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Instituto de Ciência e Tecnologia

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APLICAÇÃO DO PLASMA DE BAIXA TEMPERATURA SOB PRESSÃO ATMOSFÉRICA COMO ADJUVANTE AO TRATAMENTO DA MUCOSITE ORAL

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APLICAÇÃO DO PLASMA DE BAIXA TEMPERATURA SOB PRESSÃO ATMOSFÉRICA COMO ADJUVANTE AO TRATAMENTO DA MUCOSITE ORAL

Tese apresentada ao Instituto de Ciência e Tecnologia, Universidade Estadual Paulista (Unesp), Campus de São José dos Campos, como parte dos requisitos para obtenção do título de DOUTORA, pelo Programa de Pós-Graduação em CIÊNCIAS APLICADAS À SAÚDE BUCAL.

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Orientadora: Profa. Dra. Tit. Cristiane Yumi Koga-Ito

Coorientador: Dra. Noala Vicensoto Moreira Milhan

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IMPACTO POTENCIAL DESTA PESQUISA

Tratamento oncológico na região de cabeça e pescoço é o 7º no ranking mundial, com impacto importante na saúde pública. A quimioterapia associada ou não à radioterapia causam efeitos adversos, tais como a mucosite oral, capaz de afetar significativamente a saúde global do paciente com prejuízos ao tratamento antineoplásico. A presente pesquisa visa a utilização do plasma de baixa temperatura sob pressão atmosférica como um tratamento adjuvante para o tratamento das lesões de mucosite oral, contribuindo com a qualidade de vida e saúde global dos pacientes em terapia antineoplásica.

POTENTIAL IMPACT OF THIS RESEARCH

Malignant neoplasias in the head and neck region is 7th in the world ranking, and have considerably impact in public health. Chemotherapy associated or not to radiotherapy have adverse effects, such as oral mucositis, that has impact on the patients' global health and can interfere negatively on the antineoplastic treatment. The present research aims to evaluate the low temperature atmospheric pressure plasma as an adjuvant treatment for oral mucositis, improving the quality of life and global health of the patients undergoing antineoplastic therapy.

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Madre Teresa de Calcutá

RESUMO

Sampaio AG. Aplicação do plasma de baixa temperatura sob pressão atmosférica como adjuvante ao tratamento da mucosite oral [tese]. São José dos Campos (SP): Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia; 2023.

A mucosite oral é um quadro clínico que acomete frequentemente pacientes sob terapia antineoplásica na região de cabeça e pescoço e caracteriza-se por ulcerações na mucosa que geram intensa dor local, odinofagia, aumento do risco de infecções, do uso de antibióticos e do tempo de hospitalização. A correlação entre mucosite oral, infecção fúngica e o potencial de disseminação fúngica sistêmica foi recentemente descrita. Apesar do impacto desse quadro clínico sobre a qualidade e tempo de vida dos pacientes oncológicos, não há consenso sobre a profilaxia e o protocolo terapêutico. O plasma de baixa temperatura sobre pressão atmosférica (LTAPP) apresenta efeito antimicrobiano, anti-inflamatório e reparador tecidual, o que sugere que possa ser promissor no tratamento da mucosite oral. Os objetivos gerais deste projeto foram divididos em dois subprojetos: 1) Definir os melhores parâmetros *in vitro* com efeito antifúngico e não tóxico e avaliar o LTAPP no tratamento de lesão de mucosite oral em modelo murino de mucosite por quimioterapia e 2) avaliar se o tratamento com LTAPP pode prevenir a disseminação fúngica sistêmica em ratos a partir de infecção experimental de lesões de mucosite oral por *Candida albicans*. Para tal, foram incluídos no estudo 100 ratos (*Rattus norvegicus*) com 90 a 100 dias de idade. No subprojeto 1, a lesão de mucosite oral foi induzida por administração de 5 fluorouracila (5-FU), enquanto no subprojeto 2, utilizou-se 5-FU associada à cisplatina ambas associadas à aplicação tópica de ácido acético 50%. Para o subprojeto 1, os animais foram randomicamente divididos em 2 grupos experimentais (n=30): a) Grupo mucosite; b) Grupo mucosite tratado com LTAPP, avaliados após 1, 5 e 12 dias do tratamento. Durante o período experimental, as lesões foram fotografadas e a gravidade da mucosite classificada por meio da atribuição de escores. Após a eutanásia e o processamento, os cortes histológicos corados por hematoxilina-eosina (HE) foram analisados microscopicamente. Para o subprojeto 2, o estudo de disseminação sistêmica fúngica nos grupos de mucosite infectada com *C. albicans* tratado ou não com LTAPP foi conduzido pelo isolamento fúngico a partir de amostras de sangue total e macerado dos órgãos. Para tanto foram estudados 2 grupos de ratos (n=20): c) Grupo mucosite infectado com *C. albicans* e d) Grupo mucosite infectado com *C. albicans* tratada com LTAPP, avaliados após 24 e 72 h do tratamento. Para ambos os projetos, o melhor parâmetro *in vitro* foi selecionado, isto é aquele com maior atividade antifúngica e baixa toxicidade. Dessa forma, as lesões foram expostas ao LTAPP de hélio por 5 min na distância de 1,5 cm na potência de 1 W. Os resultados *in vitro* mostraram que o LTAPP teve efeito antifúngico e baixa toxicidade para células de mamíferos. Os resultados *in vivo* mostraram que 5-FU afetou a saúde geral dos animais, evidenciada pela perda de peso corporal. Em ambos os grupos, houve reparo tecidual após 12 dias do tratamento, com resolução quase completa da lesão, o que foi corroborado pelos achados microscópicos. O grupo LTAPP exibiu uma tendência maior de redução da lesão, após 12 dias de tratamento. Além disso,

o LTAPP apresentou efeito inibitório sobre *C. albicans* após 5 minutos, de exposição, com redução da recuperação fúngica da língua após 24 h ($p<0.05$). A disseminação fúngica sistêmica foi reduzida significativamente após 24 e 72 h do tratamento. Com base nos resultados obtidos, conclui-se que o LTAPP é uma ferramenta promissora para futura aplicação clínica em pacientes com mucosite oral.

Palavras-chave: Mucosite, *Candida albicans;* Gases em plasma.

ABSTRACT

Sampaio AG. Application of low temperature atmospheric pressure plasma as an adjuvant in treatment oral mucositis [doctorate thesis]. São José dos Campos (SP): São Paulo State University (Unesp), Institute of Science and Technology; 2022.

Oral mucositis is a clinical condition that frequently affects patients undergoing antineoplastic therapy in the head and neck region and is characterized by mucosal ulcerations that generate intense local pain, odynophagia, increased risks of infections, use of antibiotics and the length of hospital stay. The correlation among oral mucositis, fungal infection and the potential for systemic fungal dissemination has recently been described. Despite the impact of this clinical condition on the quality and life expectancy of cancer patients, there is no consensus on prophylaxis and the therapeutic protocols. Low temperature atmospheric pressure plasma (LTAPP) has antimicrobial, anti-inflammatory and tissue repairing effects, which suggests that it can be promising in the treatment of oral mucositis. The general objectives of this project were divided into two subprojects: 1) Define the best antifungal and non-toxic in vitro parameters and to evaluate the application of LTAPP in the treatment of oral mucositis in murine model for chemotherapy, and 2) to evaluate whether treatment with LTAPP can prevent systemic fungal dissemination in rats from experimental infection of oral mucositis lesions by Candida albicans. A total of 100 rats (Rattus norvegicus) aged 90 to 100 days were included in the study. In subproject 1, oral mucositis lesion was induced by administration of only 5-fluorouracil (5-FU), while in subproject 2, administration and systemic administration of 5-FU associated with cisplatin, both associated with topical application of 50% acetic acid. For subproject 1, the animals were randomly divided into 2 experimental groups (n=30):a) Mucositis group and b) Mucositis group treated with LTAPP evaluated after 1, 5 and 12 days of treatment. During the experimental period, the lesions were photographed, and the severity of mucositis was classified into scores. After euthanasia and processing, the histological cuts stained by hematoxylin-eosin (HE) were analyzed. For subproject 2, the study of fungal systemic dissemination in groups of mucositis infected with C. albicans treated or not with LTAPP was conducted by fungal isolation from whole blood and macerated organs. Therefore, 2 groups of rats (n=20) were studied: c) Mucositis group infected with C. albicans and d) Mucositis group infected with C. albicans treated with LTAPP, evaluated after 24 and 72 h of treatment. For both subprojects, the best in vitro parameter was selected, that is, the one with the greatest antifungal effect and low toxicity. Thus, the lesions were exposed to helium LTAPP for 5 min at a distance of 1.5 cm at power of 1 W. In vitro results showed that LTAPP has an antifungal effect and low toxicity. In vivo results showed that 5-FU affected the general health of animals evidenced by body weight loss. In both groups, there was tissue repair after 12 days of treatment, with almost complete resolution of the lesion, which was corroborated by the microscopic findings. LTAPP group showed a greater trend of reduction of lesion, after 12 days of the treatment. Furthermore, LTAPP showed inhibitory effect on C. albicans after 5 min of exposition, with reduction in fungal recovery from the tongue after 24 h ($p<0.05$). Reduction in fungal dissemination was observed after 24 and 72 h of

LTAPP treatment ($p<0.05$). Based on the obtained results, it was concluded that LTAPP is a promising tool for future clinical application in patients with oral mucositis.

Keywords: Mucositis, Candida albicans, Non-thermal atmospheric pressure plasma.

SUMÁRIO

1 INTRODUÇÃO

Atualmente, neoplasias na região de cabeça e pescoço é a sétima mais frequente em todo o mundo (Gormley et al., 2022; Johnson et al., 2020; Sung et al., 2021). Estudos epidemiológicos mundiais relatam a incidência crescente de neoplasias malignas na região nasofaringe e orofaringe (Ng et al., 2018; Tangjaturonrasme et al., 2018). O aumento mundial da incidência e mortalidade causada pela doença tem sido relatado nos últimos anos, sendo responsável por mais de 660.000 novos casos e 325.000 óbitos por ano (Bosetti et al., 2020; Gormley et al., 2022; Johson et al., 2020, Sung et al., 2021, Xie et al., 2017). A Sociedade Americana de Oncologia Clínica (ASCO) estima a ocorrência de 66.920 novos diagnósticos e 15.400 mortes somente nos Estados Unidos em 2023 (ASCO et al., 2023). No Brasil, em 2020, ocorreram 6.192 óbitos e estima-se 15.100 novos diagnósticos de neoplasia maligna na cavidade bucal para cada ano do triênio de 2023-2025 (INCA et al., 2022; Santos et al., 2023).

A mucosite orofaríngea é uma complicação comum do tratamento antineoplásico que pode impactar negativamente no sucesso do tratamento do paciente oncológico (Villa,Sonis, 2015). Os dados de prevalência são inconsistentes e acredita-se que a subnotificação seja frequente (Villa,Sonis, 2015). No entanto, há relatos de incidência de 20 a 100%, dependendo do tipo e protocolo de tratamento, além de variáveis paciente-dependentes (Vanhoecke et al., 2015). Sua ocorrência afeta entre 20 a 80% dos pacientes em tratamento por quimioterapia, aumentando para até 100% para radioterapia associada ou não a quimioterapia, na região de cabeça e pescoço (Lalla et al., 2014b, Panahi et al., 2010; Kashiwazaki et al., 2012, Rubenstein et al., 2004;).

Esta condição debilitante ocorre habitualmente após o tratamento oncológico e está associada a toxicidade medicamentosa por quimioterápico e acumulativa por radioterapia (Lalla et al., 2008, Villa,Sonis, 2015). Inicia-se após 5-14 dias da administração de quimioterápico, com persistência por dias a semanas (Brown et al., 2020). Na radioterapia desenvolve-se geralmente na terceira semana do tratamento, podendo durar entre 7 a 98 dias (Maria et al., 2017).

A mucosite oral pode causar dor local e odinofagia, que leva ao comprometimento nutricional e a necessidade de alimentação nasogástrica e

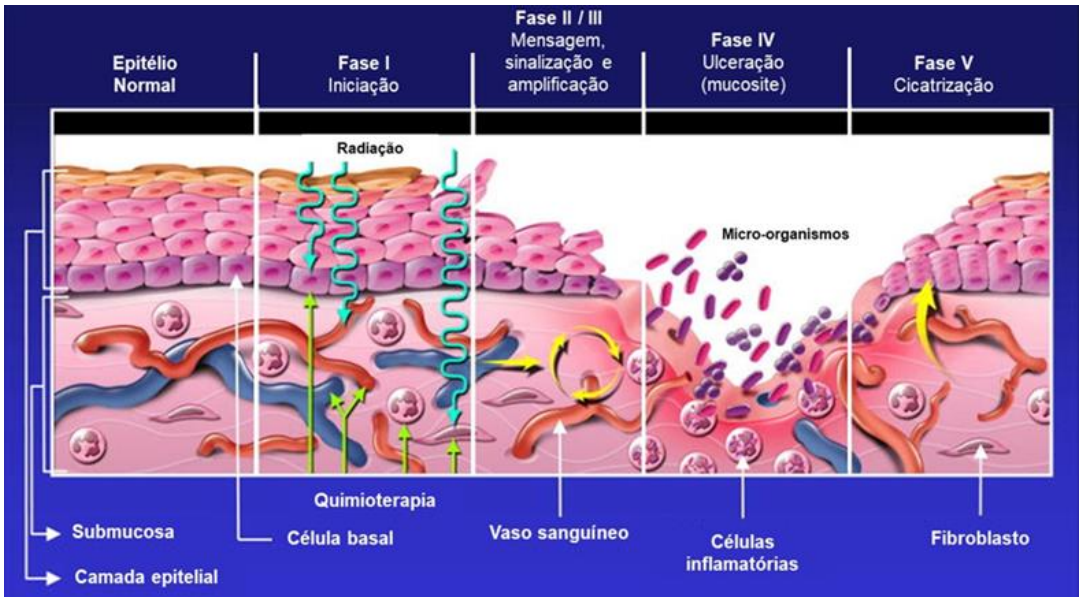
intravenosa. Também pode ocorrer dificuldade na higienização buccal, comprometendo a realização de uma escovação adequada, podendo ser intensificado com a ocorrência de xerostomia por comprometimento das glândulas salivares, impacto na qualidade de vida, alteração e interrupção da terapia anti-neoplásica, aumento do uso de analgésicos, hospitalização, risco de infecções e altos impactos econômicos (de Sanctis et al., 2019; Lalla et al., 2014a; Pereira et al., 2019; Peterson et al., 2015; Riley et al., 2016; Ruiz-Pérez et al., 2016). O efeito desse quadro clínico reduz significativamente a aderência dos pacientes ao tratamento antineoplásico, diminuindo as chances de sobrevivência (Riley et al., 2016). Cerca de 90% dos indivíduos apontam dificuldade ao ingerir água e alimentos, 85% perdem peso pela dificuldade durante a alimentação e até 1/3 dos indivíduos mencionaram dificuldade na fala (Riley et al., 2016; Yoshino et al., 2013).

A mucosite oral é caracterizada por eritema, lesões erosivas e ulceradas na região da mucosa bucal (Keef et al., 2007; Sonis et al., 2010; Treister,Sonis, 2007). O seu surgimento é consequência de um processo multifatorial de eventos biológicos que podem ser divididos em cinco etapas (Figura 1): iniciação, resposta de danos primários, extensão de sinal, ulceração e cicatrização (Yoshino et al., 2013). Durante o processo ocorre uma intensa relação entre a regulação celular de citocinas pró-inflamatórias, a lesão de células-tronco epiteliais basais e a microbiota bucal residente (Peterson et al., 2015).

As drogas anti-neoplásicas, tais como 5-fluorouracil (5-FU), cisplatina, oxiplatina, daunorrubina, ifosfamida, doxorrubicina, etoposídeo e citarabina usadas individualmente ou combinadas com outros quimioterápicos estão associadas ao desenvolvimento de mucosite oral de leve a grave, podendo variar com o modo de ação, dose e interação do tratamento (Curra et al., 2018; Suresh et al., 2010). A não especificidade às células cancerígenas por esses agentes quimioterápicos ocasionam a toxicidade que levam à perda da coesão celular e/ou alteração das células da camada basal do epitélio da cavidade bucal promovendo a formação de mucosite, que pode ocorrer em diferentes graus de severidade (Curra et al., 2018; Lima, 2011), variando de leve até grave (Peterson et al., 2015). Revisão sistemática relatou que poucos estudos avaliaram a relação entre a quimioterapia e severidade da mucosite oral em pacientes oncológicos (Curra et al., 2018), alertando para a gravidade dessa condição decorrente do uso de diferentes protocolos quimioterápicos e a necessidade de medidas preventivas. Pacientes sob terapia

mieloablativa que desenvolvem mucosite oral frequentemente necessitam de altas doses de morfina para alívio da dor (Walladbegi et al., 2018).

Figura 1: Etapas dos mecanismos de mucosite induzida por quimioterapia e radioterapia



Fonte: Adaptado de Lalla et al., 2008.

As regiões não queratinizadas, como a borda lateral da língua, mucosa jugal, soalho bucal e palato mole são os locais mais afetados (Lalla et al., 2008; Pereira et al., 2019; Stone et al., 2005). As injúrias na mucosa iniciam-se com discretas perdas de tecido da mucosa (Treister,Sonis, 2007) que posteriormente avançam para um evento celular e biológico complexo, tendo como alvo o epitélio (Treister,Sonis, 2007; Sonis, 2010). Acredita-se que os radicais livres da quimioterapia e a radioterapia atuam nas células de divisão rápida da camada basal do epitélio, impedindo o tecido de renovar-se, ocasionando a atrofia e ulceração da mucosa bucal (Lima, 2011). O processo inicia-se com danos diretos às células epiteliais basais e células do tecido conjuntivo. Estes danos resultam de efeitos diretos sobre o DNA ou outros efeitos celulares não associados ao DNA. Estes efeitos associados levam ao dano às células epiteliais que perdem a capacidade de renovação, tornando o epitélio mais fino e dando origem aos sinais e sintomas da mucosite. Os efeitos não associados ao DNA podem ser mediados por uma série de mecanismos, já que a radiação e a quimioterapia são ativadores efetivos de uma série de vias relacionados aos danos no endotélio, fibroblastos e epitélio (Sonis et al., 2004; Treister, Sonis, 2007).

O processo é facilitado por uma miríade de fatores locais e sistêmicos, como a expressão de citocinas pró-inflamatórias, a presença de micro-organismos e o infiltrado inflamatório (McGuire et al., 2002; Lalla et al., 2008; Lima, 2011; Sonis, et al., 2013). Durante o processo, sucessivos eventos envolvendo o aumento de citocinas pró-inflamatórias, principalmente de Fator de Necrose Tumoral- α (TNF-α) e interleucina-6 (IL-6), edema intercelular (Lima, 2011) e ativação da via NF-kB, podem ocasionar a morte celular, o que causa ulceração e inflamação. Sucessivamente, o infiltrado inflamatório aliado à ação de subprodutos da microbiota podem ocasionar a exposição do tecido conjuntivo e infecções secundárias, as quais podem aumentar a severidade da lesão. Por fim, ocorre a diferenciação celular e tecidual e proliferação celular epitelial que irão restaurar a integridade do epitélio lesionado (Lalla et al., 2008; Sonis, et al., 2013).

De Sanctis et al. (2019) apontam a falta de informação sobre a influência da microbiota residente no desenvolvimento da mucosite oral. A mucosite é geralmente auto-limitante quando não ocorrem complicações relacionadas à infecção. No entanto, a incidência de infecção no curso do tratamento é muito elevada (Vanhoecke et al., 2015). A entrada de patógenos por meio de ulcerações de mucosite oral pode causar infecção sistêmica que pode ser letal, principalmente em pacientes imunocomprometidos (Peterson et al., 2015; Riley et al., 2016). É importante ressaltar que a boca é considerada uma porta de entrada para infecções sistêmicas em pacientes imunocomprometidos (Senpuku et al., 2003; Yoshino et al., 2013). Indivíduos com neoplasias orais demonstram uma microbiota mais diversificada do que em indivíduos saudáveis (Gadge et al., 2019), podendo apresentar uma maior carga fúngica oral (Berkovits et al., 2016; Vadovics et al., 2021). Podem ainda apresentar elevada colonização fúngica na superfície epitelial neoplásica comparada a superfícies saudáveis adjacentes (Vadovics et al., 2021).

Dentro deste contexto, é importante destacar que a associação entre a mucosite oral e a prevalência de candidose oral pode variar de 17,9 a 50% dos casos, em pacientes oncológicos (Pereira et al., 2019). Em estudo longitudinal de pacientes com câncer sob quimioterapia com 5-fluorouracil ou doxorrubicina, Diaz et al. (2019) observaram que a candidose oral geralmente ocorre simultaneamente ou antes da mucosite.

Katagiri et al. (2018) observaram, em modelo murino, que a ocorrência de infecção por *Candida* em animais com mucosite oral induzida pode culminar com

fungemia. A ocorrência de fungemia foi verificada por hemoculturas e análises histológicas, comprovando a correlação entre mucosite oral, fungemia e o potencial de disseminação fúngica sistêmica. Este estudo chama atenção para a gravidade deste quadro clínico, que transpassa as ocorrências locais e podem levar a graves quadros de sepse nestes pacientes já imunocomprometidos.

Apesar de sua elevada frequência e graves consequências tanto locais quanto sistêmicas descritas, a falta de intervenções efetivas têm sido frustrantes para pacientes e cuidadores (Villa,Sonis, 2015). Paralelamente, medidas paliativas de prevenção e/ ou tratamento da mucosite bucal são uma necessidade médica significativa para minimizar o desconforto, aliviar os sintomas, acelerar o reparo tecidual e controlar as infecções dos pacientes oncológicos (Lalla et al., 2014a; Peterson et al., 2015).

Não há ainda um protocolo de consenso para profilaxia e tratamento da mucosite oral (Moslemi et al., 2016). Os diferentes critérios e protocolos para a avaliação dos resultados, aliada a baixa uniformidade nos resultados, nos estudos sobre mucosite bucal dificultam o consenso (Daugėlaitė et al., 2019). Os procedimentos adotados oferecem o alívio dos sintomas e controle de eventuais infecções, o que pode influenciar no reparo tecidual (Curra et al., 2018).

Atualmente, cuidados orais tradicionais, considerados paliativos, são recomendados para o tratamento dessa complicação oral (Elad et al., 2020; de Sanctis et al., 2019). Contudo, uma revisão sistemática mostrou que a clorexidina não foi efetiva na redução da severidade da mucosite, nem na prevenção da incidência de complicação da mucosite oral (Cardona et al., 2017). Além disso, a higiene oral individual não foi considerada um método eficaz para reduzir a gravidade da mucosite oral (Daugėlaitė et al., 2019; Yokota et al., 2016). O uso de enxaguatório bucal a base de cloridrato de benzidamina reduziu a gravidade da mucosite em pacientes tratados com radioterapia, contudo o mesmo efeito não foi observado em pacientes sob quimioterapia (Rastogi et al., 2017). Bioterapia com pastilhas de *Lactobacillus brevis* CD2 durante o percurso da radioterapia, também não demonstrou efeito significativo, comparado com o controle utilizando bicarbonato de sódio como enxaguatório bucal (de Sanctis et al., 2019). A aplicação de mel, zinco, glutamina e vitamina E tópica tem mostrado efeito positivo (Thomsen,Vitetta, 2018). A aplicação de Onchung-eum, uma prescrição herbal composta por 8 ervas, também mostrou efeitos positivos em animais (Park et al., 2018), necessitando

evidências baseadas em investigações clínicas. A crioterapia também apresentou efeitos positivos no tratamento e prevenção, contudo acredita-se que o efeito frio causado pelo tratamento reduz o fluxo sanguíneo o que pode reduzir a eficácia do tratamento da doença primária (Daugėlaitė et al., 2019). A fotobiomodulação como a laserterapia de baixa intensidade tem sido utilizada em estudo clínicos atuais em aplicações conjuntas com tratamentos antimicrobianos/ fotossensibilizadores ou isoladamente e tem apresentado ação positiva na prevenção e tratamento da mucosite oral (Daugėlaitė et al., 2019; Pires et al., 2020), porém a variação dos parâmetros por fotobiomoduladores e os dados indisponíveis da dosimetria limitam a consistência dos resultados para a definição de protocolos clínicos ideais (Kauark-Fontes et al., 2023).

O plasma é considerado o quarto estado da matéria e é composto por um conjunto de átomos, moléculas, íons fixos e livres gerados em diversas densidades e temperaturas, ou seja, é uma energia gerada por elétrons, a qual é responsável pelos seus efeitos (McCombs,Darby, 2010). O plasma de baixa temperatura sob pressão atmosférica (LTAPP) opera vantajosamente próximo à temperatura ambiente (McCombs,Darby, 2010) e gera um produto limpo, sem resíduos tóxicos aos fatores bióticos do biossistema. O equipamento consiste basicamente em três unidades, uma unidade responsável pela corrente direta, outra geradora de gás e uma seguinte por emitir o jato de plasma através de uma ponta de um eletrodo central por um capilar através do fluxo de gás projetado para fora do fluxo (McCombs,Darby, 2010; Weltmann, von Woedtke, 2011).

O plasma apresenta atividade antimicrobiana associada a alguns fatores, em particular as espécies reativas de oxigênio e nitrogênio (ROS e RNS) (Laroussi, Leipold, 2004; McCombs,Darby, 2010). Pesquisas demonstram que o plasma através dos mecanismos de colisão iônica gera espécies reativas com significativa interação plasma-superfície, as quais têm ação direta sobre as células microbianas (Kolb et al., 2008).

O tratamento com plasma de baixa temperatura sob pressão atmosférica (LTAPP) tem demonstrado efeito promissor em diversas aplicações médicas como no reparo de feridas, assepsia, doenças cutâneas, neoplasias e na área odontológica (Kostov et al., 2015; Lee et al., 2015; Park et al., 2014; Weltmann, von Woedtke, 2011). Von Woedtke et al. (2019) ressaltaram os efeitos promissores do plasma na área médica, os quais abrangem o efeito antimicrobiano frente a um

amplo espectro de micro-organismos, incluindo patógenos multirresistentes, eficácia em estimular a proliferação celular e angiogênese após tratamentos curtos e de baixa intensidade. Estudos *in vivo* em animais e humanos têm demonstrado eficácia do LTAPP no tratamento de feridas agudas, comprovando o efeito de estimulação do reparo tecidual (Von Woedtke et al., 2019). No entanto, os mesmos autores salientam a carência de pesquisas em feridas infectadas.

Estudos anteriores apontam para uma aplicação extremamente promissora do LTAPP em pressão atmosférica no tratamento de infecções superficiais causadas por leveduras do gênero *Candida*. A ação antifúngica frente *C. albicans* e o efeito modulatório sobre fatores de virulência, como adesão e filamentação, foram relatados (Borges et al., 2018; Yamazaki et al., 2011). Há relato sobre o efeito inibitório frente a células planctônicas de *C. albicans* após exposição de 150 segundos (Borges et al., 2018). Sun et al. (2012) relataram diminuição significativa das concentrações inibitórias mínimas de anfotericina B, fluconazol e caspofungina frente a espécies de *Candida* após exposição a plasma em pressão atmosférica. Em estudo *in vivo* utilizando modelo murino, a ação inibitória de invasão tecidual e anti-inflamatória também foi observada (Borges et al., 2018). O efeito do LTAPP sobre outras espécies fúngicas, como *Trychophyton rubrum* (Borges et al., 2019; Shapourzadeh et al., 2016) também foi relatado.

Ainda, há evidências de que o LTAPP, gerado a partir do hélio, pode contribuir na inibição da cascata inflamatória e auxiliar na reparação local com efeito na migração e proliferação de fibroblastos (Brun et al., 2014). Recentemente, relatou-se a ação positiva do LTAPP na comunicação intercelular de queratinócitos e fibroblastos, o que pode também contribuir para o reparo tecidual (Shome et al., 2020).

Estudos anteriores dos efeitos do LTAPP no reparo de feridas em pele podem trazer alguns dados interessantes que podem guiar esta investigação. Observou-se que o LTAPP altera significativamente o fator Nrf2 (*nuclear fator erythroid 2-related factor* 2), que é um dos principais envolvidos na transcrição de genes que codificam enzimas antioxidantes, além da modulação da resposta imune e inflamatória (Ahmed et al., 2017). O efeito do LTAPP sobre a via do Nrf2 foi comprovado por várias análises como *microarray*, cromatografia líquida, espectrometria de massas e análise de citocinas (Privat-Maldonado et al., 2019). Este efeito acelerou o reparo de feridas e promoveu uma resolução mais rápida de

feridas em pele devido ao aumento da proliferação basal e migração celular (Arndt et al., 2013; Schmidt et al., 2017; Schmidt et al., 2019). Observou-se que a sua expressão foi aumentada em 200 vezes em feridas de pele tratadas com LTAPP (Schmidt et al., 2019). O LTAPP também mostrou aumento na expressão dos genes HMOX1, cujo produto HO-1 é uma enzima relacionada à reposta ao dano em DNA, proliferação celular e senescência celular (Hedblom et al., 2019). Efeito sobre o NQO1, que é uma das duas maiores quinonas redutases dos sistemas dos mamíferos e tem múltiplas funções na adaptação celular ao estresse, também foi observado (Ross,Siegel, 2017).

Assim, considerando que i) não existe protocolo de consenso para profilaxia e tratamento da mucosite bucal; ii) lesões de mucosite infectadas por *C. albicans* podem ser porta de entrada para infecções sistêmicas; iii) LTAPP possui efeitos anti-inflamatórios, reparador tecidual e antifúngico e iv) não foram detectados estudos sobre a aplicação do LTAPP no controle de lesões de mucosite oral até o presente momento, esse estudo visa avaliar o potencial do LTAPP no tratamento da mucosite oral induzida por quimioterapia, em duas vertentes: nos efeitos do tratamento local de lesões de mucosite, sem associação com *C. albicans* e na prevenção da disseminação fúngica em lesões de mucosite oral associadas à candidose.

2 ARTIGO(S)

2.1 Artigo 1 – Aline da Graça Sampaio, Noala Vicensoto Moreira Milhan, Konstantin Georgiev Kostov and Cristiane Yumi Koga-Ito. Plasma de baixa temperatura sob pressão atmosférica para o tratamento de mucosite induzida por quimioterápico / *Low temperature atmospheric pressure plasma in the treatment of chemotherapy-induced mucositis**

RESUMO

A mucosite oral é uma reação adversa do tratamento antineoplásico com quimioterápicos causada pela toxicidade inespecífica do fármaco. Até o momento, não há consenso clínico para a prevenção e tratamento da mucosite, sendo que medidas paliativas são geralmente adotadas. O plasma de baixa temperatura em pressão atmosférica (LTAPP) apresenta efeito anti-inflamatório, antimicrobiano e indutor de reparo tecidual, propriedades que podem ser úteis para o tratamento da mucosite oral. Desta forma, o objetivo deste estudo foi investigar o potencial do LTAPP para o tratamento de lesões de mucosite oral induzida por quimioterápico em modelo animal. Inicialmente, os parâmetros do LTAPP com maior efeito antifúngico e melhor toxicidade para queratinócitos e fibroblastos foram determinados *in vitro*. O jato de LTAPP produzido a partir de hélio foi avaliado em 2 diferentes potências e nas distâncias de 1.5 e 2 cm e tratamentos por 1 a 5 min. Sessenta ratos Wistar foram divididos em dois grupos: mucosite tratada com LTAPP e controle negativo não tratado. Três subgrupos foram avaliados, de acordo com o período de acompanhamento pós-tratamento (1, 5 e 12 dias). Os ratos receberam 3 doses intercaladas de quimioterápico 5 fluorouracil (5-FU), seguido de indução química da mucosite oral por ácido acético na região do fórnice vestibular. O LTAPP foi aplicado em 2 dias consecutivos por 5 min. O acompanhamento da evolução clínica das lesões e a análise histopatológica foram realizados. Para a avaliação clínica foram utilizados escores de 0 a 5 (leve a grave). Para análise histopatológica, os tecidos foram preparados conforme rotina para análise por microscopia óptica (hematoxilina e eosina). Os animais foram pesados diariamente. Os dados foram analisados quanto à distribuição normal normalidade e comparados estatisticamente e o nível de significância adotado foi de 5%. Os resultados *in vitro* mostraram que o LTAPP teve efeito antifúngico e baixa toxicidade para células de mamíferos. Houve redução no peso dos animais após a administração de 5-FU, seguido de ganho de peso. A recuperação do peso inicial ocorreu no 17º dia no LTAPP e 19º dia no grupo controle. Ambos os grupos apresentaram lesões de mucosite oral de grau severo logo após a indução. O grupo LTAPP apresentou maior escore inicial (severo) e menor escore final (leve), embora não tenha sido detectada diferença significativa entre os grupos ($p > 0.05$). A avaliação histológica confirmou os achados clínicos, apontando para a resolução quase completa da lesão no último período de análise. Conclui-se que houve tendência maior de redução das lesões de mucosite oral após tratamento com LTAPP.

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Palavras-chave: mucosite; gases em plasma; cicatrização.

ABSTRACT

Oral mucositis is an adverse effect of the antineoplastic treatment due to the nonspecific toxicity of the chemotherapy drugs. There is no clinical consensus for the prevention and treatment of oral mucositis so far, and palliative measures are usually adopted. The low temperature atmospheric pressure plasma (LTAPP) shows anti-inflammatory, antimicrobial and tissue repair induction effects, and these properties can be useful for the treatment of oral mucositis. Thus, the aim of this study was to investigate the potential of LTAPP for the treatment of oral mucositis lesion induced by chemotherapy in an animal model. Firstly, the parameters of LTAPP with the best antifungal effect and lowest toxicity to keratinocytes and fibroblasts were determined in vitro. The LTAPP jet generated from helium was evaluated in 2 differents powers and distances 1.5 and 2 cm and treatment for 1 to 5 min. Sixty rats were divided into two groups: mucositis treated with LTAPP and non-treated control. Three subgroups were evaluated according to the period of post-treatment follow up (1, 5 and 12 days). The rats received 3 interspersed doses of chemotherapy 5-fluorouracil (5-FU), followed by chemical induction of mucositis with acetic acid on vestibular fornix region. LTAPP was applied on 2 consecutive days for 5 min. The clinical follow up of the lesions and histopathological analyses were performed. For the clinical assessment, clinical scores ranging from 0 to 5 (mild to severe) were used. For histopathological analysis, the tissue was routinely prepared to light microscopy analysis (hematoxylin-eosin staining). Data was tested for normality and compared statistically, with the level of significance of 5%. The results in vitro showed LTAPP had antifungal effect and low toxicity for mammalian cells. There was reduction in animals' body weight after administration of 5-FU, followed by weight gain. The recovery of the initial weight occurred on the 17th day in LTAPP treated group and on the 19th day in the control group. The results in vitro showed LTAPP. Both groups showed severe grade of oral mucositis lesions after induction that were reduced over time. The LTAPP group presented a higher initial score (severe) and lower final score (mild), although statistical differences were not detected between the groups ($p>0.05$). Histological evaluation confirmed the clinical findings showing an almost complete healing of the lesion in the last period of analysis. It could be concluded that there was a greater tendency a reduction in oral mucositis lesions after treatment with LTAPP.

Keywords: mucositis; non-thermal atmospheric pressure plasma; healing.

Low temperature atmospheric pressure plasma for the treatment of chemotherapy-induced mucositis

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Abstract: Oral mucositis is an adverse effect of the antineoplastic treatment due to the nonspecific toxicity of the chemotherapy drugs. There is no clinical consensus for the prevention and treatment of oral mucositis so far, and palliative measures are usually adopted. The low temperature atmospheric pressure plasma (LTAPP) shows anti-inflammatory, antimicrobial and tissue repair induction effects, and these properties can be useful for the treatment of oral mucositis. Thus, the aim of this study was to investigate the potential of LTAPP for the treatment of oral mucositis lesion induced by chemotherapy in an animal model. Firstly, the parameters of LTAPP with the best antifungal effect and lowest toxicity to keratinocytes and fibroblasts were determined *in vitro*. The LTAPP jet generated from helium was evaluated in 2 differents powers and distances 1.5 and 2 cm and treatment for 1 to 5 min. Sixty rats were divided into two groups: mucositis treated with LTAPP and non-treated control. Three subgroups were evaluated according to the period of post-treatment follow up (1, 5 and 12 days). The rats received 3 interspersed doses of chemotherapy 5-fluorouracil (5-FU), followed by chemical induction of mucositis with acetic acid on vestibular fornix region. LTAPP was applied on 2 consecutive days for 5 min. The clinical follow up of the lesions and histopathological analyses were performed. For the clinical assessment, clinical scores ranging from 0 to 5 (mild to severe) were used. For histopathological analysis, the tissue was routinely prepared to light microscopy analysis (hematoxylin-eosin staining). Data was tested for normality and compared statistically, with the level of significance of 5%. The results *in vitro* showed LTAPP had antifungal effect and low toxicity for mammalian cells. There was reduction in animals' body weight after administration of 5-FU, followed by weight gain. The recovery of the initial weight occurred on the 17th day in LTAPP treated group and on the 19th day in the control group. The results *in vitro* showed LTAPP. Both groups showed severe grade of oral mucositis lesions after induction that were reduced over time. The LTAPP group presented a higher initial score (severe) and lower final score (mild), although statistical differences were not detected between the groups ($p>0.05$). Histological evaluation confirmed the clinical findings showing an almost complete healing of the lesion in the last period of analysis. It could be concluded that there was a greater tendency a reduction in oral mucositis lesions after treatment with LTAPP.

Keywords: Mucositis 1; Non-thermal atmospheric pressure plasma 2; Healing 3

1. Introduction

Cancer in the head and neck region is a global public health problem that reaches a prevalence of 1.1 million cases annually [1]. Antineoplastic treatment, with chemotherapy or in combination with radiotherapy, causes toxicity capable of causing several side effects, including the emergence of oropharyngeal mucositis [2]. Epidemiological data highlights that around 20-80% of patients undergoing cancer treatment are affected by oropharyngeal mucositis [3–5].

Oral mucositis is an erosive and/or ulcerated lesion [6], caused by inflammatory and ulcerative disorders during cancer treatment and usually occurs between 7 and 10 days of therapy [7]. It causes cause pain, discomfort when talking, poor oral hygiene, dysphagia, weight loss, changes in taste and impairment of the salivary glands [8]. These conditions can lead to the use of opioid analgesics and/or interruption of the antineoplastic treatment [9,10]. Furthermore, the risk of infection is also an imminent concern [11,12], as the mouth is considered a gateway for systemic infections in immunocompromised patients [13,14].

Damaged tissues caused by oral mucositis represent an additional gateway for bacterial and fungal entry into oral connective tissue, which can lead to systemic infections, increasing the risk of death. [15–18]. The correlation among cases of fungemia, systemic infection and its lethality has already been reported [19–21]. Therefore, the search for treatments that can prevent or reduce the severity of mucositis and control symptoms, avoiding the interruption or change in the antineoplastic therapy, is extremely important [10,22].

Despite the severity and frequency of mucositis at both local and systemic levels, the lack of totally effective interventions can be frustrating for patients [23]. Therapies that involve mitigating measures and/or treatment can minimize discomfort, alleviate symptoms caused by the wound, in addition to accelerating tissue repair [8,12]. Traditional oral care is currently recommended to control the disease, which includes tooth brushing, flossing, mouthwashes, hydration and lubrication with moisturizing agents, and mouthwashes with saline solution, sodium bicarbonate and chlorhexidine [24]. Studies on the effects of photobiomodulation and photodynamic therapies, such as low-intensity laser therapy used in clinical trials during chemotherapy have demonstrated results in reducing and preventing the severity of mucositis [3,24,25]. Marques et al. [25] tested antimicrobial photodynamic therapy and observed improvements in signs and symptoms. However, a clinical study that evaluated the effectiveness of methods to the control of mucositis detected a lack of uniformity in the results among patients under the same treatment or preventive measure [3].

Low temperature atmospheric pressure plasma is considered the fourth state of matter which consists of an electrical discharge over a noble gas, generating molecules, atoms, electrons, and ions, capable of manufacturing various chemical reactions, called reactive oxygen and nitrogen species (RONS) [26–29]. Several studies have highlighted the promising effect of LTAPP on wound healing. LTAPP has demonstrated broad-spectrum antimicrobial effects [30–32] (Borges et al., 2018; Oliveira

et al., 2018; Lima et al., 2021) *in vitro* and *in vivo* studies. The action of plasma on wound healing has also been reported in the literature [33]. The application of plasma in wound studies has demonstrated positive correlations in reducing microbial load, promoting wound repairs without side effects and reactions to adjacent healthy tissues [34–36]. Study carried out by Kubinova et al. [37] showed a significant reduction in the wound area after treatment with LTAPP in an *in vivo* model of chronic wound.

It is important to highlight that the plasma device, the working gas, the parameters of the equipment and the treatment regimen used are preponderant factors for therapeutic success [38]. Biomedical investigations have reported the importance of hydrogen peroxide (H_2O_2), superoxide (O^{2-}) and nitric oxide (NO) species in antimicrobial action and wound healing [39–42]. These components showed potential to treat infections, promote angiogenesis, increase fibroblast proliferation, act on the differentiation and the migration of keratinocytes, and increase collagen synthesis [38]. However, few studies have been carried out on oral mucosa and no previous study on the effects of LTAPP in chemotherapy-induced oral mucositis was detected so far. In this context, this study aimed to evaluate the effects of low temperature atmospheric pressure plasma in the healing of chemotherapy-induced oral mucositis.

2. Materials and Methods

2.1. Low-temperature atmospheric pressure plasma (LTAPP) source

Low temperature atmospheric pressure plasma jet equipment (LTAPP) was composed by a bivolt electrical circuit (110 / 220 V) inside a cylinder (11 x 7 cm, length and width), containing a high-frequency electrode with a homemade pulsed voltage source. The system was fed with helium gas with 99.9% purity (He, Air Liquide, Brazil) and a controlled flow rate of 2.0 ± 0.1 SLM by a digital controller (N100 Horiba STEC).

2.2. Determination of the effective physical parameters of LTAPP

2.2.1. Determination of the parameters with antifungal effect

Candida albicans (ATCC 18804), stored in Sabouraud dextrose broth (SD) containing 20% glycerol at -80 °C, was reactivated in Sabouraud dextrose agar. Plates were incubated at 37 °C for 24 h, under aerobic condition. Subsequently, a standardized fungal suspension containing 10^6 cells/ml was prepared in sterile saline solution (0.9% NaCl) using a spectrophotometer (λ=530 nm; O.D.= 0.138) (AJX 3000 PC, AJ Micronal).

The evaluation of the antifungal activity of the plasma jet was based on the methodology proposed by Alkawareek et al. [43]. The variables plasma jet power, distance from the tip of plasma generator device and the surface of the agar, and exposure time were standardized to obtain the maximum antifungal effect with low cytotoxicity for mammal cells. Petri dishes containing standardized sterile Sabouraud dextrose agar in a volume of 15 mL were prepared and an aliquot of 100 µL of the fungal suspension was plated using a sterile swab. The plates were kept in a laminar flow cabinet for 15 min at room temperature for drying. Then, equidistant points on the plate were exposed to the plasma jet at different power (plasma discharges of the order of 0.3 W and 1.0 W), time (1, 2, 3 and 5 min) and distance (1.5 and 2.0 cm). After treatment,

the plates were incubated in an incubator at 37 °C for 24 h. To evaluate antifungal activity, the diameters of the inhibition areas (mm) were measured, and the area of the inhibition zone (mm^2) was calculated. The experiments were performed in triplicate at two independent times (n = 6). The control group was exposed to the helium gas flow, without ignition.

To compensate the electrical differences between *in vitro* and *in vivo* model [44], a steel metal support with the same diameter as the Petri dish was used in the experiments (**Figure 1**).

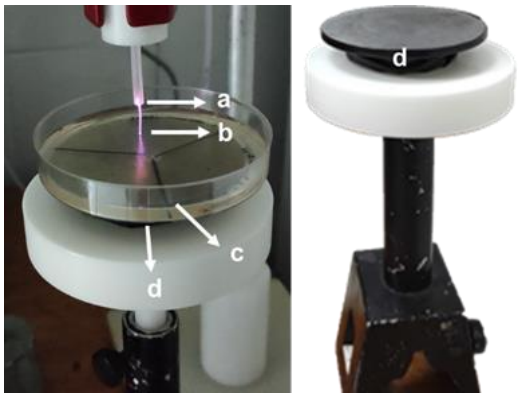

Figure 1. Experimental set used for the *in vitro* experiments. (a) nozzle, (b) plasma jet, (c) the surface of agar, and (d) the metal support.

2.2.2. Evaluation of the toxicity to mammal cells of the protocol with antifungal effect

Normal oral keratinocytes (NOK) and fibroblasts (3T3) were used for the experiments. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin (100 U/mL)/streptomycin (100 mg/mL), and maintained at 37°C and 5% CO_2. For the assay, the cells were seeded at a density of $8x10^3$ cells per well in 96-well plates and incubated 24 h to allow the cell adhesion. The cells were then exposed to the most effective parameter of LTAPP regarding antifungal activity. The amount of 30 µl of Hanks' Balanced Salt Solution (HBSS) was added to the wells to prevent their drying out during plasma exposure. Afterwards, the cells were incubated at 37 ºC for 24 h. To measure cell viability, 100 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to the wells. After 1 h, formazan crystals were dissolved with Dimethyl Sulfoxide (DMSO). The resulting optical density of the solution was obtained using a spectrophotometer at 570 nm. Absorbance data were normalized to the untreated control group (= 100%). Two independent experiments were carried out with six replicates each (n=12). The cytotoxicity threshold was set at 70%, according to ISO 10993-5.

2.3. Effect of helium LTAPP jet on the association of oral mucositis and candidiasis induced an in vivo model

2.3.1. Animals

Sixty male rats (*Rathus norvegicus*), aged 90 days, were maintained in usual housing conditions, with controlled temperature (20-22 °C), light (light/dark cycle – 12 h/each), food and water ad libitum, throughout the trial period. Prior to the test, all the animals received a single oral dose of a multipurpose dewormer (albendazole – Nova

Química, SP, Brazil) and a multivitamin (Vitagold – Fabiani, SP, Brazil) for 15 days. The animals in the control group (no treatment) and treated group (LTAPP) were randomly divided according the follow up period 1 day, 5 days and 12 days after the plasma treatment (corresponding at day 11, 15 and 22 of the experiment; n = 10).

This study was approved by the Ethics Committee on the Use of Animals (CEUA) under registration number 16/2019.

2.3.2. Experimental

The methodology for inducing chemotherapy-induced oral mucositis lesion was carried out according to Lima et al. [45]. Initially, each group received 30 mg/kg of 5-fluorouracil (5-FU – Libbs, SP, Brazil), intramuscularly, for 3 alternate days. Subsequently, on the 8th day, the animals were anesthetized with ketamine (95 mg/kg - Ceva, SP, Brazil) associated with xylazine (10 mg/kg - Ceva, SP, Brazil) to induce of mucosa ulcers. The delimitation of the mucosal ulcers was carried out with a sterile paper filter, measuring 9 mm^2, soaked in 10 μL of 50% glacial acetic acid (C_2H_3COOH), in the region of the vestibular fornix, for a period of 60 seconds, followed by washing with physiological solution (Ever Care, SP, Brazil), to reduce the chemical action of acetic acid.

For analgesia, 200 mg/kg of sodium dipyrone (Medley, SP, Brazil) were administered every 24 h for 3 subsequent days and ground food and water were provided *ad libitum*. Group treated with LTAPP was anesthetized and treated in the central region of the vestibular fornix with plasma for 5 min, at a distance of 1.5 cm and plasma generated at power of 1 W. The treatment was carried out on two consecutive days (9th and 10th days). Prior to the treatment, the area was humidified with 100 μL aliquot of sterile saline (NaCl 0.9%) to avoid dryness. The animals were then sacrificed (days 11, 15 and 22) using a triple dose of anesthetics and the vestibular fornix region was removed. The region of mucositis was photographed at a standardized distance of 18 cm in height between the camera and the animal support apparatus. The grade of oral mucositis presented per group over time was evaluated from a score pre-determined in literature (adapted from Sonis et al.,[18]). A quarter (1/4) of the tissue (top left side) was removed for histological analysis. The weight of the animals was also monitored in alternate days. **Figure 2** summarizes the steps of the experiment.

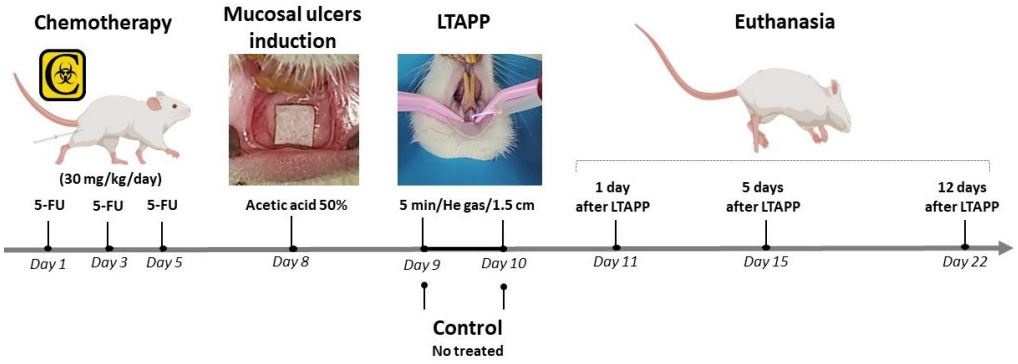

Figure 2. Experimental steps of the study on the effects of low temperature atmospheric pressure plasma on chemotherapy-induced oral mucositis lesion.

2.4. Clinical follow up of the oral mucositis lesions

The lesions were photographically monitored based on methodology used by Chen et al., [46]. The photos were taken at 11, 15 and 22 days, corresponding to periods of 1, 5 and 12 days after the last

session of treatment with LTAPP. The severity of oral mucositis and its healing was based on an adapted methodology used by Sonis et al., 2004 (**Table 1**). The follow up of the oral mucositis lesions evaluated the severity, that was classified using clinical scores that ranged from 0 to 5 (mild to severe). The analyzes of the images and designation of the scores in a metric panel was done by a single blind expert.

Table 1. Oral mucositis grades and their descriptions (Adapted from Sonis et al., [18]).

Grade	Severity	Description
0		Normal (no abnormalities).
1	Mild	No evidence of mucosal erosion (completely intact mucosa) with partial hyperemia, erythema or swelling.
2	Mild	Superficial erosion/mild sloughing, and/or overall hyperemia, erythema or swelling.
3	Moderate	Moderate mucositis characterized by frank ulcer formation (≤ 25% of vestibular fornix of lower incisors). Ulcers can present pseudomembrane formation. Hyperemia, erythema, swelling and/or epidermolysis are observed.
4	Severe	Ulcer formation affecting 25% to 90% of vestibular fornix of lower incisors. Pseudomembrane formation covers partially or totally the ulcers. Marked bleeding, erythema and/or epidermolysis are seen.
5	Severe	Extensive ulceration of the mucosa (>90%), with pseudomembrane and/or bleeding in the entire lesion area.

2.5. Microscopic analysis

After 3, 7, and 14 of mucosal ulcers induction (corresponding at day 11, 15 and 22 of the experiment), the animals were anesthetized and subsequently euthanized. The area of vestibular fornix containing the region of oral mucositis were fixed in 4% paraformaldehyde (Sigma Aldrich Chemical - Saint Louis, MO, USA) for 48 h, and subsequently processed and included in Paraplast™. Three semi-serial histological sections were then made (5 μm of thick) after which the slides were stained with hematoxylin-eosin and analyzed using light microscopy.

The characteristics of the lining epithelium, as well as the underlying connective and muscular tissue were thoroughly analyzed, with the aim of verifying the changes resulting from mucositis and the evolution of tissue repair in the groups with and without treatment.

2.6. Statistical analyses

The results obtained were analyzed using Graphpad prism v8.0 software (Graphpad Software Inc. CA, USA). Shapiro Wilk normality test was performed to evaluate the normal distribution of results. Subsequently, Man-Whitney test was performed to analyze the antifungal activity and intergroup evolution of the severity of oral

mucositis (scores). One-way ANOVA, ad hoc Kruskal-Wallis test was used to analyze the intragroup score of oral mucositis. The results are presented as mean and standard deviation (mean ± SD) or standard error median (SEM). A significance level of 5% was adopted for all the tests.

3. Results

3.1. Determination of the effective physical parameters of LTAPP

3.1.1. Antifungal activity

C. albicans exhibited different susceptibilities to the plasma jet produced under the different parameters tested. The areas of inhibition had positive correlation with the distance, power, and period of exposure (**Figure 3**).

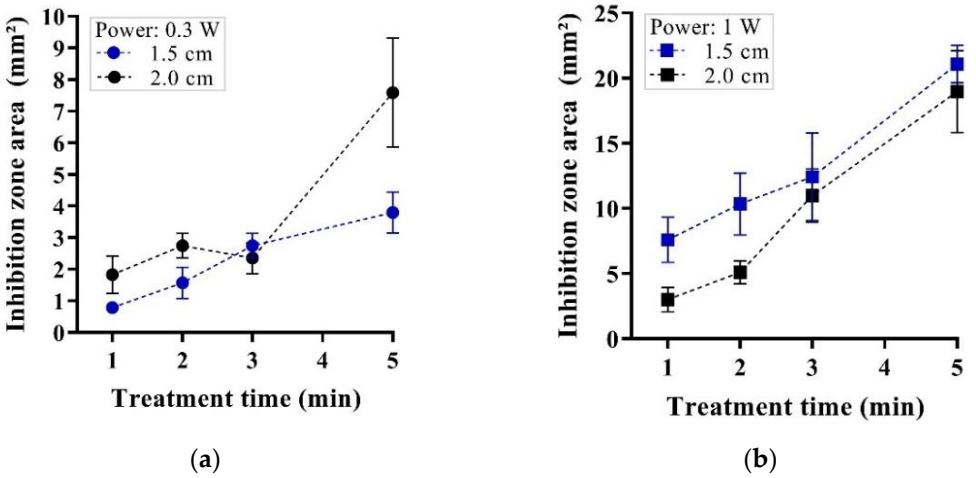

Figure 3. Mean and standard deviation values of the areas of the inhibition zones of *C. albicans* after exposition to the plasma jet at different distances from plasma device nozzle to the surface of the agar, powers and periods of exposure. In (a) different parameters under a 0.3 W power and (b) different parameters under a 1 W power condition.

An increase in the inhibition zones was observed over the time of plasma jet exposure at both distances and applied powers. However, higher antifungal activity was observed in when a power of 1 W and distance of 1.5 cm were adopted (Figure 3). After 1 minute of exposure, the areas of the inhibition zone decreased from 7.59 ± 1.72 to 3.00 ± 2.30 mm^2 when the distance was changed from 1.5 to 2.0 cm. After 5 min of exposure, values of 21.06 ± 1.44 and 18.97 ± 3.14 mm^2 were observed to 1.5 and 2.0 cm, respectively. When plasma was applied at a distance of 2.0 cm for 5 min, the growth of some fungal colonies inside the inhibition zone was observed. This effect was not observed when the distance of 1.5 cm was adopted (**Figure 4**). The control group (only gas without ignition) did not show the formation of inhibition zone at all the tested parameters.

A significant statistical difference was observed ($p = 0.0022$, Mann Whitney test) in the comparative analysis between the two parameters with higher antifungal activity, at 5 min (power 0.3 W and distance 2 cm compared to power 1 W and distance 1.5 cm). The parameter with power of 1 W and distance of 1.5 cm was the most effective at 5 min.

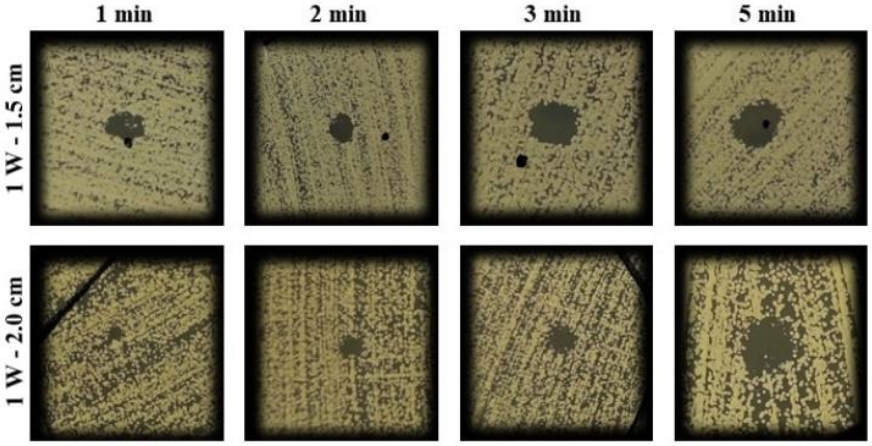

Figure 4. Zones of inhibition of *Candida albicans* after plasma jet exposure produced under power of 1 W and different distances over time.

3.1.2. Evaluation of the toxicity to mammalian cells of the protocol of LTAPP with antifungal effect

The protocol of LTAPP with the best antifungal effect (distance of 1.5 cm and exposure time of 5 min) was evaluated regarding the toxicity to fibroblasts and keratinocytes. Plasma treatment reduced the viability of both fibroblast and keratinocyte to 74.26 and 74.55%, respectively (figure 5). Considering that the cytotoxicity threshold adopted was 70%, the LTAPP was considered non-toxic.

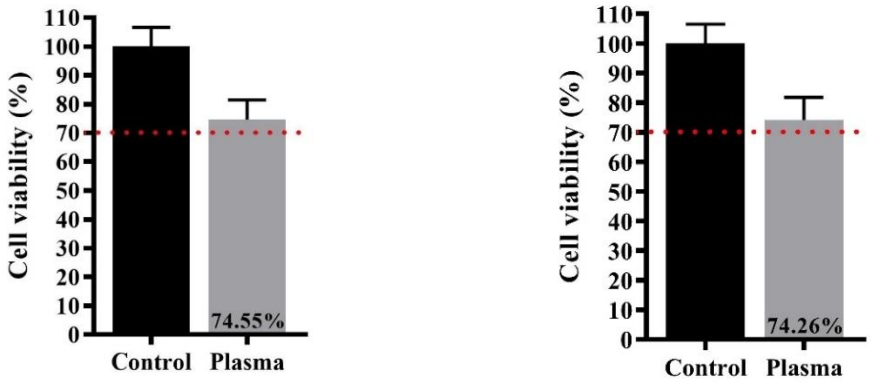

Figure 5. Percentage of cell viability obtained with MTT assay after 5 min of plasma jet exposure for (a) fibroblasts and (b) keratinocytes. Values are expressed as mean and standard deviation (mean ± SD). The dashed line indicates the cutoff point between non-toxic (≥ 70%) and toxic (≤ 70%) responses according to ISO 10.993-5 (n = 12 per group).

3.2. Action of the LTAPP jet in an in vivo model of induced oral mucositis

3.2.1. Body weight

The global health of animals was analyzed through body weight. Weight alterations were observed during the treatment. There was weight loss from the second day of the chemotherapy for both groups until the 7th day. The mean weight varied from 370.57 ± 38.08 to 333.68 ± 33.48 for the control group and from 345.70 ± 7.50 to 319.88 ± 5.85 for treated group. The treated animals showed a linear gain of weight from

days 11 to 17. Control group showed a non-linear gain of weight, with recuperation of initial weight on the 19th day.

3.2.2. Clinical findings

The photos for clinical assessment were obtained at days 11, 15 and 22, corresponding to periods of 1, 5 and 12 days after the last session of treatment with LTAPP.

The oral mucositis lesions in the fornix region were diagnosed on the 1st day, after the treatment, by ulcerated areas, covered by extensive pseudomembrane and purulent content. The lesions showed irregular edges, sometimes extending into gingival areas. The mucosa was inflamed with some spontaneous bleeding. In some animals, the ulcerations were more extensive compared to others, mainly in the LTAPP group. On the 5th day, the ulcerated areas covered by pseudomembrane were reduced in both groups, as well as minor inflammatory changes were observed compared to the previous period. On the 12th day, there was a marked improvement of the clinical signs in both groups, especially in LTAPP group. However, some areas of erosion and/or mild swelling were still observed in most animals (**Figure 6**).

Figure 6. Images of chemotherapy-induced oral mucositis lesion representing the control group and plasma treated group.

3.2.3. Follow up of the oral mucositis lesions

The evaluation of chemotherapy-induced oral mucositis lesions in rats treated or not with LTAPP was based on clinical scores. Induction of mucositis by 5-FU chemotherapy associated with 50% acetic acid induced severe mucositis lesion (**Figure 7**) in the animals. On the 11st day (1 day after the last session of LTAPP treatment), mean scores of 4.8 and 4.3 (**Table 2**) for LTAPP and control group were detected, respectively. On the 15th day (5 days after treatment), a low reduction was observed, and the groups showed similar mean scores of 3.9 for control and 4.0 for LTAPP group. A marked reduction in the score was observed after 12 days of treatment (22nd day) for both groups, mainly for the treated group. The final mean score for treated group was 2.7, while the control group presented a mean final score of 3.0. No statistically significant difference was observed between the groups in the intergroup analysis.

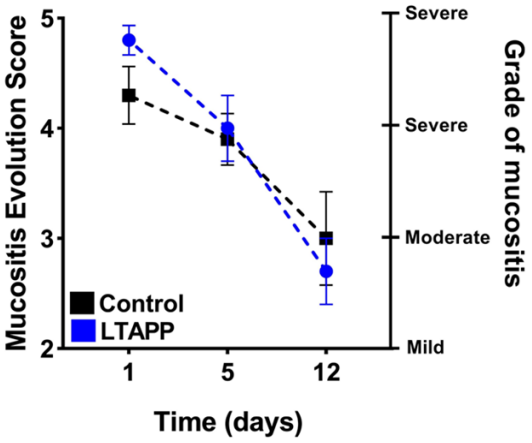

Figure 7. Mean oral mucositis scores on the 1st, 5th and 7th days after treatment with helium plasma and the control (untreated). Mean and standard error mean (mean ± SEM) data are presented.

When the values of scores noted on the 1st and 12nd days were compared intragroup, a significant reduction in the overall severity of oral mucositis lesions was detected for treated group ($p = 0.0001$) and control group ($p = 0.0395$).

Table 2. Mean scores of oral mucositis severity in rats treated with LTAPP and non-treated group.

Groups	Score		
	1 day	5 days	12 days
Control	4.3 ± 0.82	3.9 ± 0.73	3.0 ± 1.19
LTAPP	4.8 ± 0.42	4.0 ± 0.94	2.7 ± 0.94

3.4. Histopathological analysis

Below are descriptive analyses of the most frequent histological findings in the segment selected for microscopic evaluation. The characteristics of the groups will be addressed according to the period of analysis, with emphasis on tissue changes resulting from mucositis and the evolution of the repair, in the absence and presence of treatment.

3.4.1. 3 days post- mucositis induction and 1 day after the last LTAPP session

In the control group (**Figure 8**), most of the oral mucosa fragments were partially covered by keratinized stratified squamous epithelium, with an area of ulceration. The ulcer area was covered by a fibrinopurulent pseudomembrane, characterized by the presence of necrotic tissue, fibrin, and bacterial colonies. In some cases, the area of ulceration extended to the depth of the fragment, compromising the underlying connective tissue, as well as structures into this tissue, such as nerve bundles. The remaining epithelium showed areas of either atrophy or hyperplasia of the epithelium, in addition to areas of hydropic degeneration, spongiosis, hyperplasia and hyperchromasia of the basal layer, acanthosis, hyperkeratosis and sometimes mild exocytosis, especially close to the ulcerated area.

The connective tissue, which was predominantly loose, exhibited prominent edema, foci of coagulative necrosis, congested small and medium-sized blood vessels, areas of hemorrhage and mixed inflammatory infiltrate that varied in composition and intensity, depending on the region. In the superficial lamina propria, close to the ulcer, the inflammation, which varied from moderate to intense, was predominantly polymorphonuclear. In the deepest plane of the lamina propria, a mixed inflammatory infiltrate predominantly mononuclear of mild to moderate frequency was observed.

In fragments with significant inflammation extending to depth, there was disorganization of the interface between connective tissue and muscle layer. The bundles of muscle fibers cut transversely and longitudinally showed areas of cellular atrophy and sometimes degeneration, in addition to mild or moderate inflammation in the stroma. The skin appendages were preserved, with the absence or only a scarce presence of inflammatory cells in the skin area.

In the plasma group (**Figure 8**), part of the ulcerated fragments was covered by a fibrinopurulent pseudomembrane, and the other part by superficial blood clots, which were more prominent in this group. In most part of the fragments, epithelial lining was observed only in the skin area, while in others there was a remaining epithelium in the mucosal area, with areas of hyperplasia of the basal layer, acanthosis, hydropic degeneration, spongiosis and hyperkeratosis.

In this group, areas of necrosis were also observed, sometimes extending to the depth of the fragment, in addition to foci of coagulative necrosis in the lamina propria. Generalized edema was present in most fragments. The inflammatory infiltrate maintained a similar pattern to the control group. It was mixed with a greater number of polymorphonuclear cells on the surface and a predominance of mononuclear cells in depth, although in fragments with extensive tissue destruction, polymorphonuclear cells were frequent in deeper layers.

In the muscular layer, a similar pattern to the untreated group was also observed. Thus, there was mild to moderate inflammatory infiltrate, areas of degeneration and myocyte atrophy.

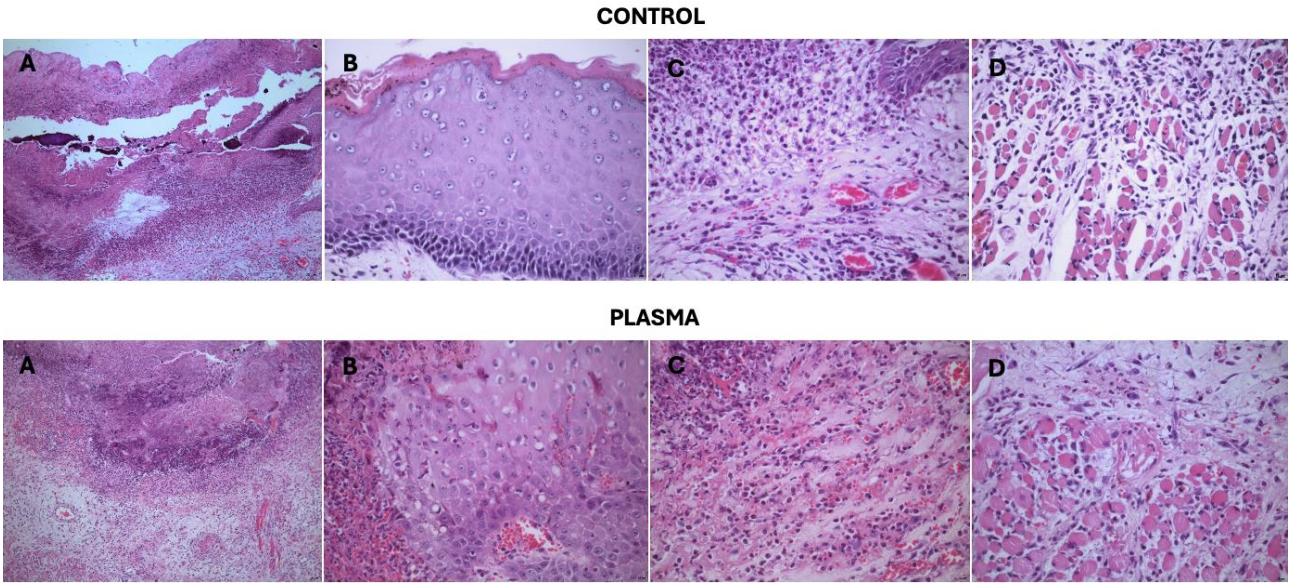

Figure 8. Photomicrograph of the vestibular fornix of lower incisors in a rat from control and plasma group, 3 days after induction of mucositis. (A) Extensive

fibrinopurulent pseudomembrane in the ulcer area. (B) Epithelium adjacent to the ulcerated area with architectural changes due to inflammation. (C) Predominantly polymorphonuclear inflammatory infiltrate in the superficial area of the lamina propria with some congested blood vessels, hemorrhage, edema, and tissue necrosis. Fibrin deposition is also evident in plasma group. (D) Disorganization of the interface between connective tissue and muscle, in the presence of diffuse mixed inflammatory infiltrate and atrophy of the muscle bundles in both groups and degeneration of muscle bundles in the plasma group. Hematoxylin and Eosin staining, original magnification of 100x (A) and 400x (B, C, D).

3.4.2. 7 days post-mucositis induction and 5 days after the last LTAPP session

During this period, the control group (**Figure 9**) presented fragments of mucosa that were sometimes completely covered by keratinized stratified squamous epithelium and other times partially ulcerated. The ulceration areas were still covered by a fibrinopurulent pseudomembrane, as in the previous period. The fully covered fragments exhibited areas of atrophy and straightening of the basal layer, and re-epithelialization could be confirmed by the association of clinical and histological findings. The epithelium, that totally or partially covered the surface, also exhibited areas of hydropic degeneration, spongiosis, acanthosis, hyperplasia and hyperchromasia of the basal layer, and exocytosis.

The connective tissue underlying the ulcer or epithelial area showed areas of liquefactive necrosis, represented by hyaline segments. Edema, congested vessels and inflammatory infiltrate, with predominance of polymorphonuclear cells on the surface and mononuclear ones in the depth of the lamina propria, were still prominent in most of the fragments. Despite this, newly formed connective tissue, also called granulation tissue, rich in capillaries and blood vessels, endothelial cells, fibroblasts and mononuclear inflammatory cells, was already present permeating areas of necrosis.

The muscular layer still showed a pattern of inflammation ranging from mild to moderate, with parenchyma showing areas of atrophy and degeneration. The cutaneous appendages were preserved, as in the previous period.

In the plasma group (**Figure 9**), most of the fragments were partially covered by epithelium, with the ulcerated region still covered by a fibrinopurulent pseudomembrane, sometimes less thick. Approximately 20% of the fragments were completely reepithelialized. Areas of atrophy and straightening of the basal layer were observed in both partially and fully covered fragments, with a clinical correlation indicating re-epithelialization. Areas of hydropic degeneration, spongiosis, hyperplasia of epithelium, hyperkeratosis and mild exocytosis were also observed.

The connective tissue exhibited edema, congested blood vessels and moderate mixed inflammatory infiltrate. In the depth of the fragments, this inflammation was predominantly mononuclear, and sometimes showed less intensity. There was newly formed connective tissue or granulation tissue that was present in a higher amount in some fragments, previously compromised by a larger area of necrosis. In the superficial planes of the lamina propria, the granulation tissue permeated areas of liquefactive necrosis.

The muscle fiber bundles exhibited areas of atrophy and degeneration with mild diffuse inflammation, although greater intensity inflammation was observed in some fragments. The skin's appendages remained preserved.

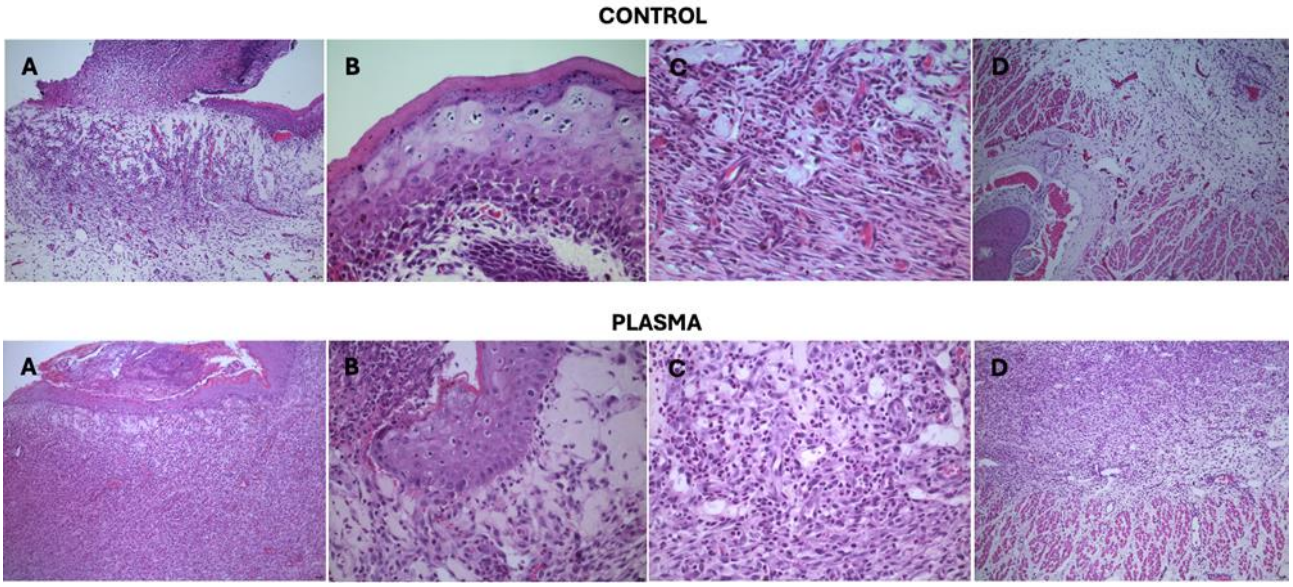

Figure 9. Photomicrograph of the buccal fornix of lower incisors in a rat from control and plasma group, 7 days after induction of mucositis. (A) Fibrinopurulent pseudomembrane detaching from an ulcerated area in control group and reepithelialized area in plasma group. In the control group, it is possible to see the migration of the epithelium to the ulcerated area, characterizing the re-epithelialization process. (B) Epithelium with architectural changes due to inflammation. In plasma group there is a subepithelial area of liquefy active necrosis. (C) Granulation tissue, intensely cellularized and rich in blood capillaries and blood vessels, filling areas of necrosis. (D) Deeper plane of the lamina propria showing edema and predominantly mononuclear inflammation. Underlying this area is the muscular layer with atrophic myocytes and mild inflammation. Hematoxylin and Eosin staining, original magnification of 100x (A, D) and 400x (B, C).

3.4.3. 14 days post-mucositis induction and 12 days after the last LTAPP session

In the control group (**Figure 10**), the fragments were completely or almost completely re-epithelialized. Architectural changes of the epithelium were scarcer than in previous periods. Reepithelialized areas exhibited atrophy and/or straightening of the basal layer, while adjacent epithelium showed areas with some degree of hyperplasia and hyperchromasia of basal cells, spongiosis and mild exocytosis.

The lamina propria presented connective tissue that showed variability in its maturation in different fragments and sometimes within the same fragment. Some fragments presented connective tissue that was mostly mature and consequently rich in thick collagen fibers. Others displayed areas of loose connective tissue with edema, with a variable amount of collagen fibers or fibrils arranged in different orientations. Areas of granulation tissue were still observed in some fragments in the subepithelial portion. A greater number of vascular-nervous bundles were observed in this period, when compared to previous ones. A

predominantly diffuse mononuclear inflammatory infiltrate was still observed, although during this period the inflammation was predominantly mild.

The bundles of muscle fibers, sometimes still atrophied, exhibited absence of inflammation or a mild mononuclear inflammatory infiltrate, which in some fragments was concentrated only in specific areas. The cutaneous appendages were completely preserved.

In the plasma group (**Figure 10**), most of the fragments were also completely or almost completely re-epithelialized. The regenerated epithelium exhibited some areas of atrophy and/or straightening of the basal layer. Some degree of hyperplasia and hyperchromasia of the basal layer, hydropic degeneration and mild exocytosis could be seen in some fragments, although in general the epithelial architecture was predominantly preserved.

The lamina propria showed areas of granulation tissue in most of the fragments, mainly in the subepithelial portion, sometimes extending deeper. In other areas, there was lower cellularity, edema, and loose connective tissue with collagen fibrils arranged in different orientations, with a predominantly mononuclear, mild and diffuse inflammation. In some regions it was possible to observe thicker collagen fibers. A greater number of vascular-nervous bundles was found in this period, compared to previous ones.

The muscle tissue, which still showed some areas of parenchymal atrophy, presented mostly discrete or absent mononuclear inflammation. The skin's appendages remained preserved.

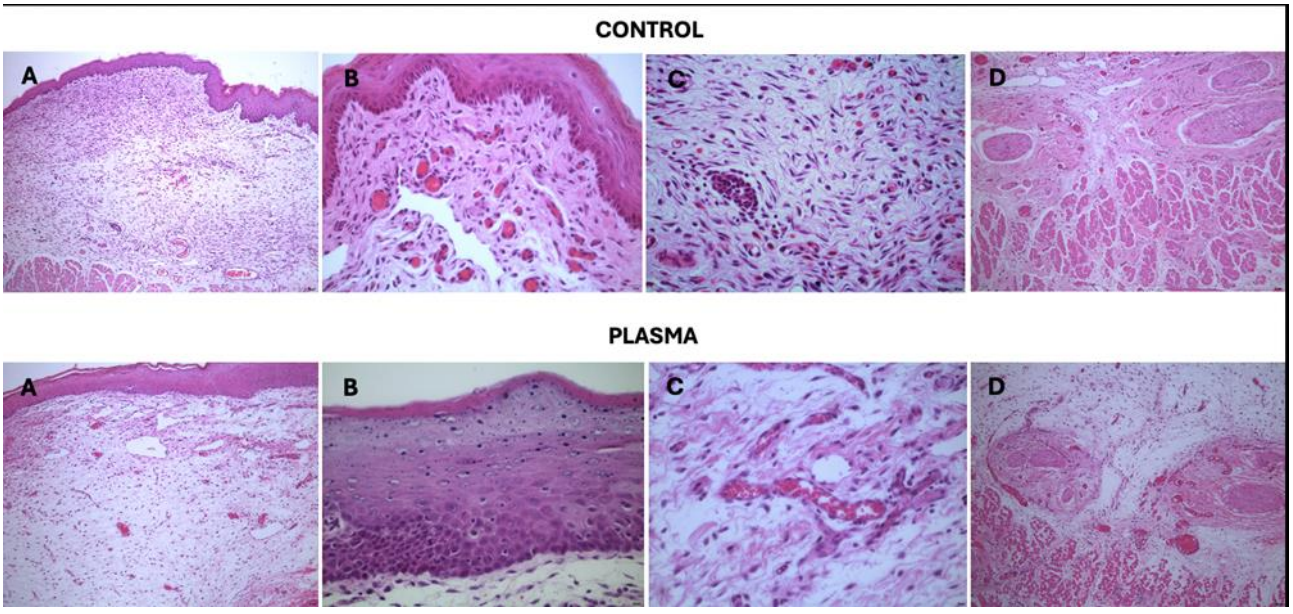

Figure 10. Photomicrograph of the buccal fornix of lower incisors in rats from control and plasma group, 14 days after induction of mucositis. (A) The mucositis region is completely reepithelialized in these fragments. In the lamina propria there are edema and congested blood vessels, in a stroma more cellularized in the control group. (B) Newly formed epithelium. In control group the subepithelial area is composed by thick collagen fibers with some congested blood vessels. (C) Deeper plane of the lamina propria showing loose connective tissue with collagen fibrils arranged in various orientations, some degree of edema and mild inflammation. Note some areas of thick collagen fibers and congested blood vessels in plasma group. (D) Interface between connective tissue and muscle, where prominent vascular-nervous bundles and a muscular

layer with slight inflammation can be seen. Hematoxylin and Eosin staining, original magnification of 100x (A and D) and 400x (B, C).

4. Discussion

To our knowledge, this is the first study that investigated the effect of helium low temperature pressure plasma (He-LTAPP) on the healing of chemotherapy-induced oral mucositis by evaluating the repair of buccal fornix mucosa. Our findings indicate that this treatment allows healing over time and tends to reduce the severity of oral mucositis.

Before performing the *in vivo* study, we have confirmed *in vitro* that LTAPP presents anti-*Candida* activity when produced at power 1 W, distance of 1.5 cm and exposure time of 5 min, parameters chosen for the subsequently *in vivo* study. Previous studies with *C. albicans* have also demonstrated the inhibitory effect generated by helium-LTAPP [30,47,48] at the same time and exposure distance [30,47]. The inactivation of *C. albicans* by LTAPP has been associated with the action of reactive oxygen and nitrogen (RONS) species generated in plasma production, such as O and N atoms and hydrogen peroxide[49]. The oxidative stress caused by the RONS has been highlighted as important elements against fungal cells [50].

The toxicity effect of LTAPP was also investigated *in vitro* in our study. It was previously described that depending on the dose, LTAPP can cause toxicity or apoptosis to fibroblasts and keratinocytes [51]. In our study, 5 min of plasma exposure was not cytotoxic to oral fibroblasts and keratinocytes, with more than 70% of cell viability. This value of cell viability is in accordance with the recommendations for medical devices [52], which indicated that the adopted parameters were safe for further analysis. Additionally, in a previous study with similar conditions of distance, gas and time, no cytotoxicity to Vero epithelial cells was observed after LTAPP exposure, with 86.33% of cell viability. After validation of the most effective antifungal condition, without cytotoxicity to oral cells, the *in vivo* experiments were carried out [53].

The methodology for inducing oral mucositis by 5 fluorouracil (5-FU) chemotherapy associated with acetic acid was successfully carried out in our study. It was validated by detection of oral mucositis lesions that were observed in the animals after 3 days of the mucositis induction. Lima et al., [45] performed a similar methodology and reported the observation of ulcerated areas covered by extensive pseudomembranes in the mucosa of the vestibular fornix. Technically, there are challenges involving the induction of mucositis in an *in vivo* model due to the numerous protocols described in the literature, in addition to various chemotherapy drugs, doses, time of treatment, natural healing time of the model, different models and regions of the oral mucosa where the lesions are induced. Therefore, for this study we chose to use a methodology already described in the literature which could provide a perspective to the evolution of mucositis in the same animal model.

A mild and gradual weight loss was observed after the start of chemotherapy. In the same way, a linear weight gain for the group treated with plasma, with recovery of initial weight on the 17th day was observed. Control group recovered the initial weight 2 days later. The adverse effect of weight loss caused by chemotherapy in animal models evaluating mucositis has been previously reported in the literature [54–57]. Mild initial weight loss was also observed by Sonis et al., [58]. In our

study, the linear and earlier weight recovery in the plasma group could suggest better general health in this group.

The follow up of the mucositis lesions by the attribution of scores allowed us to verify the effect of LTAPP on clinical repair. The methodology of choice was based on a model that did not include symptomatic criteria, since we have used an animal model. Clinical grades of mucositis can involve, in addition to wound analysis, symptomatic and subjective information related to pain and feeding [59,60].

Although severe mucositis was observed in both groups on the 1st day after the treatment, the treated group showed a higher clinical score when compared to non-treated group. Interestingly, 12 days after the last LTAPP session, treated group showed significant reduction of scores. On the other hand, there was no difference in intergroup analysis. The clinical findings were corroborated by the histologic evaluation where more fragments showed total loss of the mucosal epithelium and higher amount of superficial blood clots in the treated group one day after treatment (corresponding to the 3 days after the induction of mucositis). Additionally, in this period, extensive ulcerations covered by fibrinopurulent pseudomembrane and moderate to high degree of inflammation were observed in both groups, as previously observed by Lima et al., [45]. The clinical signs of ulcer reduction could also be confirmed by the histologic reepitelization already observed in different degrees on the 15th day (5 days after the treatment and referent to 7 days after mucositis induction), in addition to the presence of granulation tissue replacing previously ulcerated areas.

On the 22nd day (12 days after the treatment and corresponding to 14 days after mucositis induction), most of the fragments were totally reepithelialized. The inflammation in the connective tissue was predominantly mild and a greater number of vascular-nervous bundles were generally observed. The connective tissue was predominantly loose, and it still presented areas of edema, however there were thicker collagen fibers indicating the maturation of the tissue in both groups. These findings are in accordance with severity reduction observed in the clinical evaluation on the 22nd day (12 days after treatment and corresponding to 14 days after induction mucositis). It is worth mentioning that only part of the fragment was used for histological analysis, which means that this fragment does not represent the entire lesion as the clinical assessment.

Lima et al. [45] observed complete tissue healing 21 days after induction of oral mucositis with mature connective tissue in all the groups, while almost complete healing was observed after 16 days post mucositis induction in another study [54]. We did not evaluate longer periods, which can be considered a limitation of this study. However, the significant repair demonstrated clinically and histologically indicates that a complete healing, with no erosion or swelling, would occur in some days. Considering that the plasma group presented a worse initial clinical score, with more serious histological findings, the evolution to a lower score at the end is a promising clinical finding.

Future studies with the same and other LTAPP parameters should be performed to validate LTAPP as a possible treatment to oral mucositis. Besides, other protocols including more treatment sessions can also improve the clinical response. In the same way, the association of LTAPP with other therapies and/or antimicrobial agents such as

photobiomodulation should be investigated since they can potentialize their individual effects.

5. Conclusions

The helium low temperature pressure plasma tended to reduce the severity of oral mucositis. This may be an alternative treatment for chemotherapy-induced oral mucositis lesion. Future *in vivo* and *in vitro* studies are necessary to optimize parameters, such as the exposure time and number of treatment sessions, and to obtain optimal effects of this treatment alone or associated with already used therapies.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee on the Use of Animals (CEUA) under registration number 16/2019 (CEUA – UNESP / ICT).

Informed Consent Statement: Not applicable.

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Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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2.2 Artigo 2 – Aline da Graça Sampaio, Noala Vicensoto Moreira Milhan, Konstantin Kostov and Cristiane Yumi Koga-Ito. Efeito do plasma de baixa temperatura sob pressão atmosférica na prevenção de fungemia associada à mucosite oral induzida em modelo murino sob quimioterapia / *Effect of low temperature atmospheric pressure plasma for the prevention of fungemia associated with oral mucositis induced in a murine model under chemotherapy

RESUMO

A fungemia é um problema de saúde pública preocupante em pacientes oncológicos em terapia antineoplásica. A ocorrência de mucosite oral associada com a candidose oral pode ser uma das causas de candidemia. O plasma de baixa temperatura sob pressão atmosférica (LTAPP) é composto por espécies reativas de oxigênio e nitrogênio (ERONS) que apresentam efeitos antifúngico e anti-inflamatório, com baixa toxicidade para células humanas. O objetivo deste estudo foi avaliar o efeito clínico e hematológico do LTAPP na prevenção da disseminação fúngica sistêmica em modelo murino de quimioterapia com indução de lesão de mucosite oral associada à candidose bucal. Quarenta ratos Wistar foram divididos em grupo controle (não tratado) e tratado com LTAPP. Subgrupos de animais foram analisados após 24 e 72 h de tratamento (n = 10 por grupo). Os animais foram submetidos a 4 ciclos de quimioterapia com cisplatina (dia 1) e 5-fluorouracil (5-FU; 4 dias consecutivos), seguido de inoculação oral com *C. albicans* no 2º, 3º e 5º dia. A lesão de mucosite foi induzida na região lateral da língua com solução de ácido acético 50%. O tratamento com LTAPP foi realizado na região central da lesão por 5 min em dois dias consecutivos (4º e 5º). Os animais foram pesados diariamente. Para avaliação da disseminação fúngica sistêmica, os órgãos (língua, pulmão, fígado, rim e baço) foram removidos, bisseccionados, macerados e semeados em ágar Sabouraud com cloranfenicol para contagem fúngica. Hemograma completo foi obtido a partir de amostra de sangue obtida por punção cardíaca. O grupo controle não tratado foi incluído para fins comparativos. Os resultados *in vivo* mostraram que 5-FU afetou a saúde geral dos animais, evidenciada pela perda de peso corporal. O LTAPP mostrou efeito inibitório sobre *C. albicans* depois de 5 min com redução significativa na recuperação fúngica da língua depois de 24 h ($p<0.05$). A disseminação fúngica sistêmica foi reduzida significativamente após 24 e 72 h do tratamento ($p<0.05$). Baseado nos resultados obtidos, conclui-se que o LTAPP é uma ferramenta promissora para aplicação clínica futura em pacientes com mucosite oral, redução da ocorrência de candidemia.

Palavras-chave: Mucosite; Fungemia; Gases em plasma.

ABSTRACT

Fungemia is a public health problem of concern in cancer patients under antineoplastic therapy. The occurrence of oral mucositis associated with oral candidiasis can be one of the causes of systemic candidemia. Low temperature

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atmospheric pressure plasma (LTAPP) is composed by reactive oxygen and nitrogen species (RONS) and has antifungal and anti-inflammatory effects. The objective of this study was to evaluate the clinical and hematological effect of LTAPP in preventing systemic fungal dissemination in a murine model of chemotherapy, in which oral mucositis lesion associated with candidiasis was induced. Forty Wistar rats were divided into control (not-treated) and LTAPP group. Subgroups of the animals were analyzed after 24 and 72 h of treatment (n=10 per group). The animals were submitted to 4 cycles of chemotherapy with cisplatin (1st day) and 5-fluorouracil (5-FU; 4 consecutive days), followed by oral inoculation of C. albicans on the 2nd, 3rd and 5th day. Mucositis lesion on the lateral area of the tongue was induced with a solution of 50% acetic acid. LTAPP treatment was performed in the central region of the lesion for 5 min in two consecutive days (4th and 5th). Body weight was assessed daily. To evaluate the fungal dissemination, the organs (tongue, lung, liver, kidney and spleen) were removed, bisected, macerated and plated on Sabouraud agar with chloramphenicol for fungal counting. Blood sample was obtained by cardiac puncture and a complete blood count test was performed. A non-treated control group was included for comparative purposes. The results in vivo showed that 5-FU affected the general health of the animals, evidenced by the loss of body weight. LTAPP showed inhibitory effect on C. albicans after 5 min, with significant reduction in fungal recovery from the tongue after 24 h ($p<0.05$). Reduction in fungal dissemination was observed after 24 and 72 h of LTAPP treatment. Systemic fungal dissemination was significantly reduced after 24 and 72 h of treatment ($p<0.05$). Based on the obtained results, it was concluded that LTAPP is a promising tool for future clinical application in patients with oral mucositis, by reducing the risk of candidemia occurrence.

Keywords: Mucositis; Fungemia; Non-thermal atmospheric pressure plasma.

Research Article

Effect of low temperature atmospheric pressure plasma for the prevention of fungemia associated with oral mucositis induced in a murine model of chemotherapy

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Keywords: Mucositis; Fungemia; Non-thermal atmospheric pressure plasma.

Abstract

Fungemia is a public health problem of concern in cancer patients under antineoplastic therapy. The occurrence of oral mucositis associated with oral candidiasis can be one of the causes of systemic candidemia. Low temperature atmospheric pressure plasma (LTAPP) is composed by reactive oxygen and nitrogen species (RONS) and has antifungal and anti-inflammatory effects. The objective of this study was to evaluate the clinical and hematological effect of LTAPP in preventing systemic fungal dissemination in a murine model of chemotherapy, in which oral mucositis lesion associated with candidiasis was induced. Forty Wistar rats were divided into control (non-treated) and LTAPP group. Subgroups of the animals were analyzed after 24 and 72 h of treatment (n=10 per group). The animals were submitted to 4 cycles of chemotherapy with cisplatin (1st day) and 5-fluorouracil (5-FU; 4 consecutive days), followed by oral inoculation of *C. albicans* on the 2nd, 3rd and 5th day. Mucositis lesion on the lateral area of the tongue was induced with a solution of 50% acetic acid. LTAPP treatment was performed in the central region of the lesion for 5 min in two consecutive days (4th and 5th). Body weight was assessed daily. To evaluate the fungal dissemination, the organs (tongue, lung, liver, kidney and spleen) were removed, bisected, macerated and plated on Sabouraud agar with chloramphenicol for fungal counting. Blood sample was obtained by cardiac puncture and a complete blood count test was performed. A non-treated control group was included for comparative purposes. The results *in vivo* showed that 5-FU affected the general health of the animals, evidenced by the loss of body weight. LTAPP showed inhibitory effect on *C. albicans* after 5 min, with significant reduction in fungal recovery from the tongue after 24 h ($p<0.05$). Reduction in fungal dissemination was observed after 24 and 72 h of LTAPP treatment. Systemic fungal dissemination was significantly reduced after 24 and 72 h of treatment ($p<0.05$). Based on the obtained results, it was concluded that LTAPP is a promising tool for future clinical application in patients with oral mucositis, reducing the risk of candidemia occurrence.

Introduction

Chemotherapy-induced oropharyngeal mucositis is a common complication of antineoplastic treatment that can negatively impact its success [1], as it causes significant morbidity in the patients [2]. Mucositis is considered a side effect, generally caused by chemotherapy's nonspecific action on proliferative tissues. As a result, healthy cells with high turnover, such as oral epithelial cells, are commonly affected [3-11]. During the process, clinical oral lesions, that can be seen as erythema and/or ulcers occur as resultant of tissue damage caused by the harmful effect of reactive species generated by the chemotherapy drug. Additionally, oral infections can occur as a secondary effect, oral infections by consequence of the immunosuppressive action of cancer treatment [12,13]. This effect of direct toxicity by chemotherapy drug on tissues is called direct stomatotoxicity and can affect several oral regions, such as the lateral and ventral surface of the tongue, the soft palate, the pharyngeal area [1-14], jugal mucosa, the floor of the mouth and the lips [15-16].

A systematic review of oncological protocols reported that platinum derivatives (cisplatin and oxaliplatin) and alkylated (5-fluorouracil (5-FU)) chemotherapy agents are frequently associated with oral mucositis [17]. Around 20-80% of cancer patients in the head and neck region undergoing chemotherapy develop oral mucositis, and the incidence rate can vary according to the dose and pharmacological agent administered [18-20]. Prolonged treatments can increase the severity of the mucositis [17]. It was described that 1/3 of patients with mucositis reported difficulty of speaking, 90% have difficulty of ingesting water and food, and 85% have weight loss [21,22]. Additionally, they usually present poor oral hygiene, increasing the risk of infection [23,24].

Despite the high frequency of mucositis and severe local and systemic consequences, the lack of effective interventions has been frustrating for patients and caregivers [1]. There are many studies focused on preventing and treating mucositis, however, there is still no consensus protocol for prophylaxis and treatment of this condition[25]. Primary oral care such as tooth brushing, use of dental floss, and mouth rinsing with chlorhexidine solution are considered only palliative measures for the oral mucositis as they present limitations for reducing the severity of the lesions [18,26,27]. Other therapeutic interventions also have been proposed. Treatments with honey, glutamina and vitamin E [28], herbal medications, cryotherapyn [18] present good results, but clinical investigations are still needed. Photobiomodulators show good clinical results, however the use of different parameters and unavailability of limiting dosimetry represent limitations to establish a standardized protocol [29].

The oral cavity has a vast microbiome[30] and *Candida* spp. are commonly found [31-33]. Patients with oral cancer have a more diverse microbiota when compared to healthy individuals [82] and can

present increased oral fungal load [35,36], with higher fungal colonization in the neoplastic epithelial surface compared with adjacent healthy surfaces [36]. The incidence of oral lesions, such as oral candidiasis during chemotherapy treatment can vary from 7 to 52%, [37] and can be caused by *albicans* and non-*albicans* species [38].

Systemic infection can be caused by the entry of pathogens through oral mucositis ulcerations, in particular in immunocompromised patients [5,12]. *Candida* spp. Are opportunistic oral microorganisms that express invasive virulence factors [39] and can cause fungemia. An *in vivo* study conducted by Katagiri et al.[40] reported that the occurrence of *Candida* infection in mice with induced oral mucositis can culminate in fungemia, showing the correlation between oral mucositis and the potential for systemic fungal dissemination. This study draws attention to the severity of this clinical condition, which goes beyond local occurrences and has potential to lead to sepsis.

In this context, low temperature atmospheric pressure plasma (LTAPP) treatment appears as a promising alternative. It is composed by atoms, molecules, and fixed and free ions [36]. Reactive oxygen and nitrogen species (RONS) are particularly associated with the effects of plasma, that can be used for the treatment of infectious conditions, wounds, skin pathologies and neoplasia, with several applications in Medicine and Dentistry [41-44].

Previous studies have shown positive results for treating superficial infections caused by *Candida* spp. [45-48]. In a *in vivo* study, helium plasma inhibited of the tissue invasion by *C. albicans*, with low occurrence of inflammatory changes [49]. Similarly, antifungal effect without damage to the superficial epithelium was reported in BALB/c mice with candidiasis treated with plasma helium mixed with oxygen (He/O_2) for 4 min [50]. The prolonged use of plasma He/O_2 for 10 min also showed no mucosal irritation in rabbits [51].

Considering the occurrence of refractory cases of oral candidiasis in oral mucositis lesions and its possible association with the development of fungemia, this study aimed to evaluate if LTAPP can reduce the occurrence of *C. albicans* systemic dissemination in an *in vivo* model of chemotherapy-induced mucositis experimentally infected.

Methods

Low temperature atmospheric pressure plasma source

Low temperature atmospheric pressure jet equipment (LTAPP) was composed by a bivolt electrical circuit (110 / 220 V) inside a cylinder (11 x 7 cm, length, and width), containing a high-frequency electrode with a homemade pulsed voltage source. The system was fed with helium gas

with 99.9% purity (He, Air Liquide, Brazil) and a controlled flow rate of 2.0 ± 0.1 SLM by a digital controller (N100 Horiba STEC).

In vivo study in the murine model of chemotherapy: effect of LTAPP in the treatment of lesions of oral mucositis inoculated with C. albicans

Animals

The study was approved by the Ethics Committee on the Use of Animals under registration number 16/2019.

The study was conducted with 40 male rats (*Rathus norvegicus*) aged between 90 and 100 days. The animals were maintained under controlled conditions of temperature (22 °C), light and dark cycles, housed in ventilated racks (Alesco, Brazil), and with free access to water and food. Prior to the test, the animals received topical antiparasitic medication (1 drop/animal, Revolution 6% - Zoetis, SP, Brazil) and a single oral dose of dewormer (1mL/kg, Vermotrix, Lema biologic, MG, Brazil). To prevent microbial contamination, the test was conducted in a sterile environment, including the materials provided to the animals (food, water, wood shavings, bottles, and jars) and isolators previously sanitized with alcohol 70%. To avoid bacterial infection, tetracycline (0.83 g/ L) was administered in water *ad libitum*.

Two groups of rats (n=20) were studied: mucositis infected with *C. albicans* and mucositis infected with *C. albicans* treated with LTAPP, evaluated after 24 and 72 h of treatment.

Experimental

The methodology for chemotherapy induced oral mucositis lesion, associated with candidiasis was based on Katagiri et al. [40], with modifications (Figure 1).

The chemotherapy treatment was administered intraperitoneally in a single dose of 7 mg/kg cisplatin (Libbs, SP, Brasil) on the 1st day and 10 mg/kg 5-fluorouracil (5-FU, Libbs, SP, Brasil) for 4 consecutive days. The animals were weighed daily and the mean relative weight (ARW) was calculated according to Zhang et al., [52]. This formula is defined as [(dn - d0) / d0], where d0 represents the mean animal original weight on the first day and dn represents the mean animal weight after n days.

Prior the experiments, fresh fungal suspension and acetic acid solution were prepared. A standardized suspension of *C. albicans* ATCC 18804 containing 10^8 cells / ml was prepared in sterile physiological solution (0.9 % NaCl) by using a spectrophotometer (λ = 530 nm, O.D.= 1.258) (B582, Micronal). Then, in a sterile environment, the sterile swabs were soaked in the fungal suspension for 5 min. To induce mucositis lesion, 50% glacial acetic acid solution was prepared in sterile deionized water, and the swabs were moistened with the solution.

For the experimental procedures, the animals were anesthetized with ketamine (95 mg/kg, Ceva, SP, Brasil) and xylazine (10 mg/kg, Ceva, SP, Brasil).

C. albicans suspension were inoculated in two stages. Firstly, the suspension was inoculated in all regions of the oral cavity for 15 s and, after, the swab was maintained in the dorsal area of the tongue for 5 min [49]. The inoculations were performed on the 2nd, 3rd and 5th days.

The induction of mucositis lesion was performed on the 4th day of the experiment. For this purpose, the acetic acid solution was applied to the lateral region of the tongue for 60 s on each side, followed by washing with a swab moistened in physiologic solution (Ever care, SP, Brazil) [40].

Sodium dipyrone (200 mg/kg – Medley, SP, Brazil) was administered for 2 days per 24 h according with veterinary recommendation for pain control. At 5th and 6th.days, the diagnosis of candidiasis and mucositis was done, by the presence of lesions.

The treatment with plasma was performed on the 4th day after delimiting the mucositis lesion and on the 5th day after fungal inoculation. For treatment, the animals were anesthetized as described before. Prior the LTAPP application, the tongue was humidified with one drop of saline to avoid dryness during the treatment. Then, the plasma jet was applied in the central area of the lesion for 5 min. Prior the euthanasia, blood sample was collected by cardiac puncture and a complete hemogram was generated. Groups of 10 animals were euthanatized 24 and 72 h after the treatment (6th and 8th days) with a triple dose of anesthetic (Figure 2). To investigate the occurrence of systemic infection some organs were collected (lung, spleen, liver, kidney, and tongue) for microbiological analysis.

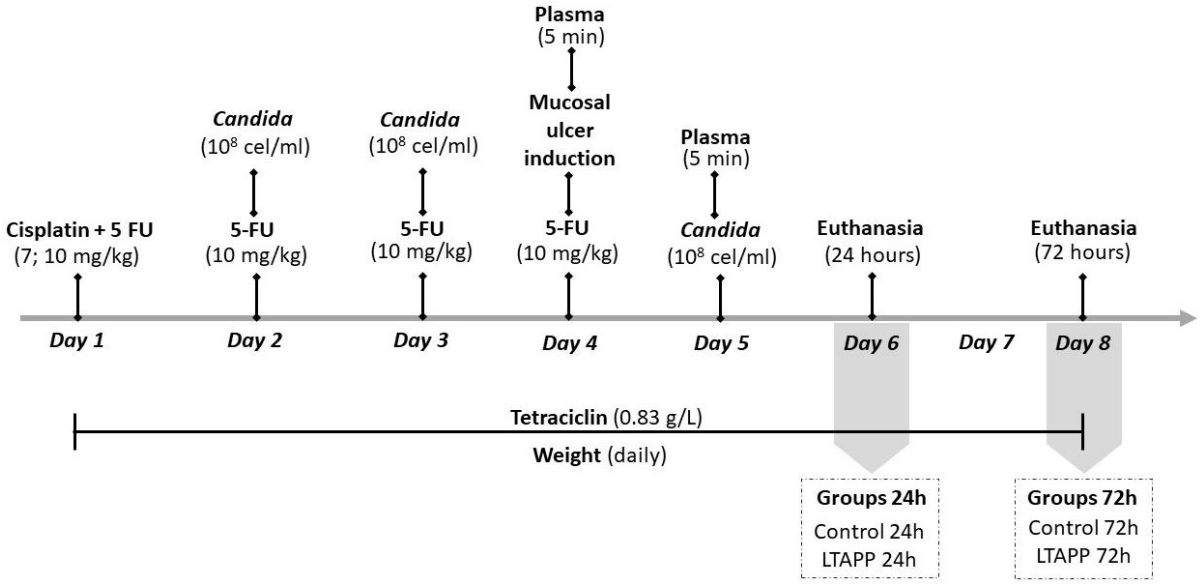


Fig. 1. Experimental timeline for the induction and treatment of oral mucositis lesions infected with *Candida albicans* in a murine model of chemotherapy.

Microbiological analyzes

The organ samples were bisected into sagittal sections (liver, kidney and tongue) and coronal sections (lung and spleen), then the left and lower halves of the organs were washed with sterile saline solution, weighed and homogenized in 1 ml of sterile saline, followed by serial dilution. After, an aliquot of 100 µL aliquot of the homogenate and dilutions were spread on Sabouraud dextrose (SD) agar with chloranfenicol and incubated at 37 °C for 48 h. Subsequently, the colony forming units (CFU) of yeasts were counted and the number of CFU per organ weight in milligrams (CFU / mg) was calculated.

Furthermore, an aliquot of 100 µL of blood sample was incubated in 5 ml of peptone yeast extract glucose broth culture medium, followed by incubation at 37 °C for 72 h. Subsequently, another aliquot of 100 µL was plated on SD agar and incubated at 37 °C for 48 h. The growth of yeasts was observed, and the number of colonies was counted.

Hematological analyses

The blood samples obtained by cardiac puncture were placed in anticoagulant tubes containing ethylenediamine tetra acetic acid (EDTA) and were stored in ice until analysis. The completed hematologic analysis was performed using an automated methodology (EXIGO H-400) at the Laboratory Diagnostic Center of the University of Vale do Paraiba (CDLAB-UNIVAP). Quantitative

analysis, consisting of erythrogram data, leukogram, and platelet count was done. Qualitative analysis to evaluate cellular morphology was also performed.

Statistical analysis

Data was statistically analyzed using the Graphpad prism version 8.0 (Graphpad Software Inc. CA, USA). A normality test was performed, and appropriate statistical tests were subsequently applied. The body weights of the animals in treated and control groups were compared by Student's t test. Fungal counts of tongue in treated and plasma group were analyzed by Mann-Whitney test. For the comparison of the number of animals positive to yeasts in test and control groups, data were analyzed by Fisher'sExact Test with aid of the software MatLab R2021a. Fungal counts of tongue in between treated group and plasma group were compared by Mann-Whitney test. The level of significance was set at 5% for all the tests.

Results

Clinical follow up post oral lesions and LTAPP jet treatment

The induction of oral mucositis lesions and candidiasis in the animal's tongue was successful. The clinical analysis revealed white spots and plaques on the dorsal, ventral and lateral surfaces of the tongue, as well as erythematous and ulcerated areas.

The oral mucositis lesions were treated with a LTAPP jet generated at power of 1 W and at a distance of 1.5 cm of distance for 5 min (Figure 6). During the experiment, 5 rats died.

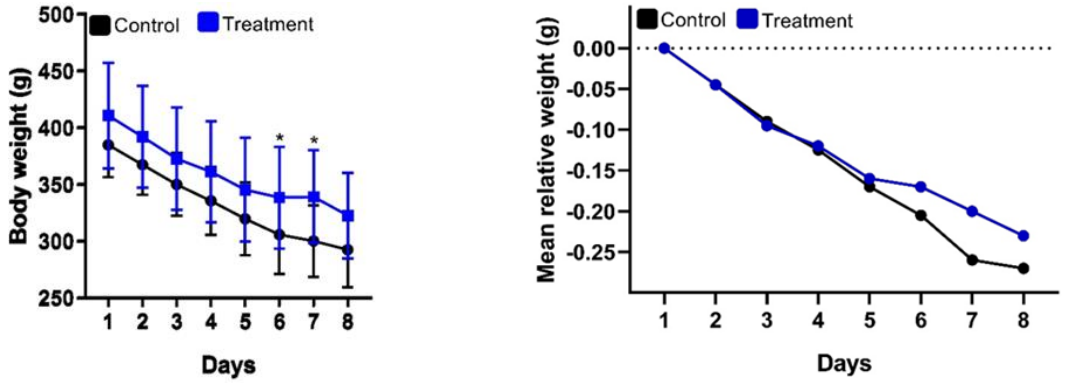
Fig. 2. Representative image of the treatment with helium low temperature atmospheric pressure plasma jet on the oral mucositis lesions.

Evaluation of the animals' body weight

All the animals showed reduction in weight during the experiment (Figure 3), from the second day of chemotherapy (figure 3a). In the control group, the weight loss of animals that

received only chemotherapy treatment was constant until the last day of the assay (8th day). A moderate weight gain was observed for the treatment group from the day 6.

Significant difference in body weight between groups was observed on the 6th (p = 0.0230) and 7th days (p = 0.0469) days, with mean relative weight of -0.17 g and -0.20 g (figure 3b) for LTAPP group, respectively. The weight loss of the control group in the same period was -0.20 (6th day) and -0.26 g (7th day).



(a) (b)

Fig. 3. Body weight of the animals under chemotherapy with oral mucositis lesion associated with fungal contamination. In (a) body weight variation during the experimental period and (b) mean relative weight of control (non-treated) and LTAPP groups. *p <0.05 (Multiple t test).

Hematological evaluation

The association among chemotherapy, candidiasis, and mucositis lesions caused alterations both in red cells and white cells counts. The results for erythrogram, leukogram, and platelets are presented in tables 1 and 2. Values of reference of complete hemogram for Wistar rats were used to compare the results [53].

Reduction in the red blood cells (RBC) counts, hemoglobin and hematocrit) was observed for the LTAPP groups compared to the reference values. The control group in the period 24 h showed values below the reference for RBC and haematocrit. RBC counts reached reference values at the period of 72 h. However, the values of hemoglobin showed reduction over time.

Table 1. Results of erythrogram and leukogram of the animals that underwent chemotherapy treated with low temperature atmospheric pressure plasma and control 24 h after the last session of treatment.

Blood Component	**Hematological values**		
	Groups		
	24 hours		
	Control	**Treatment**	**Reference values**[53]
Erythrogram			
RBC (10^6/mm3)	**6.47 ± 3.21**	**5.98 ± 2.74**	6.6-9.0
Hemoglobin (g/dL)	14.9 ± 4.6	**9.4 ± 4.0**	13-16
Haematocrit (%)	**31.8 ± 10.3**	**28.8 ±11.1**	41-51
Leukogram			
WBC (10^3/mm^3)	**1.70 ± 1.91**	**1.62 ± 1.04**	7.3-12.6
Neutrophils (%)	**20.4 ± 11.3**	**37.9 ± 16.9**	26.0-34.3
Eosinophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Basophils (10^3/mm^3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes (%)	**40.0 ± 23.7**	**58.6 ± 16.4**	59.5-68.3
Monocytes (%)	2.0 ± 1.9	3.6 ± 2.4	2.1-8.2
Platelets (mm^3)	**793.90 ± 564.65**	1159 ± 247.9	840-1240

Legenda: RBC = red blood cell; WBC = White blood cell (leukocyte); Values in bold highlight the alterations compared to reference values.

For the leukogram, alterations in white blood cells (WBC) counts were observed in all groups and periods, with marked reduction in the values when compared to the reference.

The group treated with LTAPP showed an increase in neutrophil percentage above the reference values. However, the values decreased 72 h after the treatment. The same tendency was not observed in the control group.

Lymphocyte percentage was altered only in control group at the evaluation periods of 24 and 72 h. These values were similar to the reference for the treated group. The same tendency was

observed for monocyte percentage that showed marked reduction over time in control group. The counts of platelets were below normality in the control group at 24 h.

Table 2. Results of erythrogram and leukogram of the animals that underwent chemotherapy treated with low temperature atmospheric pressure plasma and control 72 h after the last session of treatment.

Blood Component	**Hematological values**		
	Groups		
	72 hours		
	Control	**Treatment**	**Reference values** [53]
Erythrogram			
RBC (10^6/mm3)	8.16 ± 2.19	**4.78 ± 3.22**	6.6-9.0
Hemoglobin (g/dL)	**12.8 ± 3.7**	**7.6 ± 5.0**	13-16
Haematocrit (%)	**40.0 ± 11.9**	**23.8 ± 15.7**	41-51
Leukogram			
WBC (10^3/mm^3)	**1.77 ± 1.21**	**1.46 ± 1.31**	7.3-12.6
Neutrophils (%)	**4.9 ± 2.0**	**15.0 ± 6.2**	26.0-34.3
Eosinophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0-0.6
Basophils (10^3/mm^3)	0.0 ± 0.0	0.0 ± 0.0	0.00-0,03
Lymphocytes (%)	**56.6 ± 23.6**	65.40 ± 25.5	59.5-68.3
Monocytes (%)	**0.4 ± 0.7**	4.8 ± 2.2	2.1-8.2
Platelets (mm^3)	1103.8 ± 203.1	976.5 ± 349.9	840-1240

Legenda: RBC = red blood cell; WBC = White blood cell (leukocyte). Values in bold highlight the alterations compared to reference values.

Evaluation of fungal systemic dissemination

The number of animals positive for yeasts in each organ is presented in Table 1. Yeasts were isolated from all animals' tongues, independent of the group and period analyzed. For the control group, yeasts were recovered from 90% of the lungs, 50% of livers, spleens, and kidneys. In the group

treated with LTAPP, yeasts were recovered from 11.11% of the lungs and 22.22% of the kidneys. Among the animals euthanatized 72 h after LTAPP treatment, yeasts were not recovered from the organs. Differently, for the control group at the same period of analysis, yeasts were recovered from 55.55% of the lungs, 44.44% of the livers, and 33.33% of the spleens of the control group . All the blood cultures were negative to yeasts.

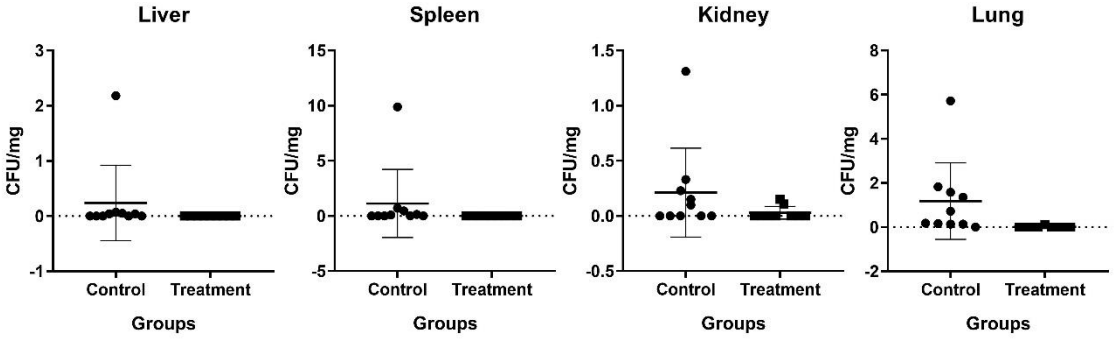
There was a significant association between the use of LTAPP treatment and the number of animals that presented a positive culture for *Candida* yeasts in the organs. The application plasma showed a statistically significant effect against systemic dissemination for the lung in both periods and also for the liver and spleen at the period of 24 h, after the last LTAPP treatment ($p < 0.05$); Table 3).

Table 3. Number of animals with positive culture for yeasts at the periods of 24 and 72 h after the second session of treatment with low temperature atmospheric pressure plasma.

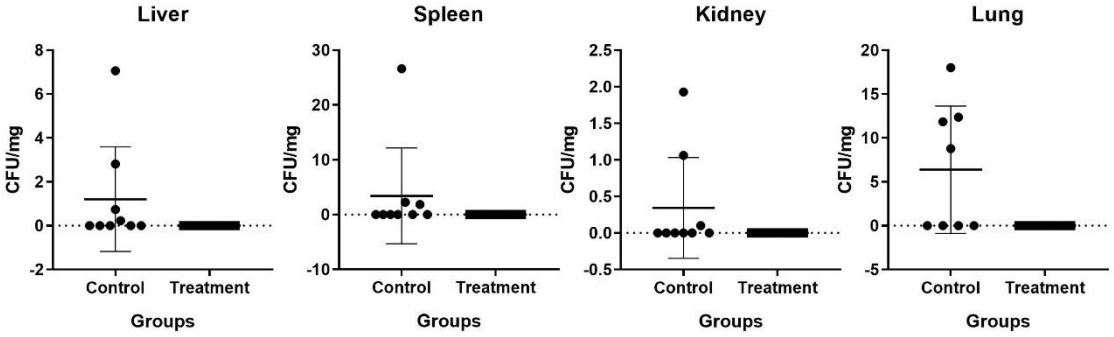
		Organs				
		Tongue	**Lung**	**Liver**	**Spleen**	**Kidney**
		24 hours				
Number of animals with positive culture to yeasts / Total animals	**Control**	10/10	9/10	5/10	5/10	5/10
	Treatment	9/9	1/9	0/9	0/9	2/9
	p value	1.0000	*0.0011	*0.0325	*0.0325	0.3498
		72 hours				
	Control	9/9	5/9	4/9	3/9	3/9
	Treatment	7/7	0/7	0/7	0/7	0/7
	p value	1	*0.0337	0.0885	0.2125	0.2125

Fisher's exact test used, *$p < 0.05$

Figure 4 shows the fungal dissemination in the organs over time. It is possible to see the effectiveness of plasma treatment on the 6th day and on the 8th day of the experiment (24 and 72 h) after the last LTAPP, respectively with low fungal counts and tendency to 0 CFU / mg for the treated group. Higher fungal counts were observed in the control group in both periods (24 h and 72 h).



(a)

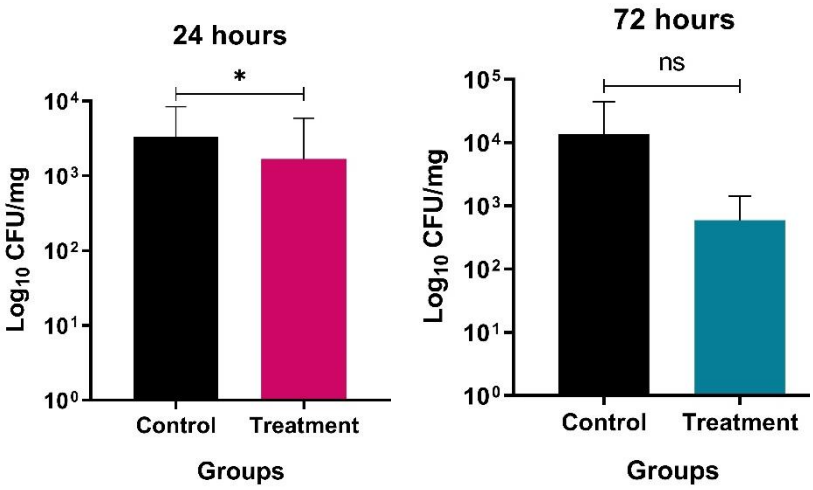


(b)

Fig. 4. Number of viable fungal cells in the organs recovered (a) 24 and (b) 72 h after the LTAPP treatment compared with the control group (non-treated). Values of the mean and standard error mean (mean ± SD) are represented.

The number of viable fungal cells was quantified from the tongues and the results are shown in Figure 5. Significant reduction in yeasts counts was detected 24 h after the second session of treatment with LTAPP ($p = 0.01$). In the period of 24 h after treatment, the mean values of CFU / mg organ were of $3.32 \times 10^3 \pm 5.01 \times 10^3$ and $1.68 \times 10^3 \pm 4.23 \times 10^3$ for the control and treated groups, respectively.

At 72 h, the mean count of yeasts in the tongue was $1.35 \times 10^4 \pm 3.08 \times 10^4$ CFU / mg. In control group. A reduction in fungal count was detected in the groups treated with LTAPP ($5.94 \times 10^2 \pm 8.35 \times 10^2$ CFU / mg), however with no statistical difference was detected.



(a) (b)

Fig 5. Counts of viable fungal cells (CFU / mg) recovered from the tongue of animals 24 h (a) and 72 h (b) after the second session of plasma jet treatment. *p < 0.05. ns – non-significant (p> 0.05). (Mann-Whitney U Statistical Test).

Discussion

Cancer patients in the head and neck region undergoing radiotherapy and chemotherapy treatment, present side effects during the therapy, such as oral mucositis, causing slight to severe buccal mucosa damages [5]. The injured region is susceptible to the secondary action of microorganisms that can aggravate mucosal tissue damage and cause systemic infection [54,55]. *Candida* spp. present high incidence in immunosuppressed individuals [35,36] and it is associated with the occurrence of systemic infection in rats with chemotherapy-induced mucositis [40].

For our knowledge, this is the first study to evaluate the effect of plasma jet on oral candidiasis associated with mucositis during chemotherapy treatment in a murine model. Our findings suggest that treatment with LTAPP was effective on the reduction of local *C. albicans* and consequently in the systemic fungal dissemination, which can indicate a significant impact on mortality by preventing candidemia. These results are promising and suggest that LTAPP can become an effective adjuvant therapy for cancer patients with simultaneous oral mucositis and candidiasis induced by immunosuppressive conditions expected during chemotherapy treatment.

The methodology for inducing mucositis and oral candidiasis in rats proved to be effective. It was validated by the clinical signs of erythematous surface and ulcers covered by pseudomembrane, features that clinically characterize oral mucositis. Additionally, whitish plaques were observed indicating the presence of oral candidiasis. It is important to highlight that the

occurrence of a disease, such as candidiasis, involves a complex microorganism-host interaction, involving both the expression of virulence factors generated by *Candida*, and the interactions of the microflora and the host's own immune system [56]. Chemotherapy associated with oral mucositis occurs under immunosuppressed conditions caused by the drug, altering hematological values [2,12,40].

In the literature, changes in red blood cell counts (RBC), hemoglobin and lymphocyte, neutrophils and monocytes has been demonstrated in association with chemotherapy, oral mucositis and infections [2,57-59]. Cancer patients with candidemia usually show leukopenia and neutropenia, which can be considered as risk factors[60], for infection [61]. Study conducted by Saftescu et al. [59], in humans, associated the use of to 5-FU and cisplatin to slight anemia, with increased effect after therapy with cisplatin. During chemotherapy, the combination of cisplatin and 5-FU leads to immunosuppression, resulting in a reduction in leukocytes (WBC) and neutrophils [62]. In the present study, the alterations of erythrogram (reduced hemoglobin, RBC, and hematocrit) and leukogram (WBC, neutrophil, lymphocyte, and platelets) were observed confirming the immunosuppression.

Chemotherapy treatment with 5-FU and cisplatin induce oral mucositis, that can cause weight loss [40,63,64]. There is a link between weight loss associated with the chemotherapy, global health of the host and occurrence of mucositis [40,63,64]. In our study, the animals of both groups showed weight loss. The control and treatment groups presented reduction of weight over time, however LTAPP group presented a significant weight gain after 24 and 48 h of treatment, which can indicate an improvement in the general health. In agreement with our study, a previous study that used a similar methodology also observed weight loss [40]. Weight loss is a common and worrying clinical sign in cancer patients, associated with several adverse side effects, including diarrhea [65,66] which can lead to hospitalization, discontinuation of treatment and lead to death. Symptoms of moderate diarrhea were observed in the animals during the study in both groups during and after chemotherapy cycles.

In this study, LTAPP was applied to oral mucositis lesions in the tongue of rats for 5 min in 2 consecutive days to evaluate the reduction of oral candidiasis and fungal dissemination. A previous study with similar methodology showed fungal presence in the tongue of all the mice [40], as observed in the rats of our study. The treatment with LTAPP showed significant fungal reduction only in the tongue of the animals euthanized 24 h after plasma exposure, although a tendency of fungal reduction was also observed after the 72h of the treatment.

The fungal dissemination of *C. albicans* was also seen in the organs, as observed by Katagiri et al. and Ninomia et al. [64]. Kidney damage by *Candida* spp. has been observed through intravenous infection [67]. Besides the tongue, our study investigated the presence of fungus in the kidney, liver,

lung, and spleen. Higher fungal counts were observed in the lung followed by the spleen in both periods of euthanasia (24h e 72h) for the control group. Katagiri et al. [40] and Ninomiya et al. [64], also detected fungal dissemination after 24 h of the last procedure. However, differently from our study, they only examined the kidney and liver.

In addition to reduce fungal count in the tongues, a significant difference between the number of animals positive and negative to yeasts in the organs was detected. Data on low variability in the fungal recovery in the treated group was observed, with a tendency towards complete fungal clearance. Ninomiya et al. [64] using similar methodology treated the tongues of mice with antifungal drugs for 3 days. They observed a reduction of white patches in the tongue and reduction of *C. albicans* dissemination for the organs. However, there is no previous study evaluating LTAPP against fungal dissemination from oral mucositis which makes the comparison of our results difficult.

Positive blood cultures were no detected in our study. One hypothesis for this finding can be there was not enough fungal recovery in the blood. Hayama et al. [68] suggested that the presence of trypsin in homogenized tissue can aid in the separation of *Candida* mycelia, increasing fungal recovery. Future studies can be carried out using this methodology to improve the recovery of the strain for blood samples. Additionally, some studies have shown that positive blood cultures occur in less than 50% of the analysis [69,70], delaying the diagnosis [71], which can be lethal for the patient. The previous studies by Katagiri et al.[40] and Ninomiya et al. [64], observed fungal presence of *C. albicans* in blood in 60% (6/10 animals, not treated) and 40% (4 / 10, treated with oral care) of the animals. However, in these studies the experimental infection was induced by the inoculation of fungal suspension, which can have facilitated fungal arrival in other organs and fungal dissemination. The induction of oral candidiasis in our work was performed with the aid of swab previously soaked in fungal suspension, like the methodology successfully employed by Borges et al 2018 [72] and Sampaio et al. [73].

Interestingly, the treatment with LTAPP in this study reduced fungal dissemination for distant organs. Our innovative findings are promising once immunocompromised patients may require additional treatment to avoid fungemia.

Conclusion

In conclusion, low temperature atmospheric pressure plasma (LTAPP) was safe and effective for the reduction of local and systemic *Candida albicans* occurrence in the murine model of

chemotherapy-induced mucositis associated with candidiasis. The treatment reduced the dissemination of *C. albicans* to different organs. These findings suggest that LTAPP represents a convenient and promising technique to prevent systemic fungal dissemination in cases of candidiasis associated with oral mucositis. At this moment, this is the first study that evaluated LTAPP for this purpose.

Statements

All papers must contain the following statements after the main body of the text and before the reference list.

Acknowledgement (optional)

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Statement of Ethics

The animal study protocol was approved by the Ethics Committee on the Use of Animals under registration number 16/2019.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

All authors contributed to the conceptualization of the manuscript and writing—original draft preparation.

Aline Sampaio, Noala Milhan, Kostantin Kostov and Cristiane Koga Ito were responsible for conceptualization, writing—original draft preparation and writing=review and editing. Aline Sampaio, Noala Milhan, and Cristiane Koga Ito were responsible for methodology, software, formal analysis and investigation. Kostantin Kostov and Cristiane Koga Ito were responsible for project administration and funding acquisition. Noala Milhan and Cristiane Koga Ito were responsible for supervision. Crisitane Koga-Ito were responsible for supervision. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data presented in this study are available from the corresponding author upon reasonable request.

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3 CONSIDERAÇÕES GERAIS

Nas últimas décadas a incidência crescente de neoplasias malignas na região nasofaringe e orofaringe têm sido relatada (Ng et al., 2018; Tangjaturonrasme et al., 2018), com registros da ocorrência de óbitos mundialmente (Xie et al., 2017; Sung et al., 2021; Johson et al., 2020; Bosetti et al., 2020; Gormley et al., 2022; ASCO et al., 2023; Santos M et al., 2023; INCA et al., 2022). A mucosite orofaríngea é um dos efeitos adversos gerados pela ação inespecífica dos tratamentos quimioterápicos, associados ou não à radioterapia, que atinge células da mucosa, causando danos teciduais (Villa, Sonis, 2015; Lalla et al., 2008). Essa condição clínica pode causar desconforto, dor, comprometimento nutricional, de glândulas salivares e higiene oral que que impactam na qualidade de vida e no tratamento do paciente, além de gerar riscos de infecção, impactos econômicos e aumento da hospitalização (Lalla et al., 2014; Pereira et al., 2019; Peterson et al., 2015; Riley et al., 2016; Ruiz-Pérez et al., 2016; De Sanctis et al., 2019). Até o momento, não existem protocolos terapêuticos de consenso para a mucosite oral e medidas paliativas têm sido adotadas. Neste contexto, a busca por novas terapias tem sido conduzida globalmente.

O plasma de baixa temperatura sob pressão atmosférica tem sido extensivamente investigado e tem demonstrado resultados promissores na área médica (Kostov et al., 2015; Lee et al., 2015; Park et al., 2014; Weltmann e von Woedtke, 2011; Bekeschus et al., 2016; Brun et al., 2014), baseados em seu efeito anti-inflamatório, antimicrobiano de amplo espectro e indutor de reparo tecidual (Borges et al., 2018; Oliveira et al., 2018; Lima et al., 2021). Essas ações são associadas à presença de espécies reativas de oxigênio e nitrogênio (RONS) geradas pela interação físico-química produzida entre o gás usado para geração do plasma, o ambiente e os parâmetros empregados (Laroussi, Leipold, 2004; McCombs, Darby, 2010).

O presente estudo objetivou avaliar o potencial do LTAPP no tratamento da mucosite oral induzida por quimioterapia, em duas vertentes: nos efeitos do tratamento local de lesões de mucosite, sem associação com *C. albicans* e na prevenção da disseminação fúngica em lesões de mucosite oral associadas à

candidose.

A investigação dos efeitos do LTAPP em mucosite oral, demonstrou que o tratamento levou à redução da gravidade da mucosite após 12 dias, demonstrados pelo acompanhamento clínico e achados histológicos. Esse potencial de redução foi um pouco mais evidente no grupo LTAPP, quando comparado ao grupo não tratado. Por outro lado, o estudo do potencial do LTAPP em reduzir a disseminação fúngica sistêmica fúngica a partir de lesões de mucosite oral induzida por quimioterápico infectadas experimentalmente com *C. albicans* demonstrou tratamento com LTAPP reduziu a disseminação fúngica para os órgãos analisados.

Os achados do uso do plasma sobre mucosite induzida por quimioterapia representam uma oportunidade para desenvolver novas terapias para pacientes submetidos a tratamento oncológico que apresentem lesões orais infectadas ou não. O efeito antifúngico e a tendência de redução das lesões mucosite, com efeito preventivo sobre a disseminação fúngica a partir das lesões, abrem para perspectivas para novos estudos com outros parâmetros como diferentes gases, equipamentos geradores de plasma, protocolos de aplicação e associação com outras terapias, ampliando conhecimentos sobre o potencial do plasma no tratamento de lesões bucais.

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ANEXO A – Certificado CEUA – Comissão de Ética no Uso de Animais

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de São José dos Campos

Instituto de Ciência e Tecnologia
São José dos Campos
UNESP

CERTIFICADO
CEUA – Comissão de Ética no Uso de Animais

CERTIFICAMOS, que o protocolo registrado sob o nº 16/2019, intitulado:- "**Aplicação do plasma de baixa temperatura sob pressão atmosférica como adjuvante ao tratamento da mucosite oral.** " sob a responsabilidade de **CRISTIANE YUMI KOGA ITO ,** tendo como colaboradora **ALINE DA GRAÇA SAMPAIO,** e que envolve a 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 **APROVADO** pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS** (CEUA – ICT – CAMPUS DE SÃO JOSÉ DOS CAMPOS-UNESP), em reunião de 17/12/2019.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da Autorização	17/12/2018 a 29/07/2022
Espécie/linhagem/raça	Ratos Wistar
Nº de Animais	120
Peso/Idade	90 a100 dias – 150grs.
Sexo	MACHO
Origem	Biotério Central – Campus de Botucatu-UNESP

São José dos Campos, 17 de dezembro de 2019

Profa.Dra. **PAULA CAROLINA KOMORI DE CARVALHO**
Coordenadora

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