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EFEITO DO TRATAMENTO SINÉRGICO ENTRE PLASMA ATMOSFÉRICO FRIO E VITAMINA C NA CANDIDOSE EXPERIMENTAL INDUZIDA EM CAMUNDONGOS

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RESUMO

Candida albicans é um fungo comensal, mas também oportunista, que pode causar doença na presença de fatores predisponentes, levando à candidose local ou invasiva. Estudos prévios in vitro e in vivo indicaram que o plasma frio em pressão atmosférica (CAP) pode exibir efeito antifúngico contra *C. albicans*, além de propriedades anti-inflamatórias. Outros estudos demonstraram efeito inibitório e modulação de fatores de virulência de *C. albicans* por vitamina C (Vit C), bem como propriedades anti-inflamatórias. Não foram detectados estudos sobre a associação de CAP e Vit C. O objetivo deste estudo é avaliar se a associação entre CAP e Vit C pode otimizar o efeito antifúngico exibido pelo CAP. Para isso, serão realizados procedimentos de imunossupressão e indução de candidose em dorso lingual de 35 camundongos, que serão aleatoriamente divididos em cinco grupos. 1) tratados com CAP (n = 7), 2) tratados com CAP e Vit C (n = 7), 3) tratados com vitamina C (n = 7), 4) tratados com nistatina (n =7) e 5) não tratados – grupo controle (n = 7). As diferentes modalidades de tratamento serão avaliadas por meio de análise clínica das lesões, no momento inicial do diagnóstico e previamente à eutanásia, análise microbiológica e microscópica das línguas, que serão hemissecionadas após a eutanásia. A avaliação clínica levará em consideração escores associados a gravidade da lesão. Já a avaliação microbiológica será feita pela determinação da quantidade de *C. albicans* (UFC/grama) em macerados de língua. Por fim, nas análises microscópicas serão analisadas alterações epiteliais do dorso lingual e o grau de inflamação do tecido conjuntivo subjacente, além da contagem do número de hifas e leveduras da superfície epitelial de diferentes áreas do fragmento. Os dados obtidos serão submetidos ao teste estatístico mais apropriado, de acordo com a distribuição amostral ($p<0.05$).

Palavras chave: Plasma frio em pressão atmosférica; Vitamina C; Candidose

1. INTRODUÇÃO

A Candidose Oral (CO) é uma doença bucal comum causada pelo fungo *Candida* spp. *C. albicans* é a espécie mais prevalente, sendo responsável por mais de 95% dos casos. Este fungo é um microrganismo comensal que habita a boca, o trato gastrointestinal e reprodutivo em mais de 80% da população (Vila et al., 2020). No entanto, *C. albicans* também é um patógeno oportunista que pode proliferar e causar doença na presença de fatores predisponentes (Singh et al., 2014; Vila et al., 2020).

Fatores locais, como higiene inadequada, uso ininterrupto de próteses, trauma na mucosa causado por próteses mal ajustadas, corticoterapia tópica, tabagismo e disfunção salivar, predispõe ao desenvolvimento de CO. Fatores sistêmicos também podem contribuir para a transição da condição comensal para a patogênica. Dentre esses fatores estão a terapia imunossupressora, a quimiorradiação, o uso de antibióticos de amplo espectro, a imunossenescência, as deficiências nutricionais e até mesmo disfunções endócrinas (Singh et al., 2014; Vila et al., 2020).

A capacidade de aderir às superfícies epiteliais orais, formar biofilmes e evadir os mecanismos de defesas do hospedeiro são fatores de virulência da *C. albicans* que contribuem para a CO (Vila et al., 2020). Clinicamente, as lesões podem apresentar-se como placas brancas removíveis à raspagem (candidose pseudomembranosa), manchas vermelhas (candidose eritematosa aguda ou crônica e queilite angular) e placas brancas aderentes (candidose hiperplásica crônica) (Lewis and Williams, 2017). Os achados microscópicos podem variar de acordo com a apresentação clínica e o grau de imunossupressão do indivíduo. Em geral, há penetração de hifas na superfície do epitélio, que geralmente apresenta exocitose e formação de microabscessos. O tecido conjuntivo apresenta reação imunológica com infiltrado inflamatório subepitelial caracterizado pela presença de macrófagos, linfócitos, granulócitos, além

hiperemia. Em indivíduos imunocomprometidos é esperada uma extensão mais profunda das hifas, além de redução do infiltrado inflamatório no tecido conjuntivo (Reichart et al., 2000).

O tratamento geralmente consiste no uso tópico ou sistêmico de agentes antifúngicos. Existem quatro tipos de antifúngicos com diferentes modos de ação e alvos, I) Polienos - ruptura das membranas celulares dos fungos (nistatina e anfotericina); II) Azóis - inibição da síntese de ergosterol (fluconazol e itraconazol); III) Equinocandinas – inibição das enzimas β-1,3 D-glucano sintetase (caspofungina); IV) Análogos da pirimidina - grupo que interfere na síntese do RNA e na replicação do DNA (flucitosina) (Costa-de-oliveira and Rodrigues, 2020; Lewis and Williams, 2017). As duas primeiras classes têm sido utilizadas para tratar CO; no entanto, o uso de polienos geralmente é limitado à administração tópica devido à má absorção através do intestino. O uso tópico também apresenta limitações pela dificuldade de manutenção de níveis adequados do medicamento no meio bucal. Diferentemente, azóis como fluconazol e itraconazol podem ser administrados sistemicamente, uma vez que são bem absorvidos pelo intestino. Entretanto, esses agentes apresentam interação medicamentosa com a varfarina (agente anticoagulante) e estatinas (inibidores da HMG-CoA redutase) (Lewis and Williams, 2017), não sendo adequados para paciente que utilizam essas medicações. Além disso, a resistência aos agentes antifúngicos é um assunto que tem emergido nos últimos anos (Costa-de-oliveira and Rodrigues, 2020; Lewis and Williams, 2017).

Diferentes mecanismos de resistência foram descritos para *C. albicans* de acordo com a classe antifúngica. Contra azóis, que representam agentes antifúngicos comumente utilizados, *C. albicans* pode atuar diminuindo a concentração do fármaco, alterando a enzima alvo, diminuindo a afinidade ao sítio de ligação, regulando positivamente a enzima alvo e na superexpressão de bombas de efluxo da membrana celular (Costa-de-oliveira and Rodrigues, 2020). Nesse contexto, tratamentos alternativos, que poderiam reduzir a frequência de

utilização dos agentes antifúngicos convencionais, têm sido investigados para tratar infecções antifúngicas na última década, incluindo a combinação de terapias (Fuentefria et al., 2018).

O plasma é conhecido como o quarto estado da matéria, o mais prevalente no universo. É uma mistura de moléculas fixas e móveis, átomos, elétrons e íons em temperaturas e densidades variadas (McCombs e Darby, 2010). O plasma pode ser produzido artificialmente e é utilizado para diversos fins industriais, como elementos eletrônicos. Esta tecnologia abrange dois grandes grupos, plasma térmico e plasma frio. O primeiro é útil para processos de fundição, incluindo síntese química ou indústria metalúrgica. Por outro lado, o plasma de pressão atmosférica frio (CAP) pode ter sido usado em materiais sensíveis ao calor para descontaminação biológica (López et al., 2019) e para aplicações biomédicas. Duas fontes de CAP têm sido comumente utilizadas no campo biomédico: descarga de barreira dielétrica (DBD) e jato de plasma de pressão atmosférica sem equilíbrio (NAPPJ) (Laroussi, 2020).

Além das propriedades antimicrobianas, o CAP pode apresentar efeito anti-inflamatório, contribuir para a cicatrização de feridas e até mesmo no tratamento do câncer (Braný et al., 2020). Dentre os mecanismos de ação do CAP, destaca-se a geração de espécies reativas de oxigênio e nitrogênio, que alteram a membrana celular das bactérias (Brun et al., 2018). As espécies reativas geradas pelo CAP também demonstraram ser eficazes contra fungos. Os possíveis efeitos do CAP nas células fúngicas são perda de permeabilidade e extravasamento celular, destruição de proteínas celulares, apoptose celular, fragmentação com liberação de DNA e deformação da ponta do micélio (Misra et al., 2019).

Estudos prévios demonstraram que o CAP apresenta propriedades antifúngicas contra *C. albicans* (Aparecida Delben et al., 2016; Borges et al., 2018; He et al., 2020). Observou-se *in vitro* que o jato de plasma operado com hélio, a uma distância de 1,5 cm, reduz a viabilidade de biofilmes de *C. albicans* após 5

minutos de exposição. Além disso, o CAP foi capaz de reduzir a invasão tecidual de *C. albicans* em modelo in vivo de candidose experimental (Borges et al., 2018).

A possibilidade de otimizar o tratamento com plasma, diminuindo seu tempo de exposição e/ou melhorando seu efeito é desejável. A Vit C, também conhecida como ácido ascórbico, é o mais poderoso agente antioxidante da pele com propriedades de formação de colágeno e clareamento cutâneo, o que confere a este composto uma ampla gama de aplicações clínicas (Al-Niaimi and Chiang, 2017). Devido ao seu efeito antioxidante, tem sido avaliado para diversas condições, como doenças inflamatórias (Lee et al., 2021; Yussif et al., 2016) e microbianas (Van Hauwenhuyse et al., 2014; LA et al., 2009), bem como para tratamento de câncer, quando utilizada em altas concentrações (Mussa et al., 2022).

Um estudo prévio demonstrou que o pré-tratamento com vit C aumenta o efeito bactericida do CAP contra biofilmes de *Staphylococcus epidermidis*, *Escherichia coli* e *Pseudomonas aeruginosa* (Helgadóttir et al., 2017). Até o momento, a associação entre Vit C e plasma para o tratamento da candidose não foi relatada na literatura. No entanto, o efeito isolado da vitamina C sobre *C. albicans* foi previamente investigado. Os autores observaram que o ácido ascórbico diminuiu a virulência e a patogenicidade *de C. albicans*, interferindo na transição levedura-hifa (LA et al., 2009).Outros autores demonstraram que a Vit C pode atuar em *C. albicans* dependente de Hsp90, no que diz respeito ao regulador transcricional Upc2. A vitamina bloqueou o crescimento de hifas, dependentes de GdA, restaurando os níveis normais de ergosterol, dependentes de Upc2 (Van Hauwenhuyse et al., 2014). Curiosamente, outro estudo demonstrou que a concentração de 90 mM de ascorbato é capaz de matar *C. albicans*. Entretanto essa eficácia foi dependente de temperatura, oxigenação, disponibilidade de fontes de energia, meio de crescimento e fase de crescimento (Avci et al., 2016).

No que diz respeito às propriedade anti-inflamatórias, a Vit C reduziu níveis de espécies reativas de oxigênio e inibiou a ativação de MAPKs, bem como a expressão de NF-κβ, AP-1 e IL-8 em células respiratórias humanas estimuladas com ácaros do pó doméstico (HDM) (Lee et al., 2021). Em outro estudo, a Vit C diminuiu a produção de espécies reativas de oxigênio, TNF-α e IL-6 em macrófagos desafiados por lipopolissacarídeos (LPS) e concomitantemente tratados por 24 horas com Vit C (100 nM). Recentemente, nosso grupo de pesquisa observou, de forma inédita, que o tratamento com Vit C induz propriedades anti-inflamatórias em queratinócitos desafiados com *C. albicans*, com redução da expressão gênica de IL-8 e IL-1β, e uma tendência de redução de outros marcadores pró-inflamatórios (dados ainda não publicados).

Estudo in vivo, que injetou a Vit C no periodonto de animais com doença periodontal e diabetes, observou que o tratamento com esse componente pode ser benéfico para reduzir os níveis de MMP-8 gengival, o estresse oxidativo, a inflamação e o acúmulo de produtos de glicação avançada (AGEs) no tecido periodontal. Dessa forma concluíram que vit C é um agente imunomodulador e antioxidante promissor, que poderá ser aplicado localmente no tratamento da periodontite para reduzir os efeitos adversos do diabetes nos tecidos periodontais (Toraman et al., 2020). Adicionalmente, estudo clínico que avaliou a ação local de Vit C injetada em gengiva, observou que esse tratamento foi capaz de reduzir vários graus de inflamação gengival crônica (Yussif et al., 2016). Esses achados indicam que a administração injetável de Vit C pode ser uma alternativa para tratamentos de ação local em mucosa oral, o que abre perspectivas para novos estudos com essa abordagem. Essa forma de administração da vitamina demonstra-se promissora uma vez que o ambiente bucal não permite a manutenção prolongada de tratamentos tópicos na área de interesse.

Atualmente, existe um grande apelo por tratamentos alternativos que possam restringir a utilização, e consequentemente a resistência aos antibióticos. Da mesma forma, a otimização de tratamentos alternativos também se faz

necessária no contexto de doenças fúngicas. Com base nos achados abordados anteriormente, indicando que a Vit C pode apresentar propriedades antifúngicas e anti-inflamatórias, bem como otimizar efeitos bactericidas gerados pelo CAP, levantamos a hipótese de que a Vit C pode também potencializar o efeito benéfico do CAP em candidose experimental em modelo murino.

2. OBJETIVOS

O objetivo geral desse estudo é avaliar o efeito do tratamento sinérgico entre Vit C e CAP na candidose experimental induzida em modelo murino. Para alcançar o objetivo geral, os objetivos específicos serão:

a) Analisar clinicamente as lesões de candidose bucal antes e após os tratamentos;
b) Quantificar *C. albicans* (CFU/g) nas línguas, maceradas após a eutanásia;
c) Avaliar microscopicamente as alterações epiteliais, a resposta inflamatória no tecido conjuntivo e o número de hifas e leveduras na superfície do epitélio.

3. MATERIAIS E MÉTODOS

3.1 Cepa e condições de crescimento microbiológico

Nesse estudo será utilizado uma cepa de referência, obtida de lesões de candidose (ATCC 18804). Os microrganismos serão cultivados em ágar Sabouraud dextrose por 24 horas a 37 ºC, em aerobiose. Para a obtenção dos inóculos, que serão utilizados nos animais, serão realizadas suspensões de *C. albicans* em solução fisiológica estéril (NaCl 0,9%) em concentração de 10^8 UFC/ml, com auxílio de espectrofotômetro (λ: 530 nm; DO: 0,138). Os inóculos serão sempre preparados poucos minutos antes da inoculação nos animais, para que a viabilidade fúngica seja preservada.

3.2 Características do jato de plasma

Será utilizado equipamento de jato de plasma operado em baixa temperatura sob pressão atmosférica (CAP), gerado por um circuito elétrico bivolt (110/220 V) dentro de um cilindro com 11 cm de comprimento e 7 cm largura. Esse equipamento apresenta eletrodo de alta frequência com fonte de tensão pulsada. Será operado com gás hélio (He, Air Liquide, Brasil) exibindo 99,9% de pureza e vazão controlada de 2,0 ± 0,1 SLM (Litros por minuto) por controlador digital (N100 Horiba STEC).

3.2 Procedimentos e grupos experimentais

A metodologia de indução da candidose oral será baseada em alguns trabalhos prévios (Borges et al., 2018; Rossoni et al., 2015). Trinta e cinco camundongos machos (*Mus musculus* Swiss) de aproximadamente 40 g serão randomicamente divididos em 5 grupos: 1) tratados com CAP (n = 7), 2) tratados com CAP e Vit C (n = 7), 3) tratados com Vit C (n = 7), 4) tratados com nistatina (n = 7) e 5) não tratados – grupo controle (n = 7).

Todos os animais serão imunossuprimidos com metilpredinisolona (Depro-Medrol; Pfizer) por via intraperitoneal na concentração de 100 mg/Kg de peso corporal, durante 4 dias intercalados. A inoculação com *C. albicans*, também abrangendo todos os animais, será realizada 2 vezes, em dias intercalados com as imunossupressões. Para isso, os animais serão sedados por via intraperitoneal com Cetamina 10% (Dopalen, 100 mg/kg) e Xilazina 2% (Anasedan, 10mg/kg). Então swabs embebidos em suspensão de *C. albicans* ATCC18804 ($1x10^8$ células/mL) serão mantidos em contato com o dorso da língua do animal durante 3 minutos. Três dias após a última inoculação, os animais serão examinados para o diagnóstico clínico das lesões. Os camundongos serão mantidos em mini-isoladores de rack ventilado com temperatura e luz controladas (22 °C; ciclo claro/escuro de 12/12h), receberão

alimentação padrão e água *ad libitum* acrescida de tetraciclina (Terramicina – Pfizer, 0,83 mg/ml).

Os camundongos serão submetidos ao tratamento com CAP duas vezes em um período de 48 horas, sendo que o primeiro tratamento será realizado 24 horas após o diagnóstico clínico da lesão. Os grupos tratados com CAP serão expostos ao jato de plasma durante 5 minutos, em dois sítios diferentes do dorso lingual, envolvendo o terço mediano anterior e posterior. Será mantida a distância de 1,5 cm entre a saída do aparato e a língua do animal. Os camundongos do grupo Vit C serão submetidos a injeções locais na mucosa com o composto, na concentração de 100 mg/kg. Já os animais do grupo CAP + Vit C receberão o tratamento com CAP, como descrito anteriormente, e injeções locais em mucosa de vitamina C (100 mg/kg), nas mesmas áreas tratadas pelo CAP, englobando o terço mediano anterior e posterior. Os tratamentos com CAP e Vit C serão realizados sob sedação. A simulação do tratamento da vitamina C, pela administração do veículo (água destilada) de forma injetável, será realizada nos grupos sem Vit C, assim como a simulação do procedimento do plasma (apenas gás), será executada nos grupos sem o CAP.

Já os animais do grupo nistatina, receberão 100 μL de suspensão de nistatina (100.000 UI/mL), em dorso de língua na região anterior, e 100 uL em dorso de língua na região posterior, por 2 dias. Os animais serão eutanasiados por excesso de anestésico, 24 horas após o último tratamento. O quadro 1 resume as etapas do experimento.

DIA	Evento
Dia 1	Imunossupressão
Dia 2	Infecção
Dia 3	Imunossupressão
Dia 4	Infecção
Dia 5	Imunossupressão
Dia 6	*
Dia 7	Diagnóstico e análise clínica da lesão
Dia 8	Tratamento
Dia 9	Tratamento
Dia 10	Análise clínica da lesão e eutanásia

Quadro 1: Esquema de execução do experimento

3.3 Avaliação clínica das lesões

Para avaliação clínica das lesões, previamente e posteriormente ao tratamento, as línguas serão observadas nos dias 7 e 10 do experimento. Com auxílio de uma lupa, as lesões serão minuciosamente observadas. Escores clínicos serão determinados com base em estudo prévio (Rossoni et al., 2015). Serão atribuídas pontuações de 0 a 4. Dessa forma, 0 corresponderá a dorso língua normal; 1: placas brancas/pseudomembranas em menos de 20% da

superfície; 2: placas brancas/pseudomembranas entre 21% e 90% da superfície; 3: placas brancas/pseudomembranas em mais de 91% da superfície; e 4: pseudomembranas espessas cobrindo mais de 91% da superfície do dorso lingual.

3.4 Análise microbiológica

Após a eutanásia as línguas serão hemissecionadas, pesadas e a partes destinadas à análise microbiológica serão maceradas em solução salina estéril (NaCl 0,9%). Serão então realizadas diluições seriadas e o conteúdo semeado em ágar Sabouraud Dextrose acrescido de cloranfenicol. Posteriormente, a quantidade de *C. albicans* será determinada em CFU/g (Borges et al., 2018)

3.5 Avaliação microscópica

As línguas hemissecionadas destinadas a análise microscópica serão fixadas em solução de formalina 10%, submetidas ao processamento histológico de rotina, e embebidas em parafina. Secções semi-seriadas de 5 micrômetros serão obtidas e coradas por HE (hematoxilina-eosina) para análise de alterações inflamatórias epiteliais e alterações inflamatórias no tecido conjuntivo.

Coloração de PAS (ácido periódico de Schiff) também será realizada para avaliar o grau de invasão do epitélio de dorso lingual por hifas e leveduras de *C. albicans*. Serão avaliados 21 campos histológicos de 2 cortes por animal, totalizando 42 campos. As análises serão realizadas com objetiva de 40 X em microscópio óptico (Zeiss). Serão atribuídos os seguintes escores: 0: ausência de hifas; 1: 1-5 hifas; 2: 6-15 hifas; 3: 16-50 hifas e 4: mais de 50 hifas (Borges et al., 2018; Rossoni et al., 2015).

3.6 Análise estatística

Os dados obtidos nos ensaios serão analisados estatisticamente com auxílio do programa GraphPad Prism, versão 6 (GraphPad Software Inc., San

Diego, CA, USA). Será realizado teste de distribuição amostral, para verificação de normalidade, e seleção do teste estatístico mais apropriado. O nível de significância adotado será de 5%.

4. PLANO DE ATIVIDADES E CRONOGRAMA PARA O PERÍODO

Abaixo estão apresentados o plano de atividades e cronograma para sua execução.

Atividades	Mês 1	Mês 2	Mês 3	Mês 4	Mês 5	Mês 6	Mês 7	Mês 8
Pesquisa bibliográfica	x	x	x	x	x	x	x	x
Etapa experimental em biotério	x	x						
Processamento dos dados dos clínicos e microbiológicos		x	x					
Processamento histopatológico			x	x	x			
Análises microscópicas					x	x	x	
Relatório final							x	x

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Apêndice A – Resultados microbiológicos e de viabilidade celular

1. Determinação da concentração inibitória mínima (MIC) e fungicida mínima (CFM) do ácido ascórbico

Observou-se que nenhuma das concentrações avaliadas de ácido ascórbico (Vit C), possui atividade antimicrobiana, uma vez que a turvação do meio foi semelhante ao controle de crescimento (grupo apenas com microrganismos) (Figura 1).

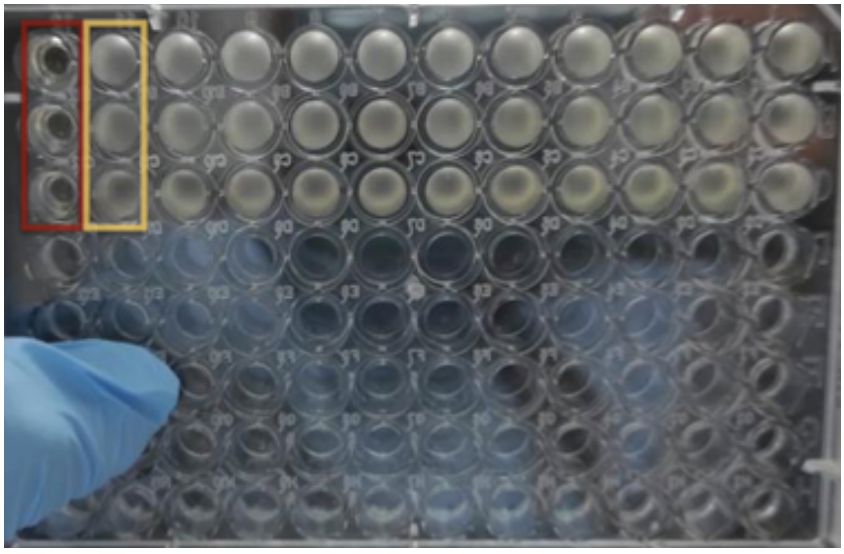

Figura 1: Imagem representativa de uma microplaca de 96 poços, onde foi realizado ensaio para estabelecimento da MIC e CFM. Os poços ilustrados com a cor vermelha indicam o controle negativo (sem turvação), enquanto os demarcados pela cor amarela correspondem ao controle de crescimento (controle positivo). Os demais poços, de turvação semelhante ao controle de crescimento, correspondem às diferentes concentrações de Vit C na faixa de 0,35 mM a 180 mM

2. Análise do tratamento proposto em biofilmes de 24 e 48 h

A avaliação dos dados de UFC/mL, após tratamento de biofilmes de 24 h, indicou diferença estatística entre os grupos ($p<0,0001$, teste Kruskal wallis) (Figura 2A). O tratamento apenas com o plasma por 2,5 min (CAP) promoveu mais de um log de redução ($P<0.05$) para o grupo controle. Houve também redução estatisticamente significativa nos grupos em que foi realizado o pré-tratamento com Ácido ascórbico na concentração de 90 mM ou o tratamento com o veículo (dH_2O), seguidos da exposição

ao CAP. O grupo Ácido ascórbico + CAP foi o grupo que apresentou o menor número de colônias, com redução de dois logs e meio em relação ao controle, embora não tenha sido diferente do grupo CAP. Já os grupos tratados apenas com Ácido ascórbico exibiram número de colônias semelhante ao grupo não tratado. A concentração de 180 mM também foi avaliada isoladamente ou em associação com o CAP, mas devido a similaridade de resultados com a concentração de 90 mM, os dados não estão apresentados.

A análise dos dados de UFC/mL, em biofilmes de 48 h, demonstrou diferença estatística entre os grupos ($p<0,0001$, teste Kruskal wallis) (Figura 2B). Tanto o grupo CAP quanto o grupo ácido Ascórbico + CAP reduziram o número de colônias ($p<0,05$ e $p<0,0001$, respectivamente). O grupo Ácido ascórbico (90 mM) + CAP foi o mais efetivo, tendo promovido uma redução de aproximadamente dois logs em relação ao grupo controle, embora não tenha mostrado diferença estatística para o grupo CAP. Já o tratamento apenas com a vitamina não reduziu o número de colônias, com resultado semelhante ao grupo não tratado.

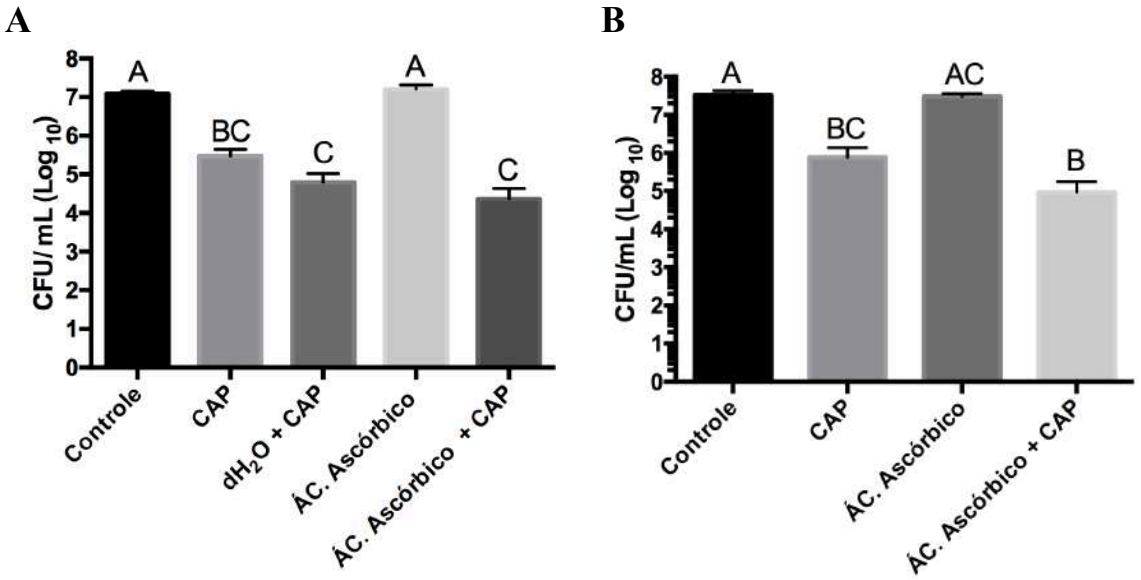

Figura 2: Gráfico representativo das médias e desvios-padrão das unidades formadoras de colônia por mL (UFC/mL) após os tratamento em biofilmes de 24 (A) e 48 horas (B) de *C. albicans*. Teste Kruskal-Wallis, comparações múltiplas (Letras diferentes indicam diferença estatística.

3. Ensaio de viabilidade celular após tratamento com CAP

A análise da viabilidade de queratinócitos expostos ao CAP indicou que o tratamento não foi citotóxico, com viabilidade celular acima de 70%, após 5 minutos de tratamento, como pode ser observado abaixo (Figura 3).

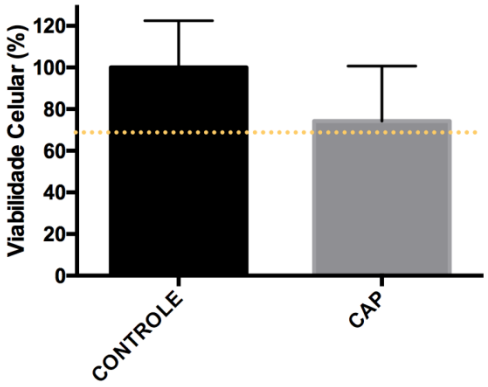

Figura 3: Gráfico representativo das médias e desvios-padrão dos dados de viabilidade celular após exposição de queratinócitos orais ao CAP 5 min. A linha pontilhada indica o limite normativo de viabilidade celular (= 70%). Note que os tratamentos não foram citotóxicos.

Apêndice B – Manuscrito aceito para publicação na Future Microbiology, contendo dados de toxicidade e imumodulação da vitamina C

Future Microbiology

Ascorbic acid as a modulator of inflammatory responseagainst Candida albicans

Journal:	*Future Microbiology*
Manuscript ID	FMB-2023-0188.R2
Manuscript Type:	Research Article
Keywords:	vitamin C, ascorbic acid, candidiasis, infection model, anti-inflammatory

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Ascorbic acid as a modulator of inflammatory response against *Candida albicans*

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ABSTRACT

Aims: To evaluate the behavior of oral keratinocytes in the presence of Vitamin C (Vit C) and its anti-inflammatory potential. **Materials & Methods:** Oral keratinocytes were initially exposed to 0.1 to 2.5 mM of Vit C and the metabolic activity and cell migration were evaluated using MTS assay and Ibidi culture inserts, respectively. After, the cells were challenged with *Candida albicans* and inflammatory markers were analyzed by qPCR. **Results:** The treatment was not cytotoxic, and the highest concentrations increased the metabolic activity at 24 h. Vit C delayed the cell migration at 48 and 72 h. Interestingly, it downregulated the genes IL-8 and IL-1b. **Conclusion:** Vit C could be an interesting adjuvant to anti-fungal treatment due to its anti-inflammatory potential.

Plain Language Summary

Vitamin C, also known as ascorbic acid, is a vitamin commonly found in fruits and vegetables. It is popular for supporting our immune system, so is commonly taken as a supplement. We looked at the action of vitamin C on cells in the mouth and its potential to reduce inflammation in a fungal disease of the mouth - oral candidiasis. We showed that vitamin C is not toxic to cells of the mouth and may reduce inflammation in cells

infected by the fungus. This suggests that vitamin C could be used as a complementary therapy for oral candidiasis.

Key words: vitamin C; ascorbic acid; candidiasis; infection model; anti-inflammatory

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1. Introduction

Oral Candidiasis (OC) is an oral disease caused by *Candida* spp. *C. albicans* is the most prevalent species, which is responsible for more than 95% of OC cases. This fungus is a commensal microorganism that inhabits the mouth, gastrointestinal, and reproductive tracts in more than 80% of the population [1]. However, *C. albicans* is an opportunistic pathogen that can proliferate and cause disease in the presence of local and/or systemic predisposing factors [1,2].

The adherence to oral epithelial cells, ability to form biofilms, evasion from host's immune defenses and the capacity to invade and destroy host tissues are considered important virulence features of *C. albicans* [1]. Polyenes and azoles have been considered the main antifungal agents to treat OC, however, the use of polyenes usually is limited to topical delivery due to the poor absorption through the gastrointestinal tract. The topical use also presents limitations because of difficulty in maintaining adequate levels of the drug in the oral environment. In contrast, azoles, such as fluconazole and itraconazole, may be administered systemically as they are well absorbed by the gastrointestinal tract. Despite this, these agents may present drug interactions with warfarin (anticoagulant agent) and statins (HMG-CoA reductase inhibitors) [3]. Moreover, the prevalence of antifungal resistance has increased significantly in recent years [3,4].

Different mechanisms of resistance have been described for *C. albicans* according to the antifungal class. Against azoles, *C. albicans* may act decreasing drug concentration, changing target enzyme, decreasing affinity to the binding site, upregulating the target enzyme and promoting the overexpression of cell membrane efflux pumps [4]. In attempt to solve these problems, alternative treatments have been investigated to treat antifungal infections in the last decade, including the combination of therapies [5].

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Vit C is a powerful antioxidant agent [6] and has been evaluated for many conditions, such as infectious diseases [7,8]. The effect of Vit C on *C. albicans* was previously investigated [8]. The authors observed that Vit C decreased the virulence and pathogenicity of *C. albicans* interfering with yeast-to-hypha transition. Van Hauwenhuyse et al. showed that Vit C may act in Hsp90-dependent *C. albicans* concerning transcriptional regulator Upc2. Vit C blocked GdA-dependent elongated growth by restoring normal ergosterol levels in a Upc2-dependent manner [7]. Interestingly, another study demonstrated that the concentration of 90 mM of Vit C can kill *C. albicans.* This effectiveness was dependent on temperature, oxygenation, availability of energy sources, growth media and growth phase [9].

Other studies have also indicated that Vit C may exert local anti-inflammatory properties, reducing ROS levels and inhibiting the activation of MAPKs, NF-κB, AP-1 and IL-8 expression in human respiratory cells [10]. In a clinical study, Vit C was able to reduce various degrees of chronic gingival inflammation [11]. Additionally, it has been considered the most powerful antioxidant agent of the skin with neocollagenesis and skin-lightening properties, which give to this compound a large range of clinical applications [6].

Considering the promising findings related to anti-inflammatory properties of Vit C as well as the modulatory effects of Vit C on *C. albicans* virulence factors, we hypothesized that Vit C could be an interesting adjunct treatment for OC. As far as we know, the effects of Vit C on in vitro model of OC have not previously been explored. The current study evaluated the effects of Vit C on oral keratinocytes, with respect to its viability/proliferation, migration properties and anti-inflammatory potential in cells challenged with *C. albicans.*

2. Material and methods

2.1 Cell culture

Primary gingival keratinocytes (PGKs) from the American Type Culture Collection (ATCC, PCS-200-014) were used in this study. The cells were cultured in Dermal Cell Basal Medium (ATCC, PCS-200-030) supplemented with keratinocyte growth kit (ATCC, PCS-200-040) and penicillin (100 U/mL). The cells were grown at 37°C in a humid environment with 5% CO_2. The media was changed every 2-3 days and cells up to 5 passages were used for all the assays.

2.2 Cell viability/proliferation assay

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PGKs were cultured in 24-well plates at a density of 20 x 10^3 cells per well (500 µL/well) and incubated at 37° for 24 h, when the treatments were performed. L-ascorbic acid (Sigma-Aldrich) was initially diluted in distilled water to a concentration of 90 mM and then filtered through a syringe filter of 0.22 mm. Afterwards, the Vit C was diluted in the cell culture media to a final concentration of 2.5, 1.25, 0.6, 0.3 and 0.1 mM, and immediately placed in contact with the cells. Cell viability was evaluated after 3 and 24 h of treatment using the colorimetric metabolic assay MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega Corp., CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay. Absorbance was measured at a wavelength of 490 nm by SoftMax Pro 6 software after 2 h of incubation. A control group (only cells and culture medium without Vit C) was performed under the same conditions and used as a normalizer. The test was carried out in two independent experiments (n=8). The cytotoxicity threshold was set at 70% according to previous studies [12,13].

2.3 Two-dimensional cell migration test (in vitro wound healing assay)

For this assay, high culture inserts with two chambers and a gap between them (Ibidi - µ-Dish 35mm; Cat. nº. 81176) were inserted in 4-well plates. PGKs were plated in the chambers at a density of 3x10^3 cells (75 mL) per chamber. The cells were cultured until reaching approximately 70% of confluence, when the inserts were removed. Subsequently, the cells were exposed to 0.1, 0.6, 1.2 mM of Vit C and the images of cells within the gap area were captured by microscopy (Leica DMI 4000B, Leica Microsystems) using Image-Pro Plus MDA, version 7.0 software. Images of the cellular migration into the gap areas were taken at 3, 24, 48 and 72 h of the starting treatment. Quantification of the distance between cells grown in the two chambers (wound area distance) was analyzed using the Wound Healing Size tool in the ImageJ/Fiji® plugin, as described by Arnedo et al. [14]. The assay was performed in two independent experiments (n=8). The treated groups were compared to control group, in which the cells were exposed only to culture medium.

2.4 Infection model

Before the infection, PGKs were plated in 24-well plates at a density of 1x10^5 cells per well (500 µL/well) and incubated at 37°C for 24 h. In the same day, *C. albicans* (SC 5314) were cultured in Sabouraud Dextrose agar plates at 37°C for 24 h. For the infection, an inoculum of *C. albicans* at a density of 5x10^5 was prepared in Dermal Cell Basal Medium (ATCC, PCS-200-

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030) supplemented with keratinocyte growth kit (ATCC, PCS-200-040) and penicillin (100 U/mL) and inserted in the wells. After 1 h of the infection, the plates were centrifuged, the spent culture medium was removed and Vit C solutions at the concentrations of 0.1, 0.6 and 1.2 mM were added to the wells. Afterward, the plates were incubated for 6 h at 37°C and the lysate was collected. To determine a suitable period of infection, the periods of 4 h and 24 h of infection were also evaluated as pilot assays. For cell lysis, a lysis solution of RNAqueous™-Micro Total RNA Isolation Kit (AM1931, Thermo Fisher Scientific) was used and the samples were stored at -80°C.

2.5 qPCR analysis

Total RNA was isolated using RNAqueous™- Micro Total RNA Isolation Kit (Thermo Fisher Scientific, AM1931). The total RNA was treated with DNase I provided with the kit to remove remnant genomic DNA in the samples. Complementary DNA (cDNA) was synthesized using High capacity cDNA reverse transcription kit (Thermo Fisher Scientific, 4368813). Quantitative real-time PCR (qPCR) was performed to quantify mRNA expression using SYBR® green PCR Master Mix (Applied Biosystems, Warringtons, UK). The oligonucleotide primer sequences for the pro-inflammatory (IL-8, GM-CSF, IL-1b, TNF-α, IL-6) and anti-inflammatory (IL-10) genes can be observed in Table 1. All samples were run in the QuantStudio 7 flex detection system. The threshold cycle (Ct) for each test gene was normalized against the 18S housekeeping gene. The housekeeping gene was unaffected by the infection or treatment with Vit C. The absolute mRNA values were calculated using the equation $\Delta Ct = Ct$ (gene of interest) – Ct (housekeeping gene).

2.6 Statistical analysis

The data were plotted and expressed as mean values and standard deviations (mean ± SD). Statistical analysis was performed using the GraphPad Prism 9 software (GraphPad Software, Inc. San Diego, CA, USA). Data were first analyzed regarding sample distribution. Parametric data were analyzed using analysis of variance (one-way ANOVA, $\alpha = 0.05$) and Tukey's post hoc test. The nonparametric data were compared by Kruskal-Wallis and Dunn's/ Dunnett's post hoc tests ($\alpha = 0.05$).

3. RESULTS

3.1 Vit C increases the metabolic activity of oral keratinocytes at 24 h of treatment in a dose dependent manner.

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After 3 h of treatment, the different concentrations of Vit C did not decrease the cell viability to levels lower than the cytotoxicity threshold (70%), although 0.3 mM reduced cell viability to a similar level (70.6%), with statistical difference in relation to the control group (Dunn's multiple comparisons test, $p<0.001$). The other concentrations of Vit C (0.1, 0.6, 1.2 and 2.5 mM) showed more than 80% cell viability with no statistical difference for in relation to the control group or among them ($p>0.05$, Dunn's multiple comparisons test) (Figure 1A). After 24 h, Vit C maintained or increased the metabolic activity, depending on the concentration. The treatment with 1.2 and 2.5 mM increased the cell viability/proliferation to 135% and 145%, respectively, with statistical differences when compared control group (Tukey's multiple comparisons test, $p<0.05$) (Figure 1B).

3.2 Vit C changes the migration properties of oral keratinocytes at 48 h and 72 h.

The analysis of the in vitro gap closure showed no statistical difference between treated and control groups at 3 h and 24 h ($p>0.05$, one-way ANOVA test). However, statistical differences were observed at 48 h and 72 h ($p<0.05$, one-way ANOVA and Kruskal-Wallis tests, respectively). A delayed gap closure kinetic (migration property) was detected in the group treated with 1.2 mM of Vit C that showed statistical differences in relation to control group at 48 h ($p<0.05$, Dunnett's multiple comparisons test) and 72 h ($p<0.05$, Dunn's multiple comparisons test). The concentrations of 0.1 and 0.6 mM of Vit C were also statistically different from control group at 48 h ($p<0.05$, Dunnett's multiple comparisons test) and 72 h ($p<0.05$, Dunn's multiple comparisons test), respectively (Figure 2).

3.3 Vit C modulates the inflammatory response of oral keratinocytes against *C. albicans*.

A range of different conditions were evaluated to find a suitable period of infection in which the control and infection groups were statistically different indicating a pro-inflammatory scenario. The period of 4 h was not sufficient to induce a pro-inflammatory response, as no statistical differences could be observed between control (only culture medium – non-infected cells) and infection groups. On the other hand, a period of 24 h of infection was very long, as most of the cells had died (data not shown).

An infection period of 7 h showed optimal conditions of infection, as can be observed in Figure 3. Statistical differences between control and infection group were observed for the genes IL-8, GM-CSF, and IL-1b ($p<0.05$, Tukey's multiple comparisons test). Vit C showed an anti-inflammatory effect with downregulation of genes IL-8 after treatment with 0.1 and 1.2 mM of

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Vit C and IL-1b after treatment with 1.2 mM of Vit C. These treatments showed statistical differences for infection group ($p<0.05$, Tukey's multiple comparisons test). The analysis of the gene GM-CSF showed no statistical differences between infection and Vit C groups ($p>0.05$, Tukey's multiple comparisons test), although lower means of absolute mRNA have been observed in the treated groups, mostly at concentration of 0.1 mM of Vit C, which also was not statistically different from negative control group (Figure 3, A, B, C).

No statistical differences were observed between groups for IL-6 and TNF-a genes, although the infection group showed a tendency of upregulation while the treated groups demonstrated a tendency of downregulation ($p>0.05$, Tukey's multiple comparisons test) (Figure 3, D and E). The anti-inflammatory gene IL-10 was not expressed at a detectable level by the cells (data not shown).

4. Discussion

The search for new anti-fungal treatments has emerged in the last decades, including the combination of therapies [5,15]. Vit C has currently been evaluated for multiple purposes [16,17] , including its action as an anti-inflammatory agent [18] . The aim of the current study was to evaluate Vit C as a possible modulator of treatment for oral candidiasis. For this, the potential effects of Vit C on the metabolic activity and migration of oral keratinocytes were first evaluated. After, an in vitro infection model in which the cells were challenged with *C. albicans* and treated with Vit C. was performed to evaluate the anti-inflammatory potential of Vit C. Oral keratinocytes were chosen for this study because they are the first line of defense against candidal infection working as a physical barrier in the oral cavity [19].

The MTS assays indicated that Vit C did not reduce the cell viability below the acceptable cytotoxicity threshold (70%) after the first hours of exposure. Additionally, the higher concentrations stimulated the cells after 24 h, as indicated by the marked increase in metabolic activity, an indicator of cell viability/proliferation. It is well known that keratinocytes can express two different transporters, which are responsible for Vit C uptake, the sodium-dependent vitamin C transporter 1 (SVCT1) and 2 (SVCT2) [20,21]. A previous study proposed a biphasic role of Vit C according to SVCT-2 expression in intestinal cancer cells. High SVCT2 expression is probably related with a pro-oxidant effect culminating in cell death while low SVCT2 expression may be related with an antioxidant effect and high cell viability/proliferation. The authors observed that the decrease in cell viability is inversely proportional to SVCT2 expression [22]. In the current study with PGKs cells, the higher metabolic activity observed after 24 h of exposure could indicate an antioxidant effect and low SVCT2 expression.

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Candidal infection often induces non-ulcerative lesions. However, in critically ill patients, this infection is commonly responsible for chronic oral ulcers [19] in which the properties of proliferation and migration of the cells are crucial for tissue repair. Keratinocytes are able to undergo an epithelial-to-mesenchymal transition, which gives them an ability to migrate toward each other by reformulating cell–cell and cell–extracellular binding. Thus, it is possible to measure the re-epithelialization stage of wound healing [23]. Therefore, we performed an in vitro migration/wound closure assay to analyze the in vitro role of Vit C in the migration of the oral keratinocytes. Our measurements indicate that Vit C delayed the wound healing kinetic after 48 h and 72 h, although Vit C did not significantly interfere with wound healing process at 3 h and 24 h. A previous study that evaluated primary epidermal keratinocytes wound healing for 24 h observed that 4.4 µg/mL of Vit C did not change the cellular gap closure after this period [24], similarly to our study after 24 h. Other studies using two-dimensional monolayer models of cells have indicated good ability of Vit C to promote in vitro wound healing, however these studies focused on different cell types [25,26]. The reasons by which vitamin C delayed the cell migration at 48 h and 72 h in our hands is still unclear and need to be investigated. It is noteworthy that this assay was not performed in the presence of infection, due to the high pathogenicity of C*. albicans* strain after 24 hours of infection, which represents a limiting factor of this in vitro study.

According to previous findings, Vit C can regulate epidermal differentiation via activation of protein kinase C (PKC). Additionally, it has been shown to protect keratinocytes against oxidative stress, particularly during the inflammatory phase of wound repair, by the improvement of the hydrophilic antioxidant status inside the cells [27]. In our study, the anti-inflammatory potential of Vit C on PGK cells challenged with *C. albicans* was investigated through the analysis of genes related to the inflammatory response. The model of infection was validated after 7 h of PGK-fungi contact via the upregulation of IL-8, GM-CSF, and IL-1b genes. These are pro-inflammatory markers that usually become upregulated when oral epithelial cells are infected with *C. albicans*, especially during direct physical contact and the germination of yeast into hyphae [28–31]. Vit C showed an anti-inflammatory potential in the conditions of this study, as observed by the statistical downregulation of IL-8 and IL-1b, and a dose-dependent downregulation tendency of the pro-inflammatory genes GM-CSF, TNF-a and IL-6.

Consistent with our findings, Vit C showed an anti-inflammatory property in UBV-damaged keratinocytes suppressing the inflammatory response via the downregulation of IL-8 and MCP-1 [32]. Respiratory epithelial cells (H292) challenged with house dust-mites (HDM) and treated with 100 or 200 mM Vit C were reported. The challenge enhanced ROS levels,

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increased IL-8 expression and activated the signaling pathways MAPKs, NF-κB, and AP-1. Interestingly, the treatment with Vit C decreased ROS levels. Additionally, it inhibited activation of MAPKs, NF-κB and AP-1 and downregulated the expression of IL-8 [10]. Other studies that have investigated different doses of Vit C also indicated that this vitamin could inhibit multiple pathways related to the activation of NF-κB transcription factor [33–35]. Cárcamo et al., 2002 pointed out the importance of the ROS in the GM-CSF signaling response and showed that the treatment with Vit C can downmodulate GM-CSF signaling responses in human monocytic (U937), kidney (293T) and human myeloid cells (HL-60) at intracellular concentrations of 5 mM [36]. In another study, Vit C decreased lipopolysaccharide (LPS)-induced ROS, TNF-α and IL-6 production in macrophages challenged and treated for 24 h with LPS and Vit C (100 nM), respectively [37]. In the present study, a dose-dependent downregulation tendency of the pro-inflammatory genes GM-CSF, TNF-α and IL-6 was observed after 6 h of treatment. Due to the high virulence of the strain used in our model of infection, it was not possible to evaluate if statistical significance would be obtained after 24 h of exposure to Vit C. Nevertheless, the anti-inflammatory potential of Vit C was seen after 6 h of treatment, resulting in the downregulation IL-8 and IL-1b.

Considering the activation of NF-kB transcription factor in the inflammatory response against *C.* albicans [38,39], Vit C may be inhibiting some signaling pathway related to NF-κB, resulting in the anti-inflammatory effects observed in this study. Corroborating our hypothesis, these findings indicate that Vit C could be in interesting adjuvant to anti-fungal treatment and highlight the importance of future in vitro and in vivo studies in this approach.

5. Conclusion

Vit C presents an anti-inflammatory effect on oral keratinocytes challenged with *C. albicans*, which suggests that it could be an interesting adjunct for the treatment of oral candidiasis. It is important to emphasize that this was an in vitro study with its limitations, and the interference of Vit C in wound repair in cases of candidiasis should be investigated in future in vivo models.

Summary points

- Vitamin C is an antioxidant agent that has been investigated for the treatment of different microbial and inflammatory conditions.
- Previous studies demonstrated that this compound may present modulatory effects on *C. albicans* virulence factors.

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- Alternative methods to reduce the use of antifungals agents are desired.
- This study evaluated for the first time the effects of Vit C on in vitro model of oral candidiasis.
- Vit C increased the metabolic activity of the cells at 24 h and delayed the cell migration at 48 and 72 h.
- Vit C downregulated the pro-inflammatory genes IL-8 and IL-1b.
- The data indicated that Vit C could be in interesting adjunct for the treatment of oral candidiasis.

Figure and Table Legends

Table 1: Oligonucleotide primer sequences

Figure 1: Cell viability of oral keratinocytes after Vit C exposure for 3 h (A) and 24 h (B). Bars represent mean and SD values after the treatments. The dotted line (red) corresponds to the toxicity threshold (ISO 10993-5). Different letters indicate statistical differences between groups (A, Kruskal-Wallis and Dunn's post hoc tests, $p<0.05$; B, one-way ANOVA and Tukey's post hoc test, $p<0.05$).

Figure 2: Analysis of cell migration or in vitro wound closure using oral keratinocytes (A) Mean data and SD error bars of the remaining scratch/gap area at 3, 24, 48 and 72 h. (B) Illustrations of the in vitro wound closure for each group at all the periods. The images were captured in an inverted microscope at 250X magnification. The asterisks (*) indicate statistical differences between control group and 0.1/1.2 mM of Vit C at 48 h and 0.6/1.2 mM of Vit C at 72 h (3, 24, 48h; one-way ANOVA and Dunnett's post hoc test, $p<0.05$), (72 h; Kruskal-Wallis and Dunn's post hoc tests, $p<0.05$).

Figure 3: Effect of infection with Candida albicans for 7 h and treatment with Vit C for 6 h on IL-8 (A), GM-CSF (B), IL-1b (C), IL-6 (D) and TNF-α (E) gene expression by oral keratinocytes. Bars represent mean and SD values after the different treatments. 18S was used as housekeeping gene. Different letters indicate statistical differences between groups (one-way ANOVA and Tukey's post hoc test, $p<0.05$).

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No writing assistance was utilized in the production of this manuscript.

Competing interest disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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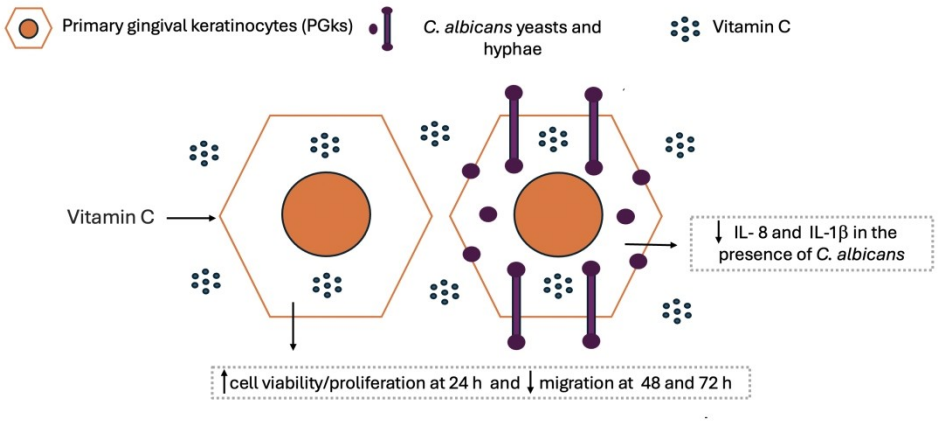

GRAPHICAL ABSTRACT

225x111mm (300 x 300 DPI)

Table 1: Oligonucleotide primer sequence

IL-8	FORWARD	TCCTGATTTCTGCAGCTCTG
	REVERSE	GTCCACTCTCAATCACTCTCAG
GM-CSF	FORWARD	ATGTGAATGCCATCCAGGAG
	REVERSE	CTTGTACAGCTCCAGGCG
IL-1β	FORWARD	TCTTCGACACATGGGATAACG
	REVERSE	GAGGTGGAGAGCTTTCAGTTC
IL-6	FORWARD	AAAAGTCCTGATCCAGTTCCTG
	REVERSE	TGAGTTGTCATGTCCTGCAG
TNF-α	FORWARD	CTCACCCACACCATCAGC
	REVERSE	GAAGACCCCTCCCAGATAGA
IL-10	FORWARD	AGGATCAGCTGGACAACTTG
	REVERSE	GATGTCTGGGTCTTGGTTCTC
18S	FORWARD	CGGCGACGACCCATTCGAAC
	REVERSE	GAATCGAACCCTGATTCCCCGTC

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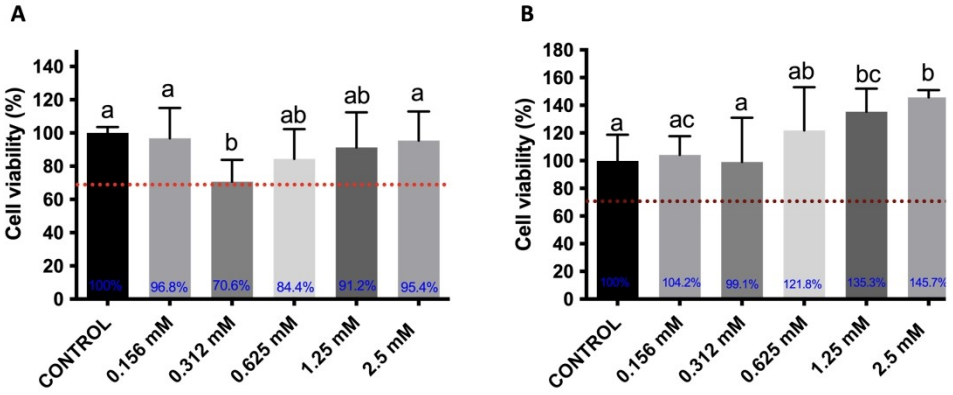

Figure 1: Cell viability of oral keratinocytes after Vit C exposure for 3 h (A) and 24 h (B). Bars represent mean and SD values after the treatments. The dotted line (red) corresponds to the toxicity threshold (ISO 10993-5). Different letters indicate statistical differences between groups (A, Kruskal-Wallis and Dunn's post hoc tests, $p<0.05$; B, one-way ANOVA and Tukey's post hoc test, $p<0.05$).

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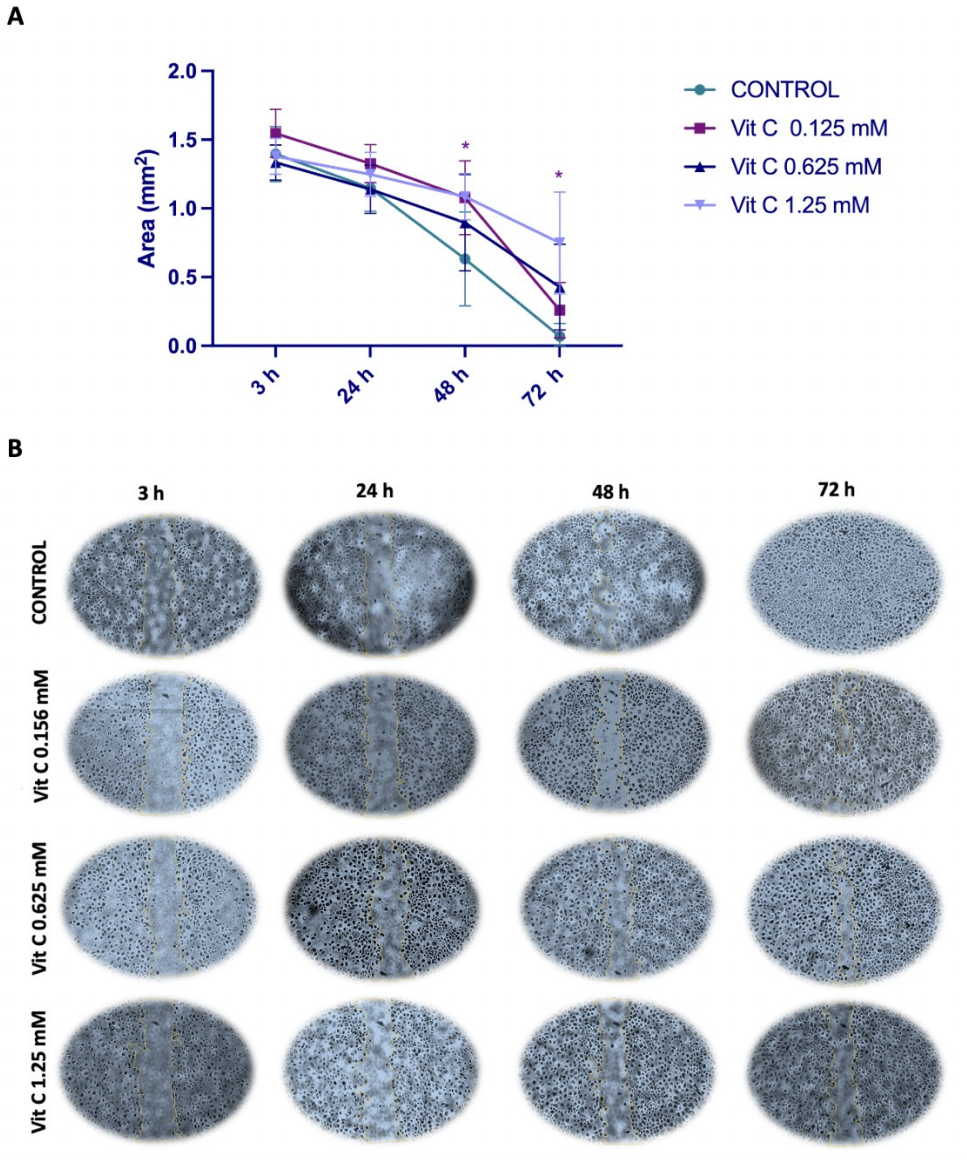

Figure 2: Analysis of cell migration or in vitro wound closure using oral keratinocytes (A) Mean data and SD error bars of the remaining scratch/gap area at 3, 24, 48 and 72 h. (B) Illustrations of the in vitro wound closure for each group at all the periods. The images were captured in an inverted microscope at 250X magnification. The asterisks (*) indicate statistical differences between control group and 0.1/1.2 mM of Vit C at 48 h and 0.6/1.2 mM of Vit C at 72 h (3, 24, 48h; one-way ANOVA and Dunnett's post hoc test, $p<0.05$), (72 h; Kruskal-Wallis and Dunn's post hoc tests, $p<0.05$).

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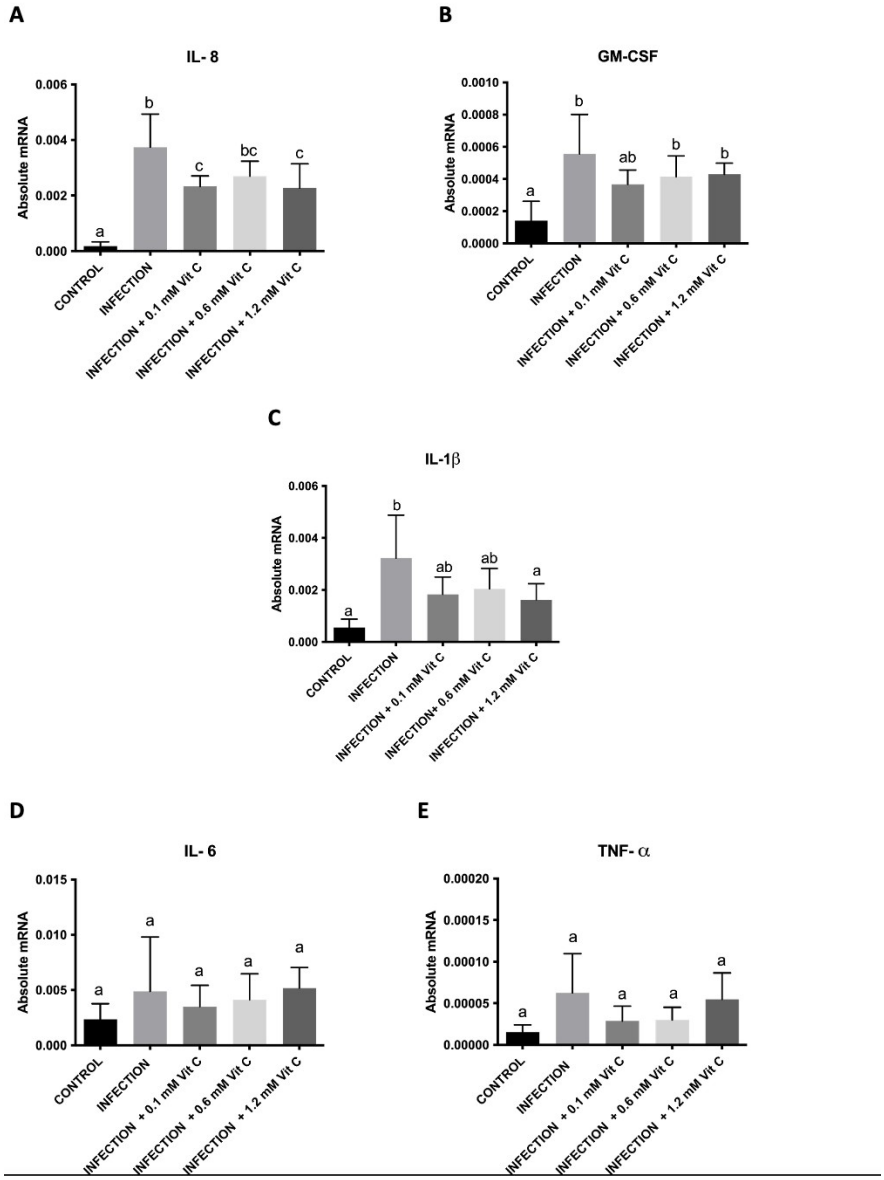

Figure 3: Effect of infection with Candida albicans for 7 h and treatment with Vit C for 6 h on IL-8 (A), GM-CSF (B), IL-1b (C), IL-6 (D) and TNF-α (E) gene expression by oral keratinocytes. Bars represent mean and SD values after the different treatments. 18S was used as housekeeping gene. Different letters indicate statistical differences between groups (one-way ANOVA and Tukey's post hoc test, $p<0.05$).

85x116mm (300 x 300 DPI)

Apêndice C – Resultados de viabilidade celular da associação CAP + Vitamina C

A análise da viabilidade de queratinócitos orais expostos ao CAP, e posteriormente a Vitamina C, por 3 horas, indicou que a associação de tratamentos não reduz a viabilidade celular, com relação ao tratamento feito exclusivamente com o CAP. Ao contrário, o grupo exposto ao CAP e tratado com a maior concentração de Vitamina C, exibiu maior viabilidade celular ($p<0.05$).

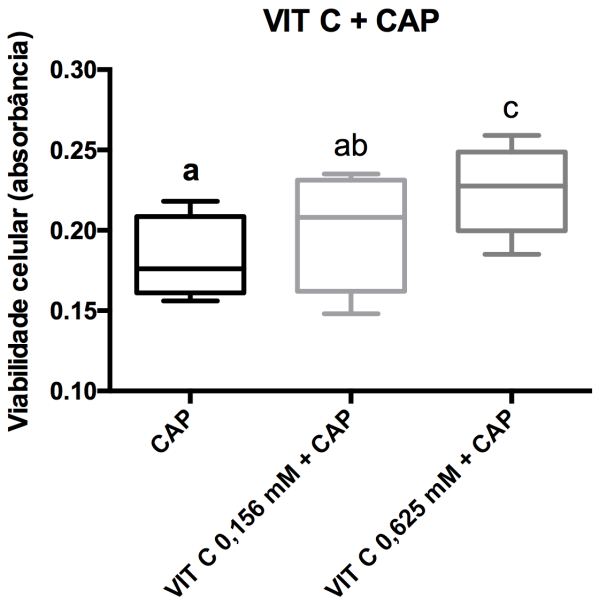

Figura 4: Gráfico representativo das médias e desvios-padrão dos dados de viabilidade celular após exposição de queratinócitos orais ao CAP e ao CAP seguido de VIT C, em diferentes concentrações, por 3 horas (Teste Kruskal-Wallis, comparações múltiplas (Letras diferentes indicam diferença estatística).