UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

LAUREN FRENZEL SCHUCH

IMPACTO DA FOTOBIOMODULAÇÃO E DA TERAPIA FOTODINÂMICA NO PROCESSO DE CARCINOGÊNSE ORAL – UMA OVERVIEW DE REVISÕES SISTEMÁTICAS E UM ESTUDO EM MODELO ANIMAL QUIMICAMENTE INDUZIDO POR 4NQO (4-NITROQUINOLINA 1-ÓXIDO)

IMPACT OF PHOTOBIOMODULATION AND PHOTODINAMIC THERAPY ON THE PROCESS OF ORAL CARCINOGENESIS – AN OVERVIEW OF SYSTEMIC REVIEWS AND AN ANIMAL MODEL STUDY INDUCED BY 4NQO (4-NITROQUINOLINE 1-OXIDE)

Piracicaba
2023

LAUREN FRENZEL SCHUCH

IMPACTO DA FOTOBIOMODULAÇÃO E DA TERAPIA FOTODINÂMICA NO PROCESSO DE CARCINOGÊNSE ORAL – UMA OVERVIEW DE REVISÕES SISTEMÁTICAS E UM ESTUDO EM MODELO ANIMAL QUIMICAMENTE INDUZIDO POR 4NQO (4-NITROQUINOLINA 1-ÓXIDO)

IMPACT OF PHOTOBIOMODULATION AND PHOTODINAMIC THERAPY ON THE PROCESS OF ORAL CARCINOGENESIS – AN OVERVIEW OF SYSTEMIC REVIEWS AND AN ANIMAL MODEL STUDY INDUCED BY 4NQO (4-NITROQUINOLINE 1-OXIDE)

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Estomatopatologia, na Área de Patologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Stomatopathology, in Pathology area.

Orientadora: Profª. Drª. Manoela Domingues Martins

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA LAUREN FRENZEL SCHUCH E ORIENTADO PELA PROFª. DRAª. MANOELA DOMINGUES MARTINS.

Piracicaba

2023

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

Sch78i

Schuch, Lauren Frenzel, 1989-
Impacto da fotobiomodulação e da terapia fotodinâmica no processo de carcinogênese oral - uma overview de revisões sistemáticas e um estudo em modelo animal quimicamente induzido por 4NQO (4-nitroquinolina 1-óxido) / Lauren Frenzel Schuch. – Piracicaba, SP : [s.n.], 2023.

Orientador: Manoela Domingues Martins.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Carcinogênese. 2. Terapia com luz de baixa intensidade. I. Martins, Manoela Domingues, 1973-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações Complementares

Título em outro idioma: Impact of photobiomodulation and photodynamic therapy on the process of oral carcinogenesis - an overview of systematic reviews and an animal model study induced by 4NQO (4-nitroquinoline 1-oxide)
Palavras-chave em inglês:
Carcinogenesis
Low-level light therapy
Área de concentração: Patologia
Titulação: Doutora em Estomatopatologia
Banca examinadora:
Manoela Domingues Martins [Orientador]
Pablo Agustin Vargas
Alan Roger dos Santos Silva
Ronell Eduardo Bologna Molina
Vivian Petersen Wagner
Data de defesa: 26-05-2023
Programa de Pós-Graduação: Estomatopatologia

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0002-0993-936X
- Currículo Lattes do autor: http://lattes.cnpq.br/5040404733798846

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 26 de maio de 2023, considerou a candidata LAUREN FRENZEL SCHUCH aprovada.

PROFª. DRª. MANOELA DOMINGUES MARTINS

PROF. DR. RONELL EDUARDO BOLOGNA MOLINA

PROFª. DRª. VIVIAN PETERSEN WAGNER

PROF. DR. PABLO AGUSTIN VARGAS

PROF. DR. ALAN ROGER DOS SANTOS SILVA

A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

DEDICATÓRIA

Dedico este trabalho aos meus queridos pais, **Beatriz** e **Rony Schuch**, pelo apoio incondicional, pela nossa parceria e pelo amor que me dedicam. Com vocês a meu lado, o mundo se torna um lugar incrível – e possível.

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)** - Código de Financiamento 001 e do **Fundo de Incentivo à Pesquisa e Eventos (FIPE)** - processo: 2021-0614.

Agradeço à **Universidade Estadual de Campinas**, na pessoa de seu Magnífico Reitor, Prof. Dr. Antonio José de Almeida Meirelles.

À **Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas**, na pessoa de seu Diretor, Prof. Dr. Flávio Henrique Baggio Aguiar

Ao **Programa de Pós-Graduação da Faculdade de Odontologia de Piracicaba**, na pessoa de seu coordenador, Prof. Dr. Valentim Adelino Ricardo Barão.

Ao **Programa de Pós-Graduação em Estomatopatologia da Faculdade de Odontologia de Piracicaba**, na pessoa de seu coordenador, Prof. Dr. Pablo Agustin Vargas.

Agradeço à **Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul** pela acolhida durante minha pesquisa.

Ao **Hospital de Clínicas de Porto Alegre** pelo suporte à pesquisa. Em especial à **Unidade de Experimentação Animal** pela estrutura, pelo cuidado com nossos animais e por nos receberem de portas abertas. **Daniela Campagnol**, **Jose Ignacio Solari**, **Marta Cioato**, **Rosalina Braga**, **Sonia Rodrigues, Tuane Garcez** e **Vera Gonçalves,** o resultado desta tese também é de vocês. Obrigada por tudo.

A todos os professores que tive durante toda minha vida.

Às professoras do Centro de Diagnóstico das Doenças da Boca da Universidade Federal de Pelotas, **Adriana Etges**, **Ana Paula Gomes** e **Sandra Tarquinio,** por terem despertado em mim o amor pela estomatologia e pela patologia. Em especial à **Prof.ª Ana Carolina Vasconcelos**, por todas as oportunidades, troca de conhecimento e por ser além de mestre, uma grande amiga.

A todos os professores do Programa de Pós-Graduação em Estomatopatologia da Faculdade de Odontologia de Piracicaba, em especial aos **Prof. Pablo Vargas**, **Prof. Alan Roger Santos-Silva** e **Prof. Marcio Lopes**. É uma honra ser formada por profissionais tão competentes e humanos. Obrigada por todos os ensinamentos e oportunidades.

Ao **Prof. Rogerio Castilho**, pelo auxílio durante a pesquisa.

À **Chris Krebs**, pela gentileza e pelo trabalho impecável no preparo das lâminas histológicas.

À Senhora **Elettra Greene**, pela parceria incansável e pelo rigor profissional em todos os meus trabalhos.

Aos colegas do **Programa de Pós-Graduação em Estomatopatologia** pela parceria e pelas trocas de aprendizado, mesmo que com alguns quilômetros de distância. Obrigada pela festa sempre que nos encontramos.

À equipe de colegas que trabalharam duro durante a execução da etapa experimental da presente tese. Sem vocês, tudo seria mais difícil e sem graça. **Carolina Michel, Daniela Campagnol**, **Grazielle Stelter** e **Tuany Schmidt** obrigada por nunca soltarem minha mão.

A nossos animais, que por algum motivo vieram com a missão de serem instrumentos para melhorarmos a ciência. Meu máximo respeito a esses seres.

Ao grupo de pesquisa ***Would Healing and Oral Cancer*** **(WHOC)**, com quem aprendi que a pesquisa, quando compartilhada, é muito melhor. Ainda mais com tanto amor e cumplicidade. Em especial à **Prof.ª Vivian Wagner**, pela generosidade e pelo auxílio durante esses últimos anos. És uma inspiração.

A **Belkiss Mármora**, **Fernanda Brochado**, **Monica Spiguel** e **Tuany Schmidt**, nossa conexão é indescritível. Com vocês essa jornada ficou muito mais leve.

Às pessoas que conheci em Porto Alegre, e que trouxeram luz para meu caminho. Em especial à **Marlise Tatsch**, pelo apoio e pela proteção.

À **Família Domingues-Martins** por ser a continuação do meu lar. Obrigada por todos os momentos compartilhados, e por ser minha família em Porto Alegre. Isa e Gabi, obrigada por me acolherem. Marco, obrigada pelo carinho e cuidado. Fred, Lola e Anna, pelo amor canino.

À minha orientadora, **Prof.ª Manoela Domingues Martins**. Nada que eu coloque no papel ou em palavras alcançariam toda gratidão que tenho por ti. Obrigada por acreditar em mim, fortalecer-me, moldar-me como pesquisadora e como ser humano. Contigo aprendi muito mais do que ciência, e o laço que formamos é o capítulo mais importante desta tese.

A tantos amigos que a vida me presenteou pelos lugares onde passei e aos que deixei em minha cidade. Levo cada um de vocês em meu coração. Em especial à **Rafaelle Stigger**, um privilégio te ter como melhor amiga há mais de 20 anos.

A **minha família**, com quem aprendo diariamente e a quem sempre posso recorrer. Em especial a meus irmãos, cunhados e sobrinhos. A certeza de que sempre terei para onde voltar é o que me impulsiona a ir cada vez mais longe. Amo vocês infinitamente.

Por fim, a **Deus**, que em sua infinita bondade, permitiu-me trilhar o caminho do conhecimento, fornecendo as ferramentas necessárias e colocando as pessoas certas ao longo dessa jornada.

“O importante na vida é a vida e não o resultado da vida”

Johann Wolfgang von Goethe, por Susan Sontag, em Diários

RESUMO

Nos últimos anos, terapias de luz têm sido introduzidas em diferentes áreas médicas, como tratamento alternativo ou adjuvante em casos de desordens potencialmente malignas e câncer. Apesar do uso clínico difundido da fotobiomodulação (FBM) como terapia preventiva para mucosite oral, seu efeito sobre a mucosa exposta a carcinógenos ainda é pouco compreendido. Em paralelo, a terapia fotodinâmica (TFD) tem sido utilizada como tratamento alternativo no manejo de lesões potencialmente malignas; entretanto, existe uma falta de embasamento pré-clínico sobre os efeitos celulares e moleculares desta terapia. Diante disso, ainda há dúvidas sobre sua segurança durante o processo de carcinogênese oral. Nesse sentido, o presente trabalho teve como objetivo avaliar o efeito de diferentes terapias utilizando luz laser – fotobiomodulação (FBM) e terapia fotodinâmica (TFD) – no desenvolvimento da carcinogênese oral quimicamente induzida. Primeiramente, foi realizada uma revisão sistemática sobre o modelo de carcinogênese oral induzido por 4NQO. Os achados dos estudos incluídos permitiram a construção de um guia de boas práticas com sugestões para futuros trabalhos de 4NQO em ratos Wistar. Em paralelo, foi feita uma Overview a respeito do impacto da TFD em CEC e desordens orais potencialmente malignas (DOPMs). Observou-se que, apesar dos efeitos adequados e seguros da TFD contra DOPM e CEC, seu suporte como tratamento de primeira linha ainda precisa de estudos de coorte mais bem desenhados. O estudo experimental resultou em outros três artigos científicos. Foram utilizados 84 ratos Wistar machos, tratados de forma sistêmica com 4NQO durante 12 e 20 semanas. Todos os animais receberam o carcinógeno 4NQO, e foram divididos em: (1) Grupo controle; (2) Grupo FBM 0.3J; (3) Grupo FBM 1J; e (4) Grupo TFD. As aplicações de FBM ocorreram três vezes na semana, e de TFD, uma vez por semana. A FBM foi realizada com laser de diodo de InGaAlP (660 nm), no modo contínuo e em contato por meio da técnica pontual. A TFD foi realizada com o animal sedado, uma vez por semana, utilizando o fotossensibilizador ácido 5-aminolevuínico (ALA) de forma tópica a 5%. Duas horas após a aplicação do 5-ALA, dois pontos da língua do animal foram irradiados com laser de comprimento de onda de 660 nm com fluência de 90 J/cm2. Os animais foram eutanasiados nas semanas 12 e 20. Todas as peças foram submetidas à análise morfológica por meio de microscopia óptica. Através desse estudo, construiu-se um protocolo modificado que, além de assegurar o desenvolvimento da carcinogênse com uma dose menor de carcinógeno, garante o bem-estar animal – o que faz com que longos estudos sejam possíveis. Avaliou-se, ainda, separadamente, o impacto das terapias de luz. Os resultados demonstram que, tanto a TFD quanto a FBM não são capazes de frear o desenvolvimento tumor, mas também não o exacerbam, não tendo impacto significativo na carcinogênese oral.

Palavras-chave: Terapia de fotobiomodulação; Terapia fotodinâmica; Carcinogênese oral; Desordem oral potencialmente maligna; Carcinoma espinocelular de boca

ABSTRACT

Light therapies have recently been adopted in a number of medical specialties as an adjunctive or alternative treatment of cancer and other potentially malignant conditions. Although photobiomodulation (PBM) is frequently used in clinical settings as a preventive treatment for oral mucositis, little is known about how it affects mucosa that has been exposed to carcinogens. Parallel to this, photodynamic therapy (PDT) has been utilized as an alternative therapy in the management of potentially cancerous lesions; however, the cellular and molecular effects of this therapy are not well supported by preclinical research. As a result, there are still concerns concerning its safety during the oral carcinogenesis process. In this regard, the goal of the current study was to assess the impact of two laser light-based therapies, photobiomodulation (PBM) and photodynamic therapy (PDT), on the emergence of chemically induced oral carcinogenesis. The 4NQO-induced oral carcinogenesis model was first thoroughly reviewed. The results of the included studies permitted the elaboration of a useful practice guide with recommendations for further research on 4NQO in Wistar rats. A summary of the effects of PDT on OSCC and potentially malignant oral diseases (PMODs) was also provided. Although PDT has acceptable and secure effects against DOPM and OSCC, we found that well-structured cohort studies are still required to justify PDT as a first-line treatment. Three further scholarly articles were produced as a result of our experimental study. Eighty-four male Wistar rats were employed and treated systemically with 4NQO for 12 and 20 weeks. Each animal was assigned to one of four groups, each receiving the 4NQO carcinogen: (1) Control group; (2) PBM 0.3J group, (3) PBM 1J group, and (4) PDT group. PBM was applied three times a week and PDT once a week. PBM was carried out with an InGaAlP diode laser operating in continuous mode and in contact while employing the punctual technique. PDT was carried out once a week on a sedated animal with topical application of 5% 5-aminolevuinic acid (5-ALA) as a photosensitizer. Two hours after 5-ALA administration, two sites on the animal's tongue were exposed to 90 J/cm^2 of 660 nm laser energy. At weeks 12 and 20, the animals were euthanized, and morphological examination was performed on each component using light microscopy. Based on this investigation, we developed a modified methodology that guarantees animal welfare and, in addition to assuring the development of carcinogenesis with a lower dose of carcinogen, allows for lengthy studies. Separately, we assessed the effects of light therapy. Our findings show that PDT and PBM have no discernible effect on oral carcinogenesis, neither inhibiting nor increasing its progression.

Keywords: Photobiomodulation; Photodynamic therapy; Oral carcinogenesis; Oral potentially malignant disorder; Oral squamous cell carcinoma

SUMÁRIO

1 INTRODUÇÃO

O carcinoma espinocelular (CEC) de boca é um problema global com incidência anual de 300.000 novos casos (D'Cruz, et al., 2018). Segundo dados brasileiros do Instituto Nacional do Câncer (INCA), o número de casos novos de câncer da cavidade oral esperados no país, para cada ano do triênio 2020-2022, é de 11.200 casos em homens, e de 4.010 em mulheres. Esses valores correspondem a um risco estimado de 10,7 casos novos a cada 100 mil homens, ocupando a quinta posição. Para as mulheres, corresponde a 3,71 para cada 100 mil mulheres, sendo a décima-terceira mais frequente entre todos os cânceres (INCA, 2020). Os principais fatores de risco são o tabagismo e o consumo excessivo de álcool, bem como a exposição ao sol. No entanto, por ser considerada uma doença multifatorial, fatores genéticos, epigenéticos e ambientais estão envolvidos (Chi et al., 2015; Ali et al., 2017).

A carcinogênese descreve uma série de mudanças genotípicas e fenotípicas que resultam na formação e na expansão clonal de uma célula maligna (Farah et al., 2019). Esse processo ocorre lentamente, podendo levar vários anos para que uma célula cancerosa prolifere e dê origem a um tumor visível. Nesse sentido, o processo de carcinogênese é descrito como um processo de múltiplas etapas que envolvem: (1) iniciação, (2) promoção e (3) progressão. A iniciação é o primeiro estágio. As células normais sofrem o efeito dos agentes carcinógenos que provocam modificações em alguns genes que regulam proliferação celular. Passam a se tornar geneticamente alteradas, porém ainda não é possível detectar um tumor clinicamente. Encontram-se "preparadas", ou seja, "iniciadas" para a ação de um segundo grupo de agentes que atuará na sequência. A promoção é o segundo estágio da carcinogênese. Nesse momento, as células já iniciadas sofrem o efeito dos agentes co-carcinogênicos (oncopromotores) - que ocorre de forma lenta e gradual. A suspensão do contato com agentes promotores muitas vezes interrompe o processo nesse estágio. No entanto, quando continua a evoluir, um terceiro estágio se inicia – progressão - e as células alteradas se multiplicam de forma descontrolada e irreversível. Nessa etapa o câncer já está instalado, evoluindo até o surgimento das primeiras manifestações clínicas da doença (Kumar & Weaver, 2009).

O mecanismo de carcinogênese é resultado de diversas alterações nos genes que atuam direta ou indiretamente no controle do ciclo celular, tais como: oncogenes,

genes supressores de tumor, genes reguladores da apoptose e genes envolvidos no reparo do DNA. Os agentes capazes de atuar na carcinogênese são denominados carcinógenos ou carcinogênicos. Os agentes oncoiniciadores são capazes de provocar diretamente o dano genético das células, iniciando o processo de carcinogênese (Exemplo: benzopireno, um dos componentes da fumaça do cigarro e alguns vírus oncogênicos, entre outros). Os agentes oncopromotores são aqueles que atuam sobre as células iniciadas, transformando-as em malignas. Enquanto o agente oncoacelerador gera a multiplicação descontrolada e irreversível das células alteradas, atuando no estágio final do processo. Dentre os diversos agentes carcinogênicos, o fumo tem sido considerado um agente completo, pois permite a exposição a carcinógenos, a formação de adutos de DNA e o acúmulo resultante de mutações somáticas permanentes em genes críticos. As mutações somáticas levam ao crescimento clonal e, por meio do acúmulo de mutações adicionais, ao desenvolvimento do câncer (CDC, 2010).

O 4-Nitroquinoline-1-Oxido (4NQO) é um carcinógeno sintético solúvel em água, que tem sido considerado o melhor carcinógeno atualmente disponível para a produção da carcinogênese oral em ratos. Tem sido evidenciado que o 4NQO forma adutos de DNA, levando à substituição de adenosina por guanosina, e induzindo ao estresse oxidativo intracelular (Sagheer et al., 2021). Os efeitos resultantes da aplicação do 4NQO são extremamente semelhantes às alterações induzidas pelo tabaco. Nesse modelo experimental, pode-se avaliar a formação dos estágios sequenciais da carcinogênesse, e estudos evidenciam que suas alterações histológicas e moleculares são similares às que ocorrem em humanos (Kanojia& Vaidya, 2006; Miki et al., 2016; Sagheer et al., 2021). Histologicamente, podem ser observadas alterações no epitélio lingual que variam de hiperplasia, displasia leve, moderada e severa antes da formação do carcinoma invasivo (Sagheer et al., 2021). Ainda, sabe-se que o espectro mutacional do CEC humano é fielmente recapitulado em tumores de animais induzidos por 4NQO (Serqueira et al., 2020). Com esse modelo, pode-se avançar no entendimento das alterações moleculares e celulares da carcinogênese, bem como no desenvolvimento de novas estratégias terapêuticas.

O uso da luz com finalidade terapêutica vem sendo há anos investigado. A terapia que envolve o uso de luz laser ou LED (do inglês, *light-emitting diode*), com finalidade de gerar efeitos biológicos sem gerar calor, é denominada terapia de fotobiomodulação (FBM). A FBM representa uma abordagem terapêutica promissora,

explorando a capacidade da luz de modular os processos bioquímicos e moleculares nas células vivas (Levchenko et al., 2018). Dependendo dos parâmetros de luz, pode ser capaz de ativar ou inibir uma série de vias de sinalização celular (Wang et al., 2017; Rupel et al., 2018). O mecanismo preciso da capacidade da FBM em induzir a proliferação ou apoptose ainda não é totalmente compreendido, embora seu efeito possa ser parcialmente explicado pela capacidade bem conhecida da luz de modular o nível de espécies reativas de oxigênio intracelular (De Freitas & Hamblin, 2016). Sabe-se que a ação dos fótons na citocromo-c oxidase é o componente primário envolvido no mecanismo de ação da FBM. De forma geral, ocorre a absorção da irradiação pelo citocromo-c oxidase na mitocôndria, causando fotoexcitação do cromóforo e aumento da produção de adenosina trifosfato (ATP) (Dompe et al., 2020). De modo geral a FBM tem sido utilizada com o objetivo de promover o reparo tecidual, modular a inflamação e produzir analgesia (De Pauli Paglioni et al., 2019).

Outra forma de utilização da luz é a terapia fotodinâmica (TFD), a qual se baseia no efeito da luz em excitar cromóforos para produzir espécies reativas de oxigênio (De Freitas & Hamblin, 2016). De forma semelhante à FBM, a TFD se caracteriza por ser uma abordagem minimamente invasiva (Chen et al., 2019). Tal técnica possui três elementos fundamentais: (1) oxigênio, (2) fotossensibilizador e (3) um comprimento de onda específico de luz visível (Olek et al., 2021). Nesse processo, o fotossensibilizador é ativado pela luz e causa uma série de reações fotoquímicas e fotobiológicas, resultando em danos irreversíveis e, eventualmente, na morte das células alvo (Hopper, 2000; Prates et al., 2011; Sharma et al., 2012; Prażmo et al., 2016; van Straten et al., 2017). Como a TFD é um processo fotoquímico a frio, não há aquecimento tecidual, e tecidos conjuntivos, como colágeno e elastina, no local tratado, não são afetados; portanto, há muito menos risco de danificar a integridade das estruturas funcionais subjacentes do que com técnicas de laser térmico e outras abordagens invasivas (Olek et al., 2021).

Tanto a FBM quanto a TFD têm sido utilizadas no manejo de lesões orais. Apesar do uso clínico difundido da FBM como terapia preventiva para mucosite oral, seu efeito sobre a mucosa exposta a carcinógenos ainda é pouco compreendido. Em paralelo, a TFD tem sido utilizada como tratamento alternativo no manejo de lesões potencialmente malignas; entretanto, existe falta de embasamento pré-clínico sobre os efeitos celulares e moleculares dessa terapia. Diante disso, o efeito da FBM e TFD na carcinogênese oral tem sido alvo de estudo em diferentes metodologias.

Uma revisão sistemática, publicada por Silveira e colaboradores (2019), analisou os efeitos da terapia com FBM no CEC de cabeça e pescoço em 15 artigos. Dos 13 estudos in vitro, sete observaram um efeito positivo da FBM na inibição ou na prevenção do efeito nas células tumorais do CEC de cabeça e pescoço, enquanto seis observaram aumento da proliferação. Apenas dois estudos fizeram avaliação em modelo animal: de C Monteiro et al. (2011) relatou aumento da progressão do CEC oral, enquanto Ottaviani et al. (2016) observaram redução da progressão tumoral. Os autores concluíram que os dados dos artigos incluídos na revisão sistemática ainda não suportam uma conclusão clara sobre os efeitos da FBM nas células de CEC de cabeça e pescoço. Já, em 2020, foi publicado um estudo por Chiang e coautores, com o objetivo de avaliar o potencial terapêutico de FBM na fibrose submucosa oral (desordem oral potencialmente maligna) em ratos induzidos por extrato de noz areca. Os resultados mostram que o FBM reduziu significativamente o desenvolvimento de fibrose submucosa oral, quantificado por mudanças na espessura da camada submucosa e deposição de colágeno (Chiang et al., 2020).

Recentemente, nosso grupo de pesquisa realizou um estudo que avaliou o impacto da FBM em CEC oral, utilizando modelo de xenoenxerto. Os resultados mostram que não houve diferenças significativas entre os grupos irradiados e não irradiados quanto a medidas volumétricas, grau histopatológico e marcação imuno-histoquímica (Silveira et al., 2022). Dessa forma, concluiu-se que a FBM não tem impacto no comportamento do câncer oral em modelos de xenoenxerto. No entanto, alguns estudos sugerem que o uso do FBM é capaz de influenciar os processos metabólicos celulares a ponto de estimular a proliferação de células malignas e modular o microambiente tumoral para aumentar seu volume (Frigo et al., 2009; de C Monteiro et al., 2011). Por outro lado, estudos de outros autores sugerem que FBM induz apoptose e morte celular em células neoplásicas malignas de forma dose-dependente, sem potencial para ativar células malignas residuais (Tsai et al., 2015; Barasch et al., 2016). De Pauli Paglioni et al. (2019) demonstram, através de uma revisão sistemática conduzida com 27 artigos, que o uso de FBM para prevenir e/ou tratar complicações associadas ao tratamento do câncer é seguro. No entanto, ressaltam que estudos futuros, usando protocolos semelhantes de aplicação de FBM e com acompanhamento de longo prazo, são necessários para confirmar a segurança do uso de FBM em pacientes com câncer.

A TFD tem sido amplamente utilizada para tratar desordens potencialmente malignas e cânceres de pele, trato digestivo e mucosa geniturinária, bem como tumores e malformações vasculares em outros órgãos (Chen et al., 2019). Olek e coautores (2021) publicaram uma revisão sistemática com o objetivo de apresentar uma visão geral do uso da TFD em estudos pré-clínicos em modelo animal. Os resultados da revisão mostram que a pesquisa tem se concentrado principalmente na avaliação dos efeitos da TFD no crescimento tumoral, na expectativa de vida dos animais e na ocorrência de efeitos adversos, bem como no papel da via de administração nos efeitos alcançados. No entanto, sua segurança em mucosa alterada pela ação carcinogênica ainda não foi comprovada, não sendo amplamente utilizada nos centros clínicos.

Uma vez que a utilização de terapia de luz ainda é contraditória na literatura, o presente trabalho tem como objetivo entender como a FBM e a TFD atuam e/ou interferem na indução da proliferação e da diferenciação celular durante o processo de carcinogênese oral.

2 ARTIGOS

2.1 Revisiting the evidence of photodynamic therapy for oral potentially malignant disorders and oral squamous cell carcinoma: an overview of systematic reviews

Artigo publicado no periódico Photodiagnosis and photodynamic therapy (2023 Mar 22;42:103531. doi: 10.1016/j.pdpdt.2023.103531) (**Anexos 1 e 2**).

Lauren Frenzel **Schuch**[1], Tuany Rafaeli **Schmidt**[2], Laura Borges **Kirschnick**[1], José Alcides Almeida **de Arruda**[3], Daniela **Champagnol**[4], Marco Antônio Trevizani **Martins**[2,5], Alan Roger **Santos-Silva**[1], Márcio Ajudarte **Lopes**[1], Pablo Agustin **Vargas**[1], Vanderlei Salvador **Bagnato**[6], Cristina **Kurachi**[6], Eliete Neves Silva **Guerra**[7], Manoela Domingues **Martins**[1,2]

[1]Oral Diagnosis Departament, Piracicaba Dental School, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil.
[2]Departament of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.
[3]Departament of Oral Surgery, Pathology and Clinical Dentistry, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.
[4]Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.
[5]Departament of Oral Medicine, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.
[6]Department of Physics and Materials Science, São Carlos Institute of Physics, Universidade de São Paulo, São Carlos, São Paulo, Brazil.
[7]Laboratory of Oral Histopathology, Health Sciences Faculty, Universidade de Brasília, Brasília, Distrito Federal, Brazil.

Corresponding author: Manoela Domingues Martins. Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, R. Ramiro Barcelos, 2492, room 503. Porto Alegre, Rio Grande do Sul, Brazil, CEP: 90035-003. Phone: +55 (51) 3308-5011. E-mail: manomartins@gmail.com; manoela.martins@ufrgs.br

Abstract

This study summarized the available evidence about the use of photodynamic therapy (PDT) for the management of oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC). An overview of systematic reviews was undertaken based on the PRISMA 2020 statement. Electronic searches were performed in five databases. Studies published up to November 2022 were included. Risk of bias was assessed with the AMSTAR 2 tool. Thirty studies enrolling 9,245 patients with OPMD (n=7,487) or OSCC (n=1,758) met the selection criteria. All studies examined the efficacy and/or safety of PDT. OPMD were investigated individually in 82.8% of the studies, the most common being oral lichen planus and actinic cheilitis. OSCC was addressed separately in 10.3% of the studies, while only 6.9% evaluated both OPMD and OSCC. Fourteen different types of photosensitizers were described. PDT was used according to the following laser setting parameters: 417-670 nm, 10-12000 mW/cm^2, 1.5-200 J/cm^2, and 0.5-143 minutes. Regarding OPMD, leukoerythroplakia showed the best response rates, while oral lichen planus showed partial or no response in nearly 75% of documented cases. A complete response was observed in 85.9% of OSCC cases, while 14.1% had no resolution. Overall, the response to PDT depended on the type of OPMD/OSCC and the parameters used. Although PDT is an emerging candidate for the treatment for OPMD and OSCC, there is heterogeneity of the methodologies used and the clinical data obtained, particularly regarding the follow-up period.

Keywords: oral cancer; oral oncology; oral potentially malignant disorder; oral squamous cell carcinoma; photodynamic therapy; photosensitizing.

1. Introduction

Almost 90% of oral cavity cancers are squamous cell carcinomas (SCC), and their global incidence was estimated at 377,713 new cases, with 177,757 deaths in 2020 [1]. The development of oral cavity SCC (OSCC) may be preceded by a significant subset of mucosal disorders recognized as oral potentially malignant disorders (OPMD) [2]. The malignant transformation rate across OPMD has been estimated at 7.9% [3].

OSCC is usually treated by surgery followed by radiotherapy and/or chemoradiation. Surgery for OSCC can be disfiguring and both surgery and radiotherapy have significant functional adverse effects, notably impairing the ability to eat, drink, and talk [4]. Moreover, a variety of therapeutic strategies have been proposed for OPMD, including application of topical and systemic drugs, CO_2 laser, cryosurgery, electrosurgery, and surgical excision [2,5,6]. Despite this, there still is no available evidence that treatment prevents malignant transformation and counterproductive adverse effects (e.g., scare formation and recurrence) have been documented in some cases [2,3,6]. In this scenario, a promising candidate appears to be photodynamic therapy (PDT), a noninvasive tool that uses the combination of a photosensitizer (PS) and oxygen that activates reactive oxygen species (ROS), causing the death of microorganisms by apoptosis or necrosis [7].

For over 30 years after its regulatory approval (e.g., Food and Drug Administration), PDT has been the subject of numerous studies and compelling evidence indicates that it is an effective therapy against cancer [8]. Systematic reviews have examined the safety and/or efficacy of PDT in SCC of the head and neck and have indicated that PDT is a promising candidate for the treatment of early-stage disease [9,10]. Similarly, a study demonstrated that interstitial PDT seems to be safe and effective for the treatment of brain tumors [11]. In cervical epithelial tumors, PDT may be a practical approach regarding their regression compared to placebo [12]. Wang et al. [13] and Collier et al. [14] endorsed that PDT is a useful method for the treatment of basal cell carcinoma, with cosmetic advantages over surgery and cryosurgery.

PDT has been approved as a modality for the treatment of cancer elsewhere, particularly in the management of superficial lesions of the skin and luminal organs [15]. For instance, PDT is approved in the United States, Canada and the European Union for the treatment of actinic keratosis and/or basal cell carcinoma [16]. While

there is currently growing interest in the development of a safe, effective, and repeatable function-sparing treatment for OSCC, there still seem to be conflicting results about the role of PDT in managing OPMD and OSCC. Previous systematic reviews have examined the efficacy and safety of PDT for OPMD and/or OSCC [17–19]; however, due to the heterogeneity of the methodologies employed, as well as the quality of the reviews performed, the conclusions are still unclear. In this sense, when there is conflicting evidence about a certain topic, overviews play an important role in bringing reviews together using a transparent and systematic method. This sort of study assists in making informed decisions by methodically gathering and examining published evidence [20]. Therefore, the purpose of the present overview was to systematically examine and critically appraise available evidence regarding the impact of PDT on the management of OPMD and OSCC.

2. Material and Methods

2.1 Study design and eligibility criteria

This overview was based on a systematic review and meta-analysis that aimed to investigate the impact of PDT on the management of OPMD and OSCC. OPMD were reported according to the consensual definition and classification proposed by Warnakulasuriya et al. [2]. Studies whose primary objective was the assessment of the impact of PDT or studies on the treatment of OPMD or OSCC that demonstrated the results of PDT separately were included. No restrictions were applied regarding publication time or language of the articles.

The acronym PICOS (Population, Intervention, Comparison, Outcomes, and Studies) was structured as follows:

(P) Participants with OPMD and/or OSCC;

(I) PDT;

(C) None or other therapies;

(O) Efficacy, safety, and response rate of PDT (the general PDT was also used) for the management of OPMD and/or OSCC;

(S) Systematic reviews and meta-analyses.

2.2 Search strategy

Electronic searches were performed in June 2022 in five databases: PubMed (National Library of Medicine), Web of Science (Thomson Reuters), Scopus (Elsevier),

Embase (Elsevier), and Cochrane (Cochrane Library). An update was carried out in November 2022. The gray literature was also examined, limited to the first 100 results through ProQuest, OpenGrey, and Google Scholar [21]. A hand search was also performed in the reference list of the included studies. **Supplementary File 1** provides the details of the search strategy employed.

2.3 Study selection and data extraction

The reference list was retrieved from the Endnote website (Clarivate Analytics, PA, USA). Titles/abstracts were read independently by three authors (L.F.S.; T.R.S.; L.B.K.). If the title/abstract met the eligibility criteria, the article was included. The full text of the articles with titles/abstracts providing insufficient information for a clear decision was obtained. After evaluation of the full text, the references that met the eligibility criteria were included. Disagreements among authors were resolved by discussion with a fourth author (M.D.M.).

The authors (L.F.S., T.R.S. and L.B.K.) extracted all data from the included studies. For each study, the following data were extracted: author's name, year and country of publication, review question and/or aim of the study, histopathological subtypes of OPMD and/or OSCC, number of included articles, study design, sample size and setting, PDT parameters, PDT response rate (i.e., complete, partial, or absent), adverse effects, follow-up period (months), recurrence rate, main results, and conclusions.

2.4 Critical appraisal

The quality assessment was performed by three authors (L.F.S., T.R.S., and L.B.K.). The methodology of selected systematic reviews was examined using the A MeaSurement Tool to Assess Systematic Reviews 2 (AMSTAR-2) checklist [22]. Risk of bias was categorized as high when the study reached up to 49% of a “yes” score, moderate when the study reached 50 to 69% of a “yes” score, and low when the study reached more than 70% of a “yes” score. Disagreements among authors were resolved by discussion with a fourth author (M.D.M.).

2.5 Data analysis

Data were tabulated in Microsoft Office Excel 2019 (Microsoft® software, Redmond, WA, USA) and analyzed descriptively.

2.6 Other information

This study was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement [23]. We also followed the key issues that are essential to assess when taking the steps in planning an overview [20]. A protocol was designed and registered in the International Prospective Register of Systematic Reviews (No. CRD42021249297). The authors declare no conflict of interests.

3. Results

3.1 Selection of studies

The search process retrieved 604 articles. After removing duplicates, 262 titles and abstracts were evaluated. Of these, 52 full texts were evaluated considering the eligibility criteria; 22 records were excluded for different reasons (**Supplementary File 2**). No articles were selected by grey literature or manual search. A total of 30 systematic reviews, covering a total of 368 primary studies, were included in this overview [9,17–19,24–49]. **Figure 1** depicts the flowchart process.

3.2 General characteristics of the articles included

A total of 9,245 individuals with OPMD (n=7,487) or OSCC (n=1,758) were analyzed. The worldwide distribution of the selected studies showed that the systemic reviews were published in 12 countries of four regions: Asia (n=15), Europe (n=8), North America (n=5), and South America (n=2). The systematic reviews investigated oral lichen planus (OLP), actinic cheilitis (AC), oral leukoplakia (OLK), oral erythroleukoplakia (OEL), oral erythroplakia (OE), oral verrucous proliferative leukoplakia (OVPL), epithelial dysplasia, including *in situ* carcinoma, as well as OSCC. **Supplementary File 3** provides the data from the included systematic reviews.

3.3 General PDT features

Different types of PS were described: aminolaevulinic acid, chlorine-e6 and dimethyl sulfoxide, chlorine-e6, foscan, hematoporphyrin derivatives, imiquimod, meta-tetrahydroxyphenylchlorin, methyl aminlevulinate, methylanomoxopentanoate, methylene blue, Photofrin® (porfimer sodium), Photosan® (hematoporphyrin derivative), polyvinylpyrrolidone, and toluidine blue.

Laser parameters were as follows: 417-670 nm wavelength, 10-12000 mW/cm^2 power density, 1.5-200 J/cm^2 energy density, and 0.5-143 minutes of irradiation (**Supplementary File 3**).

3.4 Impact of PDT for OPMD

Table 1 and **Figure 2** show the impact of PDT for OPMD according to response rate (i.e., complete, partial, or absent), follow-up period, and recurrence rate, based on the information provided. Among the informed cases, in general, OEL (93% of 100 cases) showed the best response rate (i.e., complete response), followed by AC (67.6% of 448 cases).

AC and OLP were the lesions with the highest number of publications (10 studies each). Except for the study by Carvalho et al. [30], all others demonstrated that PDT, alone or combined with other therapies (e.g., 5% imiquimod), was promising and effective for the treatment of AC (**Supplementary File 3**). However, compared to the clinical response rate of the OPMD included in this overview, AC had a higher nonresolution rate (20.3% of 448 cases) (**Figure 2**). Salgueiro et al. [35] and Trager et al. [41] showed that PDT had a high frequency of adverse effects (e.g., erythema, edema, pain, and crust formation).

Most of the studies focused on the use of PDT for the treatment of OLP and compared this alternative approach to topic corticotherapy, showing its positive effects [24,27,29,36,43,49]. However, the complete response rate observed was 42.9%. Factors such as size reduction, absence of visible scars, and reduction of signs and symptoms were pointed out by some studies, although the results were not statistically significant (**Supplementary File 3**). Hoseinpour Jajarm et al. [31] reported that PDT showed no significant effect regarding the treatment of OLP signs since no statically significant differences in lesion size were found after treatment. Moreover, Łukaszewska-Kuska et al. [40] demonstrated that the therapeutic outcomes were more advantageous for dexamethasone compared to PDT. On the other hand, Waingade et al. [47] concluded that PDT using methylene blue as a PS was found to have similar efficacy to that of conventional corticosteroid therapy in the management of OLP and may be considered an alternative of treatment when steroids are contraindicated.

Eight studies evaluated OLK, OE and/or OEL. Overall, OLK showed a lower response than OE and OEL (**Supplementary File 3**). In one study [28], OL (white lesions) did not respond satisfactorily to PDT, with complete response rates ranging

from 0 to 83%. Moreover, the complete response rate for OLK was 7.7%, compared to 91.7% for OEL [34]. Regarding size, Li et al. [32] stated that, in addition to the complete recovery of patients treated with PDT, the reduction in lesion size is also of great importance for the follow-up of treatment or resection.

3.5 Impact of PDT for OSCC

Among the informed cases, OSCC (n=411) showed complete resolution in 83.4% of cases and nonresolution in 16.6%. The recurrence rate was 12% within a follow-up of 28.4 months (**Table 1**). In the study by Cerrati et al. [17], PDT was assessed in terms of complete response to therapy, and surgery was evaluated in terms of locoregional control and recurrences. When comparing a complete response to PDT and locoregional control to the results of surgery, the authors did not detect statistically significant differences. Also, no statistically significant difference was observed regarding relapses. The authors concluded that PDT was as effective as primary surgical resection for treating early-stage OSCC and was a valid treatment approach regarding the preservation of function.

All PDT studies included by Alkindi et al. [18] showed statistically significant improvement of complete OSCC regression during the follow-up period. For PDT, the complete response ranged from 16 to 100% in OSCC cases.

Romano et al. [19] reported that the treatment of OSCC involved participants who had lesions of substantial extension or that involved regions of high cosmetic value (e.g., lower lip). In these cases, PDT was a critical therapeutic option for patients who refused to undergo traditional invasive surgery and/or general anesthesia.

Lin and colleagues [42] concluded that the efficiency of PDT, especially in the short-term for the treatment of early OSCC, was high. In parallel, PDT was highly selective with mild adverse reactions, which made it preferable to surgery when protection of the appearance and function of the target site was needed. However, it should also be noted that PDT had little effect on metastatic lesions; therefore, patients with OSCC should be carefully selected (patients at the T1N0M0/T2N0M0 stage in most cases) before PDT treatment.

3.6 Critical appraisal of systematic reviews

Risk of bias was categorized as high in six (20%) studies, moderate in 11 (36.7%), and low in 13 (43.3%) (**Supplementary File 4**).

4. Discussion

The present overview identified that PDT is a promising candidate that elicits an effective response in the management of OPMD and OSCC. However, the results clearly reveal that the response rate occurred for specific lesions and conditions, particularly for OPMD. Response rates after PDT varied according to several factors, including but not limited to, lesion location and size, morphological aspects (e.g., surface keratin, increased epithelial permeability, and accelerated epithelial cell division), type, diffusion, and retention of PS, and the parameters/protocols used for PDT. Thus, although PDT is an emerging candidate for the treatment for OPMD and OSCC, there is heterogeneity of clinical data and of the parameters used, particularly during long-term patient follow-up.

PDT for AC was reported in 10 studies with a good complete response rate in 67.6% of cases. This method yields excellent cosmetic results and is largely well-tolerated with few adverse events including erythema, edema, pain, and crusting [35,41]. About 30% of the AC cases evaluated in this overview had a partial or no response to PDT. Accordingly, it has been suggested that the treatment of AC with PDT can be used in cases of mild to moderate dysplasia and non-focal clinical presentation [26]. Unfortunately, the primary studies assessed did not explore lesion size or morphological aspects; thus, it is necessary to increase the effectiveness of PDT by optimizing the treatment parameters.

The literature states that OLP has no cure, and treatment strategies are designed to limit progression, reduce exacerbations, and alleviate pain symptoms [50]. Although a substantial number of studies have demonstrated a positive effective effect of PDT for these lesions [24,45,49], the results are conflicting. Systematic reviews have shown that PDT is less successful for OLP as the lesions only partially resolve in most cases. In turn, partial resolution involves multiple factors such as a size reduction and partial pain relief. PDT appears to be advantageous in some circumstances or when used in conjunction with conventional therapy. Moreover, it was highlighted that the range of cost per treatment session was higher for these treatment modalities when compared to topical steroids [46].

Regarding OLK, OE and OEL, the response observed in the evaluated cases was variable. Less than half the cases diagnosed as OLK have a complete response, whereas OE and OEL have a complete response in over 90% of cases. Although the

studies did not associate histopathological grading/characteristics with the outcomes, the difference in response to treatment may be possibly due to the different epithelial structure of the lesions [34]. Another important point refers to the size of the lesion. According to Gondivkar et al. [9], almost 85% of the studies included in the systematic review did not establish an association between lesion size and appearance of the lesion versus outcomes after PDT. It is noteworthy that studies that included treatment of smaller lesions [51] had a more satisfactory response rate with better follow-up than larger lesions [52,53]. This suggests, therefore, that multiple factors, including dysplasia, type, size, and surface characteristics of the lesion, may determine the effectiveness of PDT in the management of OLK, OE, and OEL. Nevertheless, future studies that address these factors are necessary for the successful management of such lesions by PDT.

In the presence of malignant lesions, PDT is essentially applied to individuals who have lesions of considerable extent or who are affected in areas of high cosmetic value (e.g., lower lip) and to patients who refuse traditional invasive surgery and/or general anesthesia [19]. We should emphasize that, whenever possible, the treatment of choice for OSCC should be surgery and/or radiotherapy. Notably, compared to surgical treatment, PDT is highly selective, minimally invasive, and easily accepted by patients, with mild adverse reactions and no cumulative toxicity [53]. However, the findings of the included articles on OSCC should be interpreted with caution. Almost all studies contained a small number of patients – especially as a direct result of the careful selection of candidates for PDT, and more positive outcomes would be expected [17]. Of note, PDT appears to be well tolerated by patients of all ages and may serve as an alternative therapy for clinically compromised patients who may not be able to withstand the adverse effects of radiotherapy or who may be too debilitated to undergo surgery. Another possible application would be in cases of compromised margins due to malignant lesions that cannot be fully resected. Concepts about the characteristics of a clinically successful PS should be taken into account, such as photodynamic reaction, localization, amphiphilicity, fluorescence, and dosimetry [54]. The type of PS, for instance, must be considered when evaluating the effectiveness of PDT. In addition, PS properties, tissue properties, and the corresponding absorption wavelength should be well evaluated when choosing laser types [55,56]. In the study by Varela-Centelles et al. [57] that compared different therapeutic approaches to AC, PDT provided ineffective clearance rates and inconsistent results. However, daylight

PDT with methyl aminolevulinate proved to be better tolerated than conventional PDT and may be specifically indicated for cases of AC associated with multiple facial actinic keratoses. Importantly, Photofrin®-based PDT has revealed promising results in reversing OSCC at early stages [28]. In contrast, aminolaevulinic acid for OL showed contradictory results, although most of them indicated that this is not a clinically effective intervention [34]. In part, a hypothesis for such a broad range of clinical response can be attributed to the variation between the clinical appearance and the underlying histology of these oral lesions [28].

There was a lack of consensus on the precise duration and frequency of PDT for the treatment of OPMD and OSCC. Therefore, the importance of these different parameters related to therapeutic protocols regarding the outcome has not yet been established. Accordingly, multiple laser sessions are known to have a significant effect on the clinical efficacy of phototherapy [43]. However, the number of sessions was poorly described in the included studies. Indeed, the potential advantages of PDT over conventional treatment for OPMD and OSCC are that this therapy is noninvasive and convenient for both the patient and the operator [9]. In general, PDT has few and moderate adverse effects and could be used as a primary cancer treatment modality, as a pre-surgical treatment for the reduction of the lesion or as a palliative intervention for late disease. Mechanistically, PDT usually has a low potential of causing DNA damage, mutations, and carcinogenesis [9]. Most PS do not accumulate in cell nuclei. Sensitizers that localize in mitochondria, such as Photofrin®, or are produced in mitochondria, such as aminolaevulinic acid-induced Protoporphyrin® IX, are likely to induce apoptosis, while sensitizers localized in the plasma membrane are likely to cause necrosis during light exposure [58,59]. The common adverse events reported included local phototoxicity and pain. Local phototoxicity usually included transient erythema, edema, erosions, crusting, blistering, scaling, itchiness, paresthesia, and mild dryness [48]. Nonetheless, the inconveniences of the technology, including specialized instrumentation and the special training required, should be considered against its potential advantages.

This overview has some limitations. The findings of this study should be interpreted with caution since the number of parameters, including laser parameters, as well as type of patients, and number of treatment sessions may affect the overall outcome of PDT in the management of OSCC. Clinicians may evaluate the availability, incidence, and severity of adverse reactions, cost-performance ratio, and local medical

insurance policy when choosing an appropriate PS. Another concern is the length of follow-up, which in some cases did not exceed six months. A longer observation period would allow a longer follow-up of the patients and, eventually, the identification of the lesion recurrence in the treated sites or in different sites. In this regard, we suggest that researchers should standardize the staging of the lesions to be treated and provide documentation with proper images taken with the use of rulers and pachymeters for future studies. Last, more than half the studies were rated as having high or moderate risk of bias based on the evaluation of their methodological quality. Methodological flaws regarded the absence of a pre-defined review protocol, the absence of a list of excluded studies and justification of the exclusions, and the absence of sources of funding.

In summary, despite the adequate and safe effects of PDT against OPMD and OSCC, its support as a first-line treatment still needs better-designed cohort studies. The lack of consistency of PDT protocols, as well as the limited number of cases included without a long-term follow-up in the primary studies, are the main reasons that prevent reaching definite conclusions. The development of standard protocols for photoactive agents, a clear understanding of dosing aspects, and the frequency of treatments for OPMD and OSCC are crucial for future studies.

References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209-249. doi: 10.3322/caac.21660

[2] Warnakulasuriya S, Kujan O, Aguirre-Urizar JM, Bagan JV, González-Moles MÁ, Kerr AR, Lodi G, Mello FW, Monteiro L, Ogden GR, Sloan P, Johnson NW. Oral potentially malignant disorders: a consensus report from an international seminar on nomenclature and classification, convened by the WHO Collaborating Centre for Oral Cancer. *Oral Dis*. 2021;27(8):1862-1880. doi: 10.1111/odi.13704

[3] Iocca O, Sollecito TP, Alawi F, Weinstein GS, Newman JG, De Virgilio A, Di Maio P, Spriano G, Pardiñas López S, Shanti RM. Potentially malignant disorders of the oral cavity and oral dysplasia: a systematic review and meta-analysis of malignant transformation rate by subtype. *Head Neck*. 2020;42(3):539-555. doi: 10.1002/hed.26006

[4] Parmar A, Macluskey M, Mc Goldrick N, Conway DI, Glenny AM, Clarkson JE, Worthington HV, Chan KK. Interventions for the treatment of oral cavity and oropharyngeal cancer: chemotherapy. *Cochrane Database Syst Rev*. 2021;12(12):CD006386. doi: 10.1002/14651858.CD006386.pub4

[5] Gurizzan C, Lorini L, Bossi P. Oral potentially malignant disorders: new insights for future treatment. *Curr Opin Otolaryngol Head Neck Surg*. 2021;29(2):138-142. doi: 10.1097/MOO.0000000000000695

[6] Lorini L, Bescós Atín C, Thavaraj S, Müller-Richter U, Alberola Ferranti M, Pamias Romero J, Sáez Barba M, de Pablo García-Cuenca A, Braña García I, Bossi P, Nuciforo P, Simonetti S. Overview of oral potentially malignant disorders: from risk factors to specific therapies. *Cancers (Basel)*. 2021;13(15):3696. doi: 10.3390/cancers13153696

[7] Abrahamse H, Hamblin MR. New photosensitizers for photodynamic therapy. *Biochem J*. 2016;473(4):347-364. doi: 10.1042/BJ20150942

[8] van Straten D, Mashayekhi V, de Bruijn HS, Oliveira S, Robinson DJ. Oncologic photodynamic therapy: basic principles, current clinical status and future directions. *Cancers (Basel)*. 2017;9(2):19. doi: 10.3390/cancers9020019

[9] Gondivkar SM, Gadbail AR, Choudhary MG, Vedpathak PR, Likhitkar MS. Photodynamic treatment outcomes of potentially-malignant lesions and malignancies of the head and neck region: a systematic review. *J Investig Clin Dent*. 2018;9(1). doi: 10.1111/jicd.12270

[10] Ibarra AMC, Cecatto RB, Motta LJ, Dos Santos Franco AL, de Fátima Teixeira da Silva D, Nunes FD, Hamblin MR, Rodrigues MFSD. Photodynamic therapy for squamous cell carcinoma of the head and neck: narrative review focusing on photosensitizers. *Lasers Med Sci*. 2022;37(3):1441-1470. doi: 10.1007/s10103-021-03462-3

[11] Leroy HA, Guérin L, Lecomte F, Baert G, Vignion AS, Mordon S, Reyns N. Is interstitial photodynamic therapy for brain tumors ready for clinical practice? a systematic review. *Photodiagnosis Photodyn Ther*. 2021;36:102492. doi: 10.1016/j.pdpdt.2021.102492

[12] Unanyan A, Pivazyan L, Davydova J, Murvatova K, Khrapkova A, Movsisyan R, Ishchenko A, Ishchenko A. Efficacy of photodynamic therapy in women with HSIL, LSIL and early stage squamous cervical cancer: a systematic review and meta-analysis. *Photodiagnosis Photodyn Ther*. 2021;36:102530. doi: 10.1016/j.pdpdt.2021.102530

[13] Wang H, Xu Y, Shi J, Gao X, Geng L. Photodynamic therapy in the treatment of basal cell carcinoma: a systematic review and meta-analysis. *Photodermatol Photoimmunol Photomed*. 2015;31(1):44-53. doi: 10.1111/phpp.12148

[14] Collier NJ, Haylett AK, Wong TH, Morton CA, Ibbotson SH, McKenna KE, Mallipeddi R, Moseley H, Seukeran D, Ward KA, Mohd Mustapa MF, Exton LS, Green AC, Rhodes LE. Conventional and combination topical photodynamic therapy for basal cell carcinoma: systematic review and meta-analysis. *Br J Dermatol*. 2018;179(6):1277-1296. doi: 10.1111/bjd.16838

[15] dos Santos AF, de Almeida DRQ, Terra LF, Baptista MS, Labriola L. Photodynamic therapy in cancer treatment - an update review. *J Cancer Metastasis Treat*. 2019;5:25. doi: 10.20517/2394-4722.2018.83

[16] Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, Hahn SM, Hamblin MR, Juzeniene A, Kessel D, Korbelik M, Moan J, Mroz P, Nowis D, Piette J, Wilson BC, Golab J. Photodynamic therapy of cancer: an update. *CA Cancer J Clin*. 2011;61(4):250-281. doi: 10.3322/caac.20114

[17] Cerrati EW, Nguyen SA, Farrar JD, Lentsch EJ. The efficacy of photodynamic therapy in the treatment of oral squamous cell carcinoma: a meta-analysis. *Ear Nose Throat J*. 2015;94(2):72-79. doi: 10.1177/014556131509400208

[18] Alkindi M. Therapeutic efficacy of photodynamic therapy in oral squamous cell carcinoma: a systematic review. *Biosc Biotech Res Comm*. 2020;13(1):187-194

[19] Romano A, Di Stasio D, Gentile E, Petruzzi M, Serpico R, Lucchese A. The potential role of photodynamic therapy in oral premalignant and malignant lesions: a systematic review. *J Oral Pathol Med*. 2021;50(4):333-344. doi: 10.1111/jop.13139

[20] Hunt H, Pollock A, Campbell P, Estcourt L, Brunton G. An introduction to overviews of reviews: planning a relevant research question and objective for an overview. *Syst Rev*. 2018;7(1):39. doi: 10.1186/s13643-018-0695-8

[21] Haddaway NR, Collins AM, Coughlin D, Kirk S. The role of Google Scholar in evidence reviews and its applicability to grey literature searching. *PLoS One*. 2015;10(9):e0138237. doi: 10.1371/journal.pone.0138237

[22] Shea BJ, Reeves BC, Wells G, Thuku M, Hamel C, Moran J, Moher D, Tugwell P, Welch V, Kristjansson E, Henry DA. AMSTAR 2: a critical appraisal tool for systematic reviews that include randomised or non-randomised studies of healthcare interventions, or both. *BMJ*. 2017;358:j4008. doi: 10.1136/bmj.j4008

[23] Page MJ, Moher D, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, Shamseer L, Tetzlaff JM, Akl EA, Brennan SE, Chou R, Glanville J, Grimshaw JM, Hróbjartsson A, Lalu MM, Li T, Loder EW, Mayo-Wilson E, McDonald S, McGuinness LA, Stewart LA, Thomas J, Tricco AC, Welch VA, Whiting P, McKenzie JE. PRISMA 2020

explanation and elaboration: updated guidance and exemplars for reporting systematic reviews. *BMJ*. 2021;372:n160. doi: 10.1136/bmj.n160

[24] Pavlic V, Vujic-Aleksic V. Phototherapy approaches in treatment of oral lichen planus. *Photodermatol Photoimmunol Photomed*. 2014;30(1):15-24. doi: 10.1111/phpp.12074

[25] Vohra F, Al-Kheraif AA, Qadri T, Hassan MI, Ahmed A, Warnakulasuriya S, Javed F. Efficacy of photodynamic therapy in the management of oral premalignant lesions. A systematic review. *Photodiagnosis Photodyn Ther*. 2015;12(1):150-159. doi: 10.1016/j.pdpdt.2014.10.001

[26] Yazdani Abyaneh MA, Falto-Aizpurua L, Griffith RD, Nouri K. Photodynamic therapy for actinic cheilitis: a systematic review. *Dermatol Surg*. 2015;41(2):189-198. doi: 10.1097/DSS.0000000000000254

[27] Akram Z, Javed F, Hosein M, Al-Qahtani MA, Alshehri F, Alzahrani AI, Vohra F. Photodynamic therapy in the treatment of symptomatic oral lichen planus: a systematic review. *Photodermatol Photoimmunol Photomed*. 2018;34(3):167-174. doi: 10.1111/phpp.12371

[28] Chau L, Jabara JT, Lai W, Svider PF, Warner BM, Lin HS, Raza SN, Fribley AM. Topical agents for oral cancer chemoprevention: a systematic review of the literature. *Oral Oncol*. 2017;67:153-159. doi: 10.1016/j.oraloncology.2017.02.014

[29] Al-Maweri SA, Ashraf S, Kalakonda B, Halboub E, Petro W, AlAizari NA. Efficacy of photodynamic therapy in the treatment of symptomatic oral lichen planus: a systematic review. *J Oral Pathol Med*. 2018;47(4):326-332. doi: 10.1111/jop.12684

[30] Carvalho MV, de Moraes SLD, Lemos CAA, Santiago Júnior JF, Vasconcelos BCDE, Pellizzer EP. Surgical versus non-surgical treatment of actinic cheilitis: a systematic review and meta-analysis. *Oral Dis*. 2019;25(4):972-981. doi: 10.1111/odi.12916

[31] Hoseinpour Jajarm H, Asadi R, Bardideh E, Shafaee H, Khazaei Y, Emadzadeh M. The effects of photodynamic and low-level laser therapy for treatment of oral lichen

planus-a systematic review and meta-analysis. *Photodiagnosis Photodyn Ther*. 2018;23:254-260. doi: 10.1016/j.pdpdt.2018.07.001

[32] Li Y, Wang B, Zheng S, He Y. Photodynamic therapy in the treatment of oral leukoplakia: a systematic review. *Photodiagnosis Photodyn Ther*. 2019;25:17-22. doi: 10.1016/j.pdpdt.2018.10.023

[33] Jin X, Xu H, Deng J, Dan H, Ji P, Chen Q, Zeng X. Photodynamic therapy for oral potentially malignant disorders. *Photodiagnosis Photodyn Ther*. 2019;28:146-152. doi: 10.1016/j.pdpdt.2019.08.005

[34] Moreschi C, Grusovin MG, Cafaro A, Gambino A, Abati S, Gherlone EF, CapparÃ P, Gastaldi G. Is topical Photodynamic Therapy with 5-ALA , using Tsai's protocol , useful in the healing of oral potentially malignant disorders? A system-atic review of the literature. *Journal of Osseointegration*. 2019;11(2),78-84. doi: 10.23805/JO.2019.11.02.01

[35] Salgueiro AP, de Jesus LH, de Souza IF, Rados PV, Visioli F. Treatment of actinic cheilitis: a systematic review. *Clin Oral Investig*. 2019;23(5):2041-2053. doi: 10.1007/s00784-019-02895-z

[36] He Y, Deng J, Zhao Y, Tao H, Dan H, Xu H, Chen Q. Efficacy evaluation of photodynamic therapy for oral lichen planus: a systematic review and meta-analysis. *BMC Oral Health*. 2020;20(1):302. doi: 10.1186/s12903-020-01260-x

[37] Lai M, Pampena R, Cornacchia L, Pellacani G, Peris K, Longo C. Treatments of actinic cheilitis: a systematic review of the literature. *J Am Acad Dermatol*. 2020;83(3):876-887. doi: 10.1016/j.jaad.2019.07.106

[38] Varela-Centelles P, Seoane-Romero J, García-Pola MJ, Leira-Feijoo Y, Seoane-Romero JM. Therapeutic approaches for actinic cheilitis: therapeutic efficacy and malignant transformation after treatment. *Int J Oral Maxillofac Surg*. 2020;49(10):1343-1350. doi: 10.1016/j.ijom.2020.02.014

[39] Bakirtzi K, Papadimitriou I, Andreadis D, Sotiriou E. Treatment options and post-treatment malignant transformation rate of actinic cheilitis: a systematic review. *Cancers (Basel).* 2021;13(13):3354. doi: 10.3390/cancers13133354

[40] Łukaszewska-Kuska M, Ślebioda Z, Dorocka-Bobkowska B. The effectiveness of topical forms of dexamethasone in the treatment of oral lichen planus- a systematic review. *Oral Dis*. 2022;28(8):2063-2071. doi: 10.1111/odi.13966

[41] Trager MH, Farmer K, Ulrich C, Basset-Seguin N, Herms F, Geskin LJ, Bouaziz JD, Lebbé C, de Masson A, Bagot M, Dobos G. Actinic cheilitis: a systematic review of treatment options. *J Eur Acad Dermatol Venereol*. 2021;35(4):815-823. doi: 10.1111/jdv.16995

[42] Lin J, Ni G, Ding T, Lei S, Zhong L, Liu N, Pan K, Chen T, Zeng X, Xu H, Li T, Dan H. Photodynamic therapy for oral squamous cell carcinoma: a systematic review and meta-analysis. *International Journal of Photoenergy*. 2021;(10):1-14. doi: 10.1155/2021/6641358

[43] Wang B, Fan J, Wang L, Chai L. Photobiomodulation therapy/photodynamic therapy versus steroid therapy for oral lichen planus: a systematic review and meta-analysis. *Photobiomodul Photomed Laser Surg*. 2021;39(3):145-154. doi: 10.1089/photob.2020.4930

[44] Binnal A, Tadakamadla J, Rajesh G, Tadakamadla SK. Photodynamic therapy for oral potentially malignant disorders: a systematic review and meta-analysis. *Photodiagnosis Photodyn Ther*. 2022;37:102713. doi: 10.1016/j.pdpdt.2022.102713

[45] Choudhary R, Reddy SS, Nagi R, Nagaraju R, Kunjumon SP, Sen R. The effect of photodynamic therapy on oral-premalignant lesions: a systematic review. *J Clin Exp Dent*. 2022;14(3):e285-e292. doi: 10.4317/jced.59348

[46] Sandhu S, Klein BA, Al-Hadlaq M, Chirravur P, Bajonaid A, Xu Y, Intini R, Hussein M, Vacharotayangul P, Sroussi H, Treister N, Sonis S. Oral lichen planus: comparative efficacy and treatment costs-a systematic review. *BMC Oral Health*. 2022;22(1):161. doi: 10.1186/s12903-022-02168-4

[47] Waingade M, Medikeri RS, Rathod P. Effectiveness of methylene blue photosensitizers compared to that of corticosteroids in the management of oral lichen planus: a systematic review and meta-analysis. *J Dent Anesth Pain Med*. 2022;22(3):175-186. doi: 10.17245/jdapm.2022.22.3.175

[48] Yang Y, Shen S, Wang P, Wang X. Efficacy of photodynamic therapy in actinic cheilitis: a systematic review. *Photodiagnosis Photodyn Ther*. 2022;38:102782. doi: 10.1016/j.pdpdt.2022.102782

[49] Romano A, Di Stasio D, Lauritano D, Lajolo C, Fiori F, Gentile E, Lucchese A. Topical photodynamic therapy in the treatment of benign oral mucosal lesions: a systematic review. *J Oral Pathol Med*. 2021;50(7):639-648. doi: 10.1111/jop.13152

[50] Huber MA. White oral lesions, actinic cheilitis, and leukoplakia: confusions in terminology and definition: facts and controversies. *Clin Dermatol*. 2010;28(3):262-268. doi: 10.1016/j.clindermatol.2009.06.011

[51] Selvam NP, Sadaksharam J, Singaravelu G, Ramu R. Treatment of oral leukoplakia with photodynamic therapy: A pilot study. *J Cancer Res Ther*. 2015;11(2):464-467. doi: 10.4103/0973-1482.147703

[52] Grant WE, Hopper C, Speight PM, Macrobert AJ, Bown SG. Photodynamic therapy of malignant and premalignant lesions in patients with 'field cancerization' of the oral cavity. *J Laryngol Otol*. 1993;107(12):1140-1145. doi: 10.1017/s0022215100125496

[53] Jerjes W, Upile T, Hamdoon Z, Mosse CA, Akram S, Hopper C. Photodynamic therapy outcome for oral dysplasia. *Lasers Surg Med*. 2011;43(3):192-199. doi: 10.1002/lsm.21036

[54] Allison RR, Moghissi K. Oncologic photodynamic therapy: clinical strategies that modulate mechanisms of action. *Photodiagnosis Photodyn Ther*. 2013;10(4):331-341. doi: 10.1016/j.pdpdt.2013.03.011

[55] Ozog DM, Rkein AM, Fabi SG, Gold MH, Goldman MP, Lowe NJ, Martin GM, Munavalli GS. Photodynamic therapy: a clinical consensus guide. *Dermatol Surg*. 2016;42(7):804-827. doi: 10.1097/DSS.0000000000000800

[56] Li X, Lovell JF, Yoon J, Chen X. Clinical development and potential of photothermal and photodynamic therapies for cancer. *Nat Rev Clin Oncol*. 2020;17(11):657-674. doi: 10.1038/s41571-020-0410-2

[57] Varela-Centelles P, Seoane-Romero J, García-Pola MJ, Leira-Feijoo Y, Seoane-Romero JM. Therapeutic approaches for actinic cheilitis: therapeutic efficacy and malignant transformation after treatment. *Int J Oral Maxillofac Surg*. 2020;49(10):1343-1350. doi: 10.1016/j.ijom.2020.02.014

[58] Kou J, Dou D, Yang L. Porphyrin photosensitizers in photodynamic therapy and its applications. *Oncotarget*. 2017;8(46):81591-81603. doi: 10.18632/oncotarget.20189

[59] Kwiatkowski S, Knap B, Przystupski D, Saczko J, Kędzierska E, Knap-Czop K, Kotlińska J, Michel O, Kotowski K, Kulbacka J. Photodynamic therapy - mechanisms, photosensitizers and combinations. *Biomed Pharmacother*. 2018;106:1098-1107. doi: 10.1016/j.biopha.2018.07.049

Figure legends

Figure 1. Flow diagram of the literature search and selection criteria based on 2020 Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA).

Figure 2. Clinical response rates for the cases of oral potentially malignant disorders (oral leukoplakia, oral erythroleukoplakia, actinic cheilitis, and oral lichen planus), and oral squamous cell carcinomas.

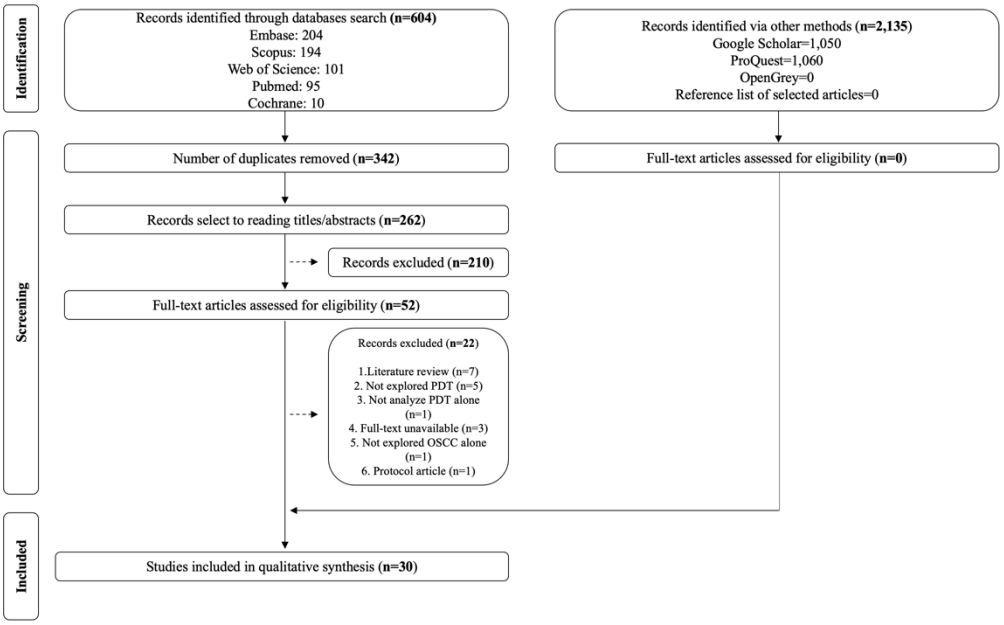


Figure 1. Flow diagram of the literature search and selection criteria based on 2020 Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA).

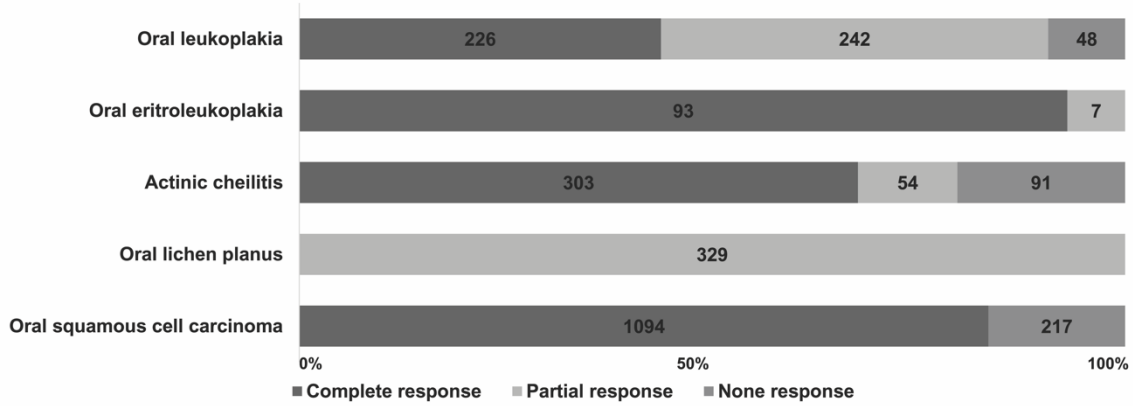


Figure 2. Clinical response rates for the cases of oral potentially malignant disorders (oral leukoplakia, oral erythroleukoplakia, actinic cheilitis, and oral lichen planus), and oral squamous cell carcinomas.

Table 1. Data regarding response rate, follow-up and recurrence rate of oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC) retrieved from systematic reviews

Variables	Response rate			Follow-up (months)	Recurrence rate
	Complete	Partial	None		
OPMD					
Actinic cheilitis (AC), *n*=448; 10 studies	67.6%	12.1%	20.3%	NI	4–15%
Oral lichen planus (OLP), *n*=576; 10 studies	42.9%	57.1%	0	0.5–48	NI
Oral leukoplakia (OLK), *n*=516; 8 studies	43.8%	46.9%	9.3%	NI	1–3%
Oral erythroleukoplakia (OEL), *n*=100; 8 studies	93%	7%	0	NI	13%
OSCC					
OSCC *n*=411; 4 studies	83.4%	0	16.6%	28.4	12%

Note: NI, not informed.

2.2 4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis in Wistar rats: a proposal for a guide for best practice based on a systematic literature review

Artigo publicado no periódico Pathology – Research and Practice (2022 Aug;236:153970, doi: 10.1016/j.prp.2022.153970) (**Anexos 3 e 4**).

Gisele Corrêa de Oliveira Zigmundo[1], Lauren Frenzel Schuch[2], Tuany Rafaeli Schmidt[1], Felipe Martins Silveira[1,3], Marco Antônio Trevizani Martins[1], Vinicius Coelho Carrard[1], Manoela Domingues Martins[1,2], Vivian Petersen Wagner[4]

[1] Department of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.
[2] Department of Oral Diagnosis, Piracicaba Dental School, Universidade de Campinas, Piracicaba, SP, Brazil.
[3] Molecular Pathology Area, School of Dentistry, Universidad de la República, Montevideo, Uruguay.
[4] Academic Unit of Oral and Maxillofacial Medicine and Pathology, Department of Clinical Dentistry, University of Sheffield, Sheffield, UK.

Corresponding Author: Vivian Petersen Wagner
Academic Unit of Oral and Maxillofacial Medicine and Pathology
19 Claremont Cres, Broomhall, Sheffield S10 2TA
Phone: +44 114 271 7801
e-mail address: vivianpetersen@hotmail.com
ORCID: 0000-0002-1447-2135
Twitter handle: @petersen_wagner

Keywords: Head and Neck Neoplasms; Oral Cancer; Chemoprevention; Animal Models; Prognosis; Precancerous Conditions

Abstract

Objective: Based on a critical review of published studies, we aimed to develop a good practice guide for using 4-nitroquinoline-1-oxide (4NQO) as an inducer of oral carcinogenesis in Wistar rats. **Design:** A systematic search was performed on Medline Ovid, PubMed, Embase, Web of Science, and Scopus databases. The SYRCLE's risk of bias tool was used to assess the quality of the studies. **Results:** Thirty-five articles met the selection criteria; 22 (62.9%) of them administered 4NQO systemically in drinking water, with a mean concentration of 30.2ppm (SD±15.9) and during a mean period of 20.8 (SD±7.8) weeks. The other 13 (37.1%) studies performed topical applications of 4NQO painting the oral mucosa of the animals three times a week (100%) with a mean period of administration of 16.8 (SD±7.0) weeks. Different 4NQO concentrations used for other periods achieved significant tumor development. Most studies didn't perform quantitative clinical analysis, and the histopathological diagnosis/grading criteria varied considerably. **Conclusions:** A poor description of solution care, adverse effects, and the number of losses were observed, and the reporting of these features needs to be improved. Suggestions to guide the development of future research are provided.

Keywords

Head and Neck Neoplasms; Oral Cancer; Chemoprevention; Animal Models; Prognosis; Precancerous Conditions

1. INTRODUCTION

Oral cancer ranks the sixth most prevalent in the global cancer incidence ranking [1,2]. Smoking, alcohol consumption, and sun exposure are reported as the main risk factors for lip and oral squamous cell carcinoma (OSCC) [3,4]. In addition, the habit's frequency and duration seem to be related to greater chances of developing tumors [5]. This fact also extends to the risk of relapse, the persistence of the disease, and the appearance of a second primary neoplasm, if maintaining habits after completing the treatment [6,7]. During oral carcinogenesis, potentially malignant oral disorders (OPMD) can arise[8]. Monitoring this type of lesion is recommended to allow an early cancer diagnosis [8,9]. Some features, such as epithelial dysplasia (ED), are recognized as poor prognostic markers for malignant transformation [10]. However, the process of oral carcinogenesis remains fully understood [11]. Also, a deeper understanding of this process can bring benefits related to the chemoprevention [12]. Heavy smokers and patients diagnosed with ED could benefit from chemopreventive measures if available [13]. Therefore, molecular biology involved in carcinogenesis is vital, and experimental animal research emerges as a valuable resource in this scenario [3,14].

4-nitroquinoline oxide (4NQO) is a mutagenic substance capable of generating changes in DNA that mimic tobacco-induced molecular events and can be used for experimental oral carcinogenesis development [15]. Once 4NQO is applied topically or systemically, an intense intracellular oxidative stress is generated, and reactive oxygen species (ROS), responsible for initiating the oral carcinogenesis process, are produced [15,16]. This model reproduces the different stages of tumor development clinically and microscopically, enabling monitoring structural and architectural changes in the epithelium until invasion occurs. Moreover, a recent study has confirmed that the mutational signatures of human OSCC and mouse 4NQO-induced OSCC broadly overlap [17]. Therefore, this model is a valuable tool for understanding oral carcinogenesis key events and valuable to study predictive markers and oncoprotective agents [3,18].

Oral carcinogenesis induced by 4NQO-induced is a classic and widely used animal model [12,15,16]. However, its use in basic research can be challenging since 4NQO is a highly toxic compound that should be used for an extended period to reach its carcinogenic effects in animals. Consequently, adverse effects and animal losses can occur during the experimental procedures, and the optimal dosage needs to

equalize adverse effects with effective tumor formation. It is essential to highlight that dose, time, and methods of application are all factors that, in conjunction, can influence the outcomes of the research. The main methodological aspects of the 4NQO-induced oral carcinogenesis model have never been critically reviewed and discussed. Therefore, this study systematically reviewed the studies published and built a good practice guide with suggestions for future reports on using 4NQO in Wistar rats. Researchers aiming to use the 4NQO method to test chemopreventive drugs or unveil basic mechanisms of oral carcinogenesis can significantly benefit from this review to determine the sample size calculation, solution care, dose and interval range, and recommendations on clinical and histological reports.

2. MATERIAL AND METHODS

2.1. Information sources and search strategies

The search strategy of this systematic review was performed in the following electronic databases: Medline Ovid (Wolters Kluwer), PubMed (National Library of Medicine), Web of Science (Thomson Reuters), Embase (Excerpta Medica database), and Scopus (Elsevier). The electronic surveys without publication date restrictions were carried out in August 2020 and updated in September 2021. The search strategy is presented in Table 1. A manual search was also performed on the reference lists of the final included studies and in the gray literature.

2.2. Eligibility criteria

Studies that evaluated 4NQO-induced oral carcinogenesis in Rattus Norvegicus (Wistar rats) were included.

Inclusion criteria consisted of studies that confirmed ED or OSCC development and presented an experimental group's results in which 4NQO was individually used.

Exclusion criteria were different strains of animals, carcinogenesis in locations outside the oral cavity, articles written in languages other than English, *in vitro* studies, abstracts, letters to the editor, and conference proceedings. When several publications from one research group using the same initial sample were available, only one study was included for data extraction.

2.3. Study selection

The initial selection by titles and abstracts was performed by two reviewers independently (GOZ and TRS). The publications initially selected were analyzed in full by three different reviewers (VPW, LFS, and FMS) to validate the framework of all the inclusion criteria provided. Divergences between reviewers' opinions were validated by a fourth reviewer (MDM), and the entire process of searching, including, and excluding information was described in detail in a data sheet.

2.4. Data extraction and synthesis

The following data were collected manually, when available:

(1) Studies: Author, year, country of origin, the purpose of the work (carcinogenesis, genotoxicity, or chemoprevention), and type of study endpoint (multiple vs. single).

(2) Animals: Sample size in the 4NQO group, number of animals per cage, gender, weight, age, losses during the study (number and reasons), adverse reactions observed, and euthanasia method and time.

(3) 4NQO: Method of substance application (topic *vs.* systemic), dilution dose, dilution vehicle, care with solution handling, and period of use. Moreover, solution exchange and consumption analysis intervals were retrieved for systemic studies; and number, interval, and method of applications (device used and standard product amount) and care after 4NQO delivery were retrieved for topic studies.

(4) Tumors: The leading site of development, clinical assessment, histological assessment (types of diagnosis and grading), time until morphological changes occurred (for studies with multiple endpoints), and percentage of architectural and morphological changes of the epithelium (epithelial hyperplasia - EH, epithelial dysplasia - ED, carcinoma in situ - CIS, and invasive OSCC) at last evaluation period.

(5) Other information: Systemic manifestations such as the presence of nodal or distant metastasis were also evaluated and retrieved if available. For data presentation, results were subdivided based on the method of 4NQO application due to the divergence of variables for collection in each type.

2.5. Quality assessment

The quality analysis of the articles included in this review was performed based on the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE) risk of bias tool [19].

2.6. Statistical analysis

The data collected were analyzed using the Statistical Package for the Social Sciences (SPSS) for Mac, version 27 (IBM Corporation, Armonk, NY). Continuous variables were compared among the application methods using the Mann-Whitney test due to a non-parametric distribution of data, and categorical variables were compared using the Chi-Square test. A p-value <0.05 was considered statistically significant.

2.7. Other information

The development of this research followed the norms described in the Declaration of Preferred Report Items for Systematic Reviews and Meta-analyzes (PRISMA), and it is registered in the International Prospective Registry of Systematic Reviews (PROSPERO) under number CRD4202120555.

3. RESULTS

3.1. Selection of studies

A total of 2638 articles were initially filtered. The electronic and manual removal of duplicates eliminated 1524 articles. The reading of the title and abstract excluded another 923 articles, and 191 publications were selected for full-text evaluation. Finally, 35 articles were included in this systematic review (Figure 1).

3.2. General characteristics of included studies

A summary of all extracted information from included studies evaluating systemic and topical 4NQO administration is present in Supplementary Table 1.

Altogether, the studies were published from 1981 to 2021. Brazil (n= 12; 34.3%) was the most common country of origin (Supplementary Figure 1). Among all studies, 22 (62.9%) used systemic administration of 4NQO, and 13 (37.1%) used topic administration of 4NQO. In the two different application methods, the main objective of studies was related to carcinogenesis (n = 22; 62.9%), followed by chemopreventive studies (n=13; 37.1%). The studies were also categorized according to endpoint type: multiple evaluation days (euthanasia periods) vs. single final analysis. It was observed that most studies (n=21; 60.0%) only performed a single evaluation at the end of the experimental period.

3.3. General characteristics of animal sample

Wistar rats were between 3 and 23 weeks old (mean=10.2; SD±5.0) at the beginning of the experimental phase, weighing between 80 and 400 g (mean= 205.4g; SD±65.9) and in most studies included only male rats (79.4%). The sample size of the 4NQO isolated group ranged from 5 to 192 animals (mean= 34.5; SD±44.6), and a mean of 3.4 (SD±1.5) animals was allocated per cage. The average time of euthanasia in the studies was 26.9 weeks (SD±20.1).

Comparing systemic and topic methods, no differences were observed for age (p=0.45; Mann-Whitney test), weight (p=0.13; Mann-Whitney test), and sample size (p=0.30; Mann-Whitney test). Interestingly, a significant difference was observed for gender (p<0.01, Chi-square test), as all systemic studies were performed with males only (21/21). Among topic studies, more variable use of genders was observed (6 – male, 4 – female, 3 – both).

Nine (25.7%) publications reported information about animal losses that occurred during the experimental periods. Among these two publications reported no losses [20,21]. In four studies [22–25], losses were described, but the causes of deaths were not reported. The reasons reported in the remaining three studies included debilitation related to the malignancy [26], death during the anesthetic procedure [27], and toxic effects of the 4NQO [28]. Based on studies with available information, the average animal loss was 14.75% in the 4NQO group. The percentage of losses was higher in the systemic method (16.6%) compared to the topic administration (10.90%), but no statistical difference was observed (p=0.38, Mann-Whitney test). Adverse reactions to carcinogen use were reported in 15 studies (42.8%), cited as animal weight loss/ malnutrition, dehydration, stress, and pain.

3.4. Administration of the carcinogenic

3.4.1. 4NQO systemic administration

The period of 4NQO administration ranged from 8 to 32 weeks (mean= 20.8 weeks; SD±7.8). In all included studies, the carcinogen was diluted and provided to the animals in drinking water *ad libitum*. The doses used ranged from 10ppm to 50ppm (mean=30.2; SD±15.9). Sixteen (45.7%) studies reported some care for solution preparation, such as freshly prepared solutions, number of solution changes during the week, and care with storage (brown bottles or use of aluminum foil).

None of the studies informed the cage's mean consumption of the 4NQO solution. Only one study reported that the mean consumption of liquid (water vs. diluted 4NQO)

was similar between the control and 4NQO groups up to 22 weeks of the experiment. Between 22 and 28 weeks, it was higher in the control group.[22] All studies reported that the leading development site of carcinoma lesions was the tongue (n = 22; 100%).

The euthanasia times (which correspond to the time that histologic evaluations were performed) in the systemic method ranged from 3 to 32 weeks with a mean of 27.7 (SD±18.8).

3.4.2. 4NQO topic administration

The mean application period was 16.8(SD±7.0) weeks, ranging from 8 to 30 weeks. In all studies, 4NQO applications occurred three times a week, at a concentration of 5000 ppm, and using propylene glycol as a dilution vehicle. All authors reported that the solution was applied locally or painted on the animals' tongues. Six (46.1%) studies describe the specific device used (microbrush, cotton swab, and precision pipette), and only five studies reported the application of a standard amount of solution (0.03 mg to 10 mg). Other precautions with 4NQO solution administration were reported, such as 2 hours of water deprivation after 4NQO application [20,27] or 10 minutes wait to reverse neuroleptanalgesia [26] to ensure the contact between the carcinogen and the oral mucosa. Care with the preparation of 4NQO solutions has not been reported in any topic study. Within this method, the lesions have been reported to occur at various oral sites (n = 5; 38.4%), tongue (n = 4; 30.8%), palate (n = 3; 23.1%), and on the buccal mucosa (n = 1; 7.7%). Euthanasia times in the topical method ranged from 1/2 to 120 weeks, with a mean of 28.2 (SD ± 32.3) weeks.

3.5. Clinical evaluation

Sixteen (45.7%) studies carried out qualitative descriptions of the appearance of the lesions, such as the presence of white plaques, exophytic lesions, and ulcers. Only Vered et al. [29] reported the number of lesions per animal, considering that each animal could have more than one. The other studies reported the total number of animals presenting clinically visible lesions. Three studies calculated the tumor volume using a digital caliper. One study digitally evaluated the tumor area using images of the tongues photographed near a reference object with known measures. In the same study, the lesions were classified based on the clinical aspect in 0 – normal surface; 1 – small papules; 2 – thick plaques; 3 – nodules; 4 – ulcerated. [25]

3.6. Histopathological evaluation

Histological evaluation was quite variable between studies; some authors used dichotomous classifications, such as ED x OSCC; and others included a broader range of diagnoses, including epithelial atrophy, EH, ED according to different grades, CIS, and OSCC.

The presence or absence of EH was mentioned in 22 (62.8%) studies; ED in 28 (80%); CIS in 5 (14.3%); and OSCC in 28 (80%). ED grading was performed in 12 (34.3%) studies and OSCC grading only 5 (14.3%). Once present, the classification systems used for ED were the binary classification system (low or high risk; n = 1), the WHO [30] classification system for epithelial dysplasia (mild, moderate, severe; n = 4), and according to the classification of Bánóczy and Csiba (mild, moderate, severe and in situ carcinoma, n=5) [31]. Rosin et al. [32] graded the lesions based on architectural and cellular epithelial changes [33]. For the OSCC grading system at the invasive tumor, the front was used in two studies and the histological classification recommended by the WHO (based on cell differentiation) in three studies [30].

Two studies with systemic administration of 4NQO evaluated tumor thickness in micrometers (μm) by measuring the distance between the deepest point of OSCC invasion to the surface of the ulcer or epithelium.

3.7. Carcinogenesis outcomes

3.7.1. Multiple endpoints

Table 2 presents the moment each epithelial alteration was initially observed and the percentage of cases (when available) for studies with multiple endpoints. Few studies have described EH as the initial morphological alteration analyzed. This epithelial alteration was observed as early as two weeks [20,34] under topical administration (5000ppm) and systemic administration with at least three weeks (20ppm) [35].

Five systemic studies have identified ED and two topic studies with multiple endpoints. This change was observed at different times, starting at two weeks for the topic administration (5000ppm) [20] and seven weeks for the systemic administration (10ppm) [22].

Most studies assessed the development of invasive OSCC, but some have included CIS in their analysis. Herein, we included CIS within the OSCC group to analyze results, as the individual analysis of CIS isolated would be hindered due to the

small sample size. Therefore, among studies with multiple endpoints, OSSC was diagnosed with a minimum of 8 weeks for the topic administration [34] and 16 weeks for the systemic administration (50ppm) [24]. Yet, some systemic studies with a single evaluation time found cases of OSCC at 12 weeks of analysis (Table 3).

3.7.2. Single/last endpoint

The percentage of animals exhibiting ED and OSCC at the last (or single) period of euthanasia is presented in Table 3. The overall percentage of ED and OSCC was highly variable, ranging from 0% to 100%. Six systemic studies reached 100% of ED at the end of the experiment. The dose administered in 5/6 studies was 20 ppm and 50ppm in 1/6. The administration time ranged from 8 weeks to 12 weeks, with the euthanasia being performed between 8 and 32 weeks. Four studies with the topic method achieved 100% of animals exhibiting ED, using 5000ppm of 4NQO during 16, 20, or 30 weeks and euthanasia being performed as early as 16 weeks and as late as 40 weeks.

Four systemic studies and five topical studies achieved 100% incidence of OSCC at the last evaluation time. Among systemic studies, three used the same protocol: 20ppm of 4NQO for 32 weeks, with the euthanasia performed simultaneously. The other study used a dose of 10ppm for 28 weeks, also with euthanasia at the same time. Many studies that used 50ppm during a shorter period (20 weeks) achieved high values of OSCC development as well, around 80%. Among topic studies, the carcinogen was applied from 16 to 24 weeks and euthanasia periods ranged from 16 to 120 weeks.

3.8. Metastasis

One study evaluated enlarged lymph nodes after topic administration in the palate [26]. Histological analysis revealed that it mainly consisted of reactive hyperplasia. Still, in one case, cervical lymph nodes metastasis was diagnosed from a moderately differentiated squamous cell carcinoma located to the gingiva of the upper jaw. Nauta et al. (topical administration, 26 weeks) reported no sign of metastasis [20]. No further reports of metastasis were mentioned in the revised studies.

3.9. Determination of the methodological reliability of the included studies

All articles had a declaration of ethics and information about the environmental conditions (temperature and light) to which the animals were submitted. Overall, most studies provided unclear methodological information on animal selection and allocation (51.4%), group randomization (80.0%), and investigator blinding (45.7%). However, most studies (80.0%) described their outcomes in detail, and, in general, the studies were free from a high risk of bias (71.4%) (Supplementary Figure 2).

4. Discussion

4NQO-induced carcinogenesis is a classic animal model used in 1960 as an in vivo tool to study oral cancer development [36,37]. The present systematic review confirmed the efficacy of 4NQO for experimental oral carcinogenesis through topical and systemic administration (Figure 4). Considerable heterogeneity was observed among systemic protocols with the mean intervals of applications and doses ranging from 10 ppm and 50 ppm, and a more uniform dosage for the topical form (5000 ppm). In general, information regarding the adverse effects of substance administration and numbers of animal losses have been poorly reported. Our discussion is presented according to the main critical aspects related to this method.

4.1. Systemic x Topic administration

The systemic administration method was the most used by the authors. It is important to note that all studies carried out after 2016 were conducted using this technique. This method is thought to have some advantages over the topic, such as the better result of tumor induction, shorter experimental periods, and better reproduction of the carcinogenesis concept, as all the mucosa is exposed to the carcinogen. In addition, lower concentrations of the substance are offered in the systemic method [38]. However, despite a clear trend towards using systemic administration, we still observed that topic use could also be effective as there were (n=5; 38.5%) studies that achieved 100% of oral carcinoma incidence with this approach. Yet, some peculiarities of this applied method include the need to sedate the animal with each application and wait for a period for the carcinogen to act on the mucosa [39,40].

On the other hand, as the animals do not receive the carcinogen systemically, fewer adverse effects would be expected within the topic administration, representing an advantage. Animals would also not develop primary tumors in other sites such as the

gastrointestinal tract, impairing the animal's systemic health. Although our review revealed no significant differences in the number of animal losses between the two methods, the limited number of authors reporting such findings does not allow us to reach a definitive conclusion.

4.2. Dose and period of administration

Due to intrinsic characteristics of different animal strains that can influence the outcomes after 4NQO administration, we decided to focus this systematic review on Wistar rats to establish the optimal dosages and time intervals for this specific strain. Unfortunately, we observed high heterogeneity in methods, especially for systemic administration in which the time and interval both varied considerably among studies. Notably, protocols using small doses such as 10ppm only achieved tumor formation after 22 weeks (17.0%; 100% OSCC) [20,22,29]. However, short analysis periods were sufficient for tumor development when higher doses were administered (16 weeks) [24]. Moreover, the most common dosage used was 50ppm (n=8) during different times such as 16, 20, and 22 weeks, which resulted in a percentage of tumor formation that ranged from 35.2% to 88.0%.

Another debatable issue is the adverse effects of the method. All the studies do not clearly describe the number of losses and adverse effects. This represents a critical limitation for data interpretation because 50ppm is a considerably high dose and could probably be associated with more adverse effects in the long-term period. Interestingly, some studies that used 4NQO at 50ppm for 16 weeks had lower losses (n=3; 9.4%) [24] and achieved a considerable OSCC incidence (52%) by performing the euthanasia in 16 weeks which suggest that this protocol could be effective and safe. The analysis of the topical studies demonstrated a greater homogeneity as all applications were carried out three times a week at a concentration of 5000ppm. The only difference observed was the period that varied from 8 to 30 weeks (mean = 16.8 weeks).

Dosage and interval determination should agree with the authors' aim within the study. If the authors desire to evaluate an intermediate moment during the oral carcinogenesis process, our systematic review suggests that ED can occur around seven weeks (systemic administration, 10ppm) to 16 weeks (systemic administration, 20ppm; topic administration, 5000ppm). For the last endpoint period, authors should consider whether they desire a high incidence of OSCC (around 100% - which can be achieved with both 20ppm for approximately 32 weeks or 50ppm for 20 weeks

systematically in an effective way) or a broader range of diagnosis. For example, some studies might benefit from final samples with different degrees of ED, early tumors, and more advanced ones. For this purpose, the dose or interval of use could be reduced (20ppm for 20 weeks used systemically, for example). Unfortunately, due to limited information on animal loss, we couldn't compare if using smaller doses for longer times is less harmful than using higher doses for shorter periods.

4.3. Solution/application care and adverse effects

Overall, poor descriptions of solution preparation care and adverse effects were observed for both methods. 4NQO is sensitive to light. Thus, it should be stored in amber bottles or bottles wrapped in aluminum foil. For systemic delivery, this also applies to the bottles used for solution delivery. Also, 4NQO is stable under specific storage conditions, including a –20°C. Therefore, it is recommended to produce a stock solution, store it in the appropriate state, and freshly prepare the solutions used for topic or systemic administration. Unfortunately, only 16 (45.7%) authors reported such care, representing a significant limitation.

The solution consumption per cage should be measured within the systemic method and indicate the mean consumption per animal. Unfortunately, individual consumption is not feasible to calculate. This happens because it is not ideal for secluding one rat per cage as they are friendly animals, and isolation is a stressful event that can influence tumor development. Verza et al. recently demonstrated that stress triggered by social isolation increased the incidence of 4NQO-induced carcinomas by 20.4% in Wistar rats compared to animals that remained grouped during the experimental period [28]. Our review observed that the cage consumption was poorly reported, representing a limitation. In addition, we observed that few studies report a standardization of the amount of substance applied for the topic method. Just three authors were careful to measure and inform the amount of carcinogen painted on the mucosa and not offer the animals water or food until 2 hours after applying 4NQO [20,26,41].

The present review observed that systemic adverse reactions were reported in 42.9% of studies. Among them, substantial loss of body weight was the most cited. Like what occurs in humans, the loss of body mass can occur due to several factors, such as difficulty in chewing and swallowing food and the increase in the metabolic rate that contributes to the decrease of energy reserve and the lack of appetite. We

identify only nine studies informing this data concerning animal losses during the experimental period. When present, the main reasons were precisely the aggressiveness of the tumors formed in some animals. The mortality rate of the 4NQO experimental group is key data, as it directly influences the sample calculation. Overall, the mean loss in the 4NQO group was 16%, yet this mean was established from studies using different doses and time intervals. Authors aiming to perform a sample size calculation should seek information concerning the percentage of losses in studies using similar methodologies, if possible.

4.4. Clinical and histopathological outcomes

Clinically, lesions induced by 4NQO can initially range from spots and white plaques (like human oral leukoplakia), followed by reddish, erosive, and ulcerative areas and nodules (Figure 2) [39]. In sixteen (45.7%) studies included in this review, the clinical description of the lesions was described, but there was no uniformity in these reports. Most authors do not detail all the aspects observed, such as the number of injuries by animals, the clinical aspect, and the size of the lesion. At present, methods for determining lesion area included photographs next to a reference object followed by the free-hand digital [25] and macroscopic analysis of tumor volume using the formula width x length x height [28]. Moreover, other methods have been reported in 4NQO studies using different animal strains, such as a threshold for clinical evaluation. Only macroscopic evident tumors with more than 0.2cm are included in the tumor volume calculation [42]. Other authors have used biweekly complete oral cavity examination and tongue photographs under local anesthesia. This exciting approach evaluates the progression of clinical aspects within each animal during the experimental [38]. Despite the method of choice, it appears to be essential to include at least one type of clinical analysis. Wagner et al., 2020 demonstrated that clinical features correlate with histopathological diagnosis in the 4NQO model [25], but further clinic-pathologic correlations could significantly increase our understanding of oral carcinogenesis.

The presence of a microscopic analysis was an inclusion criterion for our systematic review as this is the gold standard to determine the status of epithelial changes. However, we observed significant heterogeneity in how this analysis was performed. During oral carcinogenesis, stepwise cumulative changes in the epithelium range from EH, ED, CIS, and invasive OSCC (Figure 3). While some authors performed a

dichotomous analysis of ED *vs.* OSCC, others included a higher range of diagnosis, comprising histological grading. We support that ED grading is information to be included in the analysis, as there are significant differences between a mild and a severe ED in terms of clinical relevance.

OSCC grading, on the other hand, in the 4NQO model context doesn't seem to have this more considerable influence as most of the tumors are well-differentiated, presenting abundant keratin formation. Yet, in our experience, differentiated tumors can have different invasive capacities, and, in this review, we observed that none of the included studies evaluated, for example, depth of invasion. This feature is currently acknowledged as a key histologic feature associated with tumor staging and aggressiveness [43].

In the present review, some authors use the number of lesions as a sample unit (instead of the number of animals). We recognize that as the 4NQO model reproduces the cancerization concept, more than one lesion (and with a different diagnosis) can be present within the same animal's mucosa. In our opinion, this method for data presentation can be as relevant as the number of animals with lesions, and authors can choose a more appropriate or suitable way considering their aim. Yet, we recommend including the final percentage of tumors in the experimental groups (based on the more aggressive diagnosis per animal) so correlations between tumor incidence and the 4NQO protocol (dose and time) can be performed.

4.5. Metastasis

An advantage of the 4NQO-model is the possibility of metastasis development [44]. In this review, only two old studies using the topical application reported the results of assessments of the presence of metastases in the animals' [20,26]. The ability of 4NQO to induce nodal metastasis has been known since the sixties when Fujino et al. demonstrated the presence of a considerable number of metastatic lymph nodes following 4NQO topic administration at 0.25% dose in mice [36]. Moreover, the authors found one case of lung metastasis, which has also been documented in the F344 rat strain following the 4NQO administration [45]. Despite being poorly reported, metastatic lesions seem to be infrequent in the Wistar rats [46,47]. We believe further studies need to be performed, including immunohistochemical analysis of lymph nodes and lungs. Yet, until additional reports are available, authors should consider the low

risk of metastasis a possible limitation inherent to Wistar rat strain and choose other mice models if metastasis analysis is a chief outcome in the study aim.

4.6. Limitations

In the present study, we gathered important data about the 4NQO protocol to induce oral carcinogenesis; however, missing information in the original reports impaired a more robust analysis, representing an inherent limitation of systematic reviews. Another end of the present study concerns Wistar rats as an inclusion criterion. The 4NQO model is also efficient in other murine strains such as the Sprague Dawley rats and mice. To draw more accurate conclusions on the methods per se (dose *vs.* interval and how it affects the percentage of tumor development), we decided to focus our review on a specific strain, and the Wistar rat was chosen based on its widespread use. This has probably affected the worldwide distribution of studies reviewed herein, a common theme used in Brazil [48,49]. While the discussion raised concerning essential data to be reported, such as losses and adverse effects, solution care and standardization of methods are relevant to the 4NQO model in any murine strain, more specific analysis of doses and time of administration is restricted to Wistar rats' studies and should not be extrapolated to other rat strains and mice.

5. Conclusion

The data retrieved in this systematic review confirms the efficiency of 4NQO in inducing oral carcinogenesis in Wistar rats at certain time intervals and dosages. Based on a critical analysis of studies, our suggestions for further reports comprise:

- Include information on solution care, such as how often freshly prepared solutions and storage conditions.
- Report the complete administration scheme (dose, dilution vehicle, and period).
- For systemic administration: report the average solution intake per animal, calculated based on cage consumption.
- For topic application: report how the amount of substance was standardized, and which care measurements were taken to allow the carcinogen to act.
- Report adverse effects and losses in the experimental groups (if no loss occurred, state that in the methods/results).
- Report anatomical location of the lesion's development.

- Report lymph node enlargement status. Data on visual inspection could benefit our understanding of the prevalence of nodal metastasis in Wistar rats following 4NQO administration. But, if possible, the histopathological analysis should be performed to allow a more precise conclusion.
- Suppose the sample unit used for analysis is the total number of lesions (with the possibility of more than one lesion per animal). In that case, authors are also advised to include the overall incidence of tumor formation (considering the most aggressive diagnosis) to determine how dose vs. interval relates to tumor incidence accurately.
- Include a type of clinical analysis, such as descriptive (lesion type - white plaque, nodule, or ulcer) or quantitative (lesion size).
- Analyze the degree of epithelial dysplasia.

Acknowledgments

This study was supported by the Coordination of Improvement of Higher Education Personnel (CAPES, Finance Code 001) Brazil. Lauren F Schuch and Tuany R Schmidt are the recipients. Manoela D Martins is a research fellow funded by the Brazilian National Council for Scientific and Technological Development (CNPq). The authors are thankful to Tobias R. Spuldaro and Cassiano K. Rösing for the clinical and histopathological images of 4NQO-induced carcinogenesis.

Conflict of interest statement

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

6. References

[1] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries, CA. Cancer J. Clin. 71 (2021) 209–249. https://doi.org/10.3322/caac.21660.

[2] N. Cohen, S. Fedewa, A.Y. Chen, Epidemiology and Demographics of the Head and Neck Cancer Population, Oral Maxillofac. Surg. Clin. North Am. 30 (2018) 381–395. https://doi.org/10.1016/j.coms.2018.06.001.

[3] T. Tanaka, R. Ishigamori, Understanding carcinogenesis for fighting oral cancer, J. Oncol. 2011 (2011). https://doi.org/10.1155/2011/603740.

[4] International Agency for Research on Cancer, W.H. Organization, Lip, oral cavity: Globocan 2018, Glob. Cancer Obs. (2019) 2018–2019. https://gco.iarc.fr/today/data/factsheets/cancers/1-Lip-oral-cavity-fact-sheet.pdf.

[5] P.H. Montero, S.G. Patel, Cancer of the Oral Cavity, Surg. Oncol. Clin. N. Am. 24 (2015) 491–508. https://doi.org/10.1016/j.soc.2015.03.006.

[6] C. Rivera, Essentials of oral cancer, Int. J. Clin. Exp. Pathol. 8 (2015) 11884–11894. https://doi.org/10.5281/zenodo.192487.

[7] B. Du, H. Leung, K.M.F. Khan, C.G. Miller, K. Subbaramaiah, D.J. Falcone, A.J. Dannenberg, Tobacco smoke induces urokinase-type plasminogen activator and cell invasiveness: Evidence for an epidermal growth factor receptor-dependent mechanism, Cancer Res. 67 (2007) 8966–8972. https://doi.org/10.1158/0008-5472.CAN-07-1388.

[8] I. Van der Waal, Oral potentially malignant disorders: Is malignant transformation predictable and preventable?, Med. Oral Patol. Oral Cir. Bucal. 19 (2014) 1–5. https://doi.org/10.4317/medoral.20205.

[9] P. Shetty, S. Hegde, K. Vinod, S. Kalra, P. Goyal, M. Patel, Oral Leukoplakia : Clinicopathological Correlation and Its Relevance to Regional Tobacco-related Habit Index, 17 (2016) 601–608.

[10] I. Van der Waal, Potentially malignant disorders of the oral and oropharyngeal mucosa; present concepts of management, Oral Oncol. 46 (2010) 423–425. https://doi.org/10.1016/j.oraloncology.2010.02.016.

[11] K. El-Bayoumy, K.M. Chen, S.M. Zhang, Y.W. Sun, S. Amin, G. Stoner, J.B. Guttenplan, Carcinogenesis of the oral cavity: Environmental causes and potential

prevention by black raspberry, Chem. Res. Toxicol. 30 (2017) 126–144. https://doi.org/10.1021/acs.chemrestox.6b00306.

[12] A. Lan, W. Li, Y. Liu, Z. Xiong, X. Zhang, S. Zhou, O. Palko, H. Chen, M. Kapita, J.R. Prigge, E.E. Schmidt, X. Chen, Z. Sun, X.L. Chen, Chemoprevention of oxidative stress-associated oral carcinogenesis by sulforaphane depends on NRF2 and the isothiocyanate moiety, Oncotarget. 7 (2016) 53502–53514. https://doi.org/10.18632/oncotarget.10609.

[13] S. Kumar, M. Muniyandi, Tobacco Use and Oral Leukoplakia : Cross-sectional Study among the Gond Tribe in Madhya Pradesh, Asian Pacif J. Cancer Prev. 16 (2015) 1515–1518. https://doi.org/http://dx.doi.org/10.7314/APJCP.2015.16.4.1515.

[14] B. Mognetti, F. Di Carlo, G.N. Berta, Animal models in oral cancer research, Oral Oncol. 42 (2006) 448–460. https://doi.org/10.1016/j.oraloncology.2005.07.014.

[15] D. Kanojia, M.M. Vaidya, 4-Nitroquinoline-1-oxide induced experimental oral carcinogenesis, Oral Oncol. 42 (2006) 655–667. https://doi.org/10.1016/j.oraloncology.2005.10.013.

[16] I. Kaplan, J. Engelberg, D. Dayan, The effect of desalivation on 4-nitroquinoline-1-oxide-induced tongue carcinogenesis: A morphometric study of nucleolar organizer regions, J. Oral Pathol. Med. 30 (2001) 48–52. https://doi.org/10.1034/j.1600-0714.2001.300108.x.

[17] I. Sequeira, M. Rashid, I.M. Tomás, M.J. Williams, T.A. Graham, D.J. Adams, A. Vigilante, F.M. Watt, Genomic landscape and clonal architecture of mouse oral squamous cell carcinomas dictate tumour ecology, Nat. Commun. 11 (2020) 5671. https://doi.org/10.1038/s41467-020-19401-9.

[18] M. Vered, N. Yarom, D. Dayan, 4NQO oral carcinogenesis: Animal models, molecular markers and future expectations, Oral Oncol. 41 (2005) 337–339. https://doi.org/10.1016/j.oraloncology.2004.07.005.

[19] C.R. Hooijmans, M.M. Rovers, R.B.M. De Vries, M. Leenaars, M. Ritskes-Hoitinga, M.W. Langendam, SYRCLE’s risk of bias tool for animal studies, BMC Med. Res. Methodol. 14 (2014) 1–9. https://doi.org/10.1186/1471-2288-14-43.

[20] J.M. Nauta, J.L.N. Roodenburg, P.G.J. Nikkels, M.J.H. Witjes, A. Vermey, Epithelial dysplasia and squamous cell carcinoma of the Wistar rat palatal mucosa: 4NQO model, Head Neck. 18 (1996) 441–449. https://doi.org/10.1002/(sici)1097-0347(199609/10)18:5<441::aid-hed7>3.0.co;2-5.

[21] G.R. Soares, C.F.G. de Moura, M.J.D. Silva, W. Vilegas, A.B. Santamarina, L.P. Pisani, D. Estadella, D.A. Ribeiro, Protective effects of purple carrot extract (Daucus carota) against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide, Med. Oncol. 35 (2018). https://doi.org/10.1007/s12032-018-1114-7.

[22] D. Dayan, A. Hirshberg, I. Kaplan, N. Rotem, L. Bodner, Experimental tongue cancer in desalivated rats, Eur. J. Cancer Part B Oral Oncol. 33 (1997) 105–109. https://doi.org/10.1016/S0964-1955(96)00048-6.

[23] S. Helli, H. Damghani, D. Mohajeri, M. Mesgari Abbasi, R. Attaran, M. Zahed, Evaluation of the Effect of Two Different Systemic Doses of Viola Odorata on Prevention of Induced Tongue Dysplasia in Rats., J. Dent. (Shiraz, Iran). 17 (2016) 185–92. http://www.ncbi.nlm.nih.gov/pubmed/27602393.

[24] H.P. Cecilio, V.B. Valente, K.M. Pereira, G.M. Kayahara, C. Furuse, É.R. Biasoli, G.I. Miyahara, S.H.P. Oliveira, D.G. Bernabé, Beta-adrenergic blocker inhibits oral carcinogenesis and reduces tumor invasion, Cancer Chemother. Pharmacol. 86 (2020) 681–686. https://doi.org/10.1007/s00280-020-04149-2.

[25] V.P. Wagner, T.R. Spuldaro, F. Nör, E.J. Gaio, R.M. Castilho, V.C. Carrard, C.K. Rösing, Can propranolol act as a chemopreventive agent during oral carcinogenesis? An experimental animal study., Eur. J. Cancer Prev. Publish Ah (2020). https://doi.org/10.1097/CEJ.0000000000000626.

[26] A. V. Fisker, P.H. P., K. Overvad, Dose-Response Relationship in Complete Oral 4Nqo-Carcinogenesis in Rats, Acta Pathol. Microbiol. Scand. Ser. A Pathol. 95 A (1987) 281–288. https://doi.org/10.1111/j.1699-0463.1987.tb00042_95A.x.

[27] J.W. Braams, M.J.H. Witjes, C.A.A.M. Nooren, P.G.J. Nikkels, W. Vaalburg, A. Vermey, J.L.N. Roodenburg, Detection of oral dysplasia in animals with fluorine-18-fdg and carbon-11-tyrosine, J. Nucl. Med. 39 (1998) 1476–1480.

[28] F.A. Verza, V.B. Valente, L.K. Oliveira, G.M. Kayahara, M.M. Crivelini, C. Furuse, É.R. Biasoli, G.I. Miyahara, S.H.P. Oliveira, D.G. Bernabé, Social isolation stress facilitates chemically induced oral carcinogenesis, PLoS One. 16 (2021). https://doi.org/10.1371/journal.pone.0245190.

[29] M. Vered, N. Daniel, A. Hirshberg, D. Dayan, Histomorphologic and morphometric changes in minor salivary glands of the rat tongue during 4-nitroquinoline-1-oxide-induced carcinogenesis, Oral Oncol. 39 (2003) 491–496. https://doi.org/10.1016/S1368-8375(03)00011-3.

[30] K. Ranganathan, L. Kavitha, Oral epithelial dysplasia: Classifications and clinical relevance in risk assessment of oral potentially malignant disorders., J. Oral Maxillofac. Pathol. 23 (2019) 19–27. https://doi.org/10.4103/jomfp.JOMFP_13_19.

[31] J. Bánóczy, Á. Csiba, Occurrence of epithelial dysplasia in oral leukoplakia, Oral Surgery, Oral Med. Oral Pathol. 42 (1976) 766–774. https://doi.org/10.1016/0030-4220(76)90099-2.

[32] F.C.P. Rosin, A.R.P. Barcessat, G.G. Borges, L. Corrêa, Effect of 5-ALA-mediated photodynamic therapy on mast cell and microvessels densities present in oral premalignant lesions induced in rats, J. Photochem. Photobiol. B Biol. 153 (2015) 429–434. https://doi.org/10.1016/j.jphotobiol.2015.10.027.

[33] M. Bryne, H.S. Koppang, R. Lilleng, Å. Kjærheim, Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value, J. Pathol. 166 (1992) 375–381. https://doi.org/10.1002/path.1711660409.

[34] A. V. Fisker, T. Kariung, Cell population kinetics in rat palatal epithelium treated with the carcinogen 4-nitroquinoline N-oxide (4NQO), J. Oral Pathol. Med. 10 (1981) 408–416. https://doi.org/10.1111/j.1600-0714.1981.tb01292.x.

[35] M.G. Teixeira Buck, P. Souza Cabral Tuci, F.C. Perillo Rosin, A.R. Pinheiro Barcessat, L. Corrêa, Immunohistochemistry profile of p75 neurotrophin receptor in oral epithelial dysplasia and oral squamous cell carcinoma induced by 4-nitroquinoline 1-oxide in rats, Arch. Oral Biol. 96 (2018) 169–177. https://doi.org/10.1016/j.archoralbio.2018.09.010.

[36] H. Fujino, T. Chino, T. Imai, Experimental production of labial and lingual carcinoma by local application of 4-nitroquinoline N-oxide., J. Natl. Cancer Inst. 35 (1965) 907–18. http://www.ncbi.nlm.nih.gov/pubmed/5856681.

[37] K. Wallenius, U. Lekholm, Oral cancer in rats induced by the water-soluble carcinogen 4-nitrochinoline N-oxide., Odontol. Revy. 24 (1973) 39–48. http://www.ncbi.nlm.nih.gov/pubmed/4514062.

[38] R. Czerninski, P. Amornphimoltham, V. Patel, A.A. Molinolo, J.S. Gutkind, Targeting mammalian target of rapamycin by rapamycin prevents tumor progression in an oral-specific chemical carcinogenesis model., Cancer Prev. Res. (Phila). 2 (2009) 27–36. https://doi.org/10.1158/1940-6207.CAPR-08-0147.

[39] A.R.R. Barcessat, I. Huang, G.D. Rabelo, F.C.P. Rosin, L.G.V. Ferreira, J.G. de Cerqueira Luz, L. Corrêa, Systemic toxic effects during early phases of topical 4-NQO-

induced oral carcinogenesis in rats, J. Oral Pathol. Med. 43 (2014) 770–777. https://doi.org/10.1111/jop.12190.

[40] J.M. Nauta, J.L.N. Roodenburg, P.G.J. Nikkels, M.J.H. Witjes, A. Vermey, Comparison of epithelial dysplasia-the 4NQO rat palate model and human oral mucosa, Int. J. Oral Maxillofac. Surg. 24 (1995) 53–58. https://doi.org/10.1016/S0901-5027(05)80857-4.

[41] A. V. Fisker, M.J. West, H.P. Philipsen, A.H. Andersen, Quantification of oral epithelial hyperplasia in rats after topical application of the carcinogen 4-nitroquinoline 1-oxide, Acta Odontol. Scand. 48 (1990) 125–131. https://doi.org/10.3109/00016359009005868.

[42] R.K. Wali, D.P. Kunte, M. De La Cruz, A.K. Tiwari, J. Brasky, C.R. Weber, T.P. Gibson, A. Patel, S.D. Savkovic, B.E. Brockstein, H.K. Roy, Topical polyethylene glycol as a novel chemopreventive agent for oral cancer via targeting of epidermal growth factor response., PLoS One. 7 (2012) e38047. https://doi.org/10.1371/journal.pone.0038047.

[43] M.B. Amin, S. Edge, F. Greene, D.R. Byrd, R.K. Brookland, M.K. Washington, J.E. Gershenwald, C.C. Compton, K.R. Hess, D.C. Sullivan, J.M. Jessup, J.D. Brierley, L.E. Gaspar, R.L. Schilsky, C.M. Balch, D.P. Winchester, E.A. Asare, M. Madera, D.M. Gress, L.R. Meyer, AJCC Cancer Staging Manual, 8th ed., Springer International Publishing, New York, 2017.

[44] J. Tanuma, H. Shisa, H. Hiai, S. Higashi, Y. Yamada, T. Kamoto, Y. Hirayama, H. Matsuuchi, M. Kitano, Quantitative trait loci affecting 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in the rat., Cancer Res. 58 (1998) 1660–4. http://www.ncbi.nlm.nih.gov/pubmed/9563479.

[45] K. Ishida, H. Tomita, T. Nakashima, A. Hirata, T. Tanaka, T. Shibata, A. Hara, Current mouse models of oral squamous cell carcinoma: Genetic and chemically induced models, Oral Oncol. 73 (2017) 16–20. https://doi.org/10.1016/j.oraloncology.2017.07.028.

[46] G. Stenman, Malignancy of 4nqo-induced oral squamous cell carcinomas in the rat, Acta Otolaryngol. 92 (1981) 557–561. https://doi.org/10.3109/00016488109133295.

[47] R. Suzuki, H. Kohno, S. Sugie, T. Tanaka, Dietary Protocatechuic Acid During the Progression Phase Exerts Chemopreventive Effects on Chemically Induced Rat Tongue Carcinogenesis, Asian Pacific J. Cancer Prev. 4 (2003) 319–326.

[48] C.A. Rivera Martínez, 4NQO Carcinogenesis: A Model of Oral Squamous Cell Carcinoma, Int. J. Morphol. 30 (2012) 309–314. https://doi.org/10.4067/S0717-95022012000100055.

[49] S.R. Miranda, J. Noguti, J.G. Carvalho, C.T.F. Oshima, D.A. Ribeiro, Oxidative DNA damage is a preliminary step during rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide, J. Mol. Histol. 42 (2011) 181–186. https://doi.org/10.1007/s10735-011-9323-9.

[50] D.A. Ribeiro, D.M.F. Salvadori, Gingival Changes in Wistar Rats after Oral Treatment with 4-Nitroquinoline 1-Oxide, Eur. J. Dent. 01 (2007) 152–157. https://doi.org/10.1055/s-0039-1698331.

[51] M. Vered, I. Allon, A. Buchner, D. Dayan, Stromal myofibroblasts and malignant transformation in a 4NQO rat tongue carcinogenesis model, Oral Oncol. 43 (2007) 999–1006. https://doi.org/10.1016/j.oraloncology.2006.11.007.

[52] S. Ge, J. Zhang, Y. Du, B. Hu, Z. Zhou, J. Lou, Dynamic changes in the gene expression profile during rat oral carcinogenesis induced by 4-nitroquinoline 1-oxide, Mol. Med. Rep. 13 (2016) 2561–2569. https://doi.org/10.3892/mmr.2016.4883.

[53] S. Ge, H. Zhou, Z. Zhou, L. Liu, J. Lou, Serum metabolite profiling of a 4-Nitroquinoline-1-oxide-induced experimental oral carcinogenesis model using gas chromatography-mass spectrometry, PeerJ. 9 (2021) 1–14. https://doi.org/10.7717/peerj.10619.

[54] T.C. Bakker Schut, M.J.H. Witjes, H.J.C.M. Sterenborg, O.C. Speelman, J.L.N. Roodenburg, E.T. Marple, H.A. Bruining, G.J. Puppels, In vivo detection of dysplastic tissue by Raman spectroscopy, Anal. Chem. 72 (2000) 6010–6018. https://doi.org/10.1021/ac000780u.

[55] S. Balasenthil, C.R. Ramachandran, S. Nagini, Prevention of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis by garlic, Fitoterapia. 72 (2001) 524–531. https://doi.org/10.1016/S0367-326X(01)00262-3.

[56] W.D. Zavala, M.R. Foscolo, P.E. Kunda, J.C. Cavicchia, C.G. Acosta, Changes in the expression of the potassium channels TASK1, TASK3 and TRESK in a rat model of oral squamous cell carcinoma and their relation to malignancy, Arch. Oral Biol. 100 (2019) 75–85. https://doi.org/10.1016/j.archoralbio.2019.02.007.

[57] A. Madankumar, S. Jayakumar, S. Asokkumar, S. Raghunandhakumar, C. Naveenkumar, T. Devaki, Chemopreventive potential of geraniol on 4-Nitroquinoline-1 oxide induced oral carcinogenesis in rats, Int. J. Res. Pharm. Sci. 2 (2011) 531–536.

[58] G.P.P. De Jesus, F.A.P. Ribeiro, C.F.G. De Moura, A.P.B. Gollucke, C.T.F. Oshima, D.A. Ribeiro, Anti-tumor activity of grape juice concentrate in the rat tongue two-stage initiation-promotion protocol induced by 4-nitroquinoline 1-oxide, Toxicol. Mech. Methods. 24 (2014) 276–283. https://doi.org/10.3109/15376516.2014.881944.

[59] W. Lim, H. Choi, J. Kim, S. Kim, S. Jeon, K. Ni, S. yong Song, H. kyun Oh, Y. Im, G. Lee, J.Y. Lee, Y.L. Moon, J.W. You, O. Kim, Expression of cancer stem cell marker during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis, J. Mol. Histol. 45 (2014) 653–663. https://doi.org/10.1007/s10735-014-9584-1.

[60] P. Thandavamoorthy, R. Balan, J. Subramaniyan, G. Dhas, Alleviative Role of Rutin Against 4-Nitroquinoline-1-Oxide (4-NQO) Provoked Oral Squamous Cell Carcinoma in Experimental Animal Model, J. Pharm. Res. 8 (2014) 899–906.

[61] F.A.P. Ribeiro, R.C. Peres, C.T.F. Oshima, L.C. Spolidorio, L.L.S. Maluf, D.A. Ribeiro, Antioxidant activity of apple extract protects against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide, Toxicol. Mech. Methods. 25 (2015) 532–537. https://doi.org/10.3109/15376516.2015.1053651.

[62] V.B. Valente, F.A. Verza, F.Y.K. Lopes, J.Z. Ferreira, P.S.P. dos Santos, M.L.M.M. Sundefeld, É.R. Biasoli, G.I. Miyahara, A.M.P. Soubhia, M. de Andrade, S.H.P. de Oliveira, D.G. Bernabé, Stress hormones concentrations in the normal microenvironment predict risk for chemically induced cancer in rats, Psychoneuroendocrinology. 89 (2017) 229–238. https://doi.org/10.1016/j.psyneuen.2017.11.001.

[63] F. Khozeimeh, S. Ziaei, S. Khalesi, M. Allameh, G. Jahanshahi, The efficacy of photodynamic therapy in rat tongue dysplasia, J. Clin. Exp. Dent. 11 (2019) e587–e592. https://doi.org/10.4317/jced.54425.

[64] S.A. Swidan, M.M. Hassan, M.N. Elmansy, S.A. Swidan, Synergistic therapeutic effect of nano-honokiol and 5-fluorouracil on the induced-tongue cancer in rats, J. Oral Maxillofac. Surgery, Med. Pathol. 32 (2020) 556–562. https://doi.org/10.1016/j.ajoms.2020.06.003.

[65] D.J. Jiang, D.F. Wilson, P.S. Smith, A.M. Pierce, O.W. Wiebkin, Distribution of basal lamina type IV collagen and laminin in normal rat tongue mucosa and experimental oral carcinoma: Ultrastructural immunolocalisation and immunogold quantitation, Eur. J. Cancer. Part B Oral Oncol. 30 (1994) 237–243. https://doi.org/10.1016/0964-1955(94)90004-3.

[66] S. Manoharan, C.R. Ramachandran, V. Ramachandran, S. Nagini, Inhibition of 4-nitroquinoline-1-oxide-induced oral carcinogenesis by plant products, J. Clin. Biochem. Nutr. 21 (1996) 141–149. https://doi.org/10.3164/jcbn.21.141.

[67] P. Srinivasan, S. Suchalatha, P.V.A. Babu, R.S. Devi, S. Narayan, K.E. Sabitha, C.S. Shyamala Devi, Chemopreventive and therapeutic modulation of green tea polyphenols on drug metabolizing enzymes in 4-Nitroquinoline 1-oxide induced oral cancer, Chem. Biol. Interact. 172 (2008) 224–234. https://doi.org/10.1016/j.cbi.2008.01.010.

[68] A.R. Barcessat, I. Huang, F.P. Rosin, D. dos Santos Pinto, D. Maria Zezell, L. Corrêa, Effect of topical 5-ALA mediated photodynamic therapy on proliferation index of keratinocytes in 4-NQO-induced potentially malignant oral lesions, J. Photochem. Photobiol. B Biol. 126 (2013) 33–41. https://doi.org/10.1016/j.jphotobiol.2013.06.011.

[69] I. Scrobota, P. Bolfa, A.G. Filip, C. Catoi, C. Alb, O. Pop, C. Tatomir, G. Baciut, Natural chemopreventive alternatives in oral cancer chemoprevention, J. Physiol. Pharmacol. 67 (2016) 161–172.

Table 1. Search strategy

Databases	Search Strategy
Medline Ovid (Wolters Kluwer) **PubMed (National Library of Medicine)** **Web of Science (Thomson Reuters)** **Embase (Excerpta Medica database)** **Scopus (Elsevier)**	(rats OR Rat OR Rattus OR "Rattus norvegicus" OR "Rats, Norway" OR "Rats, Laboratory" OR "Laboratory Rat" OR "Laboratory Rats" OR "Rat, Laboratory") AND ("4 Nitroquinoline 1 oxide" OR "4-Nitroquinoline-N-oxide" OR "4 Nitroquinoline N oxide" OR "4-Nitroquinoline 1-oxide" 4-NQO OR 4NQO OR 4Nqo OR NQO OR NQNO)

Table 2. Time until epithelial alterations were diagnosed in studies with multiple endpoints

Author	Method	4NQO dose (ppm)	Evaluation periods	Time until development (weeks) Percentage of animals		
				EH	**ED**	**OSCC**
(Dayan et al., 1997)	Systemic	10	7, 14, 22 and 28	NI NI	7 9.7	22 17.0
(Vered et al., 2003)	Systemic	10	14, 22 and 28	NI NI	14 27.0*	22 17.0*
(D. A. Ribeiro & Salvadori, 2007)	Systemic	50	4,12 and 20	Tongue - 12 Gingiva - 20 Tongue - 70.0 Gingiva - 50.0	Tongue - 12 Gingiva - 20 Tongue - 30.0 Gingiva - 20.0	Tongue - 20 Gingiva - 20 Tongue - 70.0 Gingiva - 0.0
(Vered et al., 2007)	Systemic	10	7, 8,14,22, and 28	7.0 NA	8 NA	22 100
(Ge et al., 2016)	Systemic	20	9,13,20, 24, and 32	NI NI	9 0	24 100
(Teixeira Buck et al., 2018)	Systemic	20	3,5,7,16, and 28	3.0 NI	16 NI	28 NI
(Ge et al., 2021)	Systemic	20	9,13,20,24 and 32	NA NI	9 0	24 100
(Anders V. Fisker & Kariung, 1981)	Topic	5000	1/2, 1, 2, 3, 4, 7, 10, 14, 16, 18, 24, and 32	2 NI	NI NI	8 9.4
(A. V. Fisker et al., 1987)	Topic	5000	7 or 120	NI NI	NI NI	18 100
(Anders V. Fisker et al., 1990)	Topic	5000	1, 2, 5, 8, 12, 16, 20, 26 or 2, 6, 10, 14, 16 ,24	NI NI	8 NI	NI NI
(Jan M. Nauta et al., 1996)	Topic	5000	2,4,6,8,10,12,14,16,18, 20,22,24, and 26	2 NI	2 NI	20 100
(Braams et al., 1998)	Topic	5000	2, 4, 6, 8, 12, 16, 20, 26, and 30	NI NI	NI NI	NI NI
	Topic	5000	9 or 16	NI	9	16

(Bakker Schut et al., 2000)				NI	100	100
(A. R. R. Barcessat et al., 2014)	Topic	5000	4,5,6,15, and 16	4 NI	16 100.0	NI NA

Table 3. The percentage of epithelial dysplasia (ED) and oral squamous cell carcinoma (OSCC) at the last evaluation period.

Author	Method	Dilution dose (ppm)	Period of solution administration (weeks)	Euthanasia time	ED (%)	OSCC (%)
(Dayan et al., 1997)	Systemic	10	28	28	9.7	17
(Balasenthil et al., 2001)	Systemic	20	8	32	100	83
(Vered et al., 2003)	Systemic	10	28	28	27*	17*
(D. A. Ribeiro & Salvadori, 2007)	Systemic	50	20	20	Tongue: 30.0 Gingiva: 20.0	Tongue:70.0 Gingiva: 0
(Vered et al., 2007)	Systemic	10	28	28	NI	100
(Zavala et al., 2019)	Systemic	20	32	32	NI	100
(Madankumar et al., 2011)	Systemic	50	22	22	100	88.0
(De Jesus et al., 2014)	Systemic	20	8	8	100	NA
(Lim et al., 2014)	Systemic	50	24	24	37.0	35.0
(Thandavamoorthy et al., 2014)	Systemic	50	20	20	100	83
(F. A. P. Ribeiro et al., 2015)	Systemic	20	8	12	100	0
(Ge et al., 2016)	Systemic	20	32	32	0	100
(Helli et al., 2016)	Systemic	30	12	12	89.0	NA
(Valente et al., 2017)	Systemic	50	20	20	25.0	69.0
(Soares et al., 2018)	Systemic	50	20	12	40.0	60.0
(Teixeira Buck et al., 2018)	Systemic	20	28	28	NI	NI

(Khozeimeh et al., 2019)	Systemic	20	12	12	100.0	0
Cecilio et al. (2020)(Cecilio et al., 2020)	Systemic	50	16	16	NI	52.0
(Swidan et al., 2020)	Systemic	20	20	24	NI	70.0
(Wagner et al., 2020)	Systemic	25	20	20	45.5	54.0
(Ge et al., 2021)	Systemic	20	32	32	0	100
(Verza et al., 2021)	Systemic	50	20	20	21.0	72.0
(Anders V. Fisker & Kariung, 1981)	Topic	5000	8	34	NI	9.4
(A. V. Fisker et al., 1987)	Topic	5000	18*	120	NI	100
(Anders V. Fisker et al., 1990)	Topic	5000	8	24	NI	NI
(Jiang et al., 1994)	Topic	5000	24	24	NI	100
(Jan M. Nauta et al., 1996)	Topic	5000	26	26	NI	100
(Manoharan et al., 1996)	Topic	5000	20	40	100	100
(Braams et al., 1998)	Topic	5000	30	30	NI	NI
(Bakker Schut et al., 2000)	Topic	5000	16	16	100	100
(Srinivasan et al., 2008)	Topic	5000	8	22	Tongue:27.0 Oral cavity tumor: 27.0	Tongue: 60.0 Oral cavity tumor: 47.0
(A. R. Barcessat et al., 2013)	Topic	5000	16	16	100	NA
(A. R. R. Barcessat et al., 2014)	Topic	5000	16	16	100	NI
(Rosin et al., 2015)	Topic	5000	16	16	NI	NA

(Scrobota et al., 2016)	Topic	5000	12	16	NI	NA

Figures Legends:

Figure 1. Study selection flowchart.

Figure 2. Clinical spectrum of 4NQO-induced tongue lesions in Wistar rats (Personal archive; Porto Alegre, Brazil, Animal Use Ethics Committee approval no. 150475). (A) Thin white plaque. (B) Thicker white plaque with irregular surface and erosive areas. (C) Nodular lesion. (D) An extensive nodular lesion with ulceration.

Figure 3. Histopathological features of 4NQO-induced tongue lesions (Personal archive; Porto Alegre, Brazil, Animal Use Ethics Committee approval no. 150475). (A) Epithelial hyperplasia (Hematoxylin and Eosin (HE), 100x); (B) Mild epithelial dysplasia (HE, 100x); (C) Severe epithelial dysplasia (HE, 100x); (D) Invasive oral squamous cell carcinoma (HE, 100x).

Figure 4. The efficacy of 4NQO for experimental oral carcinogenesis through topical and systemic administration, with their respective parameters.

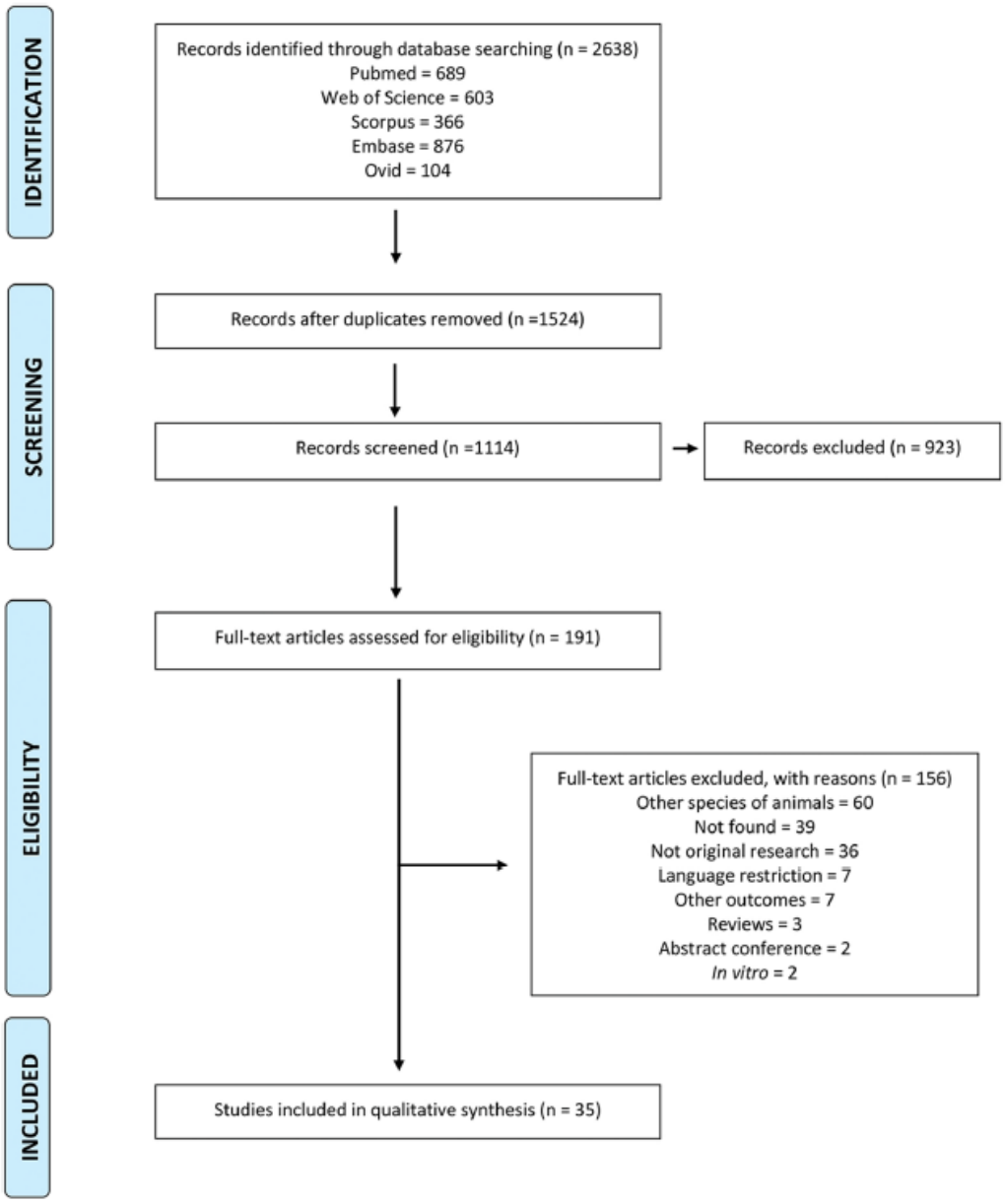


Fig. 1. Study selection flowchart.

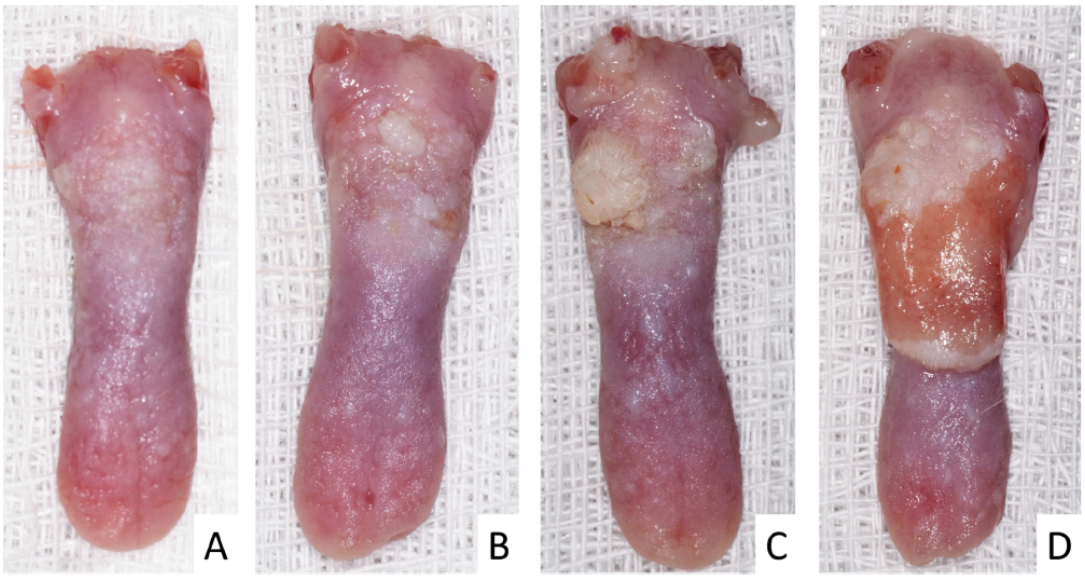


Fig. 2. Clinical spectrum of 4NQO-induced tongue lesions in Wistar rats (Personal archive; Porto Alegre, Brazil, Animal Use Ethics Committee approval no. 150475). (A) Thin white plaque. (B) Thicker white plaque with irregular surface and erosive areas. (C) Nodular lesion. (D) An extensive nodular lesion with ulceration.

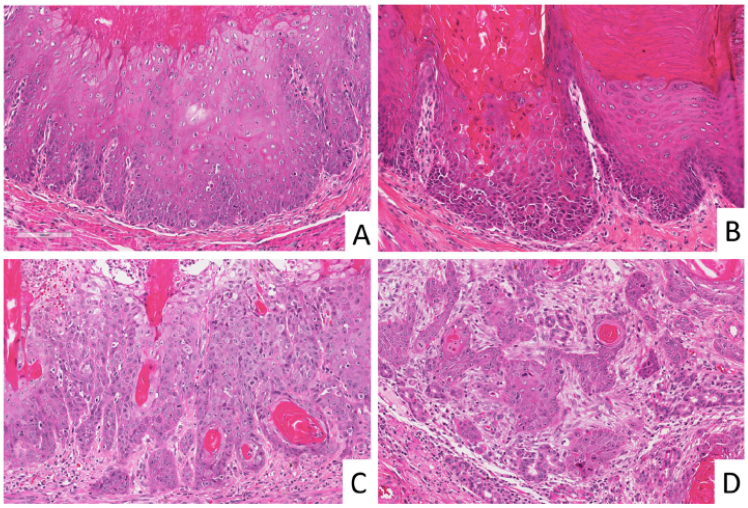


Fig. 3. Histopathological features of 4NQO-induced tongue lesions (Personal archive; Porto Alegre, Brazil, Animal Use Ethics Committee approval no. 150475). (A) Epithelial hyperplasia (Hematoxylin and Eosin (HE), 100x); (B) Mild epithelial dysplasia (HE, 100x); (C) Severe epithelial dysplasia (HE, 100x); (D) Invasive oral squamous cell carcinoma (HE, 100x).

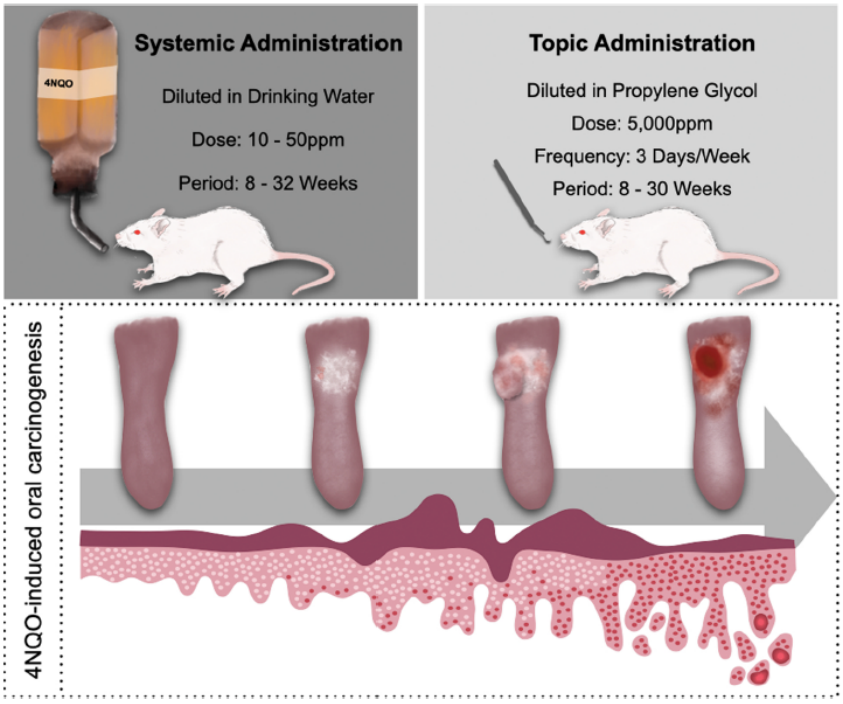


Fig. 4. The efficacy of 4NQO for experimental oral carcinogenesis through topical and systemic administration, with their respective parameters.

2.3 Proposal of a secure and efficient protocol for a murine oral carcinogenesis model induced by 4-nitroquinoline-1-oxide (4NQO)

Artigo em *'Under review'* no periódico Pathology – Research and Practice (**Anexo 5**).

Lauren Frenzel **Schuch**[1], Daniela **Campagnol**[2], Tuany Rafaeli **Schmidt**[3], Carolina Horn Troian **Michel**[4], Tuane Nerissa Alves **Garcez**[2], Chris Krebs **Danilevicz**[5], Rogerio Moraes **Castilho**[6], Marco Antonio Trevizani **Martins**[4], Pablo Agustin **Vargas**[1], Manoela Domingues **Martins**[1,3]

[1] Oral Diagnosis Departament, Piracicaba Dental School, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil
[2] Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil
[3] Departament of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[4] Departament of Edodontics, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[5] Department of Pharmacology, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[6] Michigan University, School of Dentistry, Department of Periodontics and Oral Medicine, Ann Arbor, MI, United States

Corresponding author: Manoela Domingues Martins. Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, R. Ramiro Barcelos, 2492, room 503. Porto Alegre, Rio Grande do Sul, Brazil, CEP: 90035-003. Phone: +55 (51) 3308-5011. E-mail: manomartins@gmail.com; manoela.martins@ufrgs.br

Keywords: Carcinogenesis; Experimental animal models; Oral cancer; Oral potentially malignant disorder

Abstract

An important rat model using the chemical carcinogen 4-nitroquinoline-1-oxide (4NQO) has been described for the study of the process of oral carcinogenesis. This model replicates the gradual progression seen in oral carcinoma patients. However, due to its high level of toxicity, its use in fundamental research is challenging. Here, we propose a secure and efficient modified protocol based on a lower dose of 4NQO concentration as well as an increased water supply and hypercaloric diet, in order to guarantee the animal's well-being during the process of oral carcinogenesis. Twenty-two male Wistar rats were exposed to 4NQO, evaluated clinically once a week and euthanized at 12 and 20 weeks for histopathological analysis. The protocol involves a staggered dose of 4NQO up to a concentration of 25 ppm, associated with two days of pure water, a 5% glucose solution once a week and a hypercaloric diet. This modified protocol prevents the immediate consequences of the carcinogen. At week 7, all animals displayed clinically evident tongue lesions. From a histological perspective, after 12 weeks of 4NQO exposure, 72.7% of the animals developed dysplasia and 27.3% developed *in situ* carcinoma. In the group exposed for 20 weeks, dysplasia and *in situ* carcinoma were diagnosed in one case each, whereas carcinoma was diagnosed in 81.8% of the cases. Nonsignificant modification of animal’s behavior and weight was observed. This new proposed 4NQO protocol was secure and effective for studying oral carcinogenesis and can be used to conduct lengthy investigations.

Keywords: Carcinogenesis; Experimental animal models; Oral cancer; Oral potentially malignant disorder

Introduction

Oral carcinogenesis is a multifactorial and intricate process including the expanding incidence of genetic changes that promote the inhibitory or excitatory activities of tumor oncogenes and gene suppressors and impair the histophysiology of cell division, differentiation, and cell death [1-3]. Multiple genetic, molecular, and immunological modifications are caused in normal oral mucosa by the interaction of environmental factors such as the synergistic effects of alcohol and tobacco, and hereditary predispositions [4-6]. Thus, understanding the genetic, immunological, and molecular background of oral cancer may be of help for the development of preventive medications and new treatment modalities and may enhance patient prognosis.

The process of carcinogenesis can be investigated using cell lines derived from patients with carcinoma, transgenic animals, or animal models of chemical carcinogenesis [3]. Because the chemical components of tobacco and alcohol have been shown to be a potential cause of most mouth cancers in humans, they have been selected as the principal chemical agents used in animal models of carcinogenesis [7,8]. Among the chemical carcinogens used, 4NQO is the preferred agent for the development of experimental oral carcinogenesis [8-10].

4NQO is a water-soluble synthetic carcinogen that has been considered to be the best carcinogen currently available for producing oral carcinogenesis in rats. It has been shown that 4NQO forms DNA adducts, leading to the replacement of adenosine by guanosine, thus inducing intracellular oxidative stress [9]. Histologically, changes in the oral epithelium ranging from hyperplasia to mild, moderate, and severe dysplasia can be observed before the formation of invasive carcinoma [9].

However, because 4NQO is a highly toxic substance that must be administered for a long time to have its carcinogenic effects on animals, its use in fundamental research can be difficult [10]. As a result, during the experimental procedures, adverse effects and animal losses may occur, and the ideal dosage must balance unwanted effects with efficient tumor development. In this sense, the aim of this study was to propose a new protocol using 4-NQO for oral carcinogenesis studies.

Protocol

Animal model

The present protocol was used in an animal model study approved by the Institutional Committee for Animal Care and Use (GPPG/HCPA, protocol no. 2021-

0614) according to Brazilian Law 11.794 and the Brazilian Guideline for the Care and Use of Animals of the National Council for Animal Experimentation Control.

The study was carried out on 8-week-old male Wistar albino rats (n=22) weighing approximately 250-300 grams. As an enrichment strategy, the animals were allocated to pairs or trios and received items for shelter and entertainment (tunnel, kraft paper roll, paper towel sheets, shredded kraft paper, cardboard or a rope pendulum). The animals were treated for 12 and 20 weeks.

Water consumption control

Prior to the administration of 4NQO, it is necessary to control water consumption based on the basal consumption of pure water by the animals. To this end, it is advisable to weigh the water bottles twice a week for two weeks in order to determine the average water consumption per bottle (per box/number of animals).

4NQO preparation

During the first week, the experiment was started with a lower 4NQO concentration, i.e., 10 ppm, which was then increased to 25 ppm during the second week. The carcinogen was diluted in autoclaved water until it reached 25 ppm. For this, 25 mg 4NQO powder (LOT 711645086-1-1, Toronto Research Chemicals Inc., Toronto, Canada) was dissolved in 1 ml dimethylsulfoxide (DMSO – Sigma, St. Louis, MO, USA) and then diluted in water and autoclaved until reaching the desired concentration (25ppm = 25mg of 4NQO per liter of water).

Fresh solutions must be prepared twice a week and stored in amber bottles duly identified due to the photolysis and dangerousness of the carcinogen (according to the manufacturer's instructions in the product insert).

4NQO administration

The animals received the solution in specific drinking bottles that protect the content from contact with light and the solution was supplemented once a week and fully changed weekly. The remaining 4NQO was collected into a brown one gallon container for adequate disposal. **Figure 1** illustrates the weekly administration proposed.

4NQO consumption control

Consumption control is based on the difference in liquid content of the drinking bottle between changes. In the present study, the carcinogen consumption per animal was estimated by dividing the 4NQO consumption per box/number of animals. We suggest a control once a week.

Offer of pure water, glucose solution and hypercaloric supplementation

The animals received pure water twice a week and a 5% glucose solution once a week (100 ml per animal) or more frequently if signs of dehydration were identified. During the first five weeks of the experiment, a hypercaloric diet [2 pellets (6 g) per animal] was offered once a day.

Monitoring and human endpoints

The animals were observed every day for level of mental alertness, interactions with their boxmates and the researcher, movement, and interest in food and drink [11,12], and weighed every 2 days.

Body weights and physiological and behavioral changes were recorded and evaluated together with the veterinary team at the research center. At first, a weight loss of more than 20% was considered to be an endpoint for humane euthanasia.

Histopathological analysis

Animal were euthanized with an isoflurane inhalation overdose after 12 weeks (n=11) and 20 weeks (n=11). All tongues were removed, fixed in neutral-buffered formalin solution for 24 hours and 3-µm thick sections were obtained and stained with hematoxylin and eosin for histopathological analysis.

Results

Basal water consumption

Water consumption was measured over a two-week period. A daily consumption of 35 ml per animal was observed.

4NQO consumption

4NQO consumption was determined once a week, showing an average consumption of 25 ml of carcinogen per animal per day.

Weight control

Body weight was monitored every two days as a possible sign of distress. When comparing the weekly weight, it was observed that, even with the progression of the lesions and the duration of the treatment, the animals continued to gain weight (**Figure 2**).

Clinical lesion

Oral cavities were inspected weekly for signs of lesions. The posterior dorsal surface of the rats' tongue became white with a rough appearance after 5 weeks of treatment. At week 7, all 22 animals already had areas of white plaque in the posterior region of the tongue. **Figure 3** illustrates the clinical monitoring along the 20 weeks of the experiment. **Figure 4** shows a different clinical appearance at the end of 12 weeks (A and B) and 20 weeks (C and D).

Histological changes

Table 1 demonstrates the histopathological diagnosis after the experimental period. After 12 weeks of 4NQO exposure, 72.7% of the animals developed epithelial dysplasia and 27.3% developed *in situ* carcinoma. In the 20 week group, epithelial dysplasia and *in situ* carcinoma were diagnosed in one case each, while carcinomas were observed in 81.8% of the cases. **Figure 5** illustrate some of these cases.

Adverse effects

With the offer of progressively increased 4NQO concentrations (i.e., from 10 to 25 ppm), the number of adverse reactions was minimal. During the first week of 4NQO administration, some animals eventually showed chromodacryorrhea (n=7), hypersalivation (n=11) and acute ulcers covered by a white membrane (n=4) (**Figure 6**). After the period of adaptation to 4NQO, all animals were active and interactive, and the adverse effects were resolved quickly. The animal's coat lost quality over time, being more messy than usual. At the end of the experiment, two animals in the 20 week Group had wheezing. No chronic weight loss or death was observed.

Discussion

The literature states that the 4NQO model permits useful advances in the understanding of the molecular and cellular alterations of carcinogenesis, as well as in

the development of new therapeutic strategies. The initial step was to provide clinical and histological verification that the rat 4NQO model of oral carcinogenesis mimics the development of human squamous cell carcinoma among white oral potentially malignant disorders [13]. Since this model started to be used, it has been shown that significant earlier events at the nuclear and molecular levels come before both the major clinical findings and the fine microscopic findings [13]. Although effective, the protocols described so far have reported several adverse effects combined with animal suffering [10]. In the present modified protocol, we validated a safe and successful oral carcinogenesis model that does not induce extreme adverse effects. We took special precautions that would benefit animal welfare, such as offering fresh water twice a week throughout the experiment and a hypercaloric supplementation for the first five weeks. Moreover, we started with a lower carcinogen dose and increased it until we reached the optimal amount in order to prevent acute reactions while administering 4NQO., We strongly advise researchers to use this method in order to achieve better acceptance by the animal. It is also very important to point out that 4NQO has a strong flavor and aroma which deters the animals from consuming an excessive quantity. The animals are less likely to feel disgust after drinking when a reduced concentration is administered.

Weight regulation is probably the most important factor in the assessment of animal welfare. In addition to the development of oral cancer, the administration of 4NQO to animals for the purpose of inducing cancer can also cause significant body weight loss because of decreased appetite, inability to eat, and an increase in metabolic rate. Using a methodology similar to ours, Spuldaro and coauthors (2022) exposed 26 rats to 25 ppm of the 4NQO carcinogen solution diluted in drinking water for 20 weeks [14]. A decline in animal weight was observed in both groups receiving 4NQO after 16 weeks of drug administration. Also, two animals required early euthanasia due to a 20% body weight loss caused by the drug. Using a 50 ppm 4NQO solution in drinking water, Ribeiro and Salvadori (2007) observed a marked decrease in weight gain in the experimental group, with the animals deteriorating and progressing to cachexia along the 20 weeks of the study [15]. Al-Koshab et al. (2020) administered to rats 20 ppm of 4NQO dissolved in drinking water for 8 weeks and determined the cancerous and precancerous morphological changes induced on the tongue epithelium of the animals after 22 weeks [16]. The use of 4NQO caused a significant reduction of body weight in the cancer-induced animals. Similarly, another

study showed that 4NQO caused a significant body weight loss among the cancer-induced and vehicle groups during the last weeks of the experiment [17]. In the present investigation, we gave the animals 100 ml of a 5% glucose solution per animal once a week throughout the study period plus a hypercaloric supplementation (6 g/animal/day) for the first five weeks in order to prevent body weight loss. In fact, many studies have recommended hypercaloric supplementation when necessary (e.g., to prevent severe weight loss) [18,19]. Nevertheless, none of the previous studies developed a strategy for daily supplementation to prevent animal suffering. According to our results, no chronic weight reduction was observed, highlighting the safety of our modified protocol.

Regarding the success of carcinoma induction, even with a not so aggressive 4NQO dose, all animals showed morphological changes within 12 weeks, and most of the lesions were found to be carcinomas within 20 weeks. Even with two days without 4NQO, our success rate – mainly concerning carcinoma development - was higher than that reported in several studies employing higher carcinogen doses [15,20-23]. Furthermore, we assume that the animals could be kept healthy for a few more weeks than the 20 used in the present protocol, which would undoubtedly result in more malignant neoplasms. In our research, in addition to the tongue lesion, we observed a significant clinical rise in nodular/ulcerated lesions during the final week of the experiment.

Conclusion

In conclusion, the proposed 4NQO protocol is secure and effective for studying oral carcinogenesis and can be used to conduct lengthy investigations. It was observed that, despite being exposed to the carcinogen for the full period of 20 weeks, the animals did not experience any significant behavioral or physiological changes. Thus, the most important take-home messages for researchers working on models of oral carcinogenesis are:

- Administer a staggered dose until reaching 25 ppm of 4NQO.
- Provide two days of pure water (not consecutively).
- Provide a 5% glucose solution once a week.
- Give a hypercaloric supplementation for the first five weeks.
- Continue weight management every two days.

- Weight loss greater than 15% that was not minimized after 24 hours of suspension of 4NQO, and supply of pure water was considered the endpoint for humane euthanasia.

Acknowledgments

This study was funded by the Postgraduate Research Group of Hospital de Clínicas de Porto Alegre (GPPG/FIPE: 2021-0614). The authors thank the Coordination of Improvement of Higher Education Personnel (CAPES, Finance Code 001), Brazil. L.F.S. and T.R.S. are the recipients of fellowships. Also, we would like to thank the Brazilian National Council for Scientific and Technological Development for financial support (CNPq). M.D.M. and P.A.V. are research fellows of CNPq.

Conflict of interest statement

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

References

[1] Tanaka T, Ishigamori R. Understanding carcinogenesis for fighting oral cancer. J Oncol. 2011;2011:603740. doi: 10.1155/2011/603740.

[2] Shah S, Kaur M. Biomarkers and chemopreventives in oral carcinogenesis and its prevention. J Oral Maxillofac Pathol. 2014 Jan;18(1):69-76. doi: 10.4103/0973-029X.131914.

[3] Rivera C. Essentials of oral cancer. Int J Clin Exp Pathol. 2015 Sep 1;8(9):11884-94.

[4] Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nat Rev Cancer. 2018 May;18(5):269-282. doi: 10.1038/nrc.2018.11.

[5] Alsahafi E, Begg K, Amelio I, Raulf N, Lucarelli P, Sauter T, Tavassoli M. Clinical update on head and neck cancer: molecular biology and ongoing challenges. Cell Death Dis. 2019 Jul 15;10(8):540. doi: 10.1038/s41419-019-1769-9.

[6] Canning M, Guo G, Yu M, Myint C, Groves MW, Byrd JK, Cui Y. Heterogeneity of the Head and Neck Squamous Cell Carcinoma Immune Landscape and Its Impact on Immunotherapy. Front Cell Dev Biol. 2019 Apr 9;7:52. doi: 10.3389/fcell.2019.00052.

[7] Mognetti B, Di Carlo F, Berta GN. Animal models in oral cancer research. Oral Oncol. 2006 May;42(5):448-60. doi: 10.1016/j.oraloncology.2005.07.014.

[8] Ishida K, Tomita H, Nakashima T, Hirata A, Tanaka T, Shibata T, Hara A. Current mouse models of oral squamous cell carcinoma: Genetic and chemically induced models. Oral Oncol. 2017 Oct;73:16-20. doi: 10.1016/j.oraloncology.2017.07.028.

[9] Sagheer SH, Whitaker-Menezes D, Han JYS, Curry JM, Martinez-Outschoorn U, Philp NJ. 4NQO induced carcinogenesis: A mouse model for oral squamous cell carcinoma. Methods Cell Biol. 2021;163:93-111. doi: 10.1016/bs.mcb.2021.01.001.

[10] Zigmundo GCO, Schuch LF, Schmidt TR, Silveira FM, Martins MAT, Carrard VC, Martins MD, Wagner VP. 4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis: A systematic literature review. Pathol Res Pract. 2022 Aug;236:153970. doi: 10.1016/j.prp.2022.153970.

[11] Hawkins P, Morton DB, Burman O, Dennison N, Honess P, Jennings M, Lane S, Middleton V, Roughan JV, Wells S, Westwood K; UK Joint Working Group on Refinement BVAAWF/FRAME/RSPCA/UFAW. A guide to defining and implementing protocols for the welfare assessment of laboratory animals: eleventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. Lab Anim. 2011 Jan;45(1):1-13. doi: 10.1258/la.2010.010031.

[12] Sotocinal SG, Sorge RE, Zaloum A et al. (2011). The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Molecular Pain 7: 55. doi:10.1186/1744-8069-7-55

[13] Vered M, Yarom N, Dayan D. 4NQO oral carcinogenesis: animal models, molecular markers and future expectations. Oral Oncol. 2005 Apr;41(4):337-9. doi: 10.1016/j.oraloncology.2004.07.005.

[14] Spuldaro TR, Wagner VP, Nör F, Gaio EJ, Squarize CH, Carrard VC, Rösing CK, Castilho RM. Periodontal disease affects oral cancer progression in a surrogate animal model for tobacco exposure. Int J Oncol. 2022 Jun;60(6):77. doi: 10.3892/ijo.2022.5367.

[15] Ribeiro DA, Salvadori DM. Gingival changes in wistar rats after oral treatment with 4-nitroquinoline 1-oxide. Eur J Dent. 2007 Jul;1(3):152-7.

[16] Al-Koshab M, Alabsi AM, Mohd Bakri M, Ali-Saeed R, Selvi Naicker M. Antitumor Activity of Ficus deltoidea Extract on Oral Cancer: An In Vivo Study. J Oncol. 2020 Feb 10;2020:5490468. doi: 10.1155/2020/5490468.

[17] Al-Afifi N, Alabsi A, Kaid F, Bakri M, Ramanathan A. Prevention of oral carcinogenesis in rats by Dracaena cinnabari resin extracts. Clin Oral Investig. 2019 May;23(5):2287-2301. doi: 10.1007/s00784-018-2685-6.

[18] Squarize CH, Castilho RM, Gutkind JS. Chemoprevention and treatment of experimental Cowden's disease by mTOR inhibition with rapamycin. Cancer Res. 2008 Sep 1;68(17):7066-72. doi: 10.1158/0008-5472.CAN-08-0922.

[19] Squarize CH, Castilho RM, Abrahao AC, Molinolo A, Lingen MW, Gutkind JS. PTEN deficiency contributes to the development and progression of head and neck cancer. Neoplasia. 2013 May;15(5):461-71. doi: 10.1593/neo.121024.

[20] Lim W, Choi H, Kim J, Kim S, Jeon S, Ni K, Song SY, Oh HK, Im Y, Lee G, Lee JY, Moon YL, You JW, Kim O. Expression of cancer stem cell marker during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. J Mol Histol. 2014 Dec;45(6):653-63. doi: 10.1007/s10735-014-9584-1.

[21] Valente VB, Verza FA, Lopes FYK, Ferreira JZ, Dos Santos PSP, Sundefeld MLMM, Biasoli ÉR, Miyahara GI, Soubhia AMP, de Andrade M, de Oliveira SHP, Bernabé DG. Stress hormones concentrations in the normal microenvironment predict risk for chemically induced cancer in rats. Psychoneuroendocrinology. 2018 Mar;89:229-238. doi: 10.1016/j.psyneuen.2017.11.001.

[22] Soares GR, de Moura CFG, Silva MJD, Vilegas W, Santamarina AB, Pisani LP, Estadella D, Ribeiro DA. Protective effects of purple carrot extract (Daucus carota) against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Med Oncol. 2018 Mar 15;35(4):54. doi: 10.1007/s12032-018-1114-7

[23] Cecilio HP, Valente VB, Pereira KM, Kayahara GM, Furuse C, Biasoli ÉR, Miyahara GI, Oliveira SHP, Bernabé DG. Beta-adrenergic blocker inhibits oral carcinogenesis and reduces tumor invasion. Cancer Chemother Pharmacol. 2020 Nov;86(5):681-686. doi: 10.1007/s00280-020-04149-2.

Table 1. Histological diagnosis after 12 and 20 weeks of 4NQO administration

	Epithelial dysplasia	**In situ carcinoma**	**Carcinoma**
12 weeks, n=11	8	3	0
20 weeks, n=11	1	1	9

Figure legends

Figure 1. Proposed protocol

Figure 2. Body weight variation (%) along the experimental period

Figure 3. Clinical progression of the tongue lesion during the study. (A) week 1; (B) week 8; (C) week 12; (D) week 20

Figure 4. Clinical aspects of the tongue at the end of 12 weeks (A and B) and 20 weeks (C and D)

Figure 5. Histopathological aspects of some cases. (A, B and C) Epithelial dysplasia; (D, E and F) *In situ* carcinoma; (G, H and I) Squamous cell carcinoma

Figure 6. Some adverse effects observed in the animals during the first week of the experiment. (A) Hypersalivation demonstrated by marked wetness of the hairs around the mouth; (B) Hypersalivation and chromodacryorrhea; (C and D) Acute ulcers

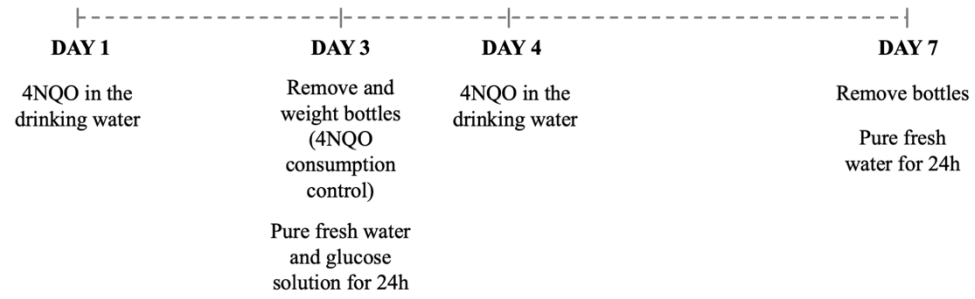


Figure 1. Proposed protocol

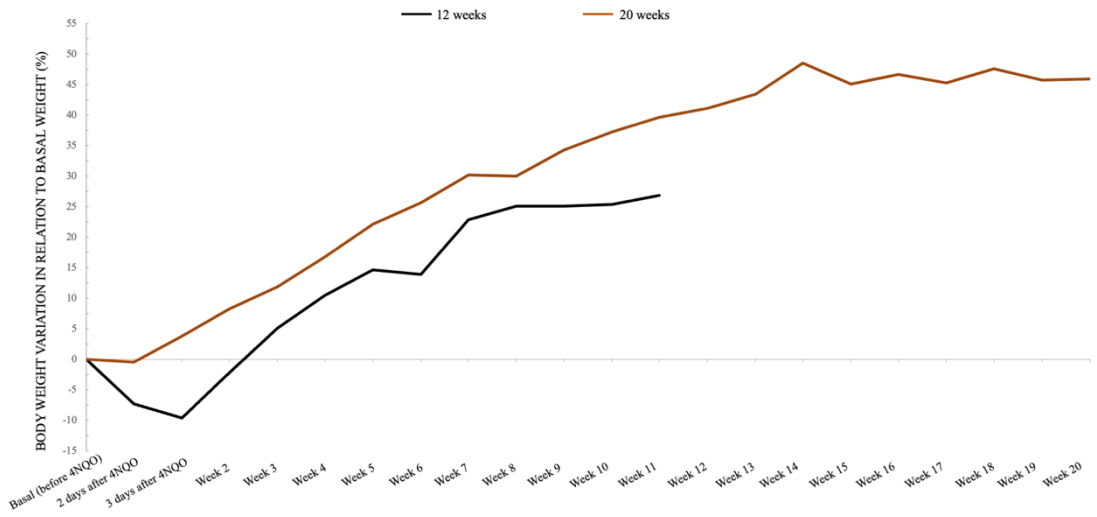


Figure 2. Body weight variation (%) along the experimental period

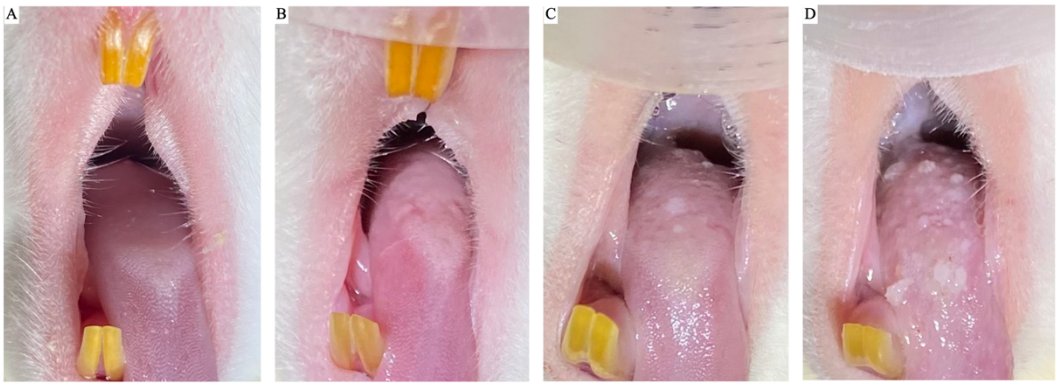


Figure 3. Clinical progression of the tongue lesion during the study. (A) week 1; (B) week 8; (C) week 12; (D) week 20

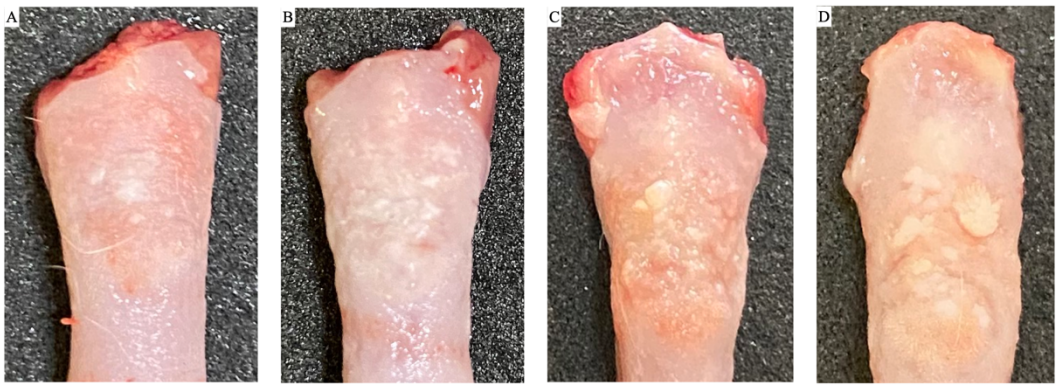


Figure 4. Clinical aspects of the tongue at the end of 12 weeks (A and B) and 20 weeks (C and D)

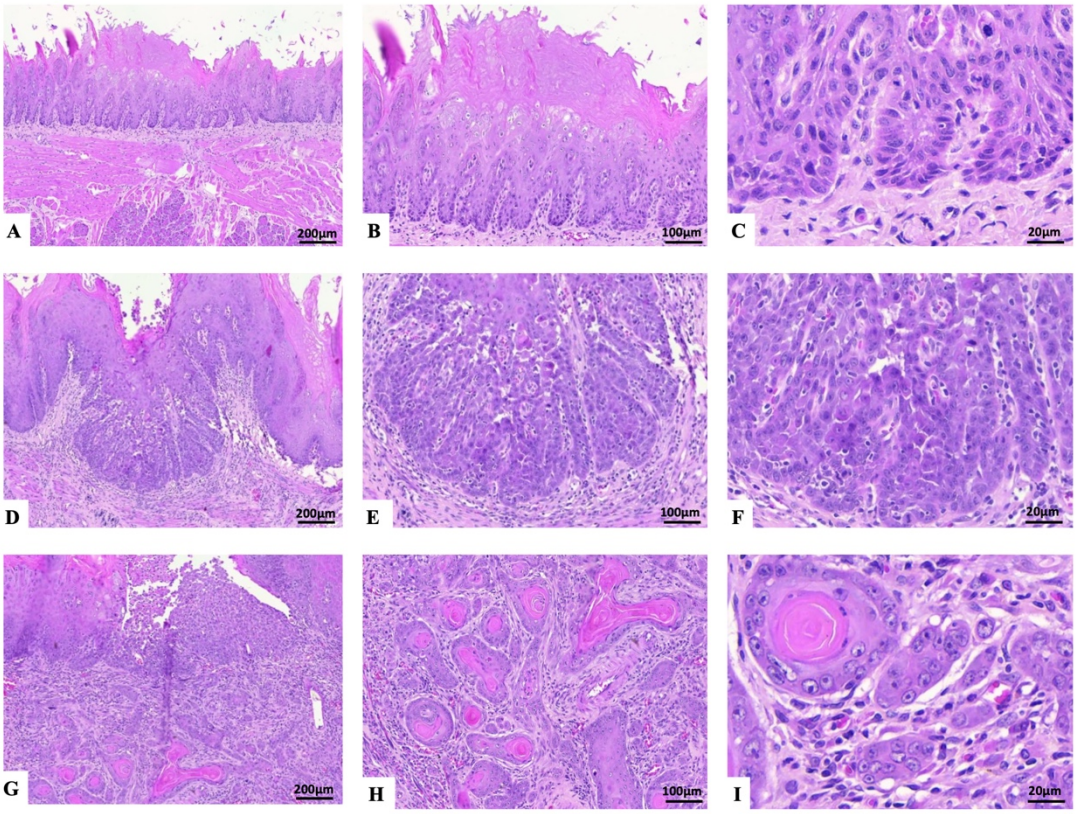


Figure 5. Histopathological aspects of some cases. (A, B and C) Epithelial dysplasia; (D, E and F) *In situ* carcinoma; (G, H and I) Squamous cell carcinoma

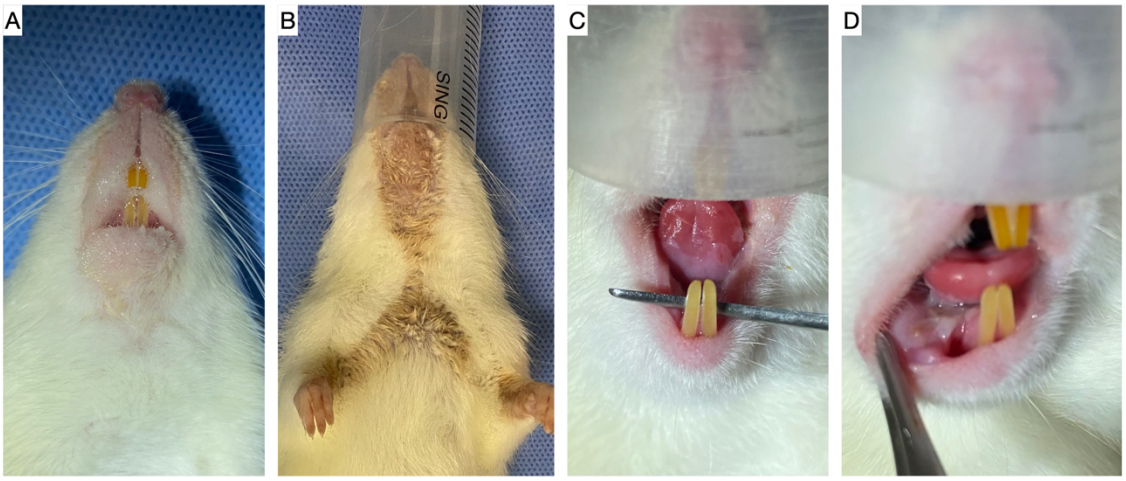


Figure 6. Some adverse effects observed in the animals during the first week of the experiment. (A) Hypersalivation demonstrated by marked wetness of the hairs around the mouth; (B) Hypersalivation and chromodacryorrhea; (C and D) Acute ulcers

2.4 The impact of photodynamic therapy on rat tongue during the carcinogenesis process induced by 4-nitroquinoline-1-oxide (4NQO)

Artigo formatado para submissão no periódico *Photodiagnosis and photodynamic therapy*.

Lauren Frenzel **Schuch**[1], Tuany Rafaeli **Schmidt**[2], Daniela **Champagnol**[3], Tuane Nerissa Alves **Garcez**[3], Alexia Antunes **Deluca**[2], Vivian Petersen **Wagner**[4], Rogerio Moraes **Castilho**[5], Marco Antonio Trevizani **Martins**[2,6], Vanderlei Salvador **Bagnato**[7], Cristina **Kurachi**[7], Pablo Agustin **Vargas**[1], Manoela Domingues **Martins**[1,2]

[1] Oral Diagnosis Departament, Piracicaba Dental School, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil.
[2] Departament of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.
[3] Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil.
[4] Academic Unit of Oral and Maxillofacial Medicine and Pathology, Department of Clinical Dentistry, University of Sheffield, Sheffield, United Kingdom
[5] Michigan University, School of Dentistry, Department of Periodontics and Oral Medicine, Ann Arbor, MI, United States
[6] Department of Oral Medicine, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[7] Department of Physics and Materials Science, São Carlos Institute of Physics, Universidade de São Paulo, São Carlos, São Paulo, Brazil

Corresponding author: Manoela Domingues Martins. Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, R. Ramiro Barcelos, 2492, room 503. Porto Alegre, Rio Grande do Sul, Brazil, CEP: 90035-003. Phone: +55 (51) 3308-5011. E-mail: manomartins@gmail.com; manoela.martins@ufrgs.br

Keywords: Oral carcinogenesis; oral dysplasia; oral potentially malignant disorders; photodynamic therapy

Abstract

Light therapies have been provided in a variety of medical fields as an alternative or supplemental treatment for cancer and other potentially malignant conditions. Regarding its effect on the oral mucosa throughout the carcinogenesis process, there are still questions. In this regard, the purpose of the current study was to assess how photodynamic treatment (PDT) affects the development of lesion during chemically induced oral carcinogenesis. For this, 4NQO was administered to 40 male Wistar rats. The carcinogen was given systemically for 12 and 20 weeks at a dose of 25 ppm diluted in distilled water. The animals were divided into two groups: Control and PDT. PDT applications were made once every week beginning in week 0. A swab impregnated with 5-Aminolevuinic acid solution was rubbed on the posterior region of tongue of the rats. Two hours after the application of 5-ALA, the same point was irradiated with a laser. All the animals from both groups developed lesions on the posterior dorsum of the tongue. The lesions began as white patches and, along the time, erosive lesions, nodule, and ulcers could be observed. Regarding histopathological diagnosis, at the end of 12 weeks, epithelial dysplasia was the most common diagnosis, whereas squamous cell carcinoma was the most frequent lesions in both groups at 20 weeks. Nonsignificant differences were observed between control and PDT groups regarding clinical appearance neither microscopic analysis in 12 nor 20 weeks. PDT neither promotes nor prevents the development of oral lesions during the process of 4NQO-induced carcinogenesis.

Keywords: Oral carcinogenesis; oral dysplasia; oral potentially malignant disorders; photodynamic therapy

Introduction

Photodynamic therapy (PDT) is a non-invasive form of therapy utilized to treat both malignancies and non-cancerous conditions [1]. It was discovered in the early 20th century by Oscar Raab, a medical student [2]. Few time later, Dr. Friedrich Meyer-Betz conducted the first clinical study on humans in 1913 [3]. He applied porphyrins on his own skin and named it photoradiation therapy. The first study on PDT effects on tumors was carried out in 1942, using hematoporphyrin-injected tumor-bearing mice that were then exposed to a quartz-halogen lamp to study tumor necrosis and fluorescence [4].

This therapy has three fundamental elements: (1) oxygen, (2) photosensitizer and (3) a specific wavelength of visible light [5]. In this process, the photosensitizer is activated by light and causes a series of photochemical and photobiological reactions, resulting in irreversible damage and, eventually, in the death of the target cells [6-10]. As PDT is a cold photochemical process, there is no tissue heating, and connective tissues, such as collagen and elastin, at the treated site, are not affected; therefore, there is much less risk of damaging the integrity of underlying functional structures than with thermal laser techniques and other invasive approaches [5].

PDT began to be employed in the palliative treatment of metastatic breast cancer in 1975 [11]. In dermatology, PDT is mostly used to treat superficial skin cancers such actinic keratoses, superficial or nodular basal cell carcinomas, Bowen's disease, and *in situ* squamous cell carcinomas [11,12]. Recently, it has also been used to treat inflammatory and infectious dermatoses like granuloma annulare, warts, and acne [11]. In oral medicine, some studies have been conducted regarding the effect of PDT on oral potentially malignant disorders (OPMD) and in oral cancer [13-17]. Our team conducted an overview to systematically examine and critically appraise available evidence regarding the impact of PDT on the management of OPMD and oral squamous cell carcinoma (OSCC) [18]. In summary, despite the adequate and safe effects of PDT against OPMD and OSCC, its support as a first-line treatment still needs better-designed cohort studies. However, its safety in mucosa changed by carcinogenic action has not yet been established, and it is not frequently employed in clinical centers. In light of this, the objective of the current study was to investigate how PDT interacts and/or interferes with the promotion of cell proliferation and differentiation during the process of chemically induced oral carcinogenesis in an animal model.

Material and Methods

Ethical approval

This study was approved by the ethics committee of Hospital de Clínicas de Porto Alegre, Brazil, under process number 2021-0614. All experiments were carried out in accordance with the Brazilian Law 11.794 and the Brazilian Guideline for the Care and Use of Animals of the National Council for Animal Experimentation Control.

Animal model

Forty male albinos Wistar rats, 8-weeks-old, weighing 250-300 g, were selected for the study. The animals were kept under controlled conditions of temperature (22±2ºC), humidity (55±5%) and a 12-hour light-dark cycle. Throughout the experiment, the animals consumed selected solid feed and autoclaved water *ad libitum*, containing or not 4NQO. The animals were kept in individually ventilated polycarbonate microisolators, with a floor lined with flocked pine shavings and a maximum density of 3 animals per box. All the handling of the boxes and the animals was of the controlled conventional type, using materials submitted to a previous sterilization process.

Sample size

The sample calculation was performed using software developed by the University of British Columbia available at *https://www.stat.ubc.ca/~rollin/stats/ssize/b2.html*. For the calculation, data on the prevalence of OSCC in the 4NQO alone and 4NQO + chemoprotective agent groups from previous studies that used similar dosage and time of application of the carcinogen were used: Soares et al., 2018 (60% vs. 0%, respectively) and Thandavamoorthy et al., 2014 (83% vs. 16%, respectively) [19,20]. A sample number of 8 animals per group was identified as necessary for a power of 0.8 and an alpha value of 0.05. Considering a risk of loss due to adverse effects of the carcinogen of up to 30% of the sample [21], the final sample number established was 11 animals per experimental group per day of euthanasia (12 and 20 weeks). The two times of euthanasia are important to the carcinogenesis process can be evaluated in different periods [22].

Experimental design

The experimental animals were divided into four groups, which were organized according to the two times of euthanasia (i.e., 12 and 20 weeks):

Control Group (n=22): Control animals were treated with normal diet and provided 4NQO drinking water for 12 weeks (n=11) and 20 weeks (n=11)

PDT Group (n=18): PDT protocol in animals treated with normal diet and provided 4NQO drinking water for 12 weeks (n=11) and 20 weeks (n=7)

Water and 4NQO consumption control

Based on the animals' baseline consumption of pure water, water consumption was monitored before the delivery of 4NQO. To determine the average water intake per box, drinkers were weighed twice a week for two weeks.

The 4QNO consumption was control by weighing the bottles between the changes, once a week.

Oral carcinogenesis model

All animals were treated with 25 ppm of 4NQO (LOT 711645086-1-1, Toronto Research Chemicals Inc., Toronto, Canada) solution diluted in the drinking water. The calculation used was: 25 mg of 4NQO, 1ml of the dimethylsulfoxide solvent (DMSO - Sigma, St. Louis, MO, USA, and 1 liter of autoclaved water.

The rats had free access to water with carcinogen during all the experimental periods. To minimize the adverse effects caused by the 4NQO, the animals received pure water twice a week and 5% glucose solution once a week (100ml per animal). Moreover, in the first five weeks of the experiment, hypercaloric diet once a day [2 pellets (2g) per animal] were offered.

After the experimental period (12 and 20 weeks), all rats were euthanized by excess isoflurane inhalation.

PDT protocol

The PDT protocol was based on previous studies [23,24]. PDT was performed with the animal sedated once a week. Inhalational anesthesia was administered with isoflurane, diluted in oxygen, supplied by a vaporizer at a concentration of 5% for induction and 1-2% for maintenance of anesthesia. The photosensitizer 5-aminolevuinic acid (ALA) was topically applied at 5% by dissolving powdered ALA

(Sigma Aldrich, USA) in EDTA (ethylenediaminetetraacetic acid), homogenized with lanolin and vaseline. A swab impregnated with ALA solution was rubbed on the posterior region of the dorsum of the tongue, covering an area of approximately 3x3mm. After sedation, 5-ALA was applied to the posterior region of the dorsum of the tongue. A time of 2 hours is necessary until the fixation of the 5-ALA in the mucosa – the rat remained anesthetized in a maintenance dose. During this period, the animals were maintained under superficial anesthesia (0.5-1% isoflurane diluted in oxygen), receiving basic care such as the use of a heating bed and compresses to avoid hypothermia. Body temperature was measured using a digital thermometer introduced in the rectal region.

After 2 hours, a point on the posterior region of the tongue demarcated by the dye was irradiated with a laser as described in **Table 1**. These irradiation parameters were adapted from another study, in which 5-ALA-mediated PDT was applied to oral potentially malignant lesions induced by 7,12 - dimethylbenzanthracene (DMBA) in hamsters [25].

Table 1. Irradiation parameters of PDT

Laser wavelength (nm)	Power (mW)	Spot size (cm^2)	Power density (W/cm^2)	Irradiation duration (s)	Energy density (J/cm^2)	Energy per point (J)
660	100	0.03	3.33	27	90	2.7

nm, nanometer; mW, miliwatt; W, watt; J, Joule; s, second

Widefield fluorescence imaging

Following the laser irradiation step, fluorescence images from the tongue were acquired in a dark room. The fluorescence image analysis aimed to evaluate the PpIX production and homogeneous distribution in the dorsum tongue surface. For the acquisition of the images, a diagnostic system for fluorescence widefield was used (EVINCE, MM Optics, Sao Carlos – Brazil). The images obtained by widefield fluorescence were assessed qualitatively through the red color visualization.

Animal monitoring

The physical and behavioral health of the animals was checked daily. Every two days, rats were weighed. Once a week, the tongue of the animals was photographed for clinical follow up.

At the end of the experiment, a blind evaluator scored the clinical appearance. The lingual mucosa was classified according to the following parameters: Score 1 – normal mucosa; Score 2 – areas of white spot/plaque with a smooth surface; Score 3 – areas of white plaques with an irregular surface; Score 4 – nodular areas; Score 5 – ulcerated areas.

Histological analysis of tongues

The whole tongue was excised and fixed in neutral-buffered formalin solution for 24 hours. The formalin-fixed tongues were then processed and embedded in paraffin. The 3-µm thick sections were stained with hematoxylin and eosin for histopathological.

An experienced oral pathologist performed microscopic analysis. All samples were blinded, both for the group and for the experiment's period. The presence or absence of epithelial dysplasia, carcinoma in situ, or invasive carcinoma was classified morphologically.

Statistical analysis

The software GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analysis. Differences between groups within each experimental time were assessed through multiple t-tests. Different lowercase letters in graphs and tables denote significant difference ($p < 0.05$).

Results

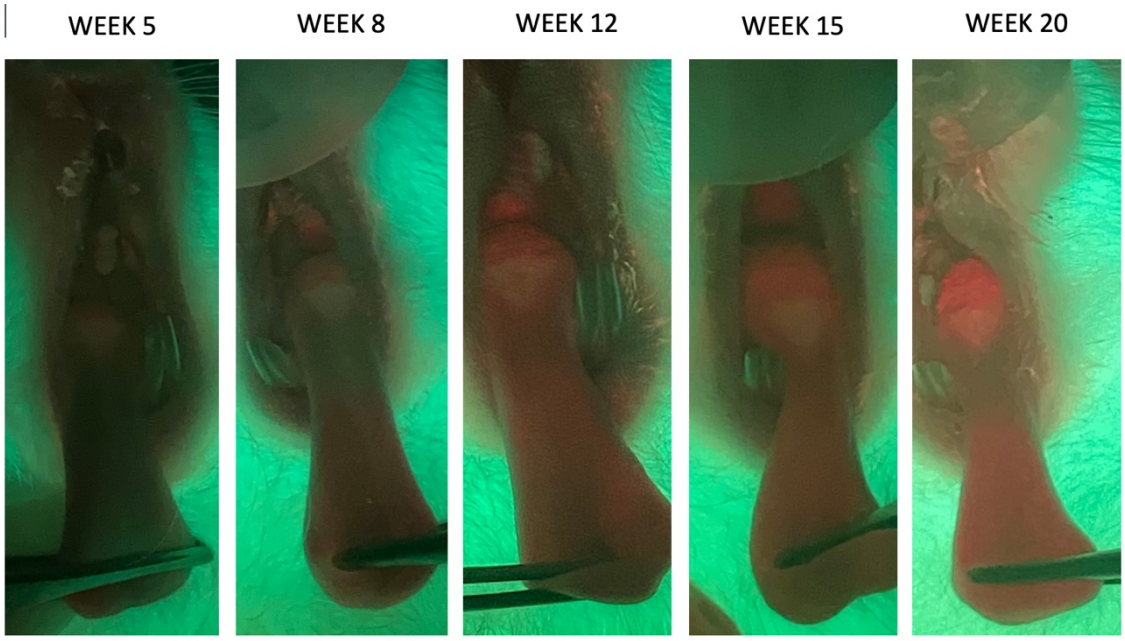
Clinical evaluation of oral lesions

All the animals from control and PDT groups exhibited lesions on tongue mucosa localized mainly along the posterior dorsum of the tongue. The lesions began on week 5, characterized by white patches. Along the time, erosive lesions, white plaques, nodules, and ulcers could be observed. Nonsignificant differences were observed in 12 weeks ($p=0.26$) and 20 weeks ($p=0.32$) between Control and PDT Group.

Widefield fluorescence imaging

During the image acquisition, clinical differences in PpIX production could be observed since the week 8. **Figure 1** shows an example of the qualitative analysis of the PpIX accumulation formed in 2 hours according to the widefield fluorescence imaging.

Figure 1. PpIX fluorescence accumulation after 2 hours of 5-ALA application



PDT does not interfere with the oral carcinogenesis process

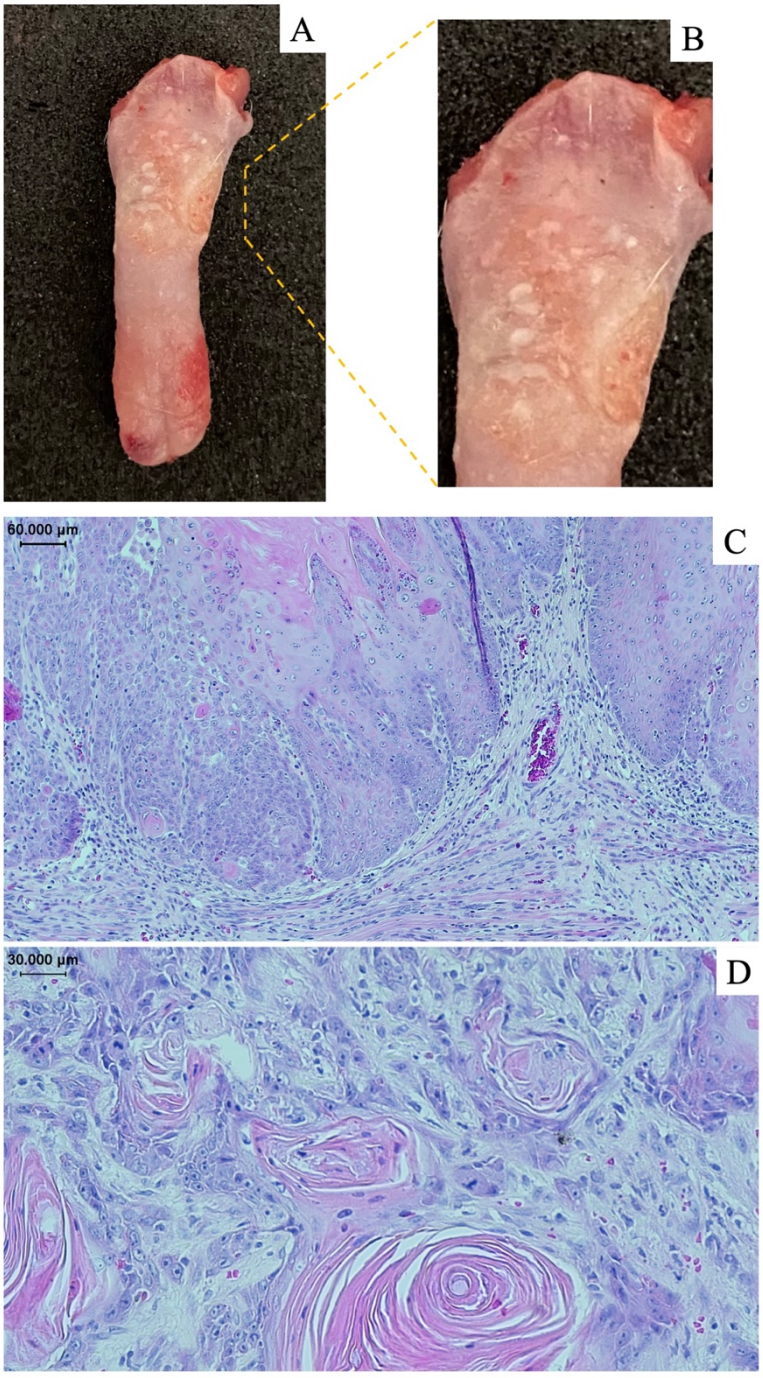
The histopathological findings are summarized in the **Table 2** and illustrated in **Figure 2**. No statistical differences were observed between control and PDT groups in 12 weeks ($p<0.54$) or 20 weeks ($p<0.43$). At the end of 12 weeks, 8 (72.7%) animals of each group (PDT and control) exhibit epithelial dysplasia. After 20 weeks of experiment, the majority lesions on control and PDT group were squamous cell carcinoma.

Table 2. Histological diagnosis after 12 and 20 weeks of experiment

	Hyperplasia, Acanthosis and Hyperkeratosis	**Epithelial dysplasia**	**In situ carcinoma**	**Carcinoma**
12 weeks				
Control Group, n=11	0	8	3	0
PDT Group, n=11	2	8	1	0

20 weeks				
Control Group, n=11	0	1	1	9
PDT Group, n=7	0	0	2	5

Figure 2. Representative case of oral squamous cell carcinoma. Reddish-white areas with an ulcer on the left side of the tongue (A and B). Invasive tumor cells (H&E, 100X magnification) (C). Islands of pleomorphic cells containing keratin pearls (H&E, 100X magnification) (D).



Animal monitoring

The animals maintained their well-being, as evidenced by daily assessments and weight gain over the weeks. Regarding adverse effects, vocalization (n=3), blood in urine (n=1), nose secretion during the sedation period probably due to the carcinogen (n=2) and acute oral ulcer in the first week of experiment (n=5) were observed. We had no losses with the 4NQO model; nevertheless, four animals died in the first week of the experiment due to a heating bed malfunction that caused hyperthermia and death.

Discussion

Since the 1970s, PDT has been acknowledged as a successful treatment for neoplastic lesions of the skin, bladder, breast, stomach, and oral cavity tissues. Animal model studies published to date have evaluated PDT as a treatment for chemically induced oral lesions [24-26]. However, little is known regarding its effect on tissues during carcinogenesis. To the best of our knowledge, this is the first study to investigate the effects of PDT on carcinogen-induced tongue in rats and its safe in a carcinogen field. It is important to highlight that our main intention was to safety in use PDT on altered mucosa, not purposing a treatment. In general, our results showed that 4NQO rat model is associated with the development of oral potentially malignant and malignant lesions; however, the PDT did not affect the clinical and histopathological aspects of these oral lesions.

In our study, all stages of oral carcinogenesis could be demonstrated by the 4NQO rat model, which has been shown to be histologically and molecularly like human oral carcinogenesis [27]. A rat model also makes it possible to maintain settings with little change, allowing for the reproducible isolation of all stages of carcinogenesis. Previous research predicted epithelial dysplasia and OSCC lesions 12 and 20 weeks after 4NQO exposure, respectively [22]. PDT did not paralyze the carcinogenic effect of 4NQO, but it did not enhance its action, according to our data. However, works that used the methodology to establish the lesion and then remove exposure to 4nqo showed promising results in the treatment. For example, Barcessat et al. [23] subjected the animals to 4NQO for 16 weeks before treatment with PDT. The cell dynamics of possibly malignant lesions following two sessions of 5-ALA-mediated PDT revealed oscillations in apoptosis and DNA repair by epithelial cells, according to the authors. In the same way, Khozeimah et al. [26] administered 4NQO for 12 weeks before

treatment with PDT. Their findings shown that ala-mediated PDT was an effective therapy approach for oral dysplasia. These results may suggest that as long as the carcinogen is administered, it does not prevent lesion progression; nevertheless, once the lesion is established and without the carcinogen stimulating the tissue, the lesion can present regression/remission.

In an *in vitro* study conducted by Jin et al. [28], the use of ALA-PDT suppressed the growth of oral precancerous cells by regulating the TGF-β signaling pathway, and its suppressive effect was enhanced using LY2109761. These findings indicate that it could be a promising alternative treatment against oral precancerous lesions. Olek and coauthors [5] published a systematic review with the objective of presenting an overview of the use of PDT in the treatment of oral squamous cell carcinoma throughout preclinical animal model studies. Their results showed that research has focused mainly on evaluating the effects of PDT on tumor growth, on the animals' life expectancy and on the occurrence of adverse effects, as well as on the role of the route of administration in the effects achieved. However, its safety in mucosa affected by carcinogenic action had not yet been proven. In this sense, our findings are promising, revealed that PDT with topical 5-ALA does not interfere with the oral carcinogenesis process.

Despite advancements in laser technology, synthetic chemistry, nanotechnology, and photobiology, PDT is still not regarded as "standard" therapy even in fields of medicine where standard therapy has not been able to produce meaningful improvements in outcomes [29,30]. However, the literature has demonstrated that PDT will eventually be incorporated into the conventional cancer treatment regimen in the clinical context, either as a component of a multimodal approach or as a stand-alone treatment for early cancer or palliative care [31] In this sense, preclinical studies like the one described in this article can support the use of therapy and the development of appropriate protocols in this regard by providing scientific evidence.

Conclusion

Our research has demonstrated that PDT is unable to change the tissue during the malignant transformation phase.

Acknowledgments

This study was funded by the Postgraduate Research Group of Hospital de Clínicas de Porto Alegre (GPPG/FIPE: 2021-0614). The authors thank the Coordination of Improvement of Higher Education Personnel (CAPES, Finance Code 001), Brazil. L.F.S. and T.R.S. are the recipients of fellowships. Also, we would like to thank the Brazilian National Council for Scientific and Technological Development for financial support (CNPq). V.S.B., C.K., M.D.M., P.A.V. are research fellows of CNPq.

Conflict of interest statement

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

References

[1] Kwiatkowski S, Knap B, Przystupski D, Saczko J, Kędzierska E, Knap-Czop K, Kotlińska J, Michel O, Kotowski K, Kulbacka J. Photodynamic therapy - mechanisms, photosensitizers and combinations. Biomed Pharmacother. 2018 Oct;106:1098-1107. doi: 10.1016/j.biopha.2018.07.049.

[2] Raab O. On the effects of fluorescent substances on infusoria. Z Biol 1900;39:524–6.

[3] Meyer-Betz, Friedrich. Untersuchungen uber die Biologische Wirkung des haematoporphyrin und anderer derivative des blut-und Gallenfarbstoffs. Dtsch Arch Klin Med 1913;112:476–503

[4] Auler H, Banzer G. Untersuchungen über die Rolle der Porphyrine bei Geschwulstkranken Menschen und Tieren. Z Krebsforsh 1942;53:65–8

[5] Olek M, Machorowska-Pieniążek A, Olek K, Cieślar G, Kawczyk-Krupka A. Photodynamic therapy in the treatment of oral squamous cell carcinoma - The state of the art in preclinical research on the animal model. Photodiagnosis Photodyn Ther. 2021 Jun;34:102236. doi: 10.1016/j.pdpdt.2021.102236.

[6] Hopper C. Photodynamic therapy: a clinical reality in the treatment of cancer. Lancet Oncol. 2000 Dec;1:212-9. doi: 10.1016/s1470-2045(00)00166-2.

[7] Prates RA, Yamada AM, Suzuki LC, França CM, Cai S, Mayer MP, Ribeiro AC, Ribeiro MS. Histomorphometric and microbiological assessment of photodynamic therapy as an adjuvant treatment for periodontitis: a short-term evaluation of inflammatory periodontal conditions and bacterial reduction in a rat model. Photomed Laser Surg. 2011 Dec;29(12):835-44. doi: 10.1089/pho.2010.2984.

[8] Sharma SK, Mroz P, Dai T, Huang YY, St Denis TG, Hamblin MR. Photodynamic Therapy for Cancer and for Infections: What Is the Difference? Isr J Chem. 2012 Sep;52(8-9):691-705. doi: 10.1002/ijch.201100062.

[9] Prażmo EJ, Kwaśny M, Łapiński M, Mielczarek A. Photodynamic Therapy As a Promising Method Used in the Treatment of Oral Diseases. Adv Clin Exp Med. 2016 Jul-Aug;25(4):799-807. doi: 10.17219/acem/32488.

[10] van Straten D, Mashayekhi V, de Bruijn HS, Oliveira S, Robinson DJ. Oncologic photodynamic therapy: basic principles, current clinical status and future directions. Cancers (Basel). 2017;9(2):19. doi: 10.3390/cancers9020019

[11] Caccavale S, Iocco A, Pieretti G, Alfano R, Argenziano G. Curettage + microneedling + topical ALA-PDT for the treatment of acral resistant warts: Our experience. Photodiagnosis Photodyn Ther. 2019 Sep;27:276-279. doi: 10.1016/j.pdpdt.2019.04.008.

[12] Jiang W, Liang M, Lei Q, Li G, Wu S. The Current Status of Photodynamic Therapy in Cancer Treatment. Cancers (Basel). 2023 Jan 18;15(3):585. doi: 10.3390/cancers15030585.

[13] Saini R, Poh CF. Photodynamic therapy: a review and its prospective role in the management of oral potentially malignant disorders. Oral Dis. 2013 Jul;19(5):440-51. doi: 10.1111/odi.12003. PMID: 24079944.

[14] Cerrati EW, Nguyen SA, Farrar JD, Lentsch EJ. The efficacy of photodynamic therapy in the treatment of oral squamous cell carcinoma: a meta-analysis. Ear Nose Throat J. 2015;94(2):72-79. doi: 10.1177/014556131509400208

[15] Alkindi M. Therapeutic efficacy of photodynamic therapy in oral squamous cell carcinoma: a systematic review. Biosc Biotech Res Comm. 2020;13(1):187-194

[16] Romano A, Di Stasio D, Gentile E, Petruzzi M, Serpico R, Lucchese A. The potential role of photodynamic therapy in oral premalignant and malignant lesions: a systematic review. J Oral Pathol Med. 2021;50(4):333-344. doi: 10.1111/jop.13139

[17] Binnal A, Tadakamadla J, Rajesh G, Tadakamadla SK. Photodynamic therapy for oral potentially malignant disorders: A systematic review and meta-analysis. Photodiagnosis Photodyn Ther. 2022 Mar;37:102713. doi: 10.1016/j.pdpdt.2022.102713

[18] Schuch LF, Schmidt TR, Kirschnick LB, de Arruda JAA, Campagnol D, Martins MAT, Santos-Silva AR, Lopes MA, Vargas PA, Bagnato VS, Kurachi C, Guerra ENS, Martins MD. Revisiting the evidence of photodynamic therapy for oral potentially malignant disorders and oral squamous cell carcinoma: An overview of systematic reviews. Photodiagnosis Photodyn Ther. 2023 Mar 22;42:103531. doi: 10.1016/j.pdpdt.2023.103531.

[19] Soares GR, de Moura CFG, Silva MJD, Vilegas W, Santamarina AB, Pisani LP, Estadella D, Ribeiro DA. Protective effects of purple carrot extract (Daucus carota) against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Med Oncol. 2018 Mar 15;35(4):54. doi: 10.1007/s12032-018-1114-7.

[20] Thandavamoorthy P, Balan R, Subramaniyan J, Dhas G. Alleviative Role of Rutin Against 4-Nitroquinoline-1-Oxide (4-NQO) Provoked Oral Squamous Cell Carcinoma in Experimental Animal Model, J. Pharm. Res. 8 (2014) 899–906.

[21] Wagner VP, Spuldaro TR, Nör F, Gaio EJ, Castilho RM, Carrard VC, Rösing CK. Can propranolol act as a chemopreventive agent during oral carcinogenesis? An experimental animal study. Eur J Cancer Prev. 2021 Jul 1;30(4):315-321. doi: 10.1097/CEJ.0000000000000626

[22] Ribeiro DA, Salvadori DM. Gingival changes in wistar rats after oral treatment with 4-nitroquinoline 1-oxide. Eur J Dent. 2007 Jul;1(3):152-7.

[23] Barcessat AR, Huang I, Rosin FP, Dos Santos Pinto D Jr, Maria Zezell D, Corrêa L (2013) Effect of topical 5-ALA mediated pho- todynamic therapy on proliferation index of keratinocytes in 4- NQO-induced potentially malignant oral lesions. J Photochem Photobiol B 126:33–41. doi:10.1016/j.jphotobiol.2013.06.011

[24] Rosin FC, Barcessat AR, Borges GG, Ferreira LG, Corrêa L. Vascular alterations after photodynamic therapy mediated by 5-aminolevulinic acid in oral leukoplakia. Lasers Med Sci. 2017 Feb;32(2):379-387. doi: 10.1007/s10103-016-2127-0.

[25] Hsu YC, Yang DF, Chiang CP, Lee JW, Tseng MK. Successful treatment of 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch precancerous lesions by topical 5-aminolevulinic acid-mediated photodynamic therapy. Photodiagnosis Photodyn Ther. 2012 Dec;9(4):310-8. doi: 10.1016/j.pdpdt.2012.03.003

[26] Khozeimeh F, Ziaei S, Khalesi S, Allameh M, Jahanshahi G. The efficacy of photodynamic therapy in rat tongue dysplasia. J Clin Exp Dent. 2019 Jul 1;11(7):e587-e592. doi: 10.4317/jced.54425.

[27] Zigmundo GCO, Schuch LF, Schmidt TR, Silveira FM, Martins MAT, Carrard VC, Martins MD, Wagner VP. 4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis: A systematic literature review. Pathol Res Pract. 2022 Aug;236:153970. doi: 10.1016/j.prp.2022.153970.

[28] Jin JQ, Wang Q, Zhang YX, Wang X, Lu ZY, Li BW. Effect of ALA-PDT on inhibition of oral precancerous cell growth and its related mechanisms. Lasers Med Sci. 2022 Dec;37(9):3461-3472. doi: 10.1007/s10103-022-03607-y.

[29] Moghissi K. PDT: The plight. Photodiagnosis Photodyn Ther. 2007 Dec;4(4):223. doi: 10.1016/j.pdpdt.2007.10.003.

[30] Benov L. Photodynamic therapy: current status and future directions. Med Princ Pract. 2015;24 Suppl 1(Suppl 1):14-28. doi: 10.1159/000362416.

[31] Algorri JF, Ochoa M, Roldán-Varona P, Rodríguez-Cobo L, López-Higuera JM. Photodynamic therapy: a compendium of latest reviews. *Cancers (Basel)*. 2021;13(17):4447. doi: 10.3390/cancers13174447

Figure legends

Figure 1. PpIX fluorescence accumulation after 2 hours of 5-ALA application

Figure 2. Representative case of oral squamous cell carcinoma. Reddish-white areas with an ulcer on the left side of the tongue (A and B). Invasive tumor cells (H&E, 100X magnification) (C). Islands of pleomorphic cells containing keratin pearls (H&E, 100X magnification) (D).

2.5 Photobiomodulation does not affect the oral carcinogenesis process induced by 4NQO in rats

Artigo formatado para submissão no periódico *Oral Diseases*.

Lauren Frenzel **Schuch**[1], Daniela **Champagnol**[2], Tuany Rafaeli **Schmidt**[3], Carolina Horn Troian **Michel**[4], Tuane Nerissa Alves **Garcez**[2], Vanessa Rodrigues **Velho**[3], Vivian Petersen **Wagner**[5], Rogerio Moraes **Castilho**[6], Marco Antonio Trevizani **Martins**[3,7], Pablo Agustin **Vargas**[1], Alan Roger **Santos-Silva**[1], Manoela Domingues **Martins**[1,3]

[1] Oral Diagnosis Departament, Piracicaba Dental School, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil

[2] Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil

[3] Departament of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[4] Departament of Edodontics, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[5] Academic Unit of Oral and Maxillofacial Medicine and Pathology, Department of Clinical Dentistry, University of Sheffield, Sheffield, United Kingdom

[6] Michigan University, School of Dentistry, Department of Periodontics and Oral Medicine, Ann Arbor, MI, United States

[7] Department of Oral Medicine, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

Corresponding author: Manoela Domingues Martins. Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, R. Ramiro Barcelos, 2492, room 503. Porto Alegre, Rio Grande do Sul, Brazil, CEP: 90035-003. Phone: +55 (51) 3308-5011. E-mail: manomartins@gmail.com; manoela.martins@ufrgs.br

Running title: Photobiomodulation does not affect oral carcinogenesis in rats

Abstract

Objective: To assess the impact of photobiomodulation (PBM) on the oral carcinogenesis process induced by 4NQO.

Material and Methods: Sixty-six male Wistar rats received systemic 4NQO for 12 (n=33) and 20 weeks (n=33). Each animal was assigned to one of three groups: (1) Control group; (2) PBM 0.3J; and (3) PBM 1J. Applications for PBM were occurred three times a week and carried out with an InGaAlP diode laser operating in continuous mode and in contact while employing the punctual technique. At weeks 12 and 20, the animals were euthanized.

Results: The PBM 1J group had higher scores since it was the only group with nodular lesions (p=0.005). Most animals in both the control (n=7) and PBM 0.3J (n=10) groups had clinically erosive lesions. The nodular form was more common in animals euthanized after 20 weeks. No significant difference was observed among the groups of 12 and 20 weeks. At 12 weeks, the most common histological diagnosis in all groups was epithelial dysplasia. After 20 weeks, most of the cases revealed squamous cell carcinoma.

Conclusion: Our results demonstrate that the exposure to PBM on a neoplastic mucosa neither promotes tumor growth nor inhibits the neoplastic process.

Introduction

Oral carcinogenesis is a multifactorial process where mutations build up over time until a malignant tumor is developed (Tanaka & Ishigamori, 2011). Neoplastic cells differ from normal cells in a variety of ways, including resistance to anti-growth signals, evasion of apoptosis, self-sufficiency in growth signals, limitless potential for replication, encouragement of angiogenesis, capacity for tissue invasion and metastasis, altered metabolic pathways, and immunity evasion (Hanahan & Weinberg, 2000; Fouad & Aanei, 2017). These features are functionally capable of enabling cancerous cells to endure, multiply, and disseminate outside of their original site. The high death rate, destructive nature of conventional treatments, frequent comorbidities, and frequent oral squamous cell carcinoma (OSCC) diagnoses necessitate a deeper comprehension of the carcinogenic process and the development of novel therapeutic approaches.

Photobiomodulation therapy (PBM) is a non-invasive modality that uses non-ionizing forms of light sources, including lasers and LEDs for producing photophysical and photochemical events. In general, the effect of PBM has been attributed to accelerated respiratory metabolism and the activation of cell-signaling mechanisms that promote cell proliferation, prevention of cell death, restoration of cellular metabolism, and reduction of pain and inflammation (Anders, Lanzafame, Arany, 2015; de Freitas, Hamblin 2016; Glass, 2021). In oral medicine, compelling evidence indicates the use of PBM, including, but not limited to, facial pain and neuromuscular disorders (e.g., orofacial pain and temporomandibular disorders), dermatologic diseases (e.g., lichen planus and pemphigus vulgaris), burning mouth syndrome, xerostomia/hyposalivation, chemo- and/or radiation-induced oral mucositis, recurrent herpes simplex lesions and recurrent aphthous ulcerations/stomatitis (Pandeshwar et al., 2016; Al-Maweri et al., 2017; Al-Maweri et al., 2018; Munguia, Jang, Salem, Clark, Enciso, 2018; Zadik et al., 2019; Amorim Dos Santos et al., 2020; Golez et al., 2022).

The increasing use of PBM in different clinical situations requires that we understand the safety of its use. Several studies using in vitro assays (Schalch et al., 2019; Silveira et al., 2019; Martins et al., 2020), animal models (Barasch, Raber-Durlacher, Epstein, Carroll, 2016; Silveira et al., 2023) and clinical (Antunes et al., 2017; de Pauli Paglioni et al., 2019) studies have shown that PBM does not interfere

with tumor behavior. However, there are some articles that claim PBM therapy can directly harm tumors (Wu et al., 2014; Lu, Zhou, Wu, Liu, Xing, 2016) or stimulate cancer cells (de Monteiro et al., 2011; AlGhamdi, Kumar, Moussa, 2012; Hamblin, Nelson Strahan, 2018). In this sense, the literature is still conflicting on whether or not PBM can intensify the impact of carcinogenesis processes. Therefore, the current study intends to assess the clinical and microscopic impact of PBM during the process of oral carcinogenesis induced by 4NQO.

Material and Methods

Animal model

Eight-week-old male Wistar rats were used in this present study. All animals were maintained in a room with a 12 h light and 12 h dark cycle (lights on from 7 a.m. to 7 p.m.). They were housed in air-conditioned quarters. The rats were given a standard pellet diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. During the first five weeks of study, the animal also received hypercaloric supplementation once a day (2g per animal) and 5% glucose solution once a week (100ml per animal).

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals and was approved by the Porto Alegre University Hospital's (HCPA, Brazil) Ethics Committee on Animal Use under protocol number 2021-0614.

Sample size calculation

Each sample size calculation for the experimental groups was performed based on previous studies with a similar methodology. The groups were composed of 11 animals to account for the expected mortality rate of ~30% (Wagner et al., 2021) due to 4NQO-related side effects during the 12 and 20 weeks of carcinogen administration.

Experimental design

The experimental animals were divided into three groups, as follows:

Control Group (n=22): Animals receiving 4NQO administration and being handled three times per week while not receiving PBM, for 12 weeks (n=11) and 20 weeks (n=11).

PBM 0.3J (n=22): Animals that, starting on the first day of 4NQO administration, will receive PBM with a protocol of 0.3J, three times per week for 12 weeks (n=11) and 20 weeks (n=11).

PBM 1J (n=22): Animals that, starting on the first day of 4NQO administration, will receive PBM with a protocol of 1J, three times per week for 12 weeks (n=11) and 20 weeks (n=11).

Oral carcinogenesis model

For oral carcinogenesis induction, all rats were treated with 25 ppm of 4NQO (LOT 711645086-1-1, Toronto Research Chemicals Inc., Toronto, Canada) solution diluted in the drinking water. The 25 mg of carcinogen was diluted in 1 ml of the dimethylsulfoxide solvent (DMSO - Sigma, St. Louis, MO, USA), and then in 1 liter of autoclaved water.

Due to photolysis and the danger of the carcinogen, fresh solutions were prepared twice a week and stored in amber bottles that were clearly labeled. Consumption was controlled by overfilling the solution between exchanges. The animals were given pure water twice per week.

Photobiomodulation protocol

PBM was performed three times a week under inhalational anesthesia with isoflurane, diluted in oxygen, supplied by a vaporizer at a concentration of 5% for induction and 1-2% for maintenance of anesthesia. An InGaAlP diode laser (660 nm; MMOptics, São Carlos, São Paulo, Brazil) was used in continuous mode and in contact using the punctual technique in the parameters described in Table 1. A point in the posterior region of the dorsum of the tongue was irradiated. Two different protocols were tested (Total radiant energy of 0.3J and 1J) to verify if there is a difference in safety during the carcinogenesis process.

Table 1. Irradiation parameters applied in the PBM

	PBM 0.3J	PBM 1J
Center wavelengths (nm)	660±10	
Operating mode	Continuous	
Peak power (W)	0.01	
Average power (mW)	100	
Spot size (cm^2)	0.03	
Beam shape	Round	
Irradiance at target (mW/cm^2)	3.333	
Fluence (J/cm^2)	10	33.3
Exposure duration (s)	3	10
Total radiant energy (J)	0.3	1
Photon Fluence ($p.J/cm^2$)	19	63
Photon Fluence (Einstein)	4.2	14
Number of points irradiated	1	
Application technique	Contact	
Frequency of sessions	3 times/week for 12 weeks	

nm, nanometer; mW, miliwatt; W, watt; J, Jaule; s, second; p.J, photon fluence

Animal monitoring

The animals were monitored daily for general health and weighed three times a week with an electronic scale. Oral examinations were performed three times a week to monitor changes in the lingual mucosa. Once a week, following isoflurane inhalation sedation, the animals' tongues were pulled, and the posterior region of the tongue photographed.

All rats were euthanized by isoflurane inhalation overdose after 12 weeks (n=33) and 20 weeks (n=33). At the end of the experiment, a calibrated researcher who was blind to the groups was in responsible of clinical classifying the lesions based on the following parameters: Score 1, normal mucosa; Score 2, areas of white spot/plaque with a smooth surface; Score 3, areas of white plaques with an irregular surface; Score 4, nodular areas; and Score 5, ulcerated areas.

Histopathological analysis

All the tongues were then fixed in 10% buffered formaldehyde for 24 hours, embedded in paraffin blocks, sectioned at 3 μm and stained with hematoxylin and eosin (H&E). The histopathological diagnosis was made by an experience oral pathologist, blinded for the groups. The microscopy features were based on no dysplasia (atrophy of the epithelium, hyperkeratosis, hyperplasia), epithelial dysplasia, *in situ* carcinoma and squamous cell carcinoma.

Statistical analysis

The software GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analysis. Differences between groups within each experimental time were assessed through multiple t-tests. Different lowercase letters in graphs and tables denote significant difference ($p<0.05$).

Results

Establishment of oral carcinogenesis animal model

The model's validity was confirmed once all 66 animals developed lesions, both clinically and histopathologically. The rats performed well throughout the experiment. Only in the first week after ingestion of 4NQO were adverse effects such as chromodacryorrhea, hypersalivation, and acute ulcers covered by a white membrane noticed in some animals. After a time of adaptation to 4NQO, all animals were energetic and interactive, and the negative effects were swiftly resolved. The animal's coat deteriorated with time, becoming more unkempt than usual. Two animals in the 20-week group wheezed at the end of the experiment. However, there was no evidence of chronic weight loss or death.

Clinical analyze of oral lesions

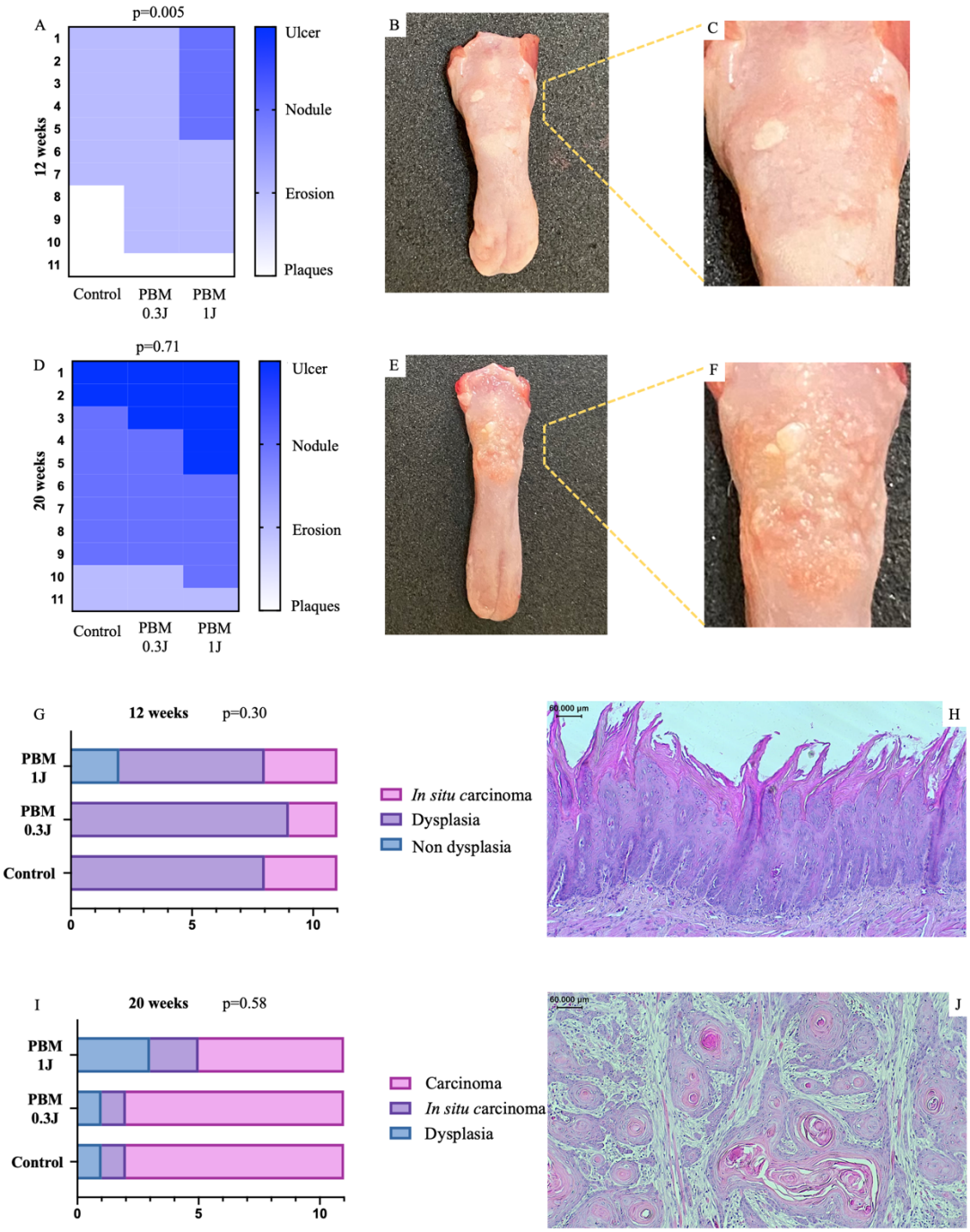
Oral lesions could be observed since the week 5 as plaques. In 12 weeks analysis, animals of PBM 1J group showed oral lesions with nodular aspect which was significant different than control and PBM 0.3J groups that exhibited more erosive lesions ($p=0.005$) (**Figure 1A-C**).

In 20 weeks evaluation, the nodular lesions was more common in both control (n=7) and PBM 0.3J (n=6) groups. Five rats in the PBM 1J group had nodular and five had ulcerated lesions. No significant difference between groups was found (p=0.05) (**Figure 1D-F**).

PBM does not change the morphological features

No significant difference in histopathological characteristics was observed among the groups of 12 (p=0.30) and 20 weeks (p=0.58) (**Figure 1G-J**). At 12 weeks, the most common histological diagnosis in all groups was epithelial dysplasia. After 20 weeks, the majority of the cases revealed squamous cell carcinoma.

Figure 1. Clinical and histopathological features of our sample. A, distribution of the cases according to clinical presentation in the 12 weeks; B and C, clinical aspect of one case at the end of 12 weeks; D, distribution of the cases according to clinical presentation in the 20 weeks; E and F, clinical aspect of one case at the end of 20 weeks; G, distribution of the cases according to histopathological diagnosis in the 12 weeks; H, epithelial dysplasia; I, distribution of the cases according to histopathological diagnosis in the 20 weeks; J, squamous cell carcinoma



Discussion

The use of PBM for therapeutic purposes has been investigated for years. This therapy represents a promising therapeutic approach, exploring the ability of light to modulate biochemical and molecular processes in living cells (Levchenko et al., 2018; Dompe et al., 2020). In the present study we evaluated the impact of PBM in carcinogenic

process in rats using a long-term administration of 4NQO with drinking water model. Our results demonstrate that PBM has no impact on the development of oral cancer.

Several studies have been conducted to elucidate the action of PBM in tumors cells in *in vitro*, *in vivo,* and clinical studies. However, its use in an altered mucosa is still controverse. Barasch et al. (2016) showed that leukemia cells exposed to PBM before being exposed to ionizing radiation did not protect against death but rather made the cells more susceptible, supporting the idea that light has a distinct impact on healthy and cancerous tissues. A systematic review published by Silveira et al. (2019) analyzed the effects of PBM therapy on head and neck SCC in 15 articles. Of them, only two studies evaluated an animal model: one of them reported increased progression of OSCC (de C Monteiro et al., 2011), while Ottaviani et al. (2016) observed a reduction in tumor progression. Our findings showed that PBM has no effect on the development of oral cancer in 4NQO model. We can infer that exposure to PBM on a neoplastic mucosa neither promotes tumor growth nor inhibits the neoplastic process. Despite of this, a study by Chiang, Lee, Chen, Chen, Wang (2020) evaluated the therapeutic potential of PBM in oral submucosal fibrosis (an oral potentially malignant disorder) in rats induced by areca nut extract. Results showed that PBM significantly reduced the development of oral submucosal fibrosis, quantified by changes in submucosal layer thickness and collagen deposition (Chiang et al., 2020).

A better knowledge of the *in vivo* biology and genetics of tumor origin, promotion, progression, and metastasis is made possible using animal models in the development and testing of novel methods to disease prevention and treatment, the identification of early diagnostic indicators, and the development of novel therapeutic targets (Onaciu et al., 2020). Carcinogenesis of the rat mucosa occurred in four stages that followed one another, resembling the situation in humans: hyperplasia, atypical hyperplasia, carcinoma in situ, and squamous cell carcinoma (Morris, Scott, Reiskin, 1961). Our results agree with this since we have different stages of carcinogenesis during our study protocol. In fact, 4NQO is a powerful inducer of oral cancers (Sagheer et al., 2021). Long-term administration of 4NQO with drinking water or its topical application led to numerous dysplastic, preneoplastic, and neoplastic lesions that closely mimicked the neoplastic transformation of the human oral cavity (Tinhofer, Braunholz, Klinghammer, 2020; Zigmundo et al., 2022). The main benefit of the 4NQO-

induced animal model is that it may be used to explore the role of genetic and carcinogenic factors in carcinogenesis.

PBM's security has been the subject of extensive investigations over the years. Bensadoun et al. (2020) published a systematic review concerning the safety and efficacy of PBM in cancer patients. Studies which investigated the effect of PBM therapy on cell proliferation/differentiation, tumor growth, recurrence rate, and/or overall survival were included. *In vivo* studies and clinical trials with a follow-up period demonstrated that PBM therapy is safe with regards to tumor growth and patient advantage in the prevention and treatment of specific cancer therapy-related complications. In this same context, our research group has also demonstrated that PBM therapy with a similar protocol to the one used in this study did not exacerbate the behavior of head and neck squamous cell carcinoma stem cell lines in terms of its proliferation, migration, survival, or percentage (Martins et al., 2020). In fact, there are no effects of light on the behavior of the OSCC, as our team recently shown through an animal model study (Silveira et al., 2023). However, the field of cancerization is one of the issues with the use of PBM in patients with head and neck cancer (Bansal et al., 2020). Our model showed that the PBM activity could not enhance the growth of neoplasms, demonstrating its safety. We comprehend that expecting a light source to be able to enhance or hinder the tumor cells' mode of action would be to magnify a feature that is not a part of PBM. When we compare antineoplastic therapies like chemotherapy and radiotherapy, for instance, we discover that these potent therapies frequently fail to stop the growth of tumor cells. Then, it is anticipated that normal oral keratinocytes under stress during wound healing would react differently than neoplastic cells. One of the most significant characteristics of cancer cells is their capacity to proliferate continuously, without the aid of proliferation signals and in the absence of outside stimuli, leading to limitless growth (Hanahan & Weinberg, 2011). In our investigation, it was evident that PBM does not appear to have an impact on the carcinogenesis process, despite its capacity to act on normal oral epithelium cells that are under metabolic stress. In other words, as demonstrated by the study of Dillenburg, Almeida, Martins, Squarize, Castilho (2014) and Silveira et al. (2023), it does not result in DNA damage in healthy oral epithelial cells.

Moreover, in the present study, animals were divided into two different protocols of PBM therapy, and it is important in preclinical studies. Ottaviani et al. (2016) also

investigated the effects of different PBM protocols on animal models of mouth cancer or melanoma (660 nm, 3 J/cm^2, 800 nm, 6 J/cm^2, and 970 nm, 6 J/cm^2). In comparison to controls, the authors found less tumor growth in all PBM-exposed mice. Recently, Neculqueo, Estrázulas, Cherubini, Koth, Salum (2022) used two different energies (1J and 9J) in leukoplakia and OSCC for seven sessions, and the authors stated that both parameters used, does not influence on clinical and histological characteristics. Interestingly, our results demonstrated that a higher energy density promoted more aggressive clinical lesions. At the end of 12 weeks, when the 1J protocol was used, the nodular aspect was more noticeable, and this was the only result that revealed a worse effect in the irradiated group. However, this was not reflected from the histological point of view since this was the group with less aggressive lesions.

It is important to highlight that the PBM parameters used in this study were based on the most recent update of the MASCC/ISOO for the prevention of OM in head and neck cancer patients (Zadik et al., 2019). The present article is a pioneering preclinical study demonstrating that the robustness of the laser did not influence tumor growth, morphological patterns, or cell proliferation outcomes.

Conclusion

Our findings from an oral carcinogenesis animal model experiment support the premise that PBM does not change the prolife of clinical and morphological response of oral tissues exposed to carcinogens.

Acknowledgments

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001), Brazil. L.F.S., T.R.S., C.H.T.M. are the recipients of fellowships. We also acknowledge the Brazilian National Council for Scientific and Technological Development (CNPq). M.D.M., A.R.S.S., P.A.V are research fellows of CNPq.

Conflict of Interest and Source of Funding

None to declare.

References

Tanaka, T., & Ishigamori, R. (2011). Understanding carcinogenesis for fighting oral cancer. *Journal of oncology*, 2011, 603740. https://doi.org/10.1155/2011/603740

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, 100(1), 57–70. https://doi.org/10.1016/s0092-8674(00)81683-9

Fouad, Y. A., & Aanei, C. (2017). Revisiting the hallmarks of cancer. *American journal of cancer research*, 7(5), 1016–1036.

Anders, J. J., Lanzafame, R. J., & Arany, P. R. (2015). Low-level light/laser therapy versus photobiomodulation therapy. *Photomedicine and laser surgery*, 33(4), 183–184. https://doi.org/10.1089/pho.2015.9848

de Freitas, L. F., & Hamblin, M. R. (2016). Proposed Mechanisms of Photobiomodulation or Low-Level Light Therapy. *IEEE journal of selected topics in quantum electronics: a publication of the IEEE Lasers and Electro-optics Society*, 22(3), 7000417. https://doi.org/10.1109/JSTQE.2016.2561201

Glass G. E. (2021). Photobiomodulation: A review of the molecular evidence for low level light therapy. *Journal of plastic, reconstructive & aesthetic surgery: JPRAS*, 74(5), 1050–1060. https://doi.org/10.1016/j.bjps.2020.12.059

Pandeshwar, P., Roa, M. D., Das, R., Shastry, S. P., Kaul, R., & Srinivasreddy, M. B. (2016). Photobiomodulation in oral medicine: a review. *Journal of investigative and clinical dentistry*, 7(2), 114–126. https://doi.org/10.1111/jicd.12148

Al-Maweri, S. A., Javed, F., Kalakonda, B., AlAizari, N. A., Al-Soneidar, W., & Al-Akwa, A. (2017). Efficacy of low level laser therapy in the treatment of burning mouth syndrome: A systematic review. *Photodiagnosis and photodynamic therapy*, 17, 188–193. https://doi.org/10.1016/j.pdpdt.2016.11.017

Al-Maweri, S. A., Kalakonda, B., AlAizari, N. A., Al-Soneidar, W. A., Ashraf, S., Abdulrab, S., & Al-Mawri, E. S. (2018). Efficacy of low-level laser therapy in management of recurrent herpes labialis: a systematic review. *Lasers in medical science*, 33(7), 1423–1430. https://doi.org/10.1007/s10103-018-2542-5

Munguia, F. M., Jang, J., Salem, M., Clark, G. T., & Enciso, R. (2018). Efficacy of Low-Level Laser Therapy in the Treatment of Temporomandibular Myofascial Pain: A Systematic Review and Meta-Analysis. *Journal of oral & facial pain and headache*, 32(3), 287–297. https://doi.org/10.11607/ofph.2032

Zadik, Y., Arany, P. R., Fregnani, E. R., Bossi, P., Antunes, H. S., Bensadoun, R. J., … Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) (2019). Systematic review of photobiomodulation for the management of oral mucositis in cancer patients and clinical practice guidelines. *Supportive care in cancer: official journal of the Multinational Association of Supportive Care in Cancer*, 27(10), 3969–3983. https://doi.org/10.1007/s00520-019-04890-2

Amorim Dos Santos, J., Normando, A. G. C., de Toledo, I. P., Melo, G., De Luca Canto, G., Santos-Silva, A. R., & Guerra, E. N. S. (2020). Laser therapy for recurrent aphthous stomatitis: an overview. *Clinical oral investigations*, 24(1), 37–45. https://doi.org/10.1007/s00784-019-03144-z

Golež, A., Frangež, I., Cankar, K., Frangež, H. B., Ovsenik, M., & Nemeth, L. (2022). Effects of low-level light therapy on xerostomia related to hyposalivation: a systematic review and meta-analysis of clinical trials. *Lasers in medical science*, 37(2), 745–758. https://doi.org/10.1007/s10103-021-03392-0

Schalch, T. D., Fernandes, M. H., Destro Rodrigues, M. F. S., Guimarães, D. M., Nunes, F. D., Rodrigues, J. C., ... Mesquita Ferrari, R. A., Bussadori, S. K., & Fernandes, K. P. S. (2019). Photobiomodulation is associated with a decrease in cell viability and migration in oral squamous cell carcinoma. *Lasers in medical science*, 34(3), 629–636. https://doi.org/10.1007/s10103-018-2640-4

Silveira, F. M., Paglioni, M. P., Marques, M. M., Santos-Silva, A. R., Migliorati, C. A., Arany, P., & Martins, M. D. (2019). Examining tumor modulating effects of photobiomodulation therapy on head and neck squamous cell carcinomas. *Photochemical & photobiological sciences: Official journal of the European Photochemistry Association and the European Society for Photobiology*, 18(7), 1621–1637. https://doi.org/10.1039/c9pp00120d

Martins, M. D., Silveira, F. M., Webber, L. P., Wagner, V. P., Martins, M. A. T., Squarize, C. H., & Castilho, R. M. (2020). The impact of photobiomodulation therapy on the biology and behavior of head and neck squamous cell carcinomas cell lines. *Journal of photochemistry and photobiology. B, Biology*, 209, 111924. https://doi.org/10.1016/j.jphotobiol.2020.111924

Barasch, A., Raber-Durlacher, J., Epstein, J. B., & Carroll, J. (2016). Effects of pre-radiation exposure to LLLT of normal and malignant cells. *Supportive care in cancer: official journal of the Multinational Association of Supportive Care in Cancer*, 24(6), 2497–2501. https://doi.org/10.1007/s00520-015-3051-8

Silveira, F. M., Schmidt, T. R., Neumann, B., Rosset, C., Zanella, V. G., Maahs, G. S., … Martins, M. D. (2023). Impact of photobiomodulation in a patient-derived xenograft model of oral squamous cell carcinoma. *Oral diseases*, 29(2), 547–556. https://doi.org/10.1111/odi.13967

Antunes, H. S., Herchenhorn, D., Small, I. A., Araújo, C. M. M., Viégas, C. M. P., de Assis Ramos, G., Dias, F. L., & Ferreira, C. G. (2017). Long-term survival of a randomized phase III trial of head and neck cancer patients receiving concurrent chemoradiation therapy with or without low-level laser therapy (LLLT) to prevent oral mucositis. Oral oncology, 71, 11–15. https://doi.org/10.1016/j.oraloncology.2017.05.018

de Pauli Paglioni, M., Araújo, A. L. D., Arboleda, L. P. A., Palmier, N. R., Fonsêca, J. M., Gomes-Silva, W., ... Santos-Silva, A. R. (2019). Tumor safety and side effects of photobiomodulation therapy used for prevention and management of cancer treatment toxicities. A systematic review. *Oral oncology*, 93, 21–28. https://doi.org/10.1016/j.oraloncology.2019.04.004

Wu, S., Zhou, F., Wei, Y., Chen, W. R., Chen, Q., & Xing, D. (2014). Cancer phototherapy via selective photoinactivation of respiratory chain oxidase to trigger a fatal superoxide anion burst. *Antioxidants & redox signaling*, 20(5), 733–746. https://doi.org/10.1089/ars.2013.5229

Lu, C., Zhou, F., Wu, S., Liu, L., & Xing, D. (2016). Phototherapy-Induced Antitumor Immunity: Long-Term Tumor Suppression Effects via Photoinactivation of Respiratory

Chain Oxidase-Triggered Superoxide Anion Burst. *Antioxidants & redox signaling*, 24(5), 249–262. https://doi.org/10.1089/ars.2015.6334

de C Monteiro, J. S., Pinheiro, A. N., de Oliveira, S. C., Aciole, G. T., Sousa, J. A., Canguss, M. C., & Dos Santos, J. N. (2011). Influence of laser phototherapy (λ660 nm) on the outcome of oral chemical carcinogenesis on the hamster cheek pouch model: histological study. *Photomedicine and laser surgery*, 29(11), 741–745. https://doi.org/10.1089/pho.2010.2896

AlGhamdi, K. M., Kumar, A., & Moussa, N. A. (2012). Low-level laser therapy: a useful technique for enhancing the proliferation of various cultured cells. Lasers in medical science, 27(1), 237–249. https://doi.org/10.1007/s10103-011-0885-2

Hamblin, M. R., Nelson, S. T., & Strahan, J. R. (2018). Photobiomodulation and Cancer: What Is the Truth?. *Photomedicine and laser surgery*, 36(5), 241–245. https://doi.org/10.1089/pho.2017.4401

Wagner, V. P., Spuldaro, T. R., Nör, F., Gaio, E. J., Castilho, R. M., Carrard, V. C., & Rösing, C. K. (2021). Can propranolol act as a chemopreventive agent during oral carcinogenesis? An experimental animal study. *European journal of cancer prevention: the official journal of the European Cancer Prevention Organisation (ECP)*, 30(4), 315–321. https://doi.org/10.1097/CEJ.0000000000000626

Levchenko, S. M., Kuzmin, A. N., Pliss, A., Ohulchanskyy, T. Y., Prasad, P. N., & Qu, J. (2019). Cellular transformations in near-infrared light-induced apoptosis in cancer cells revealed by label-free CARS imaging. *Journal of biophotonics*, 12(12), e201900179. https://doi.org/10.1002/jbio.201900179

Dompe, C., Moncrieff, L., Matys, J., Grzech-Leśniak, K., Kocherova, I., Bryja, A., … Dyszkiewicz-Konwińska, M. (2020). Photobiomodulation-Underlying Mechanism and Clinical Applications. *Journal of clinical medicine*, 9(6), 1724. https://doi.org/10.3390/jcm9061724

Ottaviani, G., Martinelli, V., Rupel, K., Caronni, N., Naseem, A., Zandonà, L., ... Zacchigna, S. (2016). Laser Therapy Inhibits Tumor Growth in Mice by Promoting Immune Surveillance and Vessel Normalization. *EBioMedicine*, 11, 165–172. https://doi.org/10.1016/j.ebiom.2016.07.028

Chiang, M. H., Lee, K. T., Chen, C. H., Chen, K. K., & Wang, Y. H. (2020). Photobiomodulation therapy inhibits oral submucous fibrosis in mice. *Oral diseases*, 26(7), 1474–1482. https://doi.org/10.1111/odi.13409

Onaciu, A., Munteanu, R., Munteanu, V. C., Gulei, D., Raduly, L., Feder, R. I., … Berindan-Neagoe, I. (2020). Spontaneous and Induced Animal Models for Cancer Research. *Diagnostics (Basel, Switzerland)*, 10(9), 660. https://doi.org/10.3390/diagnostics10090660

Morris, A. L., Scott, D. B., & Reiskin, A. B. (1961). Carcinogenesis in the hamster cheek pouch. I. Correlation of histopathology with soluble sulfhydryl groups. Cancer research, 21, 1352–1359.

Sagheer, S. H., Whitaker-Menezes, D., Han, J. Y. S., Curry, J. M., Martinez-Outschoorn, U., & Philp, N. J. (2021). 4NQO induced carcinogenesis: A mouse model for oral squamous cell carcinoma. Methods in cell biology, 163, 93–111. https://doi.org/10.1016/bs.mcb.2021.01.001

Tinhofer, I., Braunholz, D., & Klinghammer, K. (2020). Preclinical models of head and neck squamous cell carcinoma for a basic understanding of cancer biology and its translation into efficient therapies. *Cancers of the head & neck*, 5, 9. https://doi.org/10.1186/s41199-020-00056-4

Zigmundo, G. C. O., Schuch, L. F., Schmidt, T. R., Silveira, F. M., Martins, M. A. T., Carrard, V. C., … Wagner, V. P. (2022). 4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis: A systematic literature review. *Pathology, research and practice*, 236, 153970. https://doi.org/10.1016/j.prp.2022.153970

Bensadoun, R. J., Epstein, J. B., Nair, R. G., Barasch, A., Raber-Durlacher, J. E., Migliorati, C., … World Association for Laser Therapy (WALT) (2020). Safety and efficacy of photobiomodulation therapy in oncology: A systematic review. *Cancer medicine*, 9(22), 8279–8300. https://doi.org/10.1002/cam4.3582

Bansal, R., Nayak, B. B., Bhardwaj, S., Vanajakshi, C. N., Das, P., Somayaji, N. S., & Sharma, S. (2020). Cancer stem cells and field cancerization of head and neck cancer - An update. *Journal of family medicine and primary care*, 9(7), 3178–3182. https://doi.org/10.4103/jfmpc.jfmpc_443_20

Dillenburg, C. S., Almeida, L. O., Martins, M. D., Squarize, C. H., & Castilho, R. M. (2014). Laser phototherapy triggers the production of reactive oxygen species in oral epithelial cells without inducing DNA damage. *Journal of biomedical optics*, 19(4), 048002. https://doi.org/10.1117/1.JBO.19.4.048002

Neculqueo, G. W., Estrázulas, M., Cherubini, K., Koth, V. S., & Salum, F. G. (2022). Laser photobiomodulation does not alter clinical and histological characteristics of 4-NQO-induced oral carcinomas and leukoplakia in mice. *Journal of photochemistry and photobiology*. *B, Biology*, 237, 112597. https://doi.org/10.1016/j.jphotobiol.2022.112597

3 DISCUSSÃO

A carcinogênese oral representa um processo multifatorial em que mutações se acumulam ao longo de um período variável até que se estabeleça a neoplasia maligna. Em comparação com as células normais, as células neoplásicas exibem uma gama de características, incluindo resistência a sinais de anti-crescimento, evasão de apoptose, autossuficiência em sinais de crescimento, potencial replicativo ilimitado, promoção de angiogênese, capacidade de invadir tecido e metástase, vias metabólicas alteradas e capacidade de evadir o sistema imunológico (Hanahan & Weinberg, 2000; Fouad & Aanei, 2017). Tais características têm a capacidade funcional de permitir que as células neoplásicas sobrevivam, proliferem e se espalhem além de sua localização inicial. Devido à frequência de diagnósticos de CEC, ao alto índice de mortalidade, ao caráter mutilador da terapia clássica e a inúmeras complicações, entender melhor como o processo carcinogênico se estabelece, bem como propor novos métodos de tratamento, são necessários. Nos últimos anos, terapias de luz têm sido introduzidas em diferentes áreas médicas, como tratamento alternativo ou adjuvante em casos de desordens potencialmente malignas e câncer ou como auxiliares no manejo das complicações agudas e crônicas frente ao tratamento antineoplásico. No entanto, ainda há dúvidas sobre sua segurança diante de um tecido com características neoplásicas.

Apesar de alguns estudos na literatura apontarem para o uso de TFD como terapia alternativa para o CEC ou desordem oral potencialmente maligna (DOPM) e a FBM como auxiliar no manejo de efeitos adversos, a segurança de ambas as técnicas ainda não é totalmente estabelecida. Diante disso, entender o que pode afetar o processo de carcinogênese se faz necessário. Nesse sentido, a presente tese foi construída com o objetivo de identificar se a TFD e a FBM atuam e/ou interferem na indução da proliferação e diferenciação celular durante o processo de carcinogênese oral quimicamente induzida em um modelo animal. Nossos achados publicados em nossa *Overview* revelam que, apesar dos efeitos adequados e seguros da TFD contra DOPM e CEC, seu suporte como tratamento de primeira linha ainda precisa de estudos de coorte mais bem desenhados (Schuch et al., 2023). A falta de consistência dos protocolos de TFD, bem como o número limitado de casos incluídos sem seguimento a longo prazo nos estudos primários são os principais motivos que impedem conclusões definitivas. Sugerimos, portanto, o desenvolvimento de protocolos padrão para agentes fotoativos, uma compreensão clara dos aspectos de

dosagem e a frequência de tratamentos para essas lesões. Recentemente Chamoli e colaboradores (2021) realizaram uma revisão sobre o CEC, e, dentre as formas de tratamento, citam a TFD, reforçando seu potencial na morte de células tumorais ao danificar a vasculatura tumoral e ativar respostas imunes contra células tumorais. Ainda, os autores indicam que uma combinação de TFD e inibidores de EGFR gerou uma proliferação reduzida de células cancerígenas, oferecendo uma estratégia de tratamento combinada. Diante disso, reforçamos a necessidade de estudos futuros que associem a TFD com outras modalidades, principalmente para casos criteriosamente selecionados, como, por exemplo, lesões inoperáveis ou com margens comprometidas pelo tumor sendo a TFD utilizada como forma de complementar o procedimento cirúrgico.

O segundo artigo desenvolvido na presente tese teve como objetivo revisar sistematicamente a literatura e construir um guia de boas práticas com sugestões para futuros trabalhos de 4NQO em ratos *Wistar*. Os pesquisadores que pretendem usar o modelo de 4NQO para testar drogas quimiopreventivas ou desvendar os mecanismos básicos da carcinogênese oral podem se beneficiar significativamente desta revisão para determinar o cálculo do tamanho da amostra, cuidados com a solução, intervalo de dose e recomendações sobre relatórios clínicos e histológicos. Além do mais, a partir dessa revisão sistemática, conseguimos traçar as melhores alternativas para o desenvolvimento da parte experimental do estudo, visando, além do entendimento sobre o impacto de fontes de luz no processo de carcinogênese oral, garantir o bem-estar animal ao longo do experimento (Zigmundo et al., 2022).

Da parte experimental da presente tese, surgiram três trabalhos. O primeiro foi baseado no que se aprendeu com a revisão sistemática sobre o 4NQO. Nele, pode-se sugerir um protocolo modificado que, além de assegurar o desenvolvimento da carcinogênse com uma dose menor de carcinógeno, garante o bem-estar animal – o que faz com que longos estudos sejam possíveis. Em relação ao sucesso da indução do carcinoma, mesmo com uma dose não tão agressiva de 4NQO (25 ppm), todos os animais apresentaram alterações morfológicas em 12 semanas, sendo que a maioria das lesões em 20 semanas eram de carcinomas. Mesmo com dois dias sem 4NQO, a taxa de sucesso - principalmente em relação ao desenvolvimento de carcinoma - foi maior do que a relatada em vários estudos que empregaram doses mais altas de carcinógenos (Ribeiro & Salvadori, 2007; Lim et al., 2014; Valente et al., 2018; Soares et al., 2018; Cecilio et al., 2020). Além disso, assume-se que os animais poderiam ser

mantidos saudáveis por algumas semanas a mais do que as 20 utilizadas no presente protocolo, o que sem dúvida resultaria em mais neoplasias malignas. Na pesquisa, observou-se um aumento clínico significativo de lesões nodulares/ulceradas durante a última semana do experimento.

Através da TFD, teve-se como objetivo avaliar como ela afeta o desenvolvimento da lesão durante a carcinogênese oral quimicamente induzida por 4NQO. Os resultados não demonstraram diferenças significativas entre os grupos controle e TFD em relação à aparência clínica e microscópica em 12 e 20 semanas. Sabe-se que a TFD vem sendo utilizada com finalidade terapêutica em vários tumores (Wang et al., 2015; Collier et al., 2018; Gondivkar et al., 2018; Unanyan et al., 2021; Ibarra et al., 2022). Vale salientar que o objetivo no presente estudo não foi avaliar eficácia de tratamento. Apesar disso, teve-se a expectativa de que ela pudesse diminuir ou até mesmo bloquear o processo de carcinogênese oral. Isso alerta a que, enquanto o tecido estiver sofrendo exposição ao carcinógeno, não se observa o efeito terapêutico da TFD. A partir disso, sugere-se para os próximos estudos a importância de ter um grupo de animais que receba o carcinógeno sem TFD e após o estabelecimento da lesão, seja removido o carcinógeno e utilizada a TFD como tratamento. Novos protocolos de TFD podem ser testados.

A FBM vem sendo muito investigada ao longo dos anos em termos de segurança. Nosso grupo de pesquisa demonstrou recentemente, através de um estudo em modelo animal que, de fato, não há efeitos da FBM nos parâmetros utilizados para prevenção de mucosite oral no comportamento do CEC (Silveira et al., 2021). No entanto, uma das preocupações em relação ao uso de FBM em pacientes com câncer de cabeça e pescoço refere-se ao campo de cancerização. Diante disso, a ideia deste estudo foi justamente verificar o impacto da FBM durante o processo de carcinogênese oral. Este modelo demonstrou que a ação da FBM não foi capaz de potencializar o desenvolvimento neoplásico, o que mostra sua segurança. Desta forma, verificamos que as células epiteliais submetidas ao desafio carcinogênico com 4NQO não respondem à FBM e adquirem o fenótipo maligno independentemente do uso desta forma de luz.

Assim sendo, podemos - dentro das limitações deste estudo - concluir que a TFD e FBM nos parâmetros testados não modificam o perfil da carcinogênese oral induzida por 4NQO. Seguindo a visão dos *hallmarks* do câncer, inferimos que essas terapias não modificaram a capacidade do 4NQO de sustentar a sinalização

proliferativa, evitar supressores de crescimento, resistir à morte celular, possibilitar a imortalidade replicativa e ativar a invasão tendo em vista que houve o mesmo padrão de desenvolvimento de tumores em todos os grupos (Hanahan, 2022). Ao traçar um paralelo com o tratamento antineoplásico com quimioterapia e radioterapia, por exemplo, vê-se que muitas vezes esses tratamentos agressivos não são capazes de conter o mecanismo próprio das células tumorais o que mostra, mais uma vez que, as células neoplásicas malignas adquirem características de imortalidade e de escape de tratamentos antineoplásicos vigentes.

4 CONCLUSÃO

A presente tese foi construída com o objetivo de investigar o efeito de terapias de luz atuando no processo de carcinogênese oral. Os resultados demonstram que tanto a TFD quanto a FBM não são capazes de frear o desenvolvimento tumoral, mas também não o exacerbam, não tendo impacto significativo na carcinogênese oral induzida por 4NQO. Apesar de reconhecer as limitações de um estudo pré-clínico, o presente trabalho fornece evidências sobre a segurança dos tecidos da cavidade oral expostos a terapias que utilizam luz laser.

REFERÊNCIAS*

Ali J, Sabiha B, Jan HU, Haider SA, Khan AA, Ali SS. Genetic etiology of oral cancer. Oral Oncol. 2017 Jul;70:23-28. doi: 10.1016/j.oraloncology.2017.05.004.

Barasch A, Raber-Durlacher J, Epstein JB, Carroll J. Effects of pre-radiation exposure to LLLT of normal and malignant cells. Support Care Cancer 2016;6:2497–501.

Cecilio HP, Valente VB, Pereira KM, Kayahara GM, Furuse C, Biasoli ÉR, Miyahara GI, Oliveira SHP, Bernabé DG. Beta-adrenergic blocker inhibits oral carcinogenesis and reduces tumor invasion. Cancer Chemother Pharmacol. 2020 Nov;86(5):681-686. doi: 10.1007/s00280-020-04149-2.

Centers for Disease Control and Prevention (US); National Center for Chronic Disease Prevention and Health Promotion (US); Office on Smoking and Health (US). How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta (GA): Centers for Disease Control and Prevention (US); 2010.

Chamoli A, Gosavi AS, Shirwadkar UP, Wangdale KV, Behera SK, Kurrey NK, Kalia K, Mandoli A. Overview of oral cavity squamous cell carcinoma: Risk factors,

* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

mechanisms, and diagnostics. Oral Oncol. 2021 Oct;121:105451. doi: 10.1016/j.oraloncology.2021.105451.

Chen Q, Dan H, Tang F, Wang J, Li X, Cheng J et al. Photodynamic therapy guidelines for the management of oral leucoplakia. Int J Oral Sci. 2019 Apr 11;11(2):14. doi: 10.1038/s41368-019-0047-0.

Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma--an update. CA Cancer J Clin. 2015 Sep-Oct;65(5):401-21. doi: 10.3322/caac.21293.

Chiang MH, Lee KT, Chen CH, Chen KK, Wang YH. Photobiomodulation therapy inhibits oral submucous fibrosis in mice. Oral Dis. 2020 Oct;26(7):1474-1482. doi: 10.1111/odi.13409.

Collier NJ, Haylett AK, Wong TH, Morton CA, Ibbotson SH, McKenna KE, Mallipeddi R, Moseley H, Seukeran D, Ward KA, Mohd Mustapa MF, Exton LS, Green AC, Rhodes LE. Conventional and combination topical photodynamic therapy for basal cell carcinoma: systematic review and meta-analysis. Br J Dermatol. 2018 Dec;179(6):1277-1296. doi: 10.1111/bjd.16838.

D'Cruz AK, Vaish R, Dhar H. Oral cancers: Current status. Oral Oncol. 2018 Dec;87:64-69. doi: 10.1016/j.oraloncology.2018.10.013.

de C Monteiro JS, Pinheiro AN, de Oliveira SC, Aciole GT, Sousa JA, Canguss MC, Dos Santos JN. Influence of laser phototherapy (λ660 nm) on the outcome of oral chemical carcinogenesis on the hamster cheek pouch model: histological study. Photomed Laser Surg. 2011 Nov;29(11):741-5. doi: 10.1089/pho.2010.2896.

de Freitas LF, Hamblin MR. Proposed Mechanisms of Photobiomodulation or Low-Level Light Therapy. IEEE J Sel Top Quantum Electron. 2016 May-Jun;22(3):7000417. doi: 10.1109/JSTQE.2016.2561201.

de Pauli Paglioni M, Araújo ALD, Arboleda LPA, Palmier NR, Fonsêca JM, Gomes-Silva W et al. Tumor safety and side effects of photobiomodulation therapy used for prevention and management of cancer treatment toxicities. A systematic review. Oral Oncol. 2019 Jun;93:21-28. doi: 10.1016/j.oraloncology.2019.04.004

Dompe C, Moncrieff L, Matys J, Grzech-Leśniak K, Kocherova I, Bryja A, Bruska M, Dominiak M, Mozdziak P, Skiba THI, Shibli JA, Angelova Volponi A, Kempisty B, Dyszkiewicz-Konwińska M. Photobiomodulation-Underlying Mechanism and Clinical Applications. J Clin Med. 2020 Jun 3;9(6):1724. doi: 10.3390/jcm9061724.

Farah CS, Jessri M, Bennett NC, Dalley AJ, Shearston KD, Fox SA. Exome sequencing of oral leukoplakia and oral squamous cell carcinoma implicates DNA damage repair gene defects in malignant transformation. Oral Oncol. 2019 Sep;96:42-50. doi: 10.1016/j.oraloncology.2019.07.005.

Fouad YA, Aanei C. Revisiting the hallmarks of cancer. Am J Cancer Res. 2017 May 1;7(5):1016-1036.

Frigo L, Luppi JS, Favero GM, Maria DA, Penna SC, Bjordal JM, Bensadoun RJ, Lopes-Martins RA. The effect of low-level laser irradiation (In-Ga-Al-AsP - 660 nm) on melanoma in vitro and in vivo. BMC Cancer. 2009 Nov 20;9:404. doi: 10.1186/1471-2407-9-404.

Gondivkar SM, Gadbail AR, Choudhary MG, Vedpathak PR, Likhitkar MS. Photodynamic treatment outcomes of potentially-malignant lesions and malignancies of the head and neck region: A systematic review. J Investig Clin Dent. 2018 Feb;9(1). doi: 10.1111/jicd.12270.

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57–70.

Hanahan D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 2022 Jan;12(1):31-46. doi: 10.1158/2159-8290.CD-21-1059. PMID: 35022204.

Hopper C. Photodynamic therapy: a clinical reality in the treatment of cancer. Lancet Oncol. 2000 Dec;1:212-9. doi: 10.1016/s1470-2045(00)00166-2.

Ibarra AMC, Cecatto RB, Motta LJ, Dos Santos Franco AL, de Fátima Teixeira da Silva D, Nunes FD, Hamblin MR, Rodrigues MFSD. Photodynamic therapy for squamous cell carcinoma of the head and neck: narrative review focusing on

photosensitizers. Lasers Med Sci. 2022 Apr;37(3):1441-1470. doi: 10.1007/s10103-021-03462-3.

INCA. Estimativa 2020: incidência de câncer no Brasil/Instituto Nacional de Câncer José Alencar Gomes da Silva. – Rio de Janeiro: INCA, 2019.

Kanojia D, Vaidya MM. 4-nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol. 2006 Aug;42(7):655-67. doi: 10.1016/j.oraloncology.2005.10.013.

Kumar S, Weaver VM. Mechanics, malignancy, and metastasis: the force journey of a tumor cell. Cancer Metastasis Rev. 2009 Jun;28(1-2):113-27. doi: 10.1007/s10555-008-9173-4.

Levchenko SM, Kuzmin AN, Pliss A, Ohulchanskyy TY, Prasad PN, Qu J. Cellular transformations in near-infrared light-induced apoptosis in cancer cells revealed by label-free CARS imaging. J Biophotonics. 2019 Dec;12(12):e201900179. doi: 10.1002/jbio.201900179

Lim W, Choi H, Kim J, Kim S, Jeon S, Ni K, Song SY, Oh HK, Im Y, Lee G, Lee JY, Moon YL, You JW, Kim O. Expression of cancer stem cell marker during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. J Mol Histol. 2014 Dec;45(6):653-63. doi: 10.1007/s10735-014-9584-1.

Miki K, Orita Y, Gion Y, Takao S, Ohno K, Takeuchi M, Ito T, Hanakawa H, Tachibana T, Marunaka H, Makino T, Minoura A, Matsukawa A, Nishizaki K, Yoshino T, Sato Y. Regulatory T cells function at the early stage of tumor progression in a mouse model of tongue squamous cell carcinoma. Cancer Immunol Immunother. 2016 Nov;65(11):1401-1410. doi: 10.1007/s00262-016-1902-x.

Olek M, Machorowska-Pieniążek A, Olek K, Cieślar G, Kawczyk-Krupka A. Photodynamic therapy in the treatment of oral squamous cell carcinoma - The state of the art in preclinical research on the animal model. Photodiagnosis Photodyn Ther. 2021 Jun;34:102236. doi: 10.1016/j.pdpdt.2021.102236.

Ottaviani G, Martinelli V, Rupel K, Caronni N, Naseem A, Zandonà L, Perinetti G, Gobbo M, Di Lenarda R, Bussani R, Benvenuti F, Giacca M, Biasotto M, Zacchigna S. Laser Therapy Inhibits Tumor Growth in Mice by Promoting Immune Surveillance and Vessel Normalization. EBioMedicine. 2016 Sep;11:165-172. doi: 10.1016/j.ebiom.2016.07.028.

Prates RA, Yamada AM, Suzuki LC, França CM, Cai S, Mayer MP, Ribeiro AC, Ribeiro MS. Histomorphometric and microbiological assessment of photodynamic therapy as an adjuvant treatment for periodontitis: a short-term evaluation of inflammatory periodontal conditions and bacterial reduction in a rat model. Photomed Laser Surg. 2011 Dec;29(12):835-44. doi: 10.1089/pho.2010.2984.

Prażmo EJ, Kwaśny M, Łapiński M, Mielczarek A. Photodynamic Therapy As a Promising Method Used in the Treatment of Oral Diseases. Adv Clin Exp Med. 2016 Jul-Aug;25(4):799-807. doi: 10.17219/acem/32488.

Ribeiro DA, Salvadori DM. Gingival changes in wistar rats after oral treatment with 4-nitroquinoline 1-oxide. Eur J Dent. 2007 Jul;1(3):152-7.

Rupel K, Zupin L, Colliva A, Kamada A, Poropat A, Ottaviani G et al. Photobiomodulation at Multiple Wavelengths Differentially Modulates Oxidative Stress In Vitro and In Vivo. Oxid Med Cell Longev. 2018 Nov 11;2018:6510159. doi: 10.1155/2018/6510159.

Sagheer SH, Whitaker-Menezes D, Han JYS, Curry JM, Martinez-Outschoorn U, Philp NJ. 4NQO induced carcinogenesis: A mouse model for oral squamous cell carcinoma. Methods Cell Biol. 2021;163:93-111. doi: 10.1016/bs.mcb.2021.01.001.

Sequeira I, Rashid M, Tomás IM, Williams MJ, Graham TA, Adams DJ, Vigilante A, Watt FM. Genomic landscape and clonal architecture of mouse oral squamous cell carcinomas dictate tumour ecology. Nat Commun. 2020;11(1):5671. doi: 10.1038/s41467-020-19401-9.

Schuch LF, Schmidt TR, Kirschnick LB, de Arruda JAA, Campagnol D, Martins MAT, Santos-Silva AR, Lopes MA, Vargas PA, Bagnato VS, Kurachi C, Guerra ENS, Martins MD. Revisiting the evidence of photodynamic therapy for oral potentially malignant disorders and oral squamous cell carcinoma: An overview of systematic

reviews. Photodiagnosis Photodyn Ther. 2023 Mar 22;42:103531. doi: 10.1016/j.pdpdt.2023.103531.

Sharma SK, Mroz P, Dai T, Huang YY, St Denis TG, Hamblin MR. Photodynamic Therapy for Cancer and for Infections: What Is the Difference? Isr J Chem. 2012 Sep;52(8-9):691-705. doi: 10.1002/ijch.201100062.

Silveira FM, Paglioni MP, Marques MM, Santos-Silva AR, Migliorati CA, Arany P, Martins MD. Examining tumor modulating effects of photobiomodulation therapy on head and neck squamous cell carcinomas. Photochem Photobiol Sci. 2019 Jul 10;18(7):1621-1637. doi: 10.1039/c9pp00120d.

Silveira FM, Schmidt TR, Neumann B, Rosset C, Zanella VG, Maahs GS, Martins MAT, Arany P, Wagner VP, Lopes MA, Santos-Silva AR, Martins MD. Impact of photobiomodulation in a patient-derived xenograft model of oral squamous cell carcinoma. Oral Dis. 2023 Mar;29(2):547-556. doi: 10.1111/odi.13967.

Soares GR, de Moura CFG, Silva MJD, Vilegas W, Santamarina AB, Pisani LP, Estadella D, Ribeiro DA. Protective effects of purple carrot extract (Daucus carota) against rat tongue carcinogenesis induced by 4-nitroquinoline 1-oxide. Med Oncol. 2018 Mar 15;35(4):54. doi: 10.1007/s12032-018-1114-7.

Tsai SR, Yin R, Huang YY, Sheu BC, Lee SC, Hamblin MR. Low-level light therapy potentiates NPe6-mediated photodynamic therapy in a human osteosarcoma cell line via increased ATP. Photodiagn Photodyn Ther 2015;12:123–30.

Unanyan A, Pivazyan L, Davydova J, Murvatova K, Khrapkova A, Movsisyan R, Ishchenko A, Ishchenko A. Efficacy of photodynamic therapy in women with HSIL, LSIL and early stage squamous cervical cancer: a systematic review and meta-analysis. Photodiagnosis Photodyn Ther. 2021 Dec;36:102530. doi: 10.1016/j.pdpdt.2021.102530.

Valente VB, Verza FA, Lopes FYK, Ferreira JZ, Dos Santos PSP, Sundefeld MLMM, Biasoli ÉR, Miyahara GI, Soubhia AMP, de Andrade M, de Oliveira SHP, Bernabé DG. Stress hormones concentrations in the normal microenvironment predict risk for chemically induced cancer in rats. Psychoneuroendocrinology. 2018 Mar;89:229-238. doi: 10.1016/j.psyneuen.2017.11.001.

van Straten D, Mashayekhi V, de Bruijn HS, Oliveira S, Robinson DJ. Oncologic Photodynamic Therapy: Basic Principles, Current Clinical Status and Future Directions. Cancers (Basel). 2017 Feb 18;9(2):19. doi: 10.3390/cancers9020019.

Wang H, Xu Y, Shi J, Gao X, Geng L. Photodynamic therapy in the treatment of basal cell carcinoma: a systematic review and meta-analysis. Photodermatol Photoimmunol Photomed. 2015 Jan;31(1):44-53. doi: 10.1111/phpp.12148.

Wang Y, Bai X, Wang Z, Cao J, Dong Y, Dong Y et al. Various LED Wavelengths Affected Myofiber Development and Satellite Cell Proliferation of Chick Embryos via the IGF-1 Signaling Pathway. Photochem Photobiol. 2017 Nov;93(6):1492-1501. doi: 10.1111/php.12806.

Zigmundo GCO, Schuch LF, Schmidt TR, Silveira FM, Martins MAT, Carrard VC, Martins MD, Wagner VP. 4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis: A systematic literature review. Pathol Res Pract. 2022 Aug;236:153970. doi: 10.1016/j.prp.2022.153970.

ANEXOS

Anexo 1 - Comprovante de publicação do Artigo 2.1

Photodiagnosis and Photodynamic Therapy 42 (2023) 103531

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Revisiting the evidence of photodynamic therapy for oral potentially malignant disorders and oral squamous cell carcinoma: An overview of systematic reviews

Lauren Frenzel Schuch [a], Tuany Rafaeli Schmidt [b], Laura Borges Kirschnick [a], José Alcides Almeida de Arruda [c], Daniela Campagnol [d], Marco Antônio Trevizani Martins [e,i], Alan Roger Santos-Silva [a], Márcio Ajudarte Lopes [a], Pablo Agustin Vargas [f], Vanderlei Salvador Bagnato [g], Cristina Kurachi [g], Eliete Neves Silva Guerra [h], Manoela Domingues Martins [i,j,*]

[a] Oral Diagnosis Departament, Piracicaba Dental School, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil
[b] Departament of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[c] Departament of Oral Surgery, Pathology and Clinical Dentistry, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[d] Experimental Research Center, Hospital de Clínicas de Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil
[e] Department of Oral Medicine, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[f] Oral Diagnosis Departament, Piracicaba School of Dentistry, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil
[g] Department of Physics and Materials Science, São Carlos Institute of Physics, Universidade de São Paulo, São Carlos, São Paulo, Brazil
[h] Laboratory of Oral Histopathology, Health Sciences Faculty, Universidade de Brasília, Brasília, Distrito Federal, Brazil
[i] Department of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[j] Department of Oral Diagnosis, Piracicaba School of Dentistry, Universidade Estadual de Campinas, Piracicaba, São Paulo, Brazil

ARTICLE INFO

Keywords:
Oral cancer
Oral oncology
Oral potentially malignant disorder
Oral squamous cell carcinoma
Photodynamic therapy
Photosensitizing

ABSTRACT

Background: This study summarized the available evidence about the use of photodynamic therapy (PDT) for the management of oral potentially malignant disorders (OPMD) and oral squamous cell carcinoma (OSCC).
Methods: An overview of systematic reviews was undertaken based on the 2020 PRISMA statement. Electronic searches were performed in five databases. Studies published up to November 2022 were included. Risk of bias was assessed with the AMSTAR 2 tool.
Results: A total of 30 studies enrolling 9,245 individuals with OPMD ($n = 7,487$) or OSCC ($n = 1,758$) met the selection criteria. All studies examined the efficacy and/or safety of PDT. OPMD were investigated individually in 82.8% of the studies, the most common being oral lichen planus and actinic cheilitis. OSCC was addressed separately in 10.3% of the studies, while only 6.9% evaluated both OPMD and OSCC. Fourteen different types of photosensitizers were described. PDT was used according to the following setting parameters: 417–670 nm, 10–500 mW/cm^2, 1.5–200 J/cm^2, and 0.5–143 min. Regarding OPMD, leukoerythroplakia showed the best response rates, while oral lichen planus presented a partial or no response in nearly 75% of documented cases. A complete response was observed in 85.9% of OSCC cases, while 14.1% had no resolution.
Conclusion: Overall, the response to PDT depended on the type of OPMD/OSCC and the parameters used. Although PDT is an emerging candidate for the treatment of OPMD and OSCC, there is heterogeneity of the methodologies used and the clinical data obtained, particularly regarding the follow-up period.

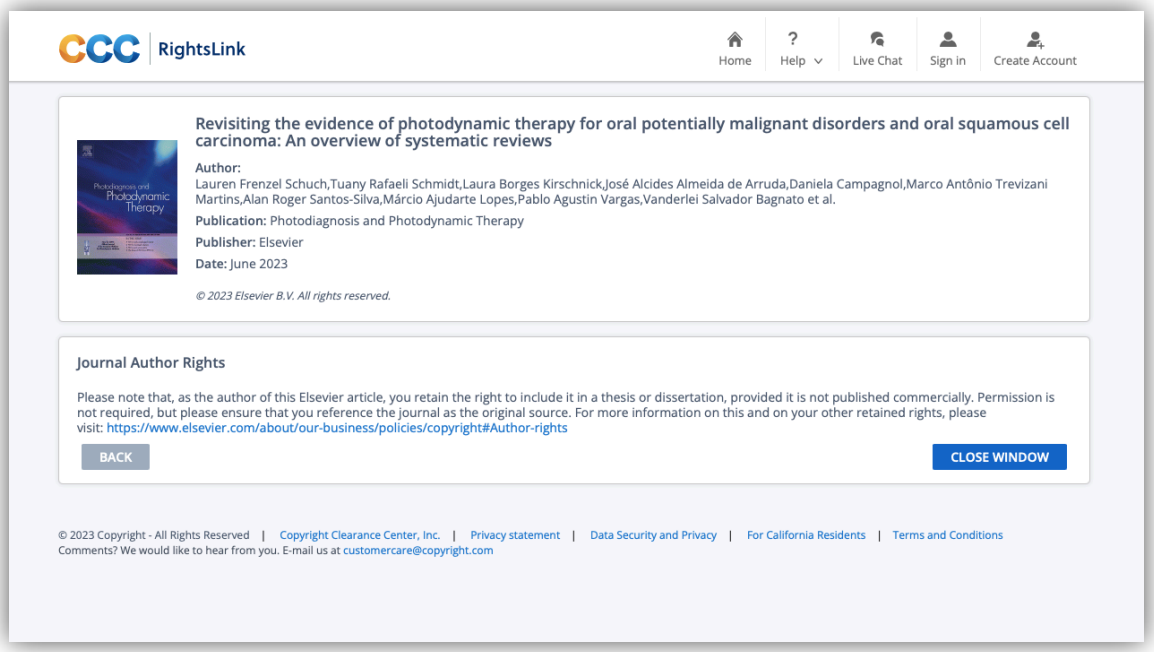
1. Introduction

Almost 90% of oral cavity cancers are squamous cell carcinomas (SCC), with a global incidence estimated at 377,713 new cases, with 177,757 deaths in 2020 [1]. The development of oral SCC (OSCC) may be preceded by a significant subset of mucosal disorders recognized as

* Corresponding author at: Universidade Federal do Rio Grande do Sul, Faculdade de Odontologia, R. Ramiro Barcelos, 2492, room 503. Porto Alegre, Rio Grande do Sul, Brazil.
E-mail address: manoela.martins@ufrgs.br (M.D. Martins).

https://doi.org/10.1016/j.pdpdt.2023.103531
Received 21 December 2022; Received in revised form 15 March 2023; Accepted 21 March 2023
Available online 22 March 2023

Anexo 2 – Autorização da Editora para a utilização do Artigo 2.1



CCC RightsLink

Home | Help | Live Chat | Sign in | Create Account

Revisiting the evidence of photodynamic therapy for oral potentially malignant disorders and oral squamous cell carcinoma: An overview of systematic reviews

Author:
Lauren Frenzel Schuch,Tuany Rafaeli Schmidt,Laura Borges Kirschnick,José Alcides Almeida de Arruda,Daniela Campagnol,Marco Antônio Trevizani Martins,Alan Roger Santos-Silva,Márcio Ajudarte Lopes,Pablo Agustin Vargas,Vanderlei Salvador Bagnato et al.

Publication: Photodiagnosis and Photodynamic Therapy

Publisher: Elsevier

Date: June 2023

© 2023 Elsevier B.V. All rights reserved.

Journal Author Rights

Please note that, as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source. For more information on this and on your other retained rights, please visit: https://www.elsevier.com/about/our-business/policies/copyright#Author-rights

BACK CLOSE WINDOW

© 2023 Copyright - All Rights Reserved | Copyright Clearance Center, Inc. | Privacy statement | Data Security and Privacy | For California Residents | Terms and Conditions
Comments? We would like to hear from you. E-mail us at customercare@copyright.com

Anexo 3 – Comprovante de publicação do Artigo 2.2

Pathology - Research and Practice 236 (2022) 153970

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Review

4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis: A systematic literature review

Gisele Corrêa de Oliveira Zigmundo [a], Lauren Frenzel Schuch [b], Tuany Rafaeli Schmidt [a], Felipe Martins Silveira [a,c], Marco Antônio Trevizani Martins [a], Vinicius Coelho Carrard [a], Manoela Domingues Martins [a,b], Vivian Petersen Wagner [d,*,1,2]

[a] Department of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Department of Oral Diagnosis, Piracicaba Dental School, Universidade de Campinas, Piracicaba, SP, Brazil
[c] Molecular Pathology Area, School of Dentistry, Universidad de la República, Montevideo, Uruguay
[d] Academic Unit of Oral and Maxillofacial Medicine and Pathology, Department of Clinical Dentistry, University of Sheffield, Sheffield, UK

ARTICLE INFO

Keywords:
Head and neck neoplasms
Oral cancer
Chemoprevention
Animal models
Prognosis
Precancerous conditions

ABSTRACT

Objective: Based on a critical review of published studies, we aimed to develop a good practice guide for using 4-nitroquinoline-1-oxide (4NQO) as an inducer of oral carcinogenesis in Wistar rats.
Design: A systematic search was performed on Medline Ovid, PubMed, Embase, Web of Science, and Scopus databases. The SYRCLE's risk of bias tool was used to assess the quality of the studies.
Results: Thirty-five articles met the selection criteria; 22 (62.9%) of them administered 4NQO systemically in drinking water, with a mean concentration of 30.2 ppm (SD±15.9) and during a mean period of 20.8 (SD±7.8) weeks. The other 13 (37.1%) studies performed topical applications of 4NQO painting the oral mucosa of the animals three times a week (100%) with a mean period of administration of 16.8 (SD±7.0) weeks. Different 4NQO concentrations used for other periods achieved significant tumor development. Most studies didn't perform quantitative clinical analysis, and the histopathological diagnosis/grading criteria varied considerably.
Conclusions: A poor description of solution care, adverse effects, and the number of losses were observed, and the reporting of these features needs to be improved. Suggestions to guide the development of future research are provided.

1. Introduction

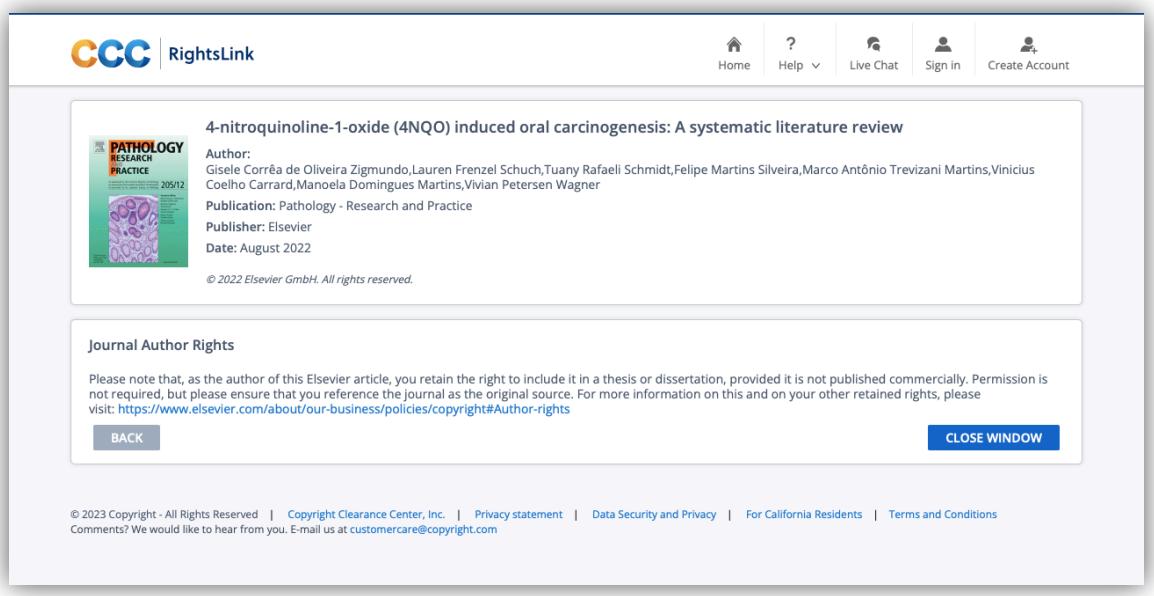
Oral cancer ranks the sixth most prevalent in the global cancer incidence ranking [1,2]. Smoking, alcohol consumption, and sun exposure are reported as the main risk factors for lip and oral squamous cell carcinoma (OSCC) [3,4]. In addition, the habit's frequency and duration seem to be related to greater chances of developing tumors [5]. This fact also extends to the risk of relapse, the persistence of the disease, and the appearance of a second primary neoplasm, if maintaining habits after completing the treatment [6,7]. During oral carcinogenesis, potentially malignant oral disorders (OPMD) can arise [8]. Monitoring this type of lesion is recommended to allow an early cancer diagnosis [8,9]. Some features, such as epithelial dysplasia (ED), are recognized as poor prognostic markers for malignant transformation [10]. However, the process of oral carcinogenesis remains fully understood [11]. Also, a deeper understanding of this process can bring benefits related to the chemoprevention [12]. Heavy smokers and patients diagnosed with ED could benefit from chemopreventive measures if available [13]. Therefore, molecular biology involved in carcinogenesis is vital, and experimental animal research emerges as a valuable resource in this scenario [3,14].

4-nitroquinoline-1-oxide (4NQO) is a mutagenic substance capable of generating changes in DNA that mimic tobacco-induced molecular events and can be used for experimental oral carcinogenesis development [15]. Once 4NQO is applied topically or systemically, an intense intracellular oxidative stress is generated, and reactive oxygen species (ROS), responsible for initiating the oral carcinogenesis process, are produced [15,16]. This model reproduces the different stages of tumor

* Correspondence to: Academic Unit of Oral and Maxillofacial Medicine and Pathology, 19 Claremont Cres, Broomhall, Sheffield S10 2TA, UK
E-mail address: v.wagner@sheffield.ac.uk (V.P. Wagner).
[1] ORCID: 0000-0002-1447-2135
[2] Twitter handle: @petersen_wagner

https://doi.org/10.1016/j.prp.2022.153970
Received 18 March 2022; Received in revised form 31 May 2022; Accepted 5 June 2022
Available online 10 June 2022
0344-0338/

Anexo 4 – Autorização da Editora para a utilização do Artigo 2.2



CCC RightsLink

Home | Help | Live Chat | Sign in | Create Account

4-nitroquinoline-1-oxide (4NQO) induced oral carcinogenesis: A systematic literature review

Author:
Gisele Corrêa de Oliveira Zigmundo,Lauren Frenzel Schuch,Tuany Rafaeli Schmidt,Felipe Martins Silveira,Marco Antônio Trevizani Martins,Vinicius Coelho Carrard,Manoela Domingues Martins,Vivian Petersen Wagner

Publication: Pathology - Research and Practice

Publisher: Elsevier

Date: August 2022

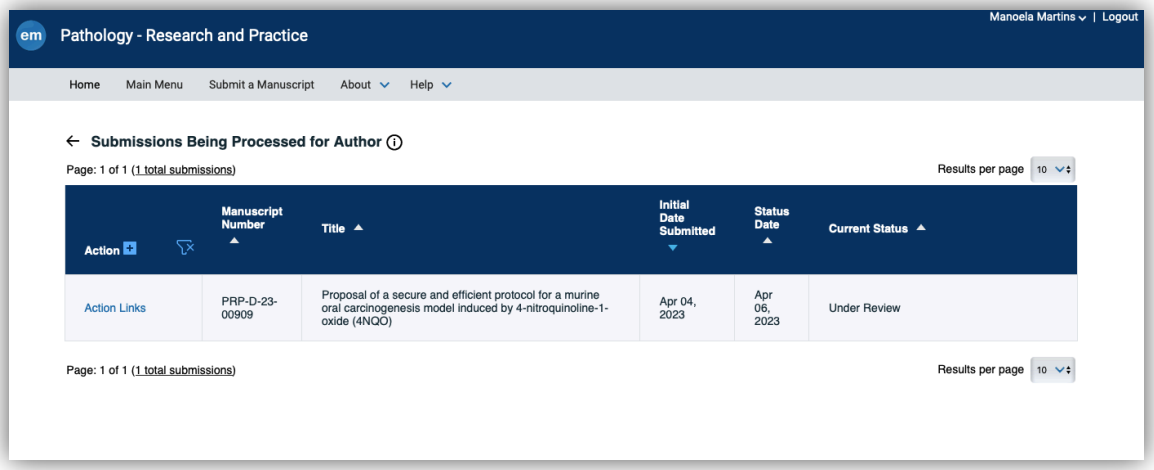
© 2022 Elsevier GmbH. All rights reserved.

Journal Author Rights

Please note that, as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source. For more information on this and on your other retained rights, please visit: https://www.elsevier.com/about/our-business/policies/copyright#Author-rights

BACK | CLOSE WINDOW

Anexo 5 – Comprovante de submissão do Artigo 2.3



Pathology - Research and Practice

Manoela Martins | Logout

Home Main Menu Submit a Manuscript About Help

Submissions Being Processed for Author

Page: 1 of 1 (1 total submissions) Results per page 10

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
Action Links	PRP-D-23-00909	Proposal of a secure and efficient protocol for a murine oral carcinogenesis model induced by 4-nitroquinoline-1-oxide (4NQO)	Apr 04, 2023	Apr 06, 2023	Under Review

Page: 1 of 1 (1 total submissions) Results per page 10

Anexo 6 - Certificado de aprovação do Comitê de Ética em Pesquisa (Hospital de Clínicas de Porto Alegre)

UFRGS
UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

HOSPITAL DE CLÍNICAS
PORTO ALEGRE RS

HOSPITAL DE CLÍNICAS DE PORTO ALEGRE

Grupo de Pesquisa e Pós Graduação

Carta de Aprovação

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), 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 de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 2021/0614

Título: IMPACTO DA FOTOBIOMODULAÇÃO E DA TERAPIA FOTODINÂMICA NO PROCESSO DE CARCINOGÊNSE ORAL QUIMICAMENTE INDUZIDA POR 4NQO (4-NITROQUINOLINA 1-ÓXIDO) EM MUCOSA LINGUAL DE RATOS WISTAR

Pesquisador Responsável: **MARCO ANTONIO TREVIZANI MARTINS**

Equipe de Pesquisa:

MANOELA DOMINGUES MARTINS LAUREN FRENZEL SCHUCH DANIELA CAMPAGNOL

Data de Aprovação: 09/02/2022

Data de Término: 01/12/2022

Espécie/Linhagem	Sexo/Idade	Quantidade	Data Reunião	Documento
RATO HETEROGÊNICO	M/8 Semana(s)	88	25/01/2022	Projeto

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

Assinado digitalmente por:
PATRICIA ASHTON PROLLA
Grupo de Pesquisa e Pós-graduação
14/02/2022 13:47:36

https://aghuse-externo.hcpa.edu.br/pesquisa/publico/cadastroapoio/conferenciaArquivo.xhtml?codarq=1960617

Impresso do sistema AGHUse-Pesquisa por CRISTIAN FIDALGO CABRAL em 09/02/2022 17:05:22