UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Lílian Mendes Borburema Cangussu

Potencial antineoplásico do ácido gálico em camundongos submetidos à exposição ao carcinógeno 4NQO e da nanopartícula AlClPc em células Scc9: duas novas propostas.

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Exame de Qualificação apresentado ao Programa de Pós-graduação em Ciências em Saúde da Universidade Estadual de Montes Claros-Unimontes, como parte das exigências para a obtenção do título de Doutora em Ciências da Saúde.

Área de Concentração:

Orientador: Prof. Dr. André Luiz Sena Guimarães

Coorientadora: Profa. Dra. Ludmilla Regina de Souza

Montes Claros
2019

FICHA CATALOGRÁFICA

Cangussu, Lílian Mendes Borburema.
C212p Potencial antineoplásico do ácido gálico em camundongos submetidos à exposição ao carcinógeno 4NQO e da nanopartícula AICIPc em células Scc9 [manuscrito] : duas novas propostas. / Lílian Mendes Borburema Cangussu. – 2019.
71 f. : il.

Inclui Bibliografia.
Tese (Doutorado) - Universidade Estadual de Montes Claros - Unimontes,

Programa de Pós-Graduação em Ciências da Saúde /PPGCS, 2019.

Orientador: Prof. Dr. André Luiz Sena Guimarães.
Coorientadora: Profa. Dra. Ludmilla Regina de Souza.

1. Terapia fotodinâmica. 2. Carcinógeno 4NQO. 3. Ação antineoplásica. I. Guimarães, André Luiz Sena. II. Souza, Ludmilla Regina de. III. Universidade Estadual de Montes Claros. IV. Título. V. Título: Duas novas propostas.

Catalogação Biblioteca Central Professor Antônio Jorge

CONSAGRAÇÃO

A Deus,

Nossa Senhora Aparecida,

São Padre Pio e

São Miguel Arcanjo,

Consagro.

DEDICATÓRIA

A Tia Paula

Laís, Tia Dora e

Ivo,

Dedico.

AGRADECIMENTOS

Agradeço a Deus, fonte de toda força, alegria e esperança em todos estes dias. Por colocar pessoas tão, tão especiais em meu caminho me fazendo lembrar que não estava só. O Senhor sempre esteve comigo! Recebe, Senhor, Tu que és Doutor dos doutores, mais este título! Porque “dEle, por Ele, e para Ele são todas as coisas”. Foi por Ti que cheguei até aqui. Fez-se a Tua vontade!

A Nossa Senhora Aparecida. Mãe, agradeço a Senhora pela doce companhia, sempre nos momentos mais difíceis e dolorosos pude, com a Senhora contar! Obrigada!

A Ivo, meu incentivo enviado por Deus na primeira vez que pensei em desistir, e em todas as outras, pois me lembrava de suas palavras. Muito obrigada, meu irmão!

A tia Paula, Laís, tia Dora e tia Vi, por terem sido mães, o colo necessário e a força de Deus manifestada fisicamente... Nunca terei palavras nem atitudes suficientes para lhes agradecer! Obrigada por cada palavra, por cada alimento espiritual e físico que não me deixaram faltar... Jamais me esquecerei que a minha dor, foi também a dor de vocês. Este título é nosso!

A Clariani que com tanta preocupação aliviava meus medos e dores e sempre me amparava com esperança. Obrigada, minha lindeza! Este título também é seu!

A Marcela, que sendo um ser humano único, ímpar, soube me fazer melhor e soube ainda me fazer ver que eu era capaz. Que todas as vezes que tentei “arremessar a bola” e não consegui, ela veio e me elevou o quanto foi preciso, em seus braços, pra que eu conseguisse. E conseguimos, Má! Conta comigo pra sempre!

A Marileide. O que teria sido de mim se não fosse você? Você aliviou as minhas dores, se doou, me ouviu e me aliviou. Lutou comigo pra que tudo desse certo. Enfim Mari, acabou! Deus te retribua cada atraso por minha culpa, cada gota de força dispensada para aliviar minhas dores. Eu amo você!

A minha madrinha, Diana, presente de Deus, que lutou comigo em oração, me encorajou e não desistiu! Muito obrigada, Dinda! És doutora também!

Aos meus irmãos do “*Terço da Divina Misericórdia Cantado da Matriz*” por cada oração. Cada palavra por vocês direcionada ao céu na tentativa de amenizar minhas dores, me alcançaram, tenham certeza! Eu não teria conseguido se não fosse vocês!

A João e Kevin, meus pocinhos de sorriso e amor. Que cada vez que corriam em minha direção sorrindo, e me abraçavam, faziam com que me sentisse mais forte e capaz de chegar até o fim. Chegamos! Obrigada meus, bebês! Eu amo vocês!
A Aninha, Bibi, Vozinha e Alex, por dividirem comigo o maior tesouro que vocês têm: tia Paula! Por me aguentarem em tantas adversidades e mesmo assim acreditarem, muitas vezes até mais que eu, que seria possível. Muito obrigada!

Aos meus amigos: Maircon, Deia, Quequel, Dinda, Kênia, Werner, Ledinha, Anielle, e todos os outros que me ouviram, tiveram paciência com as minhas ausências, rezaram e torceram por mim. A vida é mais leve porque tenho vocês!

As minhas mães: Zuíla, Tia Vi e Jô. E irmãos: Bárbara e Ícaro (meu irmão e IC), por aguentarem comigo o tranco, o cansaço e o medo de não conseguir. Amo vocês!

Aos meus pais e minha família por, mesmo sem compreenderem, através de cada palavra boa ou ruim, me impulsionarem a ser e fazer o melhor que eu conseguisse.

Aos meus colegas, especialmente Dani, Tavo, Lucas, Misa, Amandinha, Rogério, Dan, Eloá, João, Carlos Rafael, Cláudio Marcelo e Sabrina que, pacientemente respeitaram meus limites e me ajudaram, me socorreram em muitos momentos de temor e dor. Eu sou melhor porque tenho vocês! Muito obrigada!

Aos funcionários do HUCF, em especial a Valdi, Luciano, Jaque, Paula, João, Irlândia, Zezé, Luciana, Inês e Seu Tirso, que me recebiam, invariavelmente com um sorriso. De dia, de noite, de madrugada, sábados, domingos, feriados, natais, réveillons, aniversários. Se este hospital me acolheu, foi através dos abraços e sorrisos de vocês. Muito obrigada, Deus os abençoe!

A Du Carmo, que sempre, desde o início, me acolheu e pacientemente me ensinou. Foi além, se tornou amiga, confidente. Foi conselheira, intercessora. Obrigada por tudo, Du! Deus lhe retribua todo carinho!

A Dr. Wesley, amigo que a Biologia me deu, que, sendo plantonista providencialmente em todas as hospitalizações necessárias, me acolheu e incentivou, cuidou de mim com o zelo que só encontramos em profissionais raros, me ouviu e socorreu sem reservas. Amigo, estou aqui pro que precisar! Sempre! Muito obrigada!

A padre Sérgio que ali mesmo pelos corredores das enfermarias, me percebeu, acolheu, acreditou em mim, se fez pai, me ouviu, me atendeu, absolveu e instruiu. Deus lhe fortaleça sempre para que não desista nunca dos que, como eu, Deus confiou ao senhor. Muito obrigada por todas as conversas e confissões, mesmo sem espaço ou tempo, por me fazer entender que era importante e fonte de toda força, a

minha comunhão com Deus. Obrigada pelo sim que destes a Ele e por ser o cuidado dEle a me cuidar!

A minha co-orientadora, Lud, que lutou comigo. Mesmo grávida se propôs a ir tão longe para me ajudar. Obrigada por fazer tudo parecer mais leve e mais fácil do que é e por ser a doçura que falta em André. Você foi esperança em dias absolutamente nublados, compreensão nos momentos de dor, alegria nos momentos de temor e leveza quando tudo pesava demais. Deus lhe pague por tudo! Obrigada por se fazer "gente como a gente" e, com tanta humildade, acessar nossas dúvidas e saná-las com delicadeza. Cada vez que via você indo tomar café com a gente, com tanta simplicidade, repetia pra mim: eu quero ser assim! Ser um orientador acessível muda tudo e, principalmente, nosso jeito de olhar para a Ciência. Conta sempre comigo!

Ao meu orientador, André, que me ensinou a não guardar mágoa de ninguém e principalmente a colocar pra fora o que penso, sem medo. Este foi o maior ensinamento que deixou pra mim. Obrigada por pensar que eu era capaz de suportar cada vez mais. Talvez eu seja mesmo, só não descobri ainda. Deus te abençoe por compreender meus momentos de dor e tristeza, por respeitar meus limites físicos e psiquiátricos quando chegaram ao fim e por ter consideração nos piores momentos. Deus te abençoe muito e sempre!
A Universidade de Brasília e aos colaboradores do ICB-UnB por contribuírem valiosamente com a minha formação, me acolherem no campus, abrirem para mim as portas de seus laboratórios e me ensinarem tanto sobre Nanotecnologia, obrigada!

A Universidade Estadual de Montes Claros pela oportunidade de concluir um doutorado em pleno semiárido mineiro. Por se fazer uma "Universidade Sertaneja" e acolher os filhos desta terra. Por me acolher em formação por 13 anos, muito obrigada!

Aos meus ICs, especialmente Amanda, Lorena e Lincoln por terem tido paciência comigo, com minhas exigências e por me ensinarem também, obrigada!

A Bel, Lê, Anna Carolina, Ellen, Hugo, Daniel, Zaca, Carlos, Leandro e a todos que rezaram por mim, obrigada! Tudo teria sido muito mais difícil se não fosse vocês! Deus os recompense!

A todos e a cada um que passou por mim nestes anos todos e me alegraram, me ajudaram, me deram esperança, amor, café... Amo e respeito cada um pelo valor que têm em meus dias e por me ensinarem tanto.

Aos que contribuíram à sua maneira, agradável ou não, tudo é combustível. Muito obrigada!

“Se hoje enxergo longe, é porque fui colocada sobre os ombros de gigantes”

Isaac Newton

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UNIVERSIDADE ESTADUAL DE MONTES CLAROS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Unimontes

CANDIDATA: LILIAN MENDES BORBUREMA CANGUSSU

TÍTULO DO TRABALHO: "Potencial Antineoplásico do Ácido Gálico e da Nanopartícula Alumínio Cloro Ftalocianina: duas novas propostas".

ÁREA DE CONCENTRAÇÃO: Mecanismos e Aspectos Clínicos das Doenças

LINHA DE PESQUISA: Clínica, Diagnóstico e Terapêutica das Doenças

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[X] **APROVADO(A)** [ ] **REPROVADO(A)**

RESUMO

O câncer bucal causa morbidade e mortalidade significativas. Em todo o mundo, o carcinoma de células escamosas orais (CCEO) é o sexto câncer mais comum. As modalidades de tratamento atualmente disponíveis incluem cirurgia, radioterapia, quimioterapia e estratégias de prevenção, incluindo mudanças de estilo de vida, bem como quimioprevenção. A Terapia Fotodinâmica (TFD), por provocar a morte de células neoplásicas a partir da união da ação de um fotossensibilizante e de luz em comprimento de onda adequado, tem sido uma alternativa à quimioterapia e à radioterapia para o tratamento clínico do câncer devido aos seus efeitos locais que tornam o tratamento menos agressivo para o paciente. Este trabalho apresenta duas novas propostas para o tratamento de CCEO: um fotossensibilizante (AlClPc) como alternativa aos já existentes, de eficácias reduzidas e o Ácido Gálico, que por seus efeitos antioxidantes e anti-inflamatórios tem demonstrado possuir ação antineoplásica, o que o torna um agente promissor no tratamento de OSCC. A TFD foi realizada *in vitro* em células Scc9 nas quais foram realizados ensaios para avaliar o fenótipo celular após o tratamento com AlClPc. O Ácido Gálico foi administrado via gavagem em animais submetidos ao tratamento com o carcinógeno 4nqo. Os animais foram pesados diariamente para determinação da dose do AG introduzida por via oral até a morte dos animais. A TFD provocou morte celular considerável ($p<0,05$) e o Ácido Gálico amenizou a gravidade das lesões instaladas nas línguas dos animais. Os resultados deste trabalho apresentam recursos que podem ser ponto de partida para novas alternativas para o tratamento do câncer bucal.

Palavras-chave: Terapia Fotodinâmica. 4nqo. Ação antineoplásica.

ABSTRACT

Oral cancer causes significant morbidity and mortality. Around the world, squamous cell carcinoma (OSCC) is the sixth most common cancer. Treatment modalities are available in surgery, radiotherapy, chemotherapy and prevention strategies, including lifestyle changes as well as chemoprevention. Photodynamic Therapy (PDT), due to the death of neoplastic cells, has been the target of photosensitization and light in the proper wave direction. It has been an alternative to chemotherapy and radiotherapy for the clinical treatment of cancer makes the treatment less aggressive for the patient. This work presented the new measures for the treatment of OSCC: a photosensitizer (AlClPc) as once existing, of reduced sessions and the Gallic Acid, which by its own antioxidants and anti-inflammatory effects has a more effective antineoplastic action, the which makes it a promising agent in the treatment of OSCC. PDT was performed in vitro on Scc9 cells in which assays were performed to evaluate the cellular phenotype after treatment with AlClPc. Gallic acid was administered gavage in animals after treatment with the carcinogen 4nqo. The animals were weighed daily for GA oral dosing until the animals died. A PDT caused cell death ($p < 0.05$) and Gallic acid attenuated lesions in the animals' tongues. The results of this work may be the starting point for new alternatives for the treatment of oral cancer.

Keywords: Photodynamic Therapy. 4nqo. Antineoplastic action.

SUMÁRIO

1 INTRODUÇÃO

De acordo com o Instituto Nacional do Câncer (1), a estimativa 2018-2019 para Carcinoma Epidermoide de Boca (CEB) é de 14.700 novos casos, sendo 11.200 homens e 3.500 mulheres. No mundo em 2018, os novos casos de câncer da cavidade oral alcançaram 354,864 e o número de óbitos 177,384 (2).

As Diretrizes da Política Nacional de Saúde Bucal (3) orientam que deve-se estabelecer parcerias para a prevenção, diagnóstico, tratamento e recuperação do câncer bucal com Universidades e outras organizações.

O Caderno de Atenção Básica do Ministério da Saúde (4) pressupõe que o tratamento cirúrgico e radioterápico deverá ser feito em nível de média e alta complexidade e recomenda que, a reabilitação deve permear a reposição de perdas estéticas e funcionais causadas pela doença.

Mesmo com procedimentos de reconstrução cirúrgica, uma das consequências após os tratamentos é a ocorrência de algum grau de desfiguração facial, promovendo graves defeitos estéticos e funcionais (5).

Em função da heterogeneidade das mudanças genéticas, o CEB é uma neoplasia de difícil tratamento. Tendo em vista as desvantagens apresentadas, faz-se necessário o desenvolvimento de novas alternativas que possibilitem maior segurança e efetividade terapêutica no processo de tratamento de indivíduos acometidos pela doença. Considerando as possibilidades terapêuticas atuais para o tratamento das neoplasias bucais, estudos têm demonstrado a utilização de terapias alternativas no controle da doença (6-10). A terapia fotodinâmica (TFD) é uma destas alternativas para o tratamento de lesões neoplásicas dos tecidos bucais (11, 12) e o Ácido Gálico diminuiu a resposta inflamatória em células neoplásicas (13) além de possuir uma variedade de atividades farmacológicas, como atividades antioxidantes e anticancerígenas em estudos pré-clínicos (14).

A Nanopartícula Alumínio Cloro Ftalocianina (AlClPc) é um fotossensibilizante quimicamente estável de segunda geração que possui propriedades fotofísicas que são adequadas para a realização de TFD como, altos rendimentos quânticos tripletos (formação de espécies reativas de oxigênio) (15). A (bio) química da TFD não pode ser separada das suas propriedades físicas, já que o efeito biológico/clínico é resultado das interações quantitativas das duas (16). Embora

AlClPc tenha alto peso molecular, sua natureza hidrofóbica facilita a interação com as camadas bilipídicas das células (15) tornando-a ideal para a realização da TFD.

A fonte de luz utilizada neste trabalho foi o diodo emissor de luz (LED), com comprimento de onda de 660nm. O aparelho permitiu a realização do trabalho com potência de 47.2 mW/cm^2 e frequência: 28.35 Jcm^2. A morte celular é dependente da densidade de energia. Na menor densidade de energia, a apoptose foi predominante, enquanto na densidade de energia mais alta, a necrose foi o mecanismo predominante de morte celular (17).

A TFD é um procedimento terapêutico clinicamente aprovado, minimamente invasivo, que pode exercer uma atividade citotóxica seletiva para células malignas (18). É um procedimento de dois estágios: após a administração de uma substância (fotossensibilizante) sensível à luz, o tumor é irradiado com uma luz de comprimento de onda apropriado. A luz pode ser entregue a praticamente qualquer órgão do corpo por meio de dispositivos flexíveis de fibra óptica (18). Existem três mecanismos principais pelos quais a TFD medeia a destruição do tumor que podem influenciar-se mutuamente (19): 1. As espécies reativas de oxigênio geradas pela TFD podem matar células tumorais diretamente; 2. a TFD danifica a vasculatura associada ao tumor, levando-o a morte; 3. A TFD pode ativar uma resposta imune contra células tumorais (20).

Dados recentes (21) dão conta de que a AlClPc em baixas concentrações interage com poucos sítios de DNA causando uma curvatura na estrutura da fita dupla, o que fornece um ambiente favorável para a intercalação de agregados do fotossensibilizante. A combinação de todos esses componentes é necessária para o controle do tumor a longo prazo (20).

Uma vez que a droga fotossensibilizante (DF) absorve a luz, atingindo um estado excitado, pode emitir fluorescência ou fosforescência, pode reagir com diferentes moléculas circundantes, gerando radicais - fotorreação tipo 1 - ou pode catalisar a conversão de oxigênio tripleto ($^3O_2$) na fotorreação de oxigênio singlete ($^1O_2$) - tipo 2 (22).

O Ácido Gálico (AG), conhecido como ácido 3, 4, 5-tri-hidroxibenzóico, está presente amplamente em diferentes espécies de plantas, inclusive na casca do fruto da *Caryocar brasiliense* com o equivalente a 209 gramas de Ácido Gálico por kg (23). Esta capacidade permitiu que o pré-tratamento de linfócitos com AG inibisse a apoptose induzida pelo estresse oxidativo por ação direta na eliminação de radicais

livres, antioxidante mais forte que o Trolox, um análogo da vitamina E solúvel em água (24).

Além disto, este composto provoca um efeito antiproliferativo nas células SCC uma vez que contribuiu para a inibição do ciclo celular em células HaCaT e SCC-9 / SCC-4 tratadas com 1, 5 e 10 µg / ml de AG por 24, 48 e 72 h (25).

2. OBJETIVOS

2.1. Objetivo geral

Analisar o potencial antineoplásico da Nanopartícula Alumínio Cloro Ftalocianina e do Ácido Gálico.

2.2. Objetivos específicos

- Avaliar a atividade antineoplásica da nanopartícula Alumínio Cloro Ftalocianina e sua ação sobre a viabilidade das células de Carcinoma Epidermoide de Boca;

- Avaliar a eficácia do Ácido Gálico em modelos *in vivo* mediante avaliação clínica e histopatológica de camundongos *swiss* com tumores induzidos por administração 4nqo.

3. PRODUTOS

3.1. Produto 1:

Photodynamic therapy mediated by nanoparticles Aluminum Chloro Phthalocyanine in oral squamous carcinoma cells

Lilian Mendes Borburema Cangussu [1], Ludmilla Regina de Souza[1] , Marcela Gonçalves de Souza, Luis Alexandre Muehlmann[2], Paulo Narcizo de Souza[3,4], Alfredo Maurício Batista de Paula[1], Lucyana Conceição Farias[1], Sérgio Henrique Sousa Santos[5], and André Luiz Sena Guimarães[1,6#]

[1]Department of Dentistry, Universidade Estadual de Montes Claros (Unimontes), Montes Claros, Minas Gerais, Brazil

[2]Institute of Physics of the University of Brasilia; Faculty of Ceilandia, University of Brasilia, Federal District, Brazil

[3]Department of Genetics and Morphology, Institute of Biological Sciences, University of Brasilia, Federal District, Brazil

[4]Department of Physics, University of Brasilia, Federal District, Brazil

[5]Institute of Agricultural Sciences, Universidade Federal de Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil

[6]Dilson Godinho Hospital, Montes Claros, Minas Gerais, Brazil

Corresponding author:

André Luiz Sena Guimarães. Laboratório de Pesquisa em Saúde, Hospital Universitário Clemente Faria, Universidade Estadual de Montes Claros, 562 Av. Cula Mangabeira Santo Expedito, Montes Claros, Minas Gerias, 39401-001 Brazil. E-mail: andreluizguimaraes@gmail.com

ABSTRACT

Objective: oral squamous cell carcinoma (OSCC) causes significant morbidity and mortality. The current study aims to investigate the antineoplastic potential of Photodynamic Therapy with photosensitizing (PDT) with Chloro-aluminum phthalocyanine (AlClPc) in OSCC.

Methods: A basic, applied cell study was performed. Four study groups were used Group 1: Control (did not receive any treatment), Group 2: PBS + 28.3Jcm^2 (received irradiation only), Group 3: AlClPc + 28.3Jcm^2 (received the photosensitizer and irradiation) and Group 4: AlClPc (received alone the photosensitizer). Initially, the concentration of AlClPc was tested. To test the effect of PDT with AlClPc migration and cell deaths were performed. Also, the immunoexpression of Ki-67 and TP53 was performed.

Results: All AlClPc concentrations (0.7, 0.035 and 0.017nM) significantly induced OSCC cell death. The lowest concentration was chosen for the tests. Migration and Cell death assays reveal that PDT with AlClPc significantly reduced migration and increased cell death when compared to controls. Also, PDT with AlClPc reduced Ki-67 and mutated TP53 immunoexpression.

Conclusion: PDT with AlClPc proved to be effective in altering the phenotype of OSCC reducing migration and inducing cell death. Additionally, PDT with AlClPc decreased Ki-67 and muted TP53 expression.

Keywords: Photodynamic Therapy. 4nqo. Antineoplastic action.

INTRODUCTION

Cancer burden rises to 18.1 million new cases and 9.6 million deaths (26). Oral squamous cell carcinoma (OSCC) reached 354,864 cases and the 177,384 deaths in the world during 2018 (27). Brazil is among the highest rates of OSCC in Latin America (28, 29). Most of OSCC cases commit a man. However, the gender difference in OSCC rates has been decreasing over time, presumably as tobacco and alcohol consumption equalize (30). The main risk factors for OSCC development are tobacco and alcohol (31), the use of which explains 82.9% of cases (32). Oral cancer causes significant morbidity and mortality (29). Moreover, OSCC is associated with significant effects on physiological function, facial esthetics, and survival (33).

The ultimate goal of OSCC treatment is to establish effective preventive and therapeutic measures against cancer, to preserve or to restore form and function, to minimize sequelae of treatment, and ultimately to prevent subsequent further cancers (34, 35). The currently available treatment modalities to OSCC include surgery, radiotherapy, chemotherapy, combined modality treatments and primary and secondary prevention strategies including lifestyle changes as well as chemoprevention (36).

Studies have demonstrated the use of alternative therapies in the control of OSCC (10, 34, 35, 37). Photodynamic therapy (PDT) is one of these alternatives to chemotherapy and radiotherapy for the treatment of malignant neoplasm (11, 12) because due to its local effects that make treatment less aggressive to the patient. PDT works by combining a photosensitizer drug (PS) with irradiation using visible light at an appropriate wavelength, which is individually harmless (38). The PS should have a high absorption peak between 600 and 800 nanometers (nm) (39). Thus, wavelengths longer than 800 nm does not provide enough energy to excite oxygen to its singlet state and to form a significant amount of reactive oxygen species (39).

Under irradiation, PSs transfer the absorbed light to the surrounding molecular oxygen, and cause cytotoxic reactive oxygen species (ROS) such as $^1O_2$ or free radicals, thus leading to cell apoptosis and destruction of the diseased tissue (40). The efficacy of a PDT depends on the formulation of drugs, which must accumulate selectively within tumor tissues in comparison to normal ones (41). PDT can promote

direct cytotoxic effects on tumor cells (39). Additionally, PDT might damage to the tumor vasculature, and induct a robust inflammatory reaction that can lead to the development of systemic immunity against the neoplasm (39).

Light emitting diode (LED) arrays are an effective and less costly means of light delivery (42). Developments in LED technology have provided higher power and narrower spectral characteristics making them a desirable alternative to lasers (43). The region between 600 and 1200 nm is generally referred to as a woven optical window (39). The photodynamic effects that occur after the absorption of visible light lead to the production of reactive oxygen species (ROS), which act as cytotoxic agents that can inactivate tumor cells (38). In the presence of oxygen, two competing reactions of the excited sensitizer can occur: a singlet or, more commonly, a triplet, can either react with the substrate or solvent (Type I) or with oxygen (Type II) (44).

Phthalocyanine derivatives comprise the second generation of photosensitizer molecules employed in PDT and have attracted much attention due to their outstanding photosensitizing performance (41). Chloro-aluminum phthalocyanine (AlClPc) is a chemically stable photosensitizer that displays photophysical properties that are highly suitable for PDT, such as high triplet quantum yields and long triplet lifetime (45). The system composed by AlClPc presents photodynamic activity in aqueous media and may be used for anticancer PDT (46). Studies have shown the efficient antitumor activity of AlClPc (46). To analyze the antineoplastic potential of the nanoparticle Aluminum Chlorine Phthalocyanine and its action on the viability of the cells of the in oral squamous carcinoma cells. This current study aims to investigate the antineoplastic potential of the AlClPc in OSCC.

MATERIAL AND METHODS

Study Design

A basic, applied cell study was performed to test the effect of PDT with AlClPc in OSCC cells.

AlClPc Concentration

Three AlClPc concentrations were tested 0.7, 0.035 and 0.017nM. The excitation was performed at 660 nm wavelength (fluency 28.35 Jcm^2 and power density 7.2 mW/cas) described before (47, 48).

LED source

All irradiations were performed under the following conditions: by 10 minutes, 05 cm away, wavelength 660 nm, 28.35 $Jcm^{2,}$ and power density: 7.2 mW/cm^2, in an apparatus developed by Professor Paulo E. N. Souza, Prototype n.02. The light source used was composed of 20 LED light-emitting diodes (LED) model XL001WP01NRC660 (Shenzhen Sealand Optoelectronics Co., Ltd., China). The power circuit was assembled under the supervision of Prof. Dr. Paulo Souza of the Institute of Physics of the University of Brasília. The control of the power supply able to guarantee the stability of the lighting was carried out by an RCD-24-0.35 / W controller (Constant Current LED Driver, Recom Power, Inc., Germany). The emission spectrum of the LEDs was determined using a portable spectrophotometer (Ocean Optics Inc., USA). A digital potentiometer (Fieldmax II, Coherent, USA) was used to define the maximum power value of the LEDs, as well as the power values as a function of distance and the relative position of the LED bank to guarantee the reproducibility of the luminous intensity. Periodically the energy per unit of time was measured through the use of a potentiometer. The measurements recorded by the potentiometer and the detector area allowed determining that the light source has a power density of 0.04 W / cm^2. The LED apparatus used in this study was developed by Prof. Dr. Paulo Narcizo de Souza, at the University of Brasília, with low-cost

components and optimized for use in biological treatments (41) with the beginning of operation on 02/01/2015 - INCT / CNPQ University of Brasília, Institute of Physics.

Cells and experimental conditions

The Scc9 (Code. 0196) cells lines *Homo sapiens*; Tongue; Epithelial-Like were, and Cells A431, epidermal carcinoma, both cells were acquired at the Bank of Cells of Rio de Janeiro (BCRJ). Cells were cultured in DMEM/Ham's F-12 supplemented with 10% fetal bovine serum and 0.4 µg/ml hydrocortisone (Gibco, USA, Catalog number: 10437028), at 37°C with 5% CO_2 in a humidified air atmosphere. All experiments were performed in triplicate. The cells lines (2×10^6) were seeded in a 6-well plate supplemented with 10% FBS and incubated 24 h.

Cell proliferation

The cells of all groups were counted using a Newbauer chamber, 24 h after treatment. All groups were performed in triplicate. Cell proliferation assay was performed as described before (47) with necessary adaptations. A density of 2×10^5 OSCC cells was plated in 60 mm dish and incubated at 37 °C for approximately 24h to establish adherent monolayers. Then, cells were treated with the AlClPc concentration was previously defined through in the MTT assay.

Study Groups

The cells were distributed in four groups.
Group 1: Control (did not receive any type of treatment);
Group 2: PBS + 28.3Jcm^2 (received irradiation only);
Group 3: AlClPc + 28.3Jcm ^{2} (received the photosensitizer and irradiation) and
Group 4: AlClPc (received only the photosensitizer).

MTT

The cell viability was checked through assay. Cells were seeded in 96-well plate at the density of 3×10^3 cells/well and left in complete media containing 10% FBS for 24h. The cells were divided into two groups (AlClPc and AlClPc + LED), and each group was treated with AlClPc for 15 min. All groups were washed with PBS. One of the groups received irradiation. Control did not. After washing, the control group was incubated with PBS while the AlClPc group received radiation, only then the cells were placed in a dark incubator for 24h.

Migration assay

Wound healing method was used to test by cell migration (49). OSCC cells were plated in 60 mm dish at a density of 2.5×10^5 cells and maintained in culture for 24h. Before treatment, to determine the cell migration, a lesion was created on the monolayer cells using a sterile 10µl pipette tip. All groups were photographed. The cells of the Control Group and AlClPc Group have not been irradiated. While the 28.35 Jcm^2 Group and AlClPc + 28.35Jcm^2 Group was irradiated for 10 min and placed back in the incubator. The changes in the distance between the lesion edges area were monitored at 24h with the new photography. The migration rate (%) as the distance that cells migrate regarding the original distance of the lesion.
Each wound area was measured by automatic analysis software Image J. The migration ability was expressed as a percentage. Images of the wounded cell monolayers were taken using an Olympus IX81 inverted microscope (Olympus, Center Valley, PA, USA) coupled to camera SC30 (Olympus, Center Valley, PA, USA) before and at 24h after wounding.

Acridine Orange / Ethidium Bromide assay

Cell viability was analyzed in the Acridine orange / Ethidium bromide (AO/EB) assay staining used to visualize dead and viable cells (50). Pit by pit, the cells (3×10^4cells/mL) were removed from the greenhouse and had their means replaced

by a volume 20µL of a solution containing 1 part of l00 µg/mL Acridine orange in PBS; (AO, Sigma, St. Louis, USA) and 1 part of 100 µg/mL Ethidium bromide in PBS (EB, Sigma, St. Louis, USA). Cells were observed in a fluorescence microscope FSX100 (Olympus, Center Valley, PA, USA).

Immunocytochemical assay

Immunocytochemical was performed as described before (49) with necessary adaptations. A density of 1×10^5 OSCC cells was plated 60mm culture plates and submitted to the assays. Then the cells were fixed with 70% ethanol for 30 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. The following primary mouse monoclonal antibodies were used: anti-Ki-67 (1:100, SolA15, Sigma, St. Louis, MO, USA) and TP53 (1:20, PAb 122, Invitrogen). All monoclonal antibodies were incubated for 18h at 4°C. Endogenous peroxidase was blocked by incubation with 0.03% H_2O_2 in ethanol for 30 min. The primary antibodies against Ki-67 and TP53 were detected using the Universal HRP Immunostaining Kit (KP-500, Diagnostic BioSystems, Pleasanton, CA, USA). Signals were developed with 3'3-diaminobenzidine-tetrahydrochloride for 5 min and counterstained with Mayer's hematoxylin for 30 sec. Negative controls were performed by replacing the primary antibody with PBS. Slides were photographed on Brightfield microscope FSX100 (Olympus, Center Valley, PA, USA) at 20×. The manual counts were performed in merge image by ImageJ software (51). Immunocytochemistry analyses of investigated antigens were carried out by determining the percentage of positively stained viable cells in all fields counted.

The numbers of positively stained and nonstained cells were recorded separately. A negative result was defined as the absence of stained and feasible nuclei. Staining was considered positive when brown nuclear labeling was observed.

RESULTS

Chloro-aluminum phthalocyanine concentration

The effectiveness of AlClPc as a photosensitizer with and without irradiating light emitting diode (LED) was further investigated using MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide). We have observed that PDT exhibits a significant anticancer effect on 24-hour MTT treatment. We confirm that the ALClPc acts on the viability of OSCC cells at all concentrations tested (Figure 1).

After this assay, we decide to choose only one concentration for the other experiments. We chose the lowest dose of 0.017nM because it is a minimum dose of AlClPc (among those tested) capable of producing a therapeutic effect.

PDT reduce cell migration and induced cell death

Thus, the migration assay results indicated that the combined treatment of the AlClPc + 28.3 Jcm^2 considerably reduced cell migration compared with single treatments and PBS combined with LED (Figure 2).

Acridine orange penetrates living and dead cells, emitting green fluorescence as a result of the intercalation in the double-stranded DNA and red-orange fluorescence after binding with single-stranded RNA, while ethidium bromide emits red fluorescence after intercalation in the DNA of cells with altered cell membrane (at necrosis or a late stage of apoptosis) (52). Acridine Orange/Ethidium Bromide Cell death assay reveals that PDT significantly increased the number of death process cells when compared to controls (Figure 3).

Cellular development inhibition was observed when compared with controls, as can be seen in Fig. 3 OSCC cells with orange dots in cell nuclei treated with PDT. The maximum increase ($p < 0.05$) in the quantity of not viable cells was observed in treated cells. Low cytotoxicity was observed when cells were treated only with AlClPc and only LED with/or PBS.

PDT reduced Ki-67 and muted TP53 immunoexpression

Some molecules affect the process of transformation of OSCC (28, 53). Among these molecule TP53 and Ki-67, are known to significantly influence in malignization of oral potentially malignant disorders (54). OSCC cells presented a robust expression of the proliferation marker Ki-67 positive cells (Figure 3). PDT with AlClPc at 28.3 J/cm^2 group drastically reduced Ki-67 (Figure 3). Muted TP53 is associated with OSCC worse prognoses (29, 55). Our results demonstrated that the expression of the mutated TP53 protein was dramatically reduced by PDT treatment (Figure 4). Additionally, a clear hypotrophy is observed OSCC cells treated with PDT.

DISCUSSION

OSCC is the most common cancer of the head and neck (26). Moreover, even with surgical reconstruction procedures, after OSCC treatment the occurrence of some degree of facial disfigurement, leading to severe aesthetic and functional defects (56). The current study presents a possible perspective to treat OSCC with AlClPc PDT.

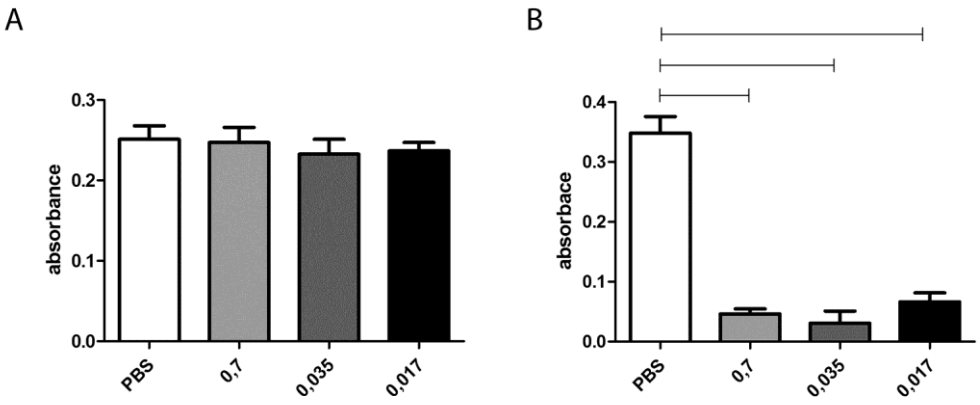
Migration is a cyclic process in which the cell changes shape produces morphological asymmetry and translocates the cell body implying death in cancer patients related to metastatic progression (57). Increased migration at the individual cell level affects local neoplasm invasion and metastasis (67). In the current study, AlClPc mediated PDT significantly reduced cell migration. The reduction of migration is essential for local growth control (58).

Previous studies have performed AO/EB staining and reported that early apoptotic cells had fragmented DNA which exhibited strong green colored nuclei (34, 35). Dual AO/EB fluorescent staining can detect fundamental morphological changes in apoptotic cells. Besides, it allows for the distinction between healthy cells, early and late apoptotic cells, and necrotic cells (34, 35). The viable and non-apoptotic cells are stained green while the apoptotic cells are stained orange or red (59). In the current study, the OSCC cells treated only with light doses 28.3 J/cm^2 were non-toxic to OSCC cells. These results are by previous demonstrations that both the light source and drug carrier are innocuous when used separately (46, 52). On the other hand, our data demonstrated that PDT with AlClPc induced cell death. Moreover, all concentrations used in the current study (0.7, 0.035 and 0.017nM) caused cell death.

Our data corroborate with evidence that demonstrated that PDT could lead to all three forms of cell death (apoptosis, necrosis, and autophagy) by different signaling pathways (60) acting induced death by arresting cell cycle progression (38).

The protein Ki-67 is only produced in actively dividing cells; it is located in the nucleus on the structure that contains most of the cell's DNA (2). The levels of this protein and location vary through the cell cycle (61) and the expression of Ki-67 is shown in all stages, except G0, whereas resting cells entering from G0 lack Ki-67 in the early part of G1(62). The level of Ki-67 expression has been used as a prognostic determination index in human cancers (54) for having variable value for detecting malignant potential (54, 63). Ki-67 scoring is essential for diagnosis for tumor grade, based on proportional of tumor-positive cells has usually used as an indication for evaluation (64). Uncontrolled proliferation is a common feature of malignant cells, therapeutic agents that target Ki-67 may be useful tools in cancer treatment (65). The over-proliferation is a crucial feature of tumor progression, and the high rate is related to lousy prognosis (66, 67). Our results showed that the PDT with AlClPc drastically reduced expression of this marker about the control groups. Also, as well as Ki-67, the TP53 expression index increased as the severity of lesion increased showing that there is a relation between these two markers (28-30, 54, 55). TP53 is a tumor suppressor gene located on chromosome 17p and mutations in this gene is one of the most common events in human carcinogenesis (68). Mutations or deletions in the TP53 gene are present in nearly 50% of human cancers (69). Mutations (70, 71) or biochemical changes (72) that cause loss of function plays an essential role in the development of OSCC. Lost of TP53 function impossibilities the proliferation control resulting in the occurrence of tumors (72). Besides this TP53 mutation probably renders more aggressive phenotype (73). These mutations occur in the expression of full-length TP53 protein, but the loss of wild type tumor suppressive function (74). Our results demonstrated that Photodynamic Therapy mediates by AlClPc caused the death of cells expressing the TP53 mutant protein thus reducing its expression.

In conclusion, PDT with AlClPc proved to be effective in altering the phenotype of OSCC reducing migration and inducing cell death. Additionally, PDT with AlClPc decreased Ki-67 and muted TP53 expression.


A
absorbance
0.3
0.2
0.1
0.0
PBS
0,7
0,035
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B
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PBS
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Figure 1.

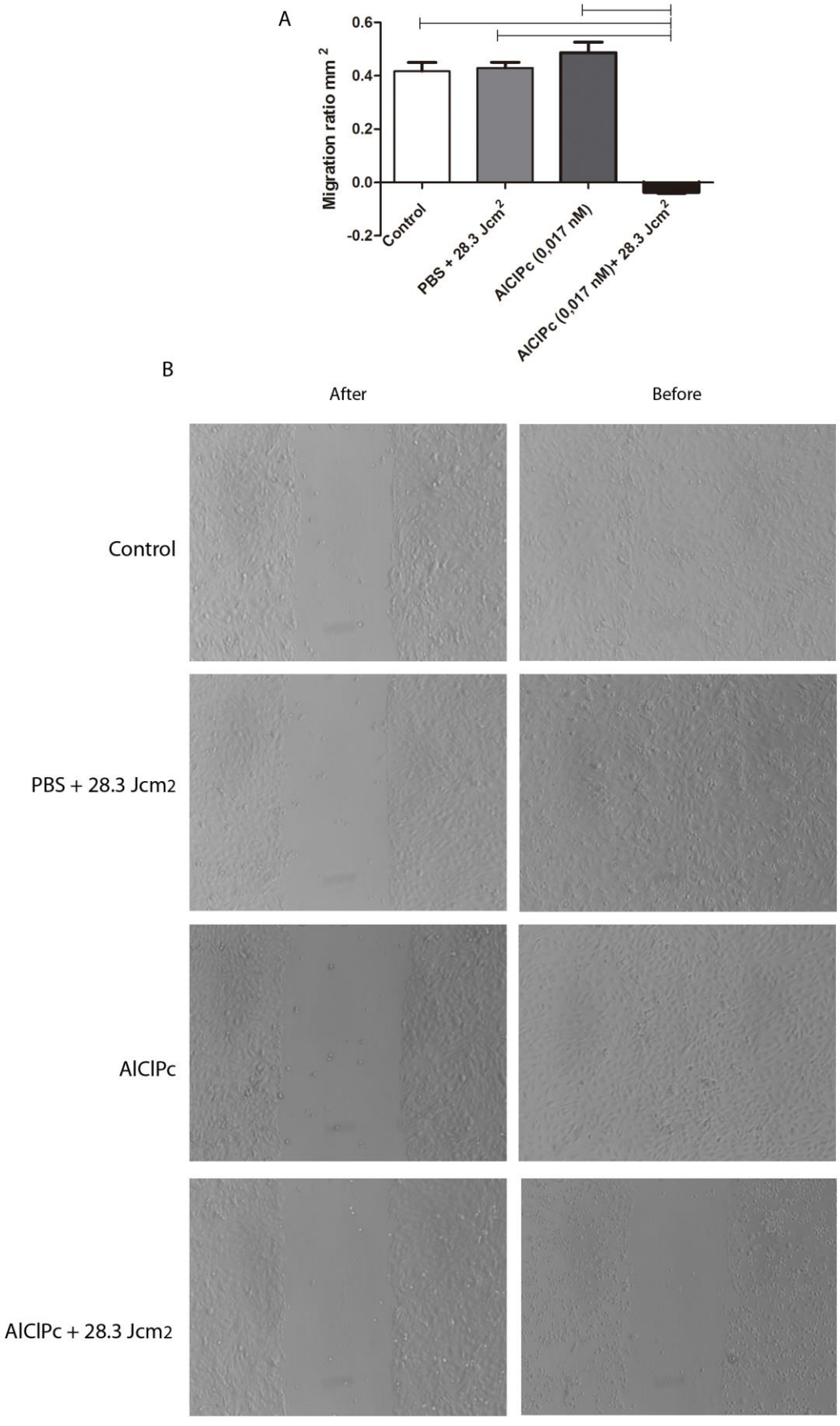
A
Migration ratio mm 2
0.6
0.4
0.2
0.0
-0.2
Control
PBS + 28.3 Jcm2
AlClPc (0,017 nM)
AlClPc (0,017 nM)+ 28.3 Jcm2
B
After
Before
Control
PBS + 28.3 Jcm2
AlClPc
AlClPc + 28.3 Jcm2

Figure 2.

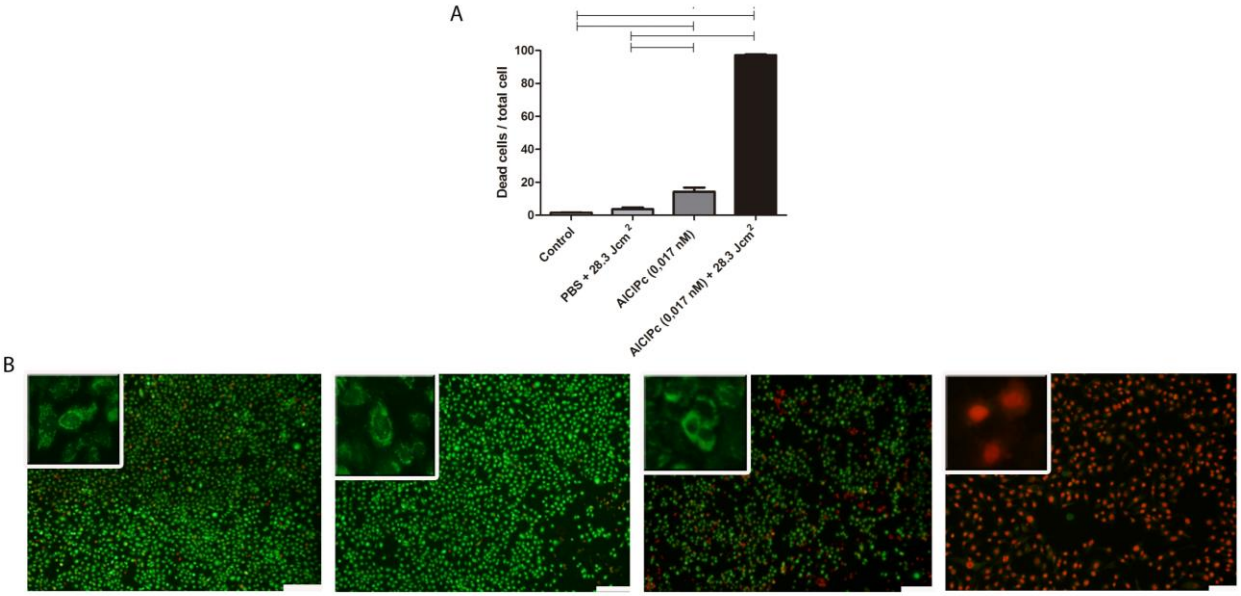

Figure 3.

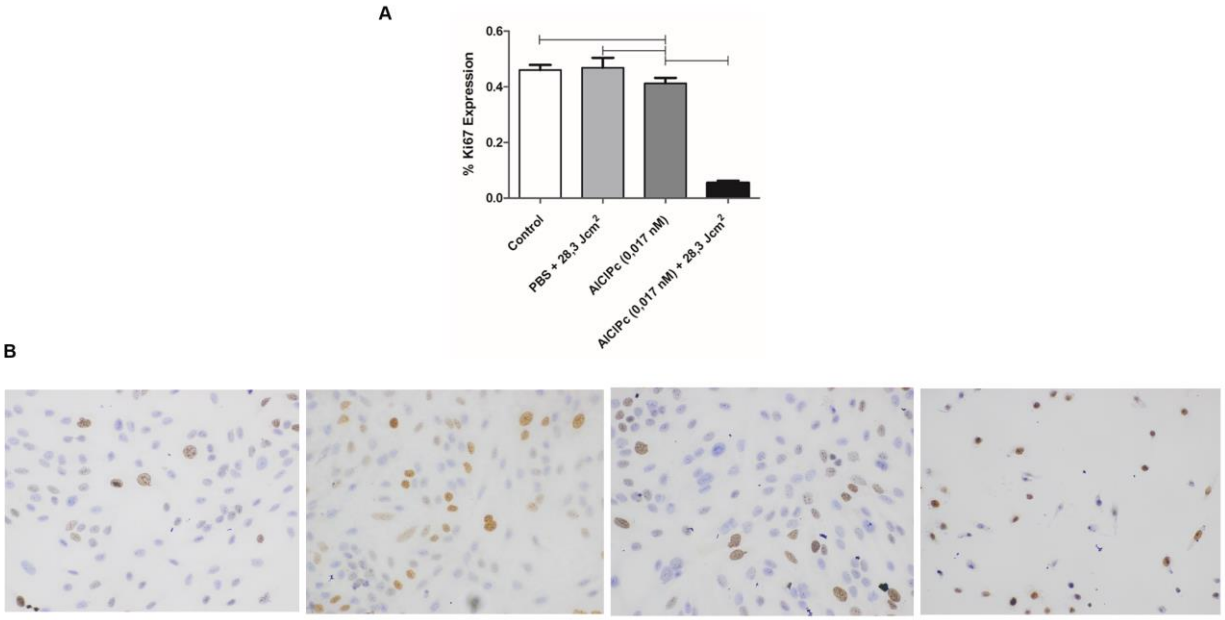

Figure 4.

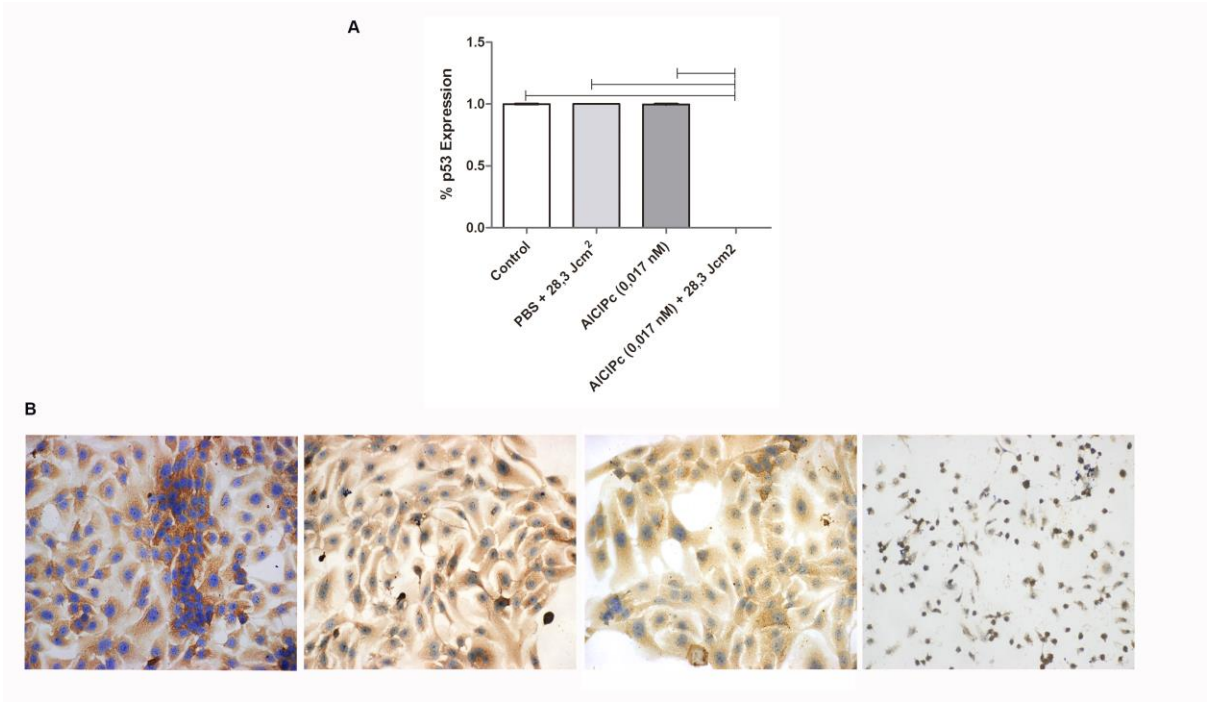

Figure 5.

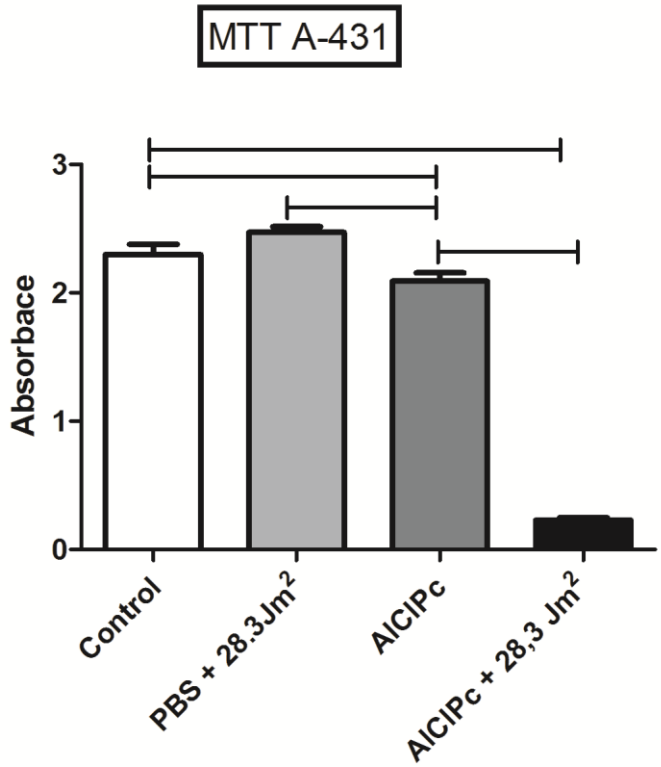

Figure 6.

Figure Legends

Figure 1. PDT suppresses the viability of SCC9 cells according to the MTT assay result. (A) Photodynamic therapy performed with PBS + LED 28.3 Jcm^2 (control). (B) Photodynamic therapy performed with AlClPc + LED (28.3 Jcm^2). The horizontal bars represent a statistical difference between the bars that express the data.

Figure 2. Effect of PDT on cell migration. Quantification of the impact of PDT on the SCC9 cells (A). PDT drastically reduced cell migration ratio (B). The horizontal bars represent a statistical difference between the bars that express the data.

Figure 3. Effect of PDT on cell death. AO/EB representative figures (A) and quantification (B) show an increase in cell death as a consequence of PDT treatment. The horizontal bars represent a statistical difference between the bars that express the data.

Figure 4. Effect of PDT on cell death. (A) Ki-67 expression (B) Ki-67 expression show a decrease in expression this marker as a consequence of PDT treatment. The horizontal bars represent a statistical difference between the bars that express the data.

Figure 5. Effect of PDT on cell death. (A) TP53 expression (B) TP53 expression show a decrease in expression these markers as a consequence of PDT treatment. The horizontal bars represent a statistical difference between the bars that express the data.

Figure 6. PDT suppresses the viability of A-431 cells according to the MTT assay result. The horizontal bars represent a statistical difference between the bars that express the data.

Acknowledgments
This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). Dr. Guimarães, Dr. Santos and Dr. de Paula are research fellows of the CNPq. Dr. Farias is a research fellow of FAPEMIG.
Disclosure of Potential Conflicts of Interest: The authors deny any conflicts of interest related to this study.

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3.2 Produto 2:

Chemopreventive effects of Gallic Acid on oral carcinogenicity induced by 4NQO in the tongue of mice

Lílian Mendes Borburema Cangussu[1], Ludmilla Regina de Souza[1], Eliane Macedo Sobrinho Santos[2], Marcela Gonçalves de Souza[1], Luís Paulo de Oliveira[1], Lincoln Valério Andrade Rodrigues[1], Carlos Ícaro de Jesus Silva[1], Guilherme Machado Xavier[4], Alfredo Maurício Batista de Paula[1], Lucyana Conceição Farias[1], Sérgio Henrique Sousa Santos[3], and André Luiz Sena Guimarães[1,5#]

[1]Department of Dentistry, Universidade Estadual de Montes Claros (Unimontes), Montes Claros, Minas Gerais, Brazil
[2] Instituto Federal do Norte de Minas Gerais - Campus Araçuaí, Minas Gerais, Brazil
[3]Institute of Agricultural Sciences, Universidade Federal de Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil
[4]Department of Orthodontics, King's College London Dental Institute, London, UK
[5]Dilson Godinho Hospital, Montes Claros, Minas Gerais, Brazil

Corresponding author:
André Luiz Sena Guimarães. Laboratório de Pesquisa em Saúde, Hospital Universitário Clemente Faria, Universidade Estadual de Montes Claros, 562 Av. Cula Mangabeira Santo Expedito, Montes Claros, Minas Gerais, 39401-001 Brazil. E-mail: andreluizguimaraes@gmail.com

ABSTRACT

Objective: As oral cancer development is a complex process, animal models represent an essential alternative to evaluate OSCC potential treatments. As such, the current study aims to investigate the GA preventive effect and potential toxicity.

Methods: The animals were kept in an environment with a controlled temperature of 21 ± 2°C and with a cycle of 12 hours of light /12 hours of dark (lights on from 12 hours, and they were fed with rations and filtered water. Animals were followed from birth to 234 days. The animals were randomly divided into four groups. A total of 39 mice *Mus musculus* males were distributed into four groups as follows: - Group 1 (Control): negative control group (N=7); - Group 2 (GA): treated with Gallic Acid (n=7); - Group 3 (GA+4NQO): received 4NQO and Gallic Acid treatment (n=7); - Group 4 (4NQO): received only 4NQO (n=7)

Results: Absent/mild epithelial dysplasia; moderate/severe epithelial dysplasia and in situ/invasive carcinoma distribution were different among groups. GA treatment statistically reduced the histopathological grade of 4NQO induced lesions. Also, GA did not promote epithelial changes in the absence of 4 NQO. Statistical differences in survival were also observed among the groups. In the presence of 4NQO, GA treatment increased the survival. Also, GA did not promote animal deaths in the absence of 4NQO.

Conclusion: AC reduced the severity of OSCC lesions, increased survival and reduced 4NQO liver toxicity in mice and decreased the mice weight loss in consequence of 4NQO.

Keywords: Antioxidant effect. Antineoplastic action. Polyphenols. Oral squamous cell carcinoma

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is worldwide common malignant neoplasm (75). OSCC main etiologically factor is tobacco consumption especially in association with alcohol (29, 76). Recently the potential role of oncogenic HPV has been extensively discussed in the literature (53, 77). Biochemical and molecular changes also can change OSCC development and prognosis (28, 30, 53, 70, 78-80). A critical issue in OSCC prevention is the absence of a deterministic biomarker to identify OSCC development (81, 82). As oral cancer development is a complex process, animal models represent an essential alternative to evaluate OSCC potential treatments (83, 84).

Experimental carcinogenesis induced by synthetic chemical 4-nitroquinoline 1-oxide (4NQO) is one of the most frequently used methods in animals for the study of OSCC (85). 4NQO is a synthetic water-soluble chemical carcinogen that forms all similar to the genetic alterations provoked by tobacco carcinogens (86). Mice exposed to 4NQO exhibit 1 to 3 carcinomas together with multiple dysplastic lesions at the end of the carcinogen exposure, thus limiting the ability to examine the benefit of preventive agents unless they also display anticancer properties (87). The tongue carcinogenesis model parallels the development of tongue cancer in humans as the dysplastic lesions are produced by long-term ingestion of small amounts of carcinogen (88). Moreover, 4NQO for 16 weeks cause hepatic and renal toxicity (89).

Currently, the treatment of OSCC is surgery, radiation, and chemotherapy alone or combined (90). Treatment strategies for OSCC vary based on the stage at the time of diagnosis (90). Patients with the localized disease typically receive surgery and radiotherapy, leading to a high probability of long-term survival but with considerable morbidity (91). Simultaneously chemotherapy and radio therapy have given significant beneficial responses at inoperable stages of the disease (92). Other potential OSCC treatments emerged, like an EGFR Inhibitors: Tyrphostin AG-1478 that improves (cisplatin sensitivity of the OSCC (93) and Nimotuzumab in combination with PDT (photodynamic therapy) that increase the antitumor effect of PDT in an oral tumor model (94). But despite all improvements in OSCC treatment, the survival of OSCC patients population remains around 50% in five years (29, 75, 77).

Currently, OSCC treatment and prevention represents a challenge to the health systems (95, 96). Significant reduction in OSCC advantage stages is achieved by a population-based screening program (97). However, a considerable decrease in OSCC incidence is not obtained only with only early detection programs (96). Moreover, completely quit smoking is difficult to achieve especially in patients with low social support, depression, greater nicotine dependence and poor social-emotional function (98). A decrease in smoking behavior was observed with multidisciplinary smoking cessation advisor team but, after 5-year follow-up appointments, 74% of patients ultimately continued to smoke (99).

Considering the difficulty in achieving the quit smoking, new therapies to minimize smoke effects and prevent OSCC are very important (81). Nimesulide decreased the incidence of tongue lesions on Fisher 344, rats with induced squamous cell carcinomas (100); but as a selective COX-2 inhibitor might represent critical adverse effects in clinical practice (101). Metformin is also a potential agent to treat OSCC (34, 102) who presents clinical risks (103).

Gallic acid (GA), 3,4,5-trihydroxybenzoic acid, ($C_6H_2(OH)_3COOH$) is one of the polyphenolic substances in plants (104). In nature, GA are present in nearly every part of the plant, such as bark, wood, leaf, fruit, root, and seed (104). They are present in different concentrations in common foodstuffs such as blueberry, blackberry, strawberry, plums, grapes, mango, cashew nut, hazelnut, walnut, tea, wine (105). Another important GA source is the Pequi (*Caryocar brasiliense* Camb.)(106). GA inhibits triggers apoptosis through the regulation of Fas/FasL, p53, and Bcl-2 family and activation of caspase cascade (107). Moreover, studies demonstrated that GA selectively induces OSCC cell death by apoptosis (108), even under hypoxia (35). GA also decrease of invasiveness (109) inhibits of NF-B (110) and have anti-angiogenesis activities (111).

The cytotoxicity shown by GA is not a frequent feature in phenolic compounds but is a reasonably specific characteristic of GA that contains three adjacent phenolics, hydroxyl groups were responsible for the cytotoxicity, and the carboxyl group that is implicated in distinguishing between normal cells and cancer cells (112). Preclinical studies to evaluate the GA toxicity and preventive effect in OSCC are scarce, despite the potential of GA to treat OSCC (35). As such, the current study aims to investigate the preventive effect of GA and potential toxicity.

MATERIALS AND METHODS

Ethical approval for this study was obtained from the relevant Institutional Animal Care and Use Committee (protocol 098/16).

Animals and experimental conditions

Where used in this study mice males swiss (*Mus musculus*) received food and water *ad libitum*. The animals were kept in an environment with a controlled temperature of 21 ± 2°C and with a cycle of 12 hours of light /12 hours of dark (lights on from 12 hours, and they were fed with rations and filtered water. Animals were followed from birth to 234 days.

Study Groups

Animals were randomly divided into four groups. A total of 39 mice *Mus musculus* males were distributed into four groups as follows:

- Group 1 (Control): negative control group (N=7)
- Group 2 (GA): treated with Gallic Acid (n=7)
- Group 3 (GA+4NQO): received 4NQO and Gallic Acid treatment (n=7)
- Group 4 (4NQO): received only 4NQO (n=7)

Cancer Induction

Induction of oral carcinogenesis was performed using 4-nitroquinoline-1-oxide (4NQO; N8141-1G, Sigma-Aldrich, St. Louis, USA) in drinking water to a final concentration of 100µg/ml as described before (113). After 39 day-olds weighing at the weight of 20 to 22 g mice received the treatment with 4NQO for 112 days. Animals were followed from birth to until the death or 234TH day.

Treatment

The establishment of Gallic Acid was according to our and other previous data (108, 113, 114). The daily administration of Gallic Acid (100 mg/Kg) by oral gavage started at the day 67^{Th} until the 234^{TH}-day lingual tissue of the animals.

End of the experiment

The endpoint of the experiment was the animal death, or at the 234^{TH} day, the remaining animals were sacrificed via decapitation through a guillotine.

Animal Macroscopic and weight evaluation

A general macroscopic evaluation (115) and body weight (116) evaluation were performed, and the groups were compared.

Histological preparation and analyses

Shortly after their sacrifices, all organs were placed in properly labeled containers and was fixed in 10% formalin solution for 48 hours. All histological techniques were performed following the protocol previously described (54, 117). Briefly, tissues were included in paraffin. The slides were stained with hematoxylin-eosin samples were covered with glass coverslips for observation and histological quantification through microscopy (Olympus Fsx100, Center Valley, Palo Alto, CA). Twenty fields of each tissue (oral mucosa, kidney, and liver) of each group were randomly photographed using a microscope (FSX100, Olympus, Center Valley, PA, USA) by a researcher who did not participate of the quantification or analyses. All quantifications were made using ImageJ software (118), which was used previously (108, 113). Histological specimens were blindly examined by a trained pathologist using the criteria recommended for histopathological diagnosis. The morphological semi-quantitatively analyses were performed in the liver and kidney to evaluate toxicity as described before (89). The OSCC lesions were graded according to previous studies (113, 119).

Statistical analysis

The PASW Statistics18-SPSS software (IBM, Armonk, NY) was used the statistical analysis. Kolmogorov–Smirnov and Shapiro-Wilk tests were used in samples that had nonparametric distribution, before, were submitted to an independent T-test, and samples that did not follow this distribution were subjected to a Mann-Whitney nonparametric test. The LSD Test was used in relative animal weight investigates. Categorical variables were analyzed by chi-square or fisher's exact. The Kaplan-Meier method was used in estimating survival relative to time, and survival differences were analyzed with the Log-rank test was do in SPSS. Confidence above 95% ($P<0.05$) was considered to be significant statistical analysis showing. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for in the construction of the graphs and $p<0.05$ was considered statistically significant.

RESULTS

The Macroscopic features of mice were used to compare the effect of GA. 4NQO (Group 3 and 4) induced weight loss in comparison to control groups (Group 1 and 2). The GA treatment (Group 2) promote weight reduction, in contrast, to control (Group 1). On the other hand, in mice treated with 4NQO (Groups 3 and 4), GA induces an increase in body weight (Group 4) (Figure 1).

Absent/mild epithelial dysplasia; moderate/severe epithelial dysplasia and in situ/invasive carcinoma distribution were different among groups (Figure 2A). GA treatment statistically reduced the histopathological grade of 4NQO induced lesions (Group 4, Figure 2A). Also, GA did not promote epithelial changes in the absence of 4 NQO (Groups 1 and 2, Figure 2A). Statistical differences in survival were also observed among the groups (Figure 2B). In the presence of 4NQO (Groups 3 and 4), GA treatment increased the survival (Group 4, Figure 2B). Also, GA did not promote animal deaths in the absence of 4NQO (Group 2, Figure 2B).

The histopathological characterization of the liver tissue of the animals in the four groups of the present study are disposed of in figure 3. 4NQO treatment (group 3)

present liver distinct cellular and histopathological changes such as steatosis in the microvascular pattern (Figure 3A). GA promote attenuation of 4NQO damage in kidney tissue (Figure 3B).

DISCUSSION

As the treatment of early staged OSCC patients is associated with higher quality of life survival (91), OSCC needs to be imminent diagnosed and treated (95-97). But OSCC patients have still been diagnosed and treated in advanced stages (29, 75, 77). Moreover, there is no preventive treatment for OSCC development in patients exposed OSCC risk factors (81). Also, no even drug could delay OSCC development in patients presented OSCC risk factors (81). The necessity to at least promote a delay in OSCC development might help to improve the local control and survival (120). Weight loss is a critical phenomenon that commits OSCC patients (121). In the absence of 4NQO GA treatment promote weight loss. It is observed that the activation of the AMPK/Sirt1/PGC1α pathway are GA target and consequently made GA a potential therapeutic intervention for insulin resistance in metabolic diseases (122) and could explain the result of the current study in group 2. Moreover, GA also reduced the weight of animals by lowering plasma glucose and triglyceride levels in addition to reducing the weight of adipose peri-renal tissues of rats (123). However, in the current study, when mice were submitted to 4NQO GA presents opposite effect in body weight. GA promoted the reduction of weight loss in group 4. It was demonstrated that GA changes OSCC reduced HIF-1α expression (35). HIF-1α is also directed related to the glycolytic pathway in the OSCC context (34). The dual effect of GA might be explained by the capacity of GA restore normal metabolism, specifically related to the glycolytic pathway, shifting to aerobic glycolysis (124). Moreover, the molecules related to metabolic syndrome such as angiotensinogen converting enzyme (ACE) might be associated with OSCC (81).

The current study demonstrated that GA attenuates the severity of the mice oral lesions. Recent studies have shown that GA can induce apoptosis as a result of the anti-inflammatory activity (125) by intrinsic mitochondrial pathway (126, 127). GA has also presented effects of DNA methyltransferases activities suggesting that GA might be useful dietary intervention strategy for tobacco-associated cancers (128). Additionally, GA treatment resulted in significant changes in cell membrane lipids and

fatty acids inducing malignant cells to apoptosis pathways (129). These findings corroborate the current study that demonstrated that systemic administration of GA has chemopreventive activity during oral carcinogenesis induced by 4NQO. Benefits of GA are perceived for other cancers such as gastric and lung cancer (130, 131). Curcumin (132) and Pecan nut shell extract, which are also rich in phenolic compounds, showed antitumor activity against MCF-7 human breast cancer cells, decreasing the viability of the cells, increasing the cell death by apoptosis and arresting cell cycle (133).

The systemic 4NQO toxicity is well established (89). GA supplementation also represents systemic benefits (114, 124).

GA provide adequate protection against oxidative stress damage in the liver in rats reducing lipid peroxidation and free radical levels and increasing enzymatic and non-enzymatic antioxidant defense in these tissues (134). At the same dose of the current study (100 mg/kg), GA is effective against chronic liver damage, induced by the administration of fluoxetine (114). GA treatment also prevented the increase in reactive species in the liver and kidney of diabetic rats (134). In corroboration with findings of the current study indicate GA was not only well tolerated but also reduced 4NQO toxicity.

The limitation of the current study is related to its design. Preclinical studies should be confirmed in humans. On the other hand for the first time, GA was tested effect and toxicity in an OSCC established model.

In conclusion: AC reduced the severity of OSCC lesions, increased survival and reduced 4NQO liver toxicity in mice. Additionally AC reduced the mice weight loss in consequence of 4NQO.

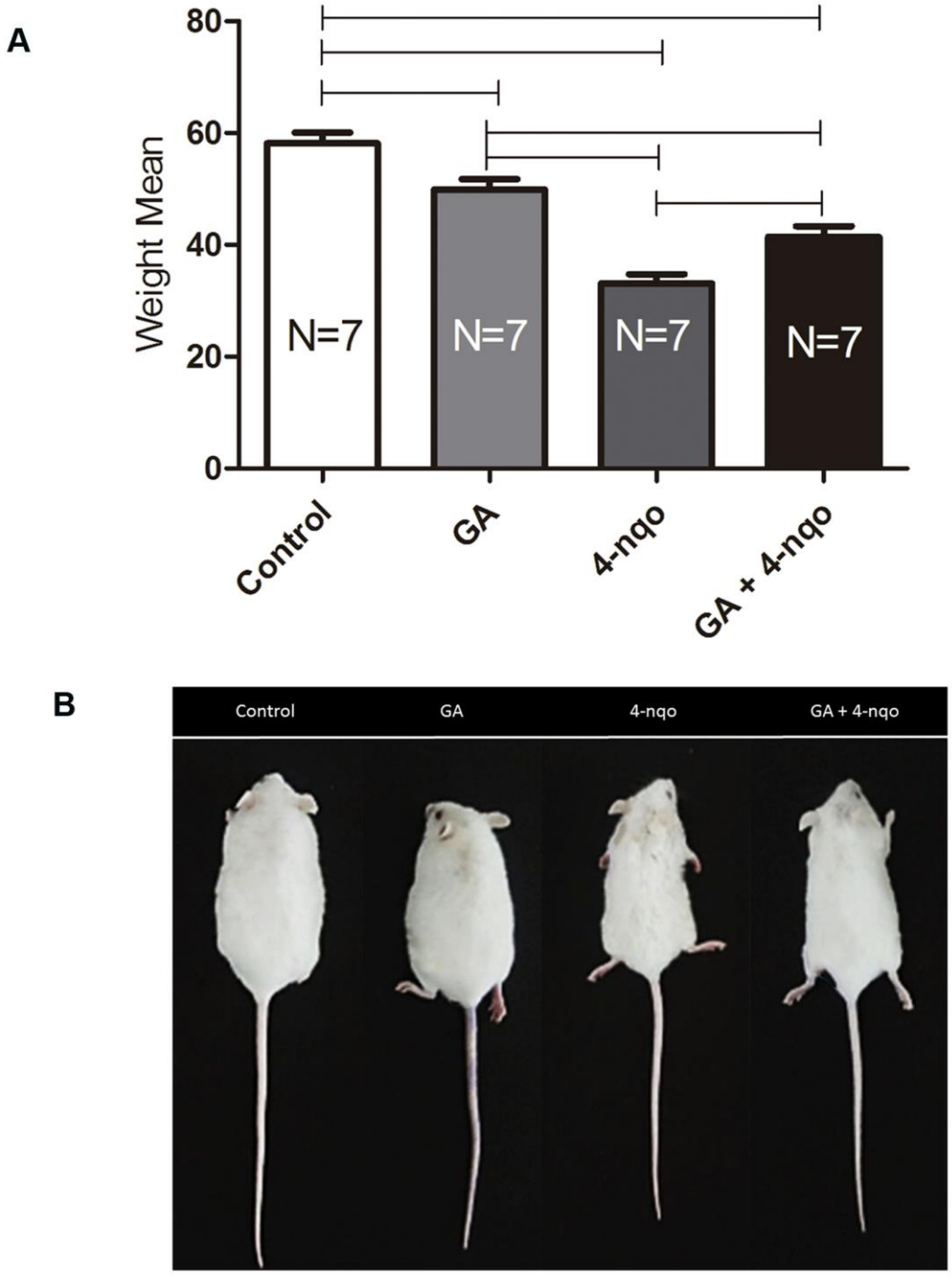
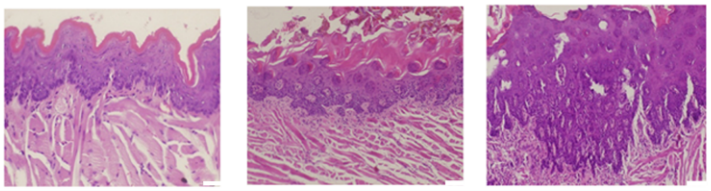

A
80
60
40
20
0
Weight Mean
N=7
N=7
N=7
N=7
Control
GA
4-nqo
GA + 4-nqo
B
Control
GA
4-nqo
GA + 4-nqo

Figure 1.

A



	Dyspplasia Absent/Mild	Dyspplasia Moderate/Severe	Carcinoma	*p* value
Control	7	0	0	
GA	7	0	0	
4NQO	1	3	3	
GA + 4NQO	3	3	1	0.006

B

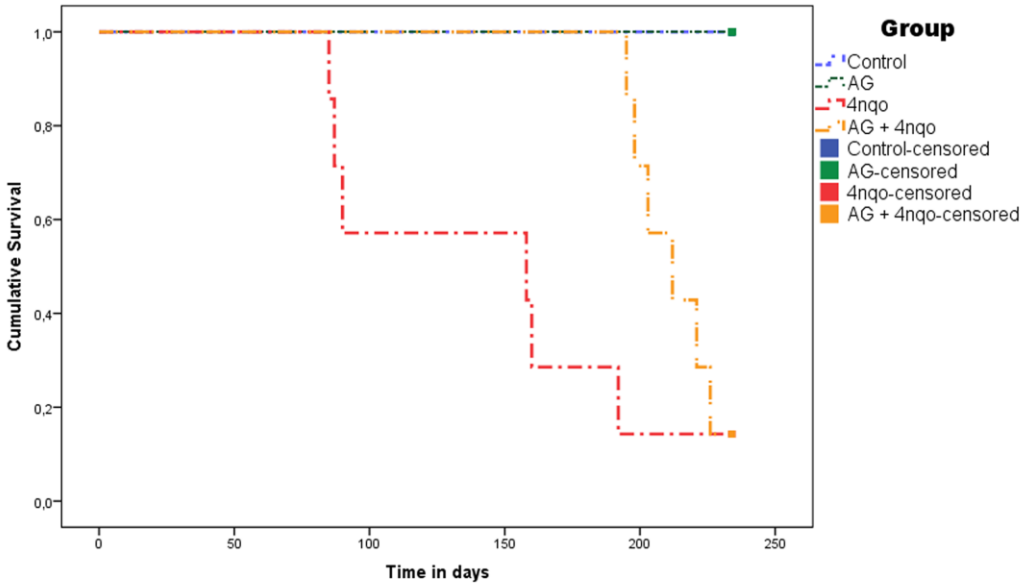
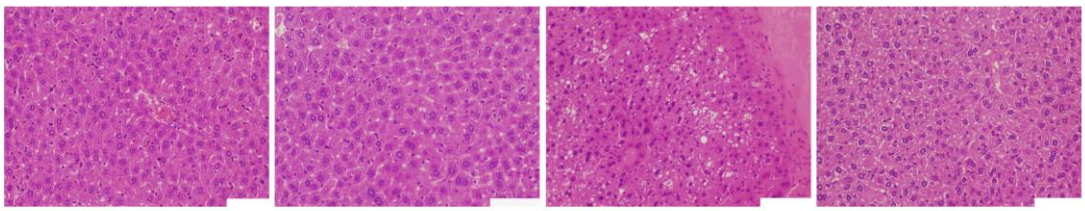


Figure 2.

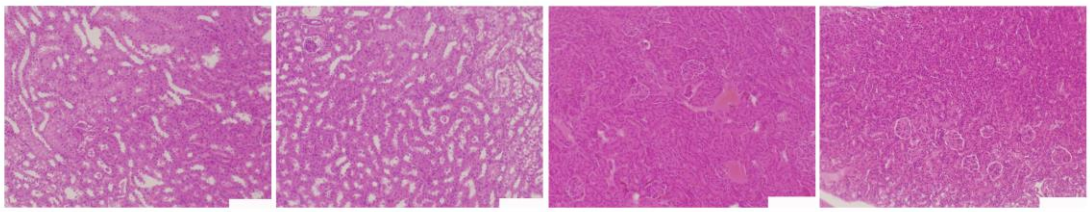
A



B

	Control	GA	4NQO	GA + 4NQO	*p* value
Undamged	20	13	0	0	
Light	0	7	0	0	
Moderate	0	0	10	16	
Severe	0	0	10	4	< 0.001

C



D

	Control	GA	4NQO	GA + 4NQO	*p* value
Undamged	20	11	0	0	
Light	0	9	0	6	
Moderate	0	0	5	14	
Severe	0	0	15	0	< 0.001

Figure 3.

Figure Legends

Figure 1. Effect of Gallic Acid in animals' weight

Weight average between groups of animals. Mice treated with 4NQO (Group 3 and 4) had lower body weight than the control groups (Group 1 and 2) ($p<0.05$). The treatment with GA provided gain in body weight compared to the group receiving only 4NQO. However, treated animals (Group 3) did not reach the same weight level as the control group (Group 1). The horizontal bars represent a statistical difference ($p < 0.05$) between the bars that express the data.

Figure 2: Effect of Gallic Acid in the incidence of Oral cancer and mice survival
In A, Gallic acid reduced the severity of 4NQO treatment in the histological grading ($p=0.006$). Scale bar represent 64 µm. The rate of mortality was significantly lower in in mice receiving 4NQO and GA than mice that received only 4NQO ($p<0.05$) in the Kaplan-Meiertes (B).

Figure 3 Effect of GA under the expected 4NQO in the animals' livers (A). Gallic acid alone did not cause hepatic tissue damage. Moreover, Gallic acid also reduced 4NQO liver toxicity ($p<0.05$). Similarly, in kidney Gallic acid also protected against 4NQO toxicity (B, $p<0.05$). Scale bar represent 10 µm.

Acknowledgments

This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). Dr. Guimarães, Dr. Santos and Dr. de Paula are research fellows of the CNPq. Dr. Farias is a research fellow of FAPEMIG.

Disclosure of Potential Conflicts of Interest: The authors deny any conflicts of interest related to this study.

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CONCLUSÕES

O objetivo principal deste trabalho foi avaliar o potencial antineoplásico da nanopartícula Alumínio Cloro Ftalocianina (AlClPc) e do Ácido Gálico (AG).
Os dois produtos propostos apresentaram resposta positiva nos experimentos até aqui realizados.

Ao serem avaliados os níveis de morte celular provocados pelo fotossensibilizante AlClPc, através da TFD, é possível inferir sobre a eficácia do procedimento proposto. Portanto, concluímos que a Terapia Fotodinâmica se mostrou eficiente no tratamento de OSCC ao ser realizada com a nanopartícula AlClPc.

Em nosso trabalho, o AG colaborou para que as lesões presentes nas línguas e fígado dos animais tivessem sua gravidade amenizada. Além disto, se mostrou eficiente na manutenção do peso e aparência dos animais, provocando considerável aumento de peso quando comparado com o grupo que recebeu apenas o carcinógeno. O Ácido Gálico apresentou capacidade de atenuar a gravidade das lesões apesar de não impedir a instalação das mesmas nas línguas dos animais tratados.

Estes resultados podem permitir que novos protocolos sejam desenvolvidos para estudos posteriores *in vivo*.

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ANEXOS

UNIVERSIDADE ESTADUAL DE MONTES CLAROS
COMISSÃO DE ÉTICA EM EXPERIMENTAÇÃO E BEM-ESTAR ANIMAL **Unimontes**

PARECER CONSUBSTANCIADO

Montes Claros, 19 de fevereiro de 2016.

Processo N. º **098**
Título do Projeto: **Avaliação da atividade antineoplásica de compostos metabólicos secundários em carcinoma de escamosas de boca: uma abordagem *in vivo***

Pesquisador responsável: **Dr. André Luiz Sena Guimarães**

Histórico

O carcinoma de células escamosas de boca (CCEB) é a neoplasia maligna mais frequente da região de cabeça e pescoço, representa um dos principais problemas de saúde pública. No Brasil é o sexto tipo de câncer mais prevalente, mas também em muitos países do mundo devido a sua alta mortalidade e constante mutilação. Evidências demonstraram que diversas alterações genéticas, epigenéticas e metabólicas estão associadas à patogênese desta neoplasia. Como consequência, o organismo perde o controle da proliferação celular sobre as células do CCEB. Como característica importante possui áreas hipóxicas, o que dificulta o efeito da radioterapia. Por isso, atualmente a combinação entre quimioterapia e radioterapia têm se mostrado necessário para o tratamento do CCEB. Porém, provoca mais efeitos colaterais nos pacientes com CCEB e apresenta custo elevado. Assim, faz-se necessário o desenvolvimento de pesquisas que busquem o desenvolvimento de novas estratégias terapêuticas. Estudos recentes demonstraram que o ácido gálico (AG) têm se mostrado agente terapêutico em potencial para o CCEB, uma vez que, apresenta propriedades antiproliferativa e citotóxica em vários tipos células neoplásicas

Mérito

O projeto justifica-se, por buscar uma nova abordagem de tratamento com o objetivo de amenizar o comportamento invasivo da doença. Objetivando avaliar a atividade antineoplásica dos metabólitos secundários: Ácido gálico, Metformina, Ácido Arjunólico (AA) e Ácido Betulínico (AB) no CCEB em modelos *in vivo*.

Parecer

A Comissão de Ética em Experimentação e Bem-Estar Animal da Unimontes analisou o processo **098** e entende que o protocolo de procedimentos preenche todos os requisitos éticos do CEEBEA/Unimontes enquadrando na categoria de **Aprovado**.

Prof. Orlando Raphael Lopasso Júnior
Presidente da Comissão de Ética em Experimentação
e Bem-Estar Animal da UNIMONTES