25]. All treatment modalities (ethanol, 4NQO, and 4NQO + ethanol) showed an increase in H3K9/K14ac, whereas the 4NQO treatment alone was not sufficient to cause alterations in H3K27ac expression. These authors, however, did not analyze the acetylation pattern based on the histopathological classification of the tongue lesions, but only based on the treatments. To the author's knowledge, this is the first study investigating histone acetylation changes in different stages of chemically induced oral carcinogenesis, as well as in Nos2-knockout animals. Such analysis is difficult and subjective, however, due to the lack of standardized morphological parameters for the grading of epithelial dysplasia in the murine epithelium, as well as in the distinction between severe dysplasia and microinvasive carcinomas.

Our findings suggest that •NO play an important role modulating histone acetylation in early stages of carcinogenesis when the tongue epithelium becomes dysplastic. Our study, however, is limited to a single observation period of 8 weeks following the 16-week treatment with 4-NQO at 50μg/mL. More prominent alterations of histone acetylation profile might be expected in advanced tumors, as reported for humans[14]. In this context, further analyses at 16 weeks following the 4NQO treatment are warranted.

It is important to consider that differences between our results and those of the current literature can be attributed to methodological variations in 4NQO treatment, such as concentration and treatment period. We provided 4NQO diluted in the drinking water at 50μg/mL for 16 weeks, whilst Urvalek *et al*.[25], for example, administered the carcinogen at 100μg/mL for 10 weeks. This reflects the complex relationship between epigenetic modifications and environmental factors, which can be dependent on dose and exposure, thus difficult to perceive at individual levels even under controlled conditions.

Considering the state of the art for the association between •NO and HPTMs, the fact that H3K9ac expression in moderate dysplasia was higher in animals lacking Nos2 was unexpected. Although •NO has been shown to inhibit histone deacetylases activity[7], and activate histone acetyltransferase[8], it is important to consider its effect on other epigenetic modifications and epigenome-modifying enzymes, such as histone methylation and histone demethylases[2,6].

Both permissive (H3K9ac and H3K27ac) and repressive (H3K9me2-3 and H3K27me3) chromatin histone marks are affected by •NO exposure[2]. Since they are mutually excluding, the lower histone acetylation observed in $Nos2^{+/+}$ moderate dysplasia might reflect their lysine methylation status. In fact, Kusumoto *et al.*[26] identified that most OSCC patients had hypoacetilated and dimethylated H3K9, showing that these two histone modifications do not co-occur. In contrast, Urvalek *et al*.[25] reported an increase in H3K9me3 and H3K27me3 following 4NQO and ethanol treatment in C57BL/6J mice, in addition to the increased acetylation marks. Since the analyses of their work were based on treatment groups, not on histopathological diagnosis, we hypothesize that the interplay

between acetylation and methylation of histone lysine residues is distinctively manifested in different stages of oral carcinogenesis, which may vary from animal to animal along the experiment. Further investigation on the expression of H3K9me2, H3K9me3 and H3K27me3 is therefore necessary to clarify their association to acetylation marks and nitric oxide in murine models and human samples.

Although not statistically significant, it was possible to observe in both animals strains an initial increase in the expression of histone acetylation marks from normal mucosa to epithelial dysplasia, and then a decrease from epithelial dysplasia to squamous cell carcinoma. A similar pattern is also described in the work of Webber *et al*.[14], where the percentage of H3K9ac-positive cells in human normal mucosa, oral leukoplakia and OSCC was 82.1%, 94.4% and 78.2%, respectively. The opposite occurs in the acetylation profile of human lip carcinogenesis, in which H3K9ac and H4K12ac significantly reduces from normal tissue to actinic cheilitis, and then increases again from the latter to lip SCC[17]. Such observations indicate that changes in acetylation pattern during carcinogenesis are not unidirectional and might be specific to certain stages of the disease natural history.

The few significant differences in the expression of acetylation marks observed between $Nos2^{+/+}$ and $Nos2^{-/-}$ mouse strains suggest that •NO influence the acetylation pattern of histone H3 only in the development of epithelial dysplasia, and that other NOS isoforms might be involved. Endothelial nitric oxide synthase, for example, has also been detected in OSCC samples and is associated to tumor angiogenesis and metastasis[27-28]. Further

investigation of eNOS expression in 4NQO-induced carcinogenesis might aid in the elucidation of its effect on the histone acetylation profile.

Taken together, our results suggest that the pattern of histone acetylation changes in the course 4NQO-induced murine oral carcinogenesis, with significant alterations detected at early stages, when the epithelial lining of the tongue becomes dysplastic, and that such epigenetic modifications might be mediated by iNOS-produced nitric oxide.

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TABLES

Table 1. Number of areas analyzed in each mouse strain and antibody.

Histopathological class	H3K9ac		H3K27ac	
	$Nos2^{+/+}$	$Nos2^{-/-}$	$Nos2^{+/+}$	$Nos2^{-/-}$
Normal mucosa	8	6	9	6
Epithelial dysplasia	21	24	29	29
Mild	6	7	10	10
Moderate	6	7	6	7
Severe	9	10	13	12
Carcinoma	7	10	8	13

$Nos2^{+/+}$: wild-type mice; $Nos2^{-/-}$: Nos2-knockout mice.

Table 2. Median (range) of the quickscores for the histone acetylation marks in $Nos2^{+/+}$ and $Nos2^{-/-}$ mice treated with 4NQO.

	H3K9ac			H3K27ac		
Histopathological class	$Nos2^{+/+}$	$Nos2^{-/-}$	*p*-value	$Nos2^{+/+}$	$Nos2^{-/-}$	*p*-value
Normal mucosa	8 (15)	11 (12)	0.337	3 (5)	3 (3)	> 0.9
Epithelial dysplasia	15 (15)	12 (15)	0.626	6 (11)	5 (11)	0.570
Mild	17 (14)	5 (8)	0.058	8 (8)	7 (10)	0.777
Moderate	8 (12)	15 (9)	**0.025***	6 (9)	8 (7)	0.892
Severe	18 (14)	16 (15)	0.754	7 (11)	5 (9)	0.830
Carcinoma	8 (14)	12 (15)	0.909	5 (9)	3 (4)	0.321

Mann Whitney U test, $p<0.05$, *statistically significant.

Table 3. Number of negative and positive cases for H3K9ac and H3K27ac in $Nos2^{+/+}$ and $Nos2^{-/-}$ mice treated with 4NQO.

Histopathological class	H3K9ac					H3K27ac				
	$Nos2^{+/+}$		$Nos2^{-/-}$			$Nos2^{+/+}$		$Nos2^{-/-}$		
	P	N	P	N	*p*-value	P	N	P	N	*p*-value
Normal mucosa	8	0	6	0	-	9	0	6	0	-
Mild dysplasia	6	0	7	0	-	8	2	2	8	**0.023***
Moderate dysplasia	6	0	7	0	-	5	1	3	4	0.55
Severe dysplasia	9	0	10	0	-	9	4	6	6	0.42
Carcinoma	7	0	10	0	-	5	3	7	6	>0.99

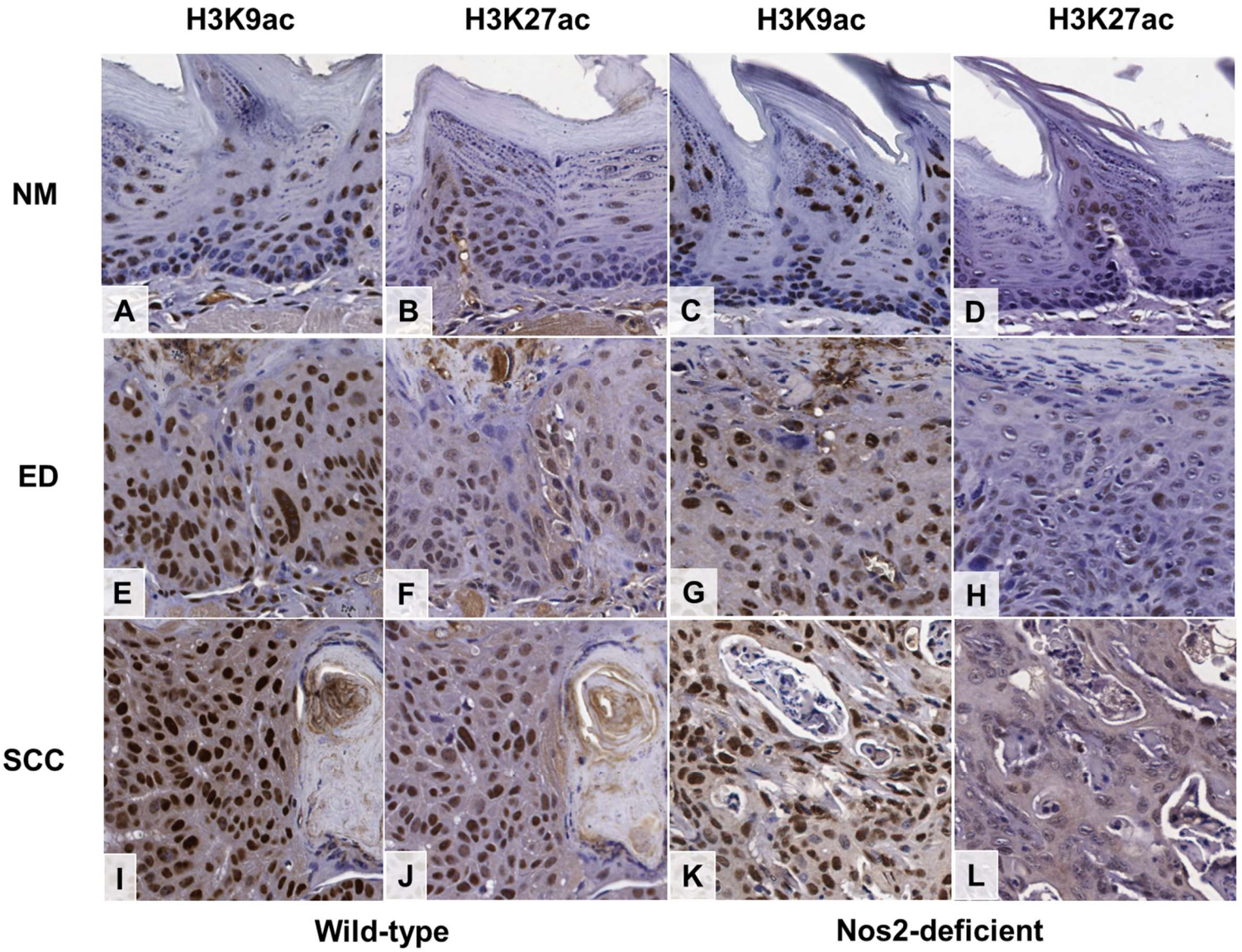
P: positive; N: negative. Fisher's exact test, $p<0.05$, *statistically significant.

FIGURE CAPTIONS

Fig. 1 Photomicrographs of the immunohistochemical expression of H3K9ac and H3K27ac in the murine tongue epithelium. **Figs. 1a to 1d** H3K9ac and H3K27ac expression in the normal epithelial lining of $Nos2^{+/+}$and $Nos2^{-/-}$ mouse tongue. **Figs. 1e to 1h** H3K9ac and H3K27ac expression in severe epithelial dysplasia of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. **Figs. 1i to 1l** H3K9ac and H3K27ac expression in frankly invasive oral squamous cell carcinoma of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice. Hematoxylin and DAB, 400x original magnification.

Fig. 2 Scatter plots of the quickscores for H3K9ac and H3K27ac expression in 4NQO-induced murine oral carcinogenesis. **Figs. 1a and 1b** illustrates the QS values for H3K9ac in normal mucosa (NM), epithelial dysplasia (ED) and tongue squamous cell carcinoma (SCC) of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice, respectively. **Figs. 1c and 1d** illustrates the QS values for H3K9ac in mild (ML), moderate (MD) and severe (SD) epithelial dysplasia of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice, respectively. **Figs. 1e and 1f** illustrates the QS values for H3K27ac in normal mucosa (NM), epithelial dysplasia (ED) and tongue squamous cell carcinoma (SCC) of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice, respectively. **Figs. 1g and 1h** illustrates the QS values for H3K27ac in mild (ML), moderate (MD) and severe (SD) epithelial dysplasia of $Nos2^{+/+}$ and $Nos2^{-/-}$ mice, respectively. Kruskal-Wallis with Dunn's uncorrected *post-hoc* test, $p<0.05$).

Figure 1


H3K9ac
H3K27ac
H3K9ac
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NM
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Wild-type
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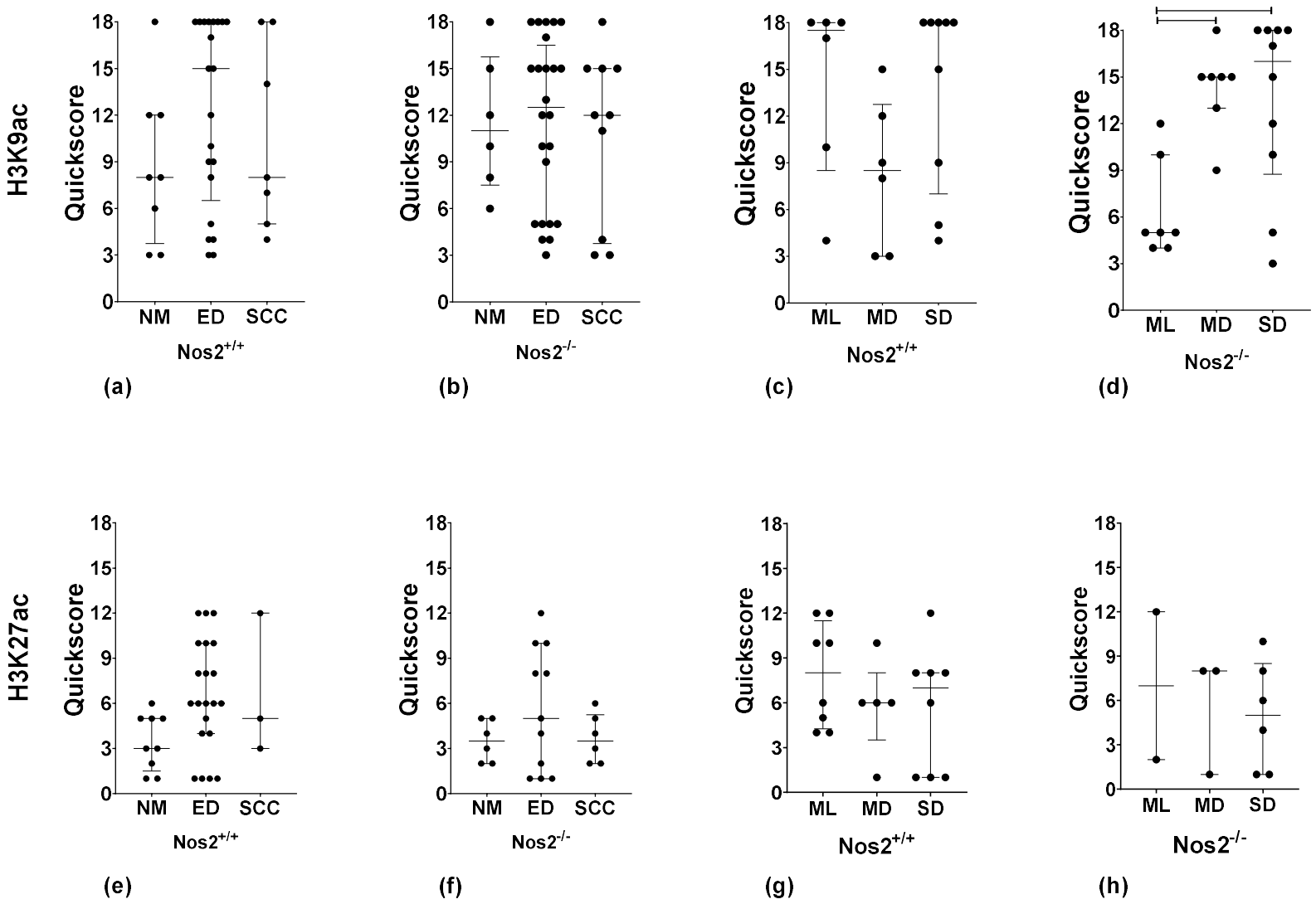
Figure 2


H3K9ac
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Nos2+/+
Nos2-/-
(a)
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(d)
H3K27ac
(e)
(f)
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(h)

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5. CONCLUSÃO

Concluiu-se que a enzima óxido nítrico sintase induzível possui um papel relevante na progressão do carcinoma de células escamosas bucal, pois a sua ausência em modelo animal geneticamente modificado implicou em menor profundidade de invasão nas lesões analisadas. Esse efeito pode estar associado a uma alteração na dinâmica entre células envolvidas na resposta inflamatória e imunológica frente ao tumor, considerando as correlações identificadas entre profundidade de invasão e biomarcadores do sangue periférico nos animais desprovidos da enzima. Embora a participação da iNOS na carcinogênese bucal experimental não seja crítica a ponto de sua ausência reduzir de maneira significativa a incidência de lesões induzidas pelo carcinógeno, esta enzima pode estar associada a alterações epigenéticas necessárias para o desenvolvimento de displasia nos estágios iniciais da tumorigênese epitelial. Tais achados sugerem o óxido nítrico e a enzima óxido nítrico sintase induzível como possíveis alvos moleculares visando a quimioprevenção do câncer bucal, embora mais estudos sejam necessários para melhor determinar as vias de sinalização nas quais possam estar envolvidos.

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ANEXO I

Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –

Comissão de Ética na Utilização de Animais CEUA

CERTIFICADO

Certificamos que o projeto intitulado "Efeito da Óxido Nítrico Sintase Induzível nos Mecanismos Genéticos e Epigenéticos de Diferentes Etapas da Carcinogênese Bucal Experimental Induzida por 4-NQO em Camundongos Nos2+/+ e Nos2-/-", protocolo nº 100/18, sob a responsabilidade de **Adriano Mota Loyola** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião **29 de Março de 2019.**

(We certify that the project entitled "Efeito da Óxido Nítrico Sintase Induzível nos Mecanismos Genéticos e Epigenéticos de Diferentes Etapas da Carcinogênese Bucal Experimental Induzida por 4-NQO em Camundongos Nos2+/+ e Nos2-/-.", protocol 100/18, under the responsibility of Adriano Mota Loyola - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of March 29th, 2019).

Vigência do Projeto	Início: 12/08/2019 Término: 01/02/2021
Espécie / Linhagem / Grupos Taxonômicos	Camundongo isogênico C57BL/6/ Camundongo *Knockout* APPSwDI/NOS2-/-
Número de animais	180
Peso / Idade	20g – 25g/ 6 semanas
Sexo	Macho
Origem / Local	Rede de Biotérios de Roedores da UFU (REBIR-UFU)
Local onde serão mantidos os animais:	Rede de Biotérios de Roedores da UFU (REBIR-UFU)

Uberlândia, 15 de Abril de 2019.

Prof. Dr. Lúcio Vilela Carneiro Girão
Coordenador da CEUA
SEI Nº 1201, DE 12 DE DEZEMBRO DE 2018