UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

MARINA CURRA

EFEITO DE DIFERENTES TERAPIAS NA PATOBIOLOGIA DA MUCOSITE QUIMIOINDUZIDA POR 5-FLUOROURACIL EM HAMSTER

Porto Alegre
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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de Cirurgião-Dentista.

Orientadora: Profª. Drª. Manoela Domingues Martins

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“A mente que se abre a uma nova ideia

jamais voltará ao seu tamanho original.”

Albert Einstein

RESUMO

CURRA, Marina. **Efeito de diferentes terapias na patobiologia da mucosite quimioinduzida em hamster.** 2013. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

A mucosite bucal é uma complicação comum no tratamento do câncer e o desenvolvimento de intervenções efetivas para sua prevenção e tratamento são vistos como prioridade nos cuidados de suporte ao paciente oncológico. O objetivo do presente estudo foi investigar diferentes terapias na patobiologia da mucosite quimioinduzida em hasmter. Para isso, serão abordados dois trabalhos: o primeiro avaliando o perfil imunoistoquímico das citocinas pró-inflamatórias (IL-1β e TNF- α) em mucosite quimioinduzida tratada com camomila e corticóides; e, o segundo, o efeito da fototerapia laser (FTL) na patobiologia da mucosite bucal induzida por 5-fluorouracil (5-FU) em hamsters. Trabalho 1: foram utilizados blocos de espécimes obtidos de *hamsters* sírios dourados de experimento prévio, nos quais induziu-se mucosite com o quimioterápico 5-FU divididos em 3 grupos: Grupo I: sem tratamento (controle); Grupo II: tratamento com camomila; Grupo III: tratamento com corticóide. Os tempos de análise foram de 0, 5, 10 e 14 dias após a infusão do quimioterápico. Cortes histológicos de 3μm foram submetidos à técnica imunoistoquímica para detecção das citocinas pró-inflamatórias IL-1β e TNF- α. Foi realizada análise qualitativa descritiva do padrão de distribuição da imunomarcação, semiquantitativa por sistema de escore. O tratamento com camomila tópica reduziu os níveis de IL-1β e TNF- α, demonstrando sua ação antiinflamatória. Trabalho 2: foram utilizados hamsters sírios dourados para investigar diferentes protocolos de FTL divididos em 4 grupos (n=6): Grupo Controle, Grupo Preventivo; Grupo Terapêutico e Grupo Conjugado (Preventivo + Terapêutico); induziu-se mucosite com o quimioterápico 5-FU e escarificação da mucosa. Os animais receberam irradiação com laser de diodo (InGaAlP), 660 nm, 40 mW de potência, 6,0 J/cm^2 por 6 segundos/ponto, dose total de energia por ponto de 0,24J. Os animais receberam 1,44J de energia diária de acordo com o grupo. Nos dias 0, 5, 10 e 15 foram sacrificados seis animais de cada grupo, pesados e a mucosa jugal foi removida para análise clínica. As amostras foram submetidos à análise semi-quantitativa do NF-κB através da técnica western blot. Os animais que receberam laser em qualquer dos protocolos apresentaram menor escore de mucosite quando comparados ao grupo controle e maior nível de NF-κB ativado. Conclui-se que tanto a camomila como o FTL atuam de forma positiva no controle da mucosite e agem modulando a resposta inflamatória e acelerando o reparo tecidual.

Palavras-chave: Mucosite. Patobiologia. Camomila. Laser. Citocinas.

ABSTRACT

CURRA, Marina. **Effect of different therapies in the pathobiology of 5-fluorouracil-induced bucal mucositis in hamsters**. Final Paper (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

Buccal mucositis is a common complication in cancer treatment. The development of effective interventions for prevention and treatment are seen as a priority in supportive care to cancer patients. The aim of this study was to investigate different kind of therapy in the pathobiology of 5-fluorouracil-induced bucal mucositis in hamsters. For this, two studies have been analysed: the first assessing the effect of topical chamomile and corticosteroid treatment on the profile of tissue cytokines (IL-1β and TNF-α) in 5-fluorouracil-induced bucal mucositis in hamsters; and, the second, the effect of low power laser (LBP) in the pathobiology of 5-fluorouracil-induced bucal mucositis in hamsters. Study 1: were used blocks from Golden Syrian hamsters obtained of previous experiment in which 5-FU- induced bucal. The animals were divided into 3 groups: Group I: no treatment (control), Group II: treatment with chamomile, Group III: treatment with corticosteroids. The analysis times were 0, 5, 10 and 14 days after administration of 5-FU. Histological sections of 3μm be submitted to immunohistochemistry for the detection of pro-inflammatory cytokines IL-1β and TNF-α. The evaluation was performed by a single observer blinded and calibrated. We performed descriptive qualitative analysis of the distribution pattern of immunostaining, by semiquantitative scoring system. Treatment with topical chamomile reduced the tissue levels of IL-1β and TNF-α, thereby demonstrating anti-inflammatory action in buccal mucositis in hamsters. Study 2: were used Syrian golden hamsters divided into 4 groups (n = 6): control group, preventive group, therapy group and conjugate group (preventive + therapy); The 5-FU-induced bucal mucositis and the mucosa was scratched. The animals received irradiation with LPT, diode (InGaAlP), 660 nm, 40 mW, 10.0 J/cm2 for 6 sec / point according to the group. On days 0, 5, 10 and 15 six animals were sacrificed, weighed and the bucal mucosa was removed for clinical analysis. The samples were removed to quantitative analysis of NF-kB by Western blot. The animals that received LPT had lower mucositis scores than the control group and higher levels of activated NF-kB. It was concluded that both chamomile LPT act as a positive control mucositis and act by modulating the inflammatory response and accelerating tissue repair.

Keywords: Mucositis. Pathobiology. Chamomile. Laser. Cytokines.

SUMÁRIO

1 ANTECEDENTES E JUSTIFICATIVA

A mucosite bucal é uma complicação decorrente da toxicidade da terapia antineoplásica, seja esta radioterapia ou quimioterapia. Ambas as modalidades terapêuticas são inespecíficas, pois interferem não somente na homeostasia de células malignas, mas também de células normais (SONIS*,* 2011). Sua incidência é variável, entretanto, é especialmente comum em pacientes que recebem quimioterapia mieloblástica agressiva como, por exemplo, durante o condicionamento para transplante de medula óssea (TMO), terapia de infusão contínua para câncer de mama e cólon e em terapia para tumores da cabeça e pescoço (KEEFE et al*.,* 2007; VERA-LLONCH et al*.,* 2007; SONIS, 2013).

Clinicamente, a mucosite é classificada em 4 estágios, que variam desde lesões eritematosas pouco sintomáticas a quadros severos de ulceração e dor que podem levar à modificação do tratamento anti-neoplásico ou até mesmo a necessidade da interrupção do mesmo, promovendo assim, uma redução da qualidade de vida e/ou a sobrevida do paciente (SONIS*,* 2004; ELTING et al*.,* 2003; BARASCH et al*.,* 2006). O sistema de graduação mais utilizado para avaliação clínica da mucosite é o da Organização Mundial de Saúde (OMS) o qual considera critérios objetivos e subjetivos, envolvendo o estado físico e nutricional do paciente, assim como o aspecto clínico da boca (PARULEKAR et al., 1998). De acordo com esta classificação a mucosite varia de grau 0 a 4 como descrito a seguir: 0-ausência de alterações na mucosa; 1- inflamação e eritema; 2- eritema e ulceração (o paciente consegue engolir sólidos); 3- ulceração (o paciente pode apenas ingerir líquidos) e 4-não é possível se alimentar pela boca. Paralelamente a isto, a mucosite severa pode ocasionar aumento no tempo de permanência do paciente internado no hospital, necessidades de cuidados especiais, incluindo infusão intravenosa de barbitúricos, outros fármacos, nutrição parenteral acarretando um custo econômico mais elevado para o tratamento (ELTING et al*.,* 2003).

Os mecanismos exatos por meio dos quais as drogas quimioterápicas citotóxicas e a radioterapia provocam a mucosite não foram completamente elucidados. Entretanto, estes parecem resultar de uma série de interações dinâmicas, de eventos moleculares e celulares que envolvem todos os elementos da mucosa (epitélio e conjuntivo). A classificação atual descreve 5 fases na patobiologia da mucosite denominadas de: 1)Iniciação, 2)Super-regulação, 3)Amplificação,

4)Ulceração e 5)Cicatrização. Na primeira, a quimioterapia e/ou radioterapia iniciam a quebra de fitas de DNA e desencadeiam a produção de espécies reativas de oxigênio (ROS do inglês reactive oxygen species) sendo denominada de iniciação. Neste momento, clinicamente a mucosa exibe aspecto de normalidade, entretanto, mudanças significativas ao nível molecular e celular estão ocorrendo. A segunda fase do processo, denominada de super-regulaçao, se caracteriza pela ativação de fatores de transcrição como o NF-κB (do inglês nuclear factor kappa b) e produção de citocinas pró-inflamatórias (TNF-α e IL-1B) por produtos da fase anterior (SONIS, 2007; SONIS, 2013). A ativação do NF-κB requer a fosforilação de seus inibidores fisiológicos (particularmente o IκBα) em resíduos específicos de serina. Esta fosforilação é mediada por um complexo proteico. O complexo kinase kappa B (IKKs) é composto de três subunidades, duas unidades catalíticas IKK-α, IKK-β, e uma unidade reguladora IKKγ (NF-κB modulador essencial). Após a fosforilação ocorre a subsequente degradação das IκBs através das ubiquitinas formando um proteossoma 26S (YAMAOKA et al., 1998). A degradação proteolítica dos IKBs permite a translocação do NF-κB ao núcleo, onde é regulada a expressão de centenas de genes que são importantes à resposta imune inflamatória que por sua vez, também estimulam enzimas que produzem a apoptose e acabam por bloquear o mecanismo de crescimento e diferenciação celular. Associado a isto tem sido descrito que o dano gerado ao tecido conjuntivo e ao endotélio reduz a oxigenação epitelial, resultando na morte de células epiteliais.

A fase seguinte, denominada de amplificação de sinal, promove um *feedback* positivo das citocinas inflamatórias e amplifica o dano iniciado pela radiação ou agente quimioterápico causando agressões aicionais ao tecido. Clinicamente podem ser visualizado eritema e dor (SONIS, 2004). Desta forma, com as modificações epiteliais e conjuntivas ocorre perda de integridade da mucosa e o aparecimento de ulcerações. A fase ulcerativa da mucosite é bastante significativa do ponto de vista clínico. A perda da integridade da mucosa resulta em lesões dolorosas e propensas a colonização bacteriana superficial. No caso de pacientes neutropênicos, a ruptura da mucosa serve como porta de entrada para numerosos microorganismos da cavidade bucal e que muitas vezes podem levar a bacteremia e sepse. Além disso, com a penetração das bactérias na submucosa, ocorre ativação de intenso infiltrado inflamatório, composto por macrófagos, plasmócitos e linfócitos, que por sua vez

potencializa a lesão tecidual. Clinicamente a lesão ulcerada é dolorosa e pode estar coberta por exsudato rico em fibrina (SONIS, 2004; SONIS, 2011).

No momento em que o estímulo agressor for interrompido, ou seja, não houver mais exposição a radiação ou o quimioterápico for eliminado,o estímulo de produção de ROS e a ativação da reação inflamatória é cessada permitindo que o reparo se estabeleça. Esta última fase portanto, representa a ativação de mecanismos envolvidos na reparação tecidual e resulta no estímulo de migração, proliferação e diferenciação de células epiteliais e mesenquimais que culminam com o fechamento das lesões em boca (SCULLY, 2003; LOGAN et al*.,* 2008; SONIS, 2011).

Estudos prévios mostram que se os pacientes em tratamento oncológico desenvolverem mucosite bucal na sua forma ulcerativa (Grau 2 a 4), uma média de 5,8 dias adicionais de uso de narcóticos e 1,9 dias adicionais de nutrição parenteral são necessários. Além disto, a presença de mucosite bucal tem sido associada ao aumento de infecções sistêmicas e fadiga, o que faz com que o paciente necessite de cuidados de suporte adicionais pela equipe de enfermagem, médica e dentistas especialistas em oncologia. O custo total destes cuidados em pacientes com mucosite grau 3 e 4 pode ser superior a US$42.749 por paciente. Nos casos de pacientes submetidos a quimioterapia sem transplante, se os mesmos apresentarem mucosite grau 3 e 4, cerca de 35% receberão a dose do quimioterápico com atraso, 60% reduzirão a dose e 30% terão sua quimioterapia suspensa fatos que podem inteferir no prognóstico destes pacientes (SONIS et al., 2001; LALLA et al., 2006; ELTING et al., 2003).

Baseado no exposto acima, a prevenção e o controle da mucosite bucal está se tornando cada vez mais importante no manejo de pacientes oncológicos e o desenvolvimento de intervenções efetivas são vistos como de alta prioridade nos protocolos de suporte a estes doentes. Os tratamentos para mucosite bucal, de modo geral, são apenas paliativos, visando diminuir a severidade da manifestação, controlar os sintomas e possíveis quadros infecciosos e/ou hemorrágicos. Os tipos mais comuns de tratamentos para mucosite são: antimicrobianos tópicos, citocinas para estimulação da medula, vitaminas, fatores de crescimento, bochechos com corticóides e colutórios não alcoólicos, fitoterápicos, aminoácidos suplementares, crioterapia, e mais recentemente, tratamento com fototerapia a laser (FTL) (ANTUNES et al*.,* 2007; KEEFE et al*.,* 2007; SHUBERT et al*.*, 2007; EDUARDO et

al., 2009; KHOURI et al., 2009; LOPES et al., 2009; ZANIN et al.,2010; CHOR et al., 2010; MIGLIORATI et al., 2013).

Apesar de existirem inúmeros estudos clínicos para comparar a efetividades de diferentes protocolos de tratamento de mucosite (SHUBERT et al., 2007) os modelos animais ainda são muito utilizados especialmente compreender a patobiologia da mucosite e para verificar os efeitos celulares e moleculares das terapias empregadas para prevenção e/ou tratamento desta doença (CLARKE et al., 2002; MORVAN et al., 2004; LIMA et al., 2005; MITSUHASHI et al., 2006; LEITÃO et al., 2007; PAVESI et al., 2008; FRANÇA et al., 2009; LOPES et al., 2010; PAVESI et al., 2010; LOPEZ et al., 2013). Estudos investigando o papel das citocinas pró-inflamatórias e de fatores de crescimento envolvidos na angiogênese e na apoptose ainda não estão completamente elucidados na mucosite. Existem dúvidas quanto ao papel destas moléculas no desenvolvimento da mucosite e como se comportam ao longo de todo o curso clínico da doença. Além disso, ainda não está esclarecida a ação dos diferentes tratamentos na síntese de citocinas e de fatores de crescimento na mucosite bucal. A caracterização dos eventos biológicos que ocorrem no contexto da mucosite conduzirá inevitavelmente a descoberta de melhores protocolos de prevenção e tratamento da mucosite resultando em melhora qualidade de vida dos pacientes em tratamento de câncer. Neste sentido, nosso grupo tem estudado diferentes protocolos de tratamento/prevenção para mucosite com base nos seus efeitos clínicos e na patobiologia dessa condição (SONIS, 2011; SONIS, 2013)

Abaixo revisaremos os tratamentos que foram utilizados no manejo da mucosite bucal nos trabalhos que serão a seguir apresentados a saber: camomila e FTL.

Camomila

O uso da medicina baseada em plantas (fitoterapia) tem sido proposto no tratamento da dor e na promoção do reparo tecidual. Estudos recentes descrevem a composição do extrato preparado a partir de rizomas ou flores, e as ações que estas substâncias possam ter isoladamente. Como é o caso dos flavonóides, que parecem interferir nas propriedades funcionais em células de mamíferos, como por exemplo: mastócito, basófilo, linfócito, músculo liso e plaquetas. Alguns autores crêem que, com base nestas interferências, estes compostos possam apresentar atividades

antiinflamatórias, antialérgicas, antivirais e anticarcinogênicas (DUARTE et al., 2011; ZANOLI, et al., 2000)

A camomila é uma planta medicinal conhecida há milênios que possui ação antiinflamatória, antimicrobiana e possui propriedades cicatriciais no tratamento de lesões ulceradas. Os principais constituintes das flores da camomila incluem diversos compostos fenólicos, primeiramente os flavonóides, aspergenina, quercetina, patuletina e seus glicosídeos. Os componentes principais do óleo essencial extraído das flores são o alfa-bisabol dos terpenóides, seus óxidos e azulenos, incluindo o camazuleno (JAKOVLEV et al., 1979; JAKOVLEV et al., 1983; GLOWANIA et al., 1987; ZANOLI, et al., 2000; KYOKONG et al., 2002).

Estudos *in vitro* demonstraram que a camomilla possui propriedades antioxidantes, antimicrobiana e significativa ação anti-agregação plaquetária. Estudos em modelos animais indicam potente ação antiinflamatória, algumas propriedades mutagênicas e capacidade de reduzir os níveis de colesterol (JAKOVLEV et al., 1979; JAKOVLEV et al., 1983; ZANOLI et al., 2000).

Ramos-e-Silva et al. (2006) avaliaram o efeito do extrato de camomila recutita (Ad-Muc) em estomatite aftosa recorrente e verificaram efeito analgésico, boa tolerância pelos pacientes e que este fitoterápico acelera a involução dos quadros de ulceração em boca. Martins et al. (2009) estudaram a citotoxicidade e o efeito reparador do extrato fluido de camomila em úlceras em comparação com os corticóides. *In vitro,* a camomila diminuiu a viabilidade celular em 36%. Entretanto, *in vivo* se revelou um excelente agente cicatrizador de úlceras quando comparada aos corticóides que atrasaram o reparo tecidual devido à infecção secundária na ferida. A eficácia da camomila como protetor de mucosa bucal em mucosite foi avaliada em poucos estudos e os resultados são controversos especialmente por não haver métodos de avaliação padronizados (LALLA et al., 2006; PETERSON et al., 2010; FIDLER et al., 1996; MAZOKOPAKIS et al., 2005).

Baseado nas propriedades antiinflamatórias e antibacterianas da camomila, hipotetizamos que este fitocomposto exibe um perfil seguro para a tratamento da mucosite bucal sendo, portanto, uma nova alternativa para o tratamento desta doença. Assim sendo, nosso grupo estudou o efeito clínico e histopatológico da camomila comparado aos corticóides em mucosite em hamesters (PAVESI et al., 2010). O grupo tratado com corticóide mostrou quadro clínico mais severo, enquanto que o grupo tratado com camomila demonstrou quadro clínico leve de

mucosite ao longo de todos os períodos experimentais. O grupo tratado com camomila exibiu 12 vezes mais chance de atingir o escore zero (ausência de mucosite) do que o grupo corticóide. A análise dos escores histopatológicos demonstrou que a grupo tratado com camomila exibiu ao longo dos períodos experimentais o menor grau de mucosite quando comparado ao controle e ao corticóide. Todavia, os mecanismos celulares e bioquímicos que levaram a estas modificações clínicas e histopatológicas ainda não foram elucidados.

2. Fototerapia Laser

A palavra LASER é um acrônimo de “Light Amplification by Stimulated Emission of Radiation” (amplificação da luz por emissão estimulada de radiação) (GENOVESE et al., 2007). O laser é um dispositivo que emite luz através de um processo de amplificação óptica baseado na emissão estimulada de radiação eletromagnética. A luz laser possui propriedades únicas como a monocromacidade (um único comprimento de onda), a colimação (uma única direção, sem convergências) e a coerência (propagação na mesma direção de fótons com a mesma freqüência) (MATIC et al., 2003). Estas propriedades permitem que a luz laser penetre na superfície da pele de maneira não invasiva e precisa (MATIC et al., 2003; SCHINDL e NEUMANN, 1999).

De acordo com os seus efeito biológico nos tecidos o laser tem sido classifcado em laser de baixa potência e de alta potência. Os lasers de alta potência emitem radiações que produzem efeito térmico no tecido alvo e são utilizados para incisões, vaporização (GONTIJO et al., 2005), ablação (FREITAS et al., 2007), coagulação e esterilização (GENOVESE, 2007; GOUW-SOARES et al., 2001) dos tecidos biológicos alvo. Os lasers de baixa potência têm sido utilizados na terapêutica de várias condições patológicas principalmente de ordem inflamatória como mucosite (MIGLIORATI et al., 2013; SCHUBERT et al., 2007), reparo de feridas (HOPKINS et al., 2004; WAGNER et al. 2013), ulcerações (DE SOUZA et al., 2010), DTM (EMSHOFF et al.,2008), herpes (SCHINDL e NEUMANN, 1999) etc. Vários outros nomes têm sido dado a esses lasers, incluindo laser mole, laser de baixa reatividade (do inglês Low reactive-level laser), laser de baixa energia, laser frio, e laser em baixa intensidade (do inglês Low level laser therapy). A terapia feita com esses lasers é atualmente denominada de fototerapia laser (FTL) e corresponde ao uso de laser com comprimentos de onda que variam do espectro visível da luz (λ = 400 nm) ao infravermelho (λ = 1,064 nm) (DAMANTE et al. 2008) e

com potências abaixo de 500 mW, onde a irradiação propriamente dita vai gerar um efeito biológico. Nesta faixa de comprimento os lasers são atérmicos, ou seja, não ocorre transferência de calor, pois a energia absorvida pelos fotóns é transferida diretamente para as células-alvo, onde será transformada em efeitos fotoquímicos, fotofísicos e/ou fotobiológicos/fotobioestimulação (MATIC et al., 2003; LINS et al., 2010; BAPTISTA et al., 2010). As baixas densidades de energia e comprimentos de onda dos LBP fazem com que haja capacidade da luz laser penetrar em profundidade nos tecidos (HENRIQUES, CAZAL, CASTRO, 2010).

Os mecanismos de ação que geram a fotoestimulação nos tecidos são bastante complexos e ainda não estão totalmente esclarecidos. Vários modelos têm sido descritos na tentativa de explicar os mecanismos o efeito da FTL nas células entretanto, a explicação mais fundamentada é a denominada de teoria fotoquímica que procura explicar a sensibilidade das células à luz laser. Essa teoria considera que a conversão da FTL em energia fotoquímica ocorre com a estimulação das moléculas fotorreceptoras ou cromóforos por energia eletromagnética (KARU, 1989) que uma vez absorvida, interage com as estruturas moleculares e celulares gerando efeitors biológicos. Porém, cada indivíduo possui quantidades e organização diferentes de moléculas o que pode interferir no resultado final do efeito terapêutico.

Uma vez absorvida a FTL pode gerar diferentes efeitos que podem ser classificados em (1) primários, (2) secundários e (3) terciários (DYSON, 2006). Os efeitos denominados de primários dizem respeito a absorção de energia pelos tecidos nas camadas superficiais e posterior depósito desta energia nos tecidos para ser transformada imediatamente em efeitos biológicos. Nos efeitos primários (1) ocorre a absorção de fotóns pelos cromóforos. Esses citocromos, presentes na mitocôndria, são os responsáveis por converter o ADP em ATP, fornecendo energia para a célula (MATIC et al., 2003), e, por serem fotossensíveis, a irradiação do laser é aborvida e convertida em energia (LABBE et al., 1990). Este processo acaba por acarretar em alterações na permeabilidade da membrana, melhora na sinalização entre mitocôndria, núcleo e citosol, além de formação de ácido cítrico e aumento do metabolismo oxidativo para produzir mais ATP (KARU, 1989). Portanto, estes efeitos primários são aqueles que ocorrem na presença da luz laser que após sua absorção irão gerar uma cascata de reações bioquímicas resultando nos efeitos secundários.

Os efeitos secundários ocorrem sem a presença da luz laser e podem ocorrer horas ou dias após a irradiação (GENOVESE, 2007). Esses efeitos resultam das

mudanças fisiológicas a nível celular (DYSON, 2006). Com a irradiação há um aumento no fluxo de cátions (Ca+) intracelular, o que acaba por estimular o metabolismo celular e algumas vias de sinalização envolvidas no processo de reparo de feridas e proliferação celular (KARU, 1989; SMITH, 1991). Nos efeitos terciários (3), a resposta é influenciada pelo ambiente interno e externo e também pela interação intracelular através do aumento dos níveis de citocinas ou de fatores de crescimento (SMITH, 1991). Esse mecanismo pode explicar porque o tratamento de uma lesão pode estimular a resolução desta lesão e de alguma outra localizada em outro sítio próximo a ela (DYSON, 2006).

A FTL tem mostrado melhores efeitos sobre os tecidos que mostrem alterações funcionais ou lesão tecidual. De acordo com Karu, Pyatibrat e Kalendo (1989), o laser é mais efetivo em baixas concentrações de oxigênio e queda de pH pois estes estados levam a alteração do estado redox celular, influenciando na resposta biológica.

Os estudos até então conduzidos mostram que os resultados obtidos com a FTL em nível tecidual e celular estão baseados no aumento da proliferação de diversos tipos celulares (PEPLOW et al. 2010; RIBEIRO et al. 2009; PEREIRA et al. 2002), migração celular (PELLICIOLI et al 2013), promoção da síntese de pré-colageno e colágeno (de SOUZA et al. 2011; PEREIRA et al. 2002), potencial antiinflamatório (LIM et al. 2007), aumento da neo-vascularização (CORAZZA et al. 2007; PEREIRA et al. 2010), redução da expressão de espécies reativas de oxigêncio (LIM et al. 2007; DILLENBURG et al., 2013), liberação de fatores de crescimento (DAMANTE et al. 2009), regulação de citocinas pró-inflamatórias e mediadores químicos álgicos (SAKURAI, YAMAGUCHI, ABIKO, 2000).

Baseado nos efeitos acima descritos a FTL vem sendo muito utilizada na prática clínica diária para tratamento de diversos processos patológicos, especialmente em reparo e em condições inflamatórias como a mucosite e úlceras em geral (SCHUBERT et al. 2007). Os principais efeitos descritos são: modulação da inflamação resultando em redução da severidade de lesões ulceradas (de SOUZA et al., 2010; WAGNER et al., 2013), assim como, potencializar o reparo de feridas (MARTINS et al., 2012) e gerar diminuição da sintomatologia dolorosa (de SOUZA et al 2010).

Conhecendo os efeitos da FTL no reparo de lesões (ANTUNES, 2013), sabemos que este é uma alternativa na área da saúde para prevenir, modular e/ ou

tratar doenças que possuam na sua patobiologia um processo inflamatório (EDUARDO et al., 2007; BAPTISTA et al., 2010), sendo assim, é importante entender como o FTL age na patobiologia da mucosite. Inúmeros estudos clínicos tem demonstrado a efetividade da FTL isolada ou em em combinação com outras terapêuticas para tratamento e prevenção da mucosite bucal. Os resultados destes estudos mostram não somente a diminuição da intensidade da dor, como a diminuição da severidade da mucosite, sem apresentar efeitos colaterais (ANTUNES et al., 2007; ARUN MAIYA et al., 2006; BARASCH et al., 1995; BENSADOUN et al., 1999; COWEN et al., 1997; CRUZ et al., 2007; JAGUAR et al., 2007; MIGLIORATI et al., 2001; SANDOVAL et al., 2003; SCHUBERT et al., 2007; EDUARDO et al., 2009; KHOURI et al., 2009; ZANIN et al., 2010; CHOR et al., USUMEZ et al., 2011; ARBABI-KALATI, ARBABI-KALATI, MORIDI, 2013). Em junho de 2011, foi publicado um estudo de revisão sistemática em que BJORDAL et al. (2006) mostram a validade da FTL, aplicado com doses de 1 a 6 J por ponto, mostrando a efetividade desta terapia na prevenção da mucosite bucal, promovendo redução da severidade, dor e tempo de duração das lesões. Muitos estudos demonstram a eficácia clínica da FTL na prevenção e no tratamento da mucosite bucal, no entanto os mecanismos pelos quais a FTL promove tais efeitos benéficos não são bem compreendidos, os estudos que comprovem essa ação por meio de mecanismos celulares e moleculares são escassos (MIGLIORATI et al., 2013).

2 ARTIGO CIENTíFICO 1

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ORIGINAL ARTICLE

Effect of topical chamomile on immunohistochemical levels of IL-1β and TNF-α in 5-fluorouracil-induced oral mucositis in hamsters

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Abstract
Purpose The aim of the present study was to evaluate the effect of topical chamomile and corticosteroid treatment on the profile of tissue cytokines (IL-1β and TNF-α) in 5-fluorouracil-induced oral mucositis in hamsters.
Methods Thirty-six hamsters were randomly separated into three groups (12 animals each): Group I—without treatment (control); Group II—treatment with chamomile (Ad-Muc®); and Group III—treatment with corticosteroid (betamethasone elixir- Celestone®). The animals received an intraperitoneal injection of 5-fluorouracil on Days 0 and 2. On Days 3 and 4, the buccal mucosa was scratched and therapy was initiated on Day 5. Three animals from each group were killed on Days 0, 5, 10, and 14 and the buccal mucosa was removed. The streptavidin–biotin complex method was used to delineate the in situ distribution, localization, and semiquantitative analysis of IL-1β and TNF-α. Data from the semiquantitative analysis of immunohistochemical staining were comparatively analyzed using the Kruskal–Wallis test, followed by Dunn's multiple comparisons test.
Results The distribution and localization of IL-1β and TNF-α immunolabeling were similar. These proteins exhibited a diffuse pattern distributed throughout the connective tissue. The epithelium and adipose tissue were negative for both proteins. The semiquantitative analysis revealed that immunolabeling of IL-1β and TNF-α increased in all groups with the development of mucositis. On Day 10 (period of peak mucositis), the group treated with chamomile had lower scores for both pro-inflammatory cytokines.
Conclusions Treatment with topical chamomile reduced the tissue levels of IL-1β and TNF-α, thereby demonstrating anti-inflammatory action in oral mucositis in hamsters.

Keywords Oral mucositis · Chamomile · IL-1β · TNF-α · Inflammatory cytokines

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Introduction

Oral mucositis manifests as usually painful, erythematous, erosive, and/or ulcerative lesions of the oral mucosa related to toxicity and myelosuppression stemming from cancer treatment, such as chemotherapy and head and neck radiotherapy [1, 2]. The exact mechanisms by which cytotoxic

chemotherapy drugs and radiotherapy cause mucositis have not been fully clarified. However, this condition seems to result from a series of dynamic interactions among molecular and cellular events involving all elements of the mucosa (epithelium and connective tissue).

The pathobiology of oral mucositis has been modeled as a five-phase process: initiation, message generation, signaling/amplification, ulceration, and healing [1, 3–6]. In the initial phase, DNA damage following chemotherapy or radiotherapy occurs in endothelia, fibroblasts, and epithelia through the generation of oxidative stress and reactive oxygen species. The activation of transcription factors, such as nuclear factor-κB (NF-κB), leads to the up-regulation of many genes, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), initiating an inflammatory response and an increase in subepithelial vascularity. These cytokines also participate in a positive-feedback loop that amplifies the initial response and could culminate in the formation of painful mucosal ulcers [7].

The roles of NF-kB and cytokines in the pathobiology of mucositis have increasingly been reported in the literature. However, the dynamics of inflammatory cytokines in oral mucositis and their particular influence on this process have not been entirely specified. Studies on mucositis using animal models have demonstrated that different types of drugs, such as irinotecan, methotrexate (MTX), and 5-fluorouracil (5-FU), can lead to alimentary tract mucositis. However, these studies report differences in the timing of the histological changes as well as in the timing and intensity of the tissue expression of pro-inflammatory cytokines depending on the chemotherapy protocol [7, 8]. In humans, studies report an increase in pro-inflammatory cytokines in the blood and/or saliva of patients during cancer treatment [9–11]. Moreover, animals treated with the anti-inflammatory cytokine interleukin-11 (IL-11) demonstrated a significant reduction in mucosal injury accompanied by reduced levels of TNF-α and IL-1β [4].

Treatment for oral mucositis is generally palliative, with the aim of relieving symptoms and controlling possible infections and/or hemorrhage. Efforts are focused on the continuing search for new therapies for mucositis [12, 13]. The use of plant-based medicine (phytotherapy) has been proposed for pain treatment and the promotion of tissue repair. Chamomile is among the most studied plants and has long been used internally and externally to relieve or cure a wide range of disorders [14]. Our group previously studied the clinical and histopathological effects of chamomile on mucositis in hamsters in comparison with the use of a corticosteroid [15]. The group treated with the corticosteroid exhibited greater mucositis severity, whereas the chamomile-treated group exhibited a mild clinical manifestation of mucositis throughout the entire experimental period. However, the cellular and biochemical mechanisms that lead to these clinical and histopathological changes have not yet been clarified.

The aims of the present study were to evaluate the tissue level of pro-inflammatory cytokines (IL-1β and TNF-α) during the development of oral mucositis following chemotherapy and determine whether topical chamomile and corticosteroid treatment modify this cytokine profile in 5-FU-induced oral mucositis in hamsters.

Materials and methods

The methodology employed in the present study was designed in compliance with Brazilian National Health Council Resolution 196/96 and received approval from the Ethics Committee of the *Universidade Nove de Junho* (Sao Paulo, Brazil). The experimental protocol obeyed the principles for the ethical treatment for animals drafted by the Brazilian College of Animal Experimentation, affiliated with the International Council of Laboratory Animal Science, which establishes proper conduct in animal experimentation based on basic principles of sensitivity, good sense, and good science.

Thirty-six female golden Syrian hamsters (8 weeks of life; body mass: approximately 150 g) were used. The animals were kept in groups of five per plastic container, with food and water ad libitum. The animals were randomly divided into three groups (each with 12 animals): Group I—without treatment (control); Group II—treatment with chamomile (Ad-Muc®); and Group III—treatment with corticosteroid (betamethasone elixir—Celestone®). Each gram of chamomile ointment contained 100 mg of fluid extract of *Chamomilla recutita* (L.) Rauschert. The ointment also contained the following ingredients: glycerol, lanolin alcohols, cetostearyl alcohol, white soft petroalatum, sodium, xanthan gum, methylparaben, essence of peppermint, tincture of myrrh, mineral oil, sodium saccharin, and purified water.

For the induction of mucositis, 60 mg/Kg of the chemotherapy drug 5-FU was administered to each animal intraperitoneally on Day 0 and 40 mg/Kg was administered on Day 2, following the protocol proposed by Sonis et al. [4] and modified by Leitão et al. [16]. The right buccal mucosa was scratched twice with the tip of a sterile needle by the same operator on Days 3 and 4. Treatment with the test substances was initiated on Day 5, with application twice per day (morning and evening) using flexible cotton swabs. Three animals from each group were killed on Day 0, 5, 10, and 14 with an overdose of anesthetic and the buccal mucosa was removed for immunohistochemical analysis.

Immunohistochemistry

For immunohistochemical staining, the samples were serially sectioned into 3-μm sections. Antigen retrieval was performed using a preheated solution of citrate buffer pH 6.0 (low-pH retrieval solution, S1699, DakoCytomation, Carpinteria, CA, USA) in a steamer during 25 min. The primary antibodies, sources, dilutions, and incubation times were as follows: IL-1B (ABR-Affinity BioReagents, batch: MA1-20820, 1:200) and TNF-α (ABR-Affinity BioReagents, batch: PA1-40281, 1:200) were incubated for 1 h. The detection system used was EnVision+® (DakoCytomation, Carpinteria, CA, USA). The sections were then incubated with diaminobenzidine tetrahydrochloride (DAB, Novocastra, Newcastle, UK) and counterstained with Mayer's hematoxylin. Negative controls were obtained by replacing the primary antibodies with non-immune serum. Sections containing normal skin served as positive controls for the immunohistochemical reactions.

Qualitative analysis

The qualitative analyses were performed by two examiners using a conventional light microscope. The examiners had previously undergone a calibration process. Intra-examiner agreement was determined performed by a second analysis of one out of every 10 fields observed, using the intraclass correlation coefficient ($p < 0.01$) and the Kappa coefficient test ($p > 0.7$) to determine the level of agreement on the quantitative and qualitative analyses, respectively. The examiner was unaware of which slides belonged to the different experimental groups. The distribution and localization of the IL-1β and TNF-α proteins were recorded.

Semiquantitative analysis

IL-1β and TNF-α labeling was analyzed by two previously trained observers using light microscopy. The examiners were blinded to the group and experimental time. The entire oral mucosa was analyzed, and quantification was performed near the injury site. In cases without lesion, the normal oral mucosa was evaluated. The semiquantitative analysis of IL-1β and TNF-α immunolabeling was based on the percentage of positively stained tissue, as described by Grundtman et al. (2007) [17], using the following scoring system: negative ( ) = 0 %, 1 + = 1–10 % positive staining/mm^2, 2 + = 11–25 % positive staining/mm^2, 3 + = 26–50 % positive staining/mm^2, 4 + = 51–75 % positive staining/mm^2, and 5 + = > 76 % positive staining/mm^2.

Statistical analysis

The data on the semiquantitative immunohistochemical staining of IL-1β and TNF-α in the three groups (control, chamomile, and corticosteroid) examined at four different times (Days 0, 5, 10, and 14) were comparatively analyzed using the Kruskal–Wallis test, followed by Dunn's multiple comparisons test. Multiple comparisons were made through the decomposition of the interaction by applying Tukey's test. The level of significance was set to 5 % ($p < 0.05$) on all tests. All analyses were performed using the SAS program for Windows, version 9.2.

Results

Qualitative analysis of IL-1β and TNF-α

The distribution and localization of IL-1β (Fig. 1) and TNF-α (Fig. 2) immunolabeling were similar. These proteins exhibited a diffuse pattern distributed throughout the connective tissue. The epithelium and adipose tissue were negative for both proteins.

Semiquantitative analysis of IL-1β

All groups exhibited a significant increase in the immunolabeling for IL-1β after the infusion of 5-FU and the development of oral mucositis (Days 5–14) (Table 1). The highest scores were seen within 10 days in all groups.

In the intra-group analysis over time, significant differences in IL-1β were found in both the control group ($p = 0.0298$) and corticosteroid group ($p = 0.188$). In the group treated with chamomile, an increase in tissue levels of IL-1B was found after 5 days, but the expression of this protein did not vary significantly in the subsequent evaluation periods ($p = 0.0609$).

In the inter-group analysis at each evaluation time, mean IL-1β immunostaining was similar in all groups on Day 0 (Fig. 1a, b, c) and increased significantly in all groups on Day 5, with the highest score in the chamomile group and the lowest in the corticosteroid group (Fig. 1d, e, f). However, this difference was not statistically significant. The highest IL-1β scores occurred on Day 10 in all groups. However, the chamomile group had a significantly lower score than the other groups (Fig. g, h, i). Lower IL-1B scores were found on Day 14 in all groups, with the lowest score in the chamomile group (Fig. 1 j, k, l).

Semiquantitative analysis of TNF-α

All groups exhibited a significant increase in immunolabeling for TNF-α following infusion with 5-FU and the development of oral mucositis (Days 5–14) (Table 2). The highest scores were seen within 5 days in all groups.

In the intra-group analysis over time, significant differences in TNF-α were found in all three groups (control:

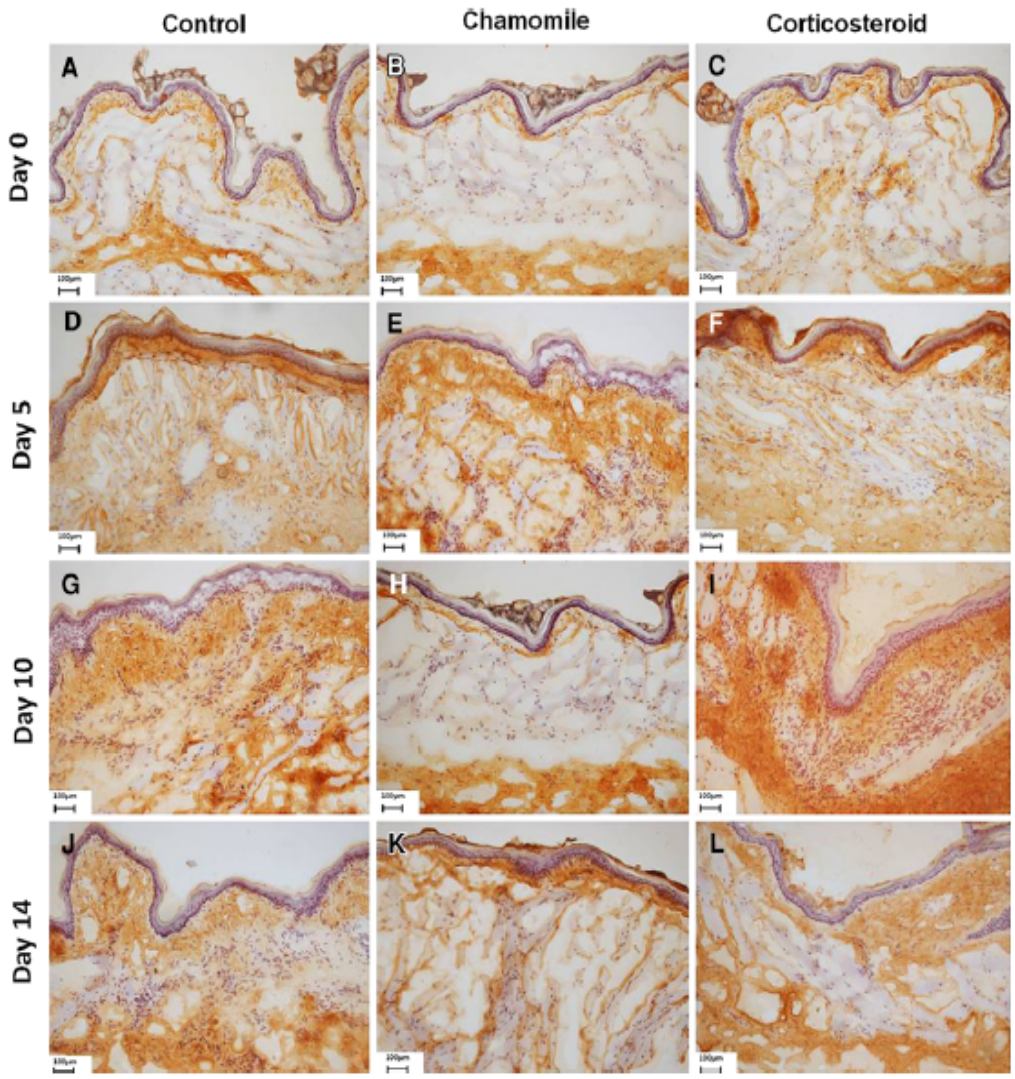

Fig. 1 Immunohistochemical distribution of IL-1β according to experimental group and evaluation time; Note the reduction in this protein in the chamomile group on Day 10 (original magnification: ×200)

$p = 0.0249$; chamomile: $p = 0.0188$; and corticosteroid: $p = 0.0254$). In the chamomile group, an increase in tissue levels of TNF-α was found on Day 5, followed by a significant decrease on Day 10 and an additional increase on Day 14.

In the inter-group analysis at each evaluation time, mean TNF-α immunostaining was similar in all groups on Day 0 (Fig. 2a, b, c) and increased significantly in all groups on Day 5 (Fig. 2d, e, f), with no statistically significant differences. On Day 10, the corticosteroid group had the highest TNF-α score ($p = 0.0304$) and the chamomile group had the lowest score, with no significant difference between the chamomile and control groups (Fig. 2g, h, i). No statistically significant differences were found among groups on Day 14, as an increase in TNF-α immunolabeling occurred in the chamomile group and the other groups maintained similar scores to those observed in previous evaluation time (Fig. 2j, k, l).

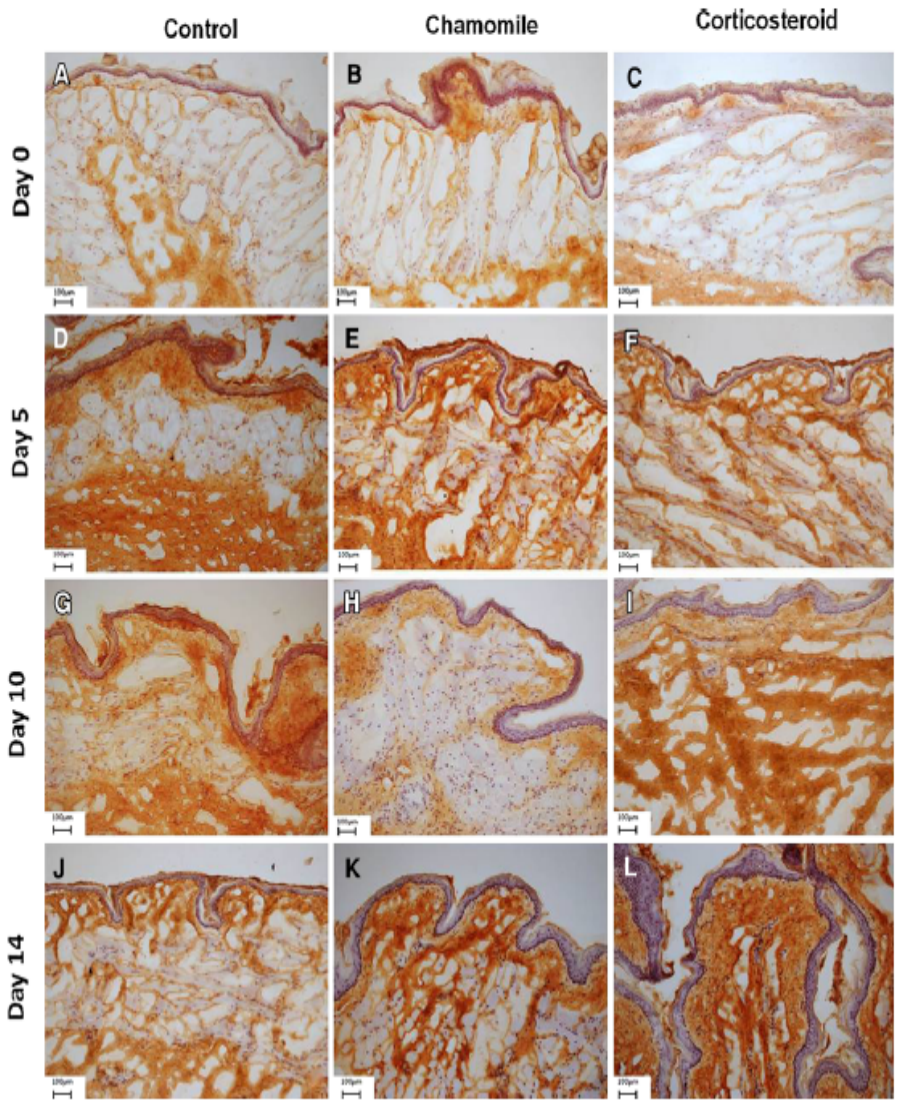

Fig. 2 Immunohistochemical distribution of TNF-α according to experimental group and evaluation time; Note the reduction in this protein in the chamomile group on Day 10 (original magnification: ×200)

Table 1 Mean expression of IL-1β in each group at each evaluation time

Group/Time	0	5	10	14
Control	1.33 ± 0.57^{aA}	3.66 ± 0.58^{bA}	5.00 ± 0.00^{cA}	$4.33 + 1.15^{bAB}$
Chamomile	1.33 ± 0.58^{aA}	4.00 ± 0.00^{bA}	4.00 ± 0.73^{bB}	3.00 ± 0.00^{bA}
Corticosteroid	1.33 ± 0.58^{aA}	3.00 ± 0.00^{bA}	5.00 ± 0.00^{cA}	4.00 ± 0.00^{bB}

Different lowercase letters on lines (intra group analysis) denote significant difference ($p < 0.05$, Tukey's test); Different uppercase letters in columns (inter group analysis) denote significant difference ($p < 0.05$, Tukey's test)

as IL-1β and TNF-α [14, 22]. Further studies investigating the action of chamomile on other cytokine pathways are needed. Moreover, randomized, double-blind, clinical trials involving humans, including a sufficient number of patients, should be carried out to reach clinically relevant conclusions regarding the use of herbal medicine for mucositis.

In conclusion, the present immunohistochemical study reveals an increase in pro-inflammatory cytokines with the development of mucositis. Topical treatment with chamomile leads to a decline in IL-1β and TNF-α levels, associated with lesser oral mucositis severity.

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3 ARTIGO CIENTíFICO 2

EFFECT OF LASER LOW INTENSITY IN PATOBIOLOGY OF 5-FLUOR-OURACIL-INDUCED BUCAL MUCOSITIS IN HAMSTER

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Abstract

Purpose The aim of the present were to evaluate the tissue levels of NF-kB activated during the development of oral mucositis and determine whether preventive and/or treatment LPT modality influence this transcription factors in 5-FU induced oral mucositis in hamster.

Methods Ninety-six male golden Syrian hamsters were randomly separated into four groups (24 animals each): Control group (without treatment); Therapeutic Group (LPT from D+5 to D+15); Conjugate Group (LPT from D-7 to D+15) and Preventive Group (LPT application 7 days before (D-7) 5-FU induction until day +5 (D+5). The animals received an intraperitoneal injection of 5-fluorouracil on Days 0 and 2. On Days 3 and 4, the buccal mucosa was scratched. The irradiation was carried out using diode LPT, 660 nm, 40mW of power, 6,0J/cm2 during 6seconds/point. Six animals from each group were killed on Days 0, 5, 10, and 15 and the buccal mucosa was removed to clinical analysis. Statistical analysis was made considering the time x treatment interaction through the SPSS Statistics 18.0® software. The multiple comparison was made by the Generalized Estimation Equation test and adjusted by the Bonferroni test. The oral mucosa removed had extraction of proteins to NF-kB evaluation by western blot technique.

Results On D+5 all groups presented similar clinical scores. The LPT groups presented lower mucosits scores than control group on D+10. The NF-kB analysis show on D+5 a similar arbitrary numbers indicating no difference between protein level of activated NF-kB in all groups. On D+10 the LPT groups showed higher levels of activated NF-kB protein than control group.

Conclusion: The LPT in preventive or treatment protocol reduced the severity of oral mucositis activating the NF-kB pathway.

Keywords Laser therapy – mucositis – NF-kB – angiogenisis - 5-FU

Introduction

Oral mucositis is an important side effect in patients with cancer who receive chemoradiotherapy or head and neck radiotherapy. Clinical manifestation of oral mucositis varies from erythematous, erosive and/or ulcerative lesions with mild to severe pain.[1] The mucositis could lead to significant impairment of quality of life, prolongation of hospital stay, increases in re-admission rates, compromises the patient's nutritional status as well as discontinuation of cancer therapy and occasionally death.[2,3,] The exact mechanisms by which cytotoxic chemotherapy drugs and radiotherapy cause mucositis have not been fully clarified. However, this condition seems to result from a series of dynamic interactions among molecular and cellular events involving all elements of the mucosa (epithelium and connective tissue).[4]

The pathobiology of oral mucositis has been described as a five-phase process: initiation, message generation, signaling and amplification, ulceration and healing.[2] The initiation occurrs after administration of cytotoxic chemotherapy or radiotherapy which promote DNA and non-DNA damage and the generation of reactive oxygen species (ROS). The second phase is the message generation that involves the up-regulation of transcription factors including NF-kB and STAT3 and activation of several cytokine. The signaling and amplification phase represent the production of several proteins, such as tumour necrosis factor (TNF-α), interleukin-1β (IL-1 β) and interleukin-6 (IL-6), which cause direct tissue damage and provide positive feedback to amplify the process. The tissue alterations results in the loss of epithelium resulting in painful lesions, bacterial infiltration and an influx of macrophages and other inflammatory cells characterizing the ulceration phase. After the cessation of stimuli (chemotherapy or radiotherapy) the healing process can be established.[2,4]

Some studies have shown that activation of NF-kB and subsequent upregulation of proinflammatory cytokines may be a potentially important factor in the pathobiology of mucositis.[5,6] These studies found that changes in serum levels preceded the development of oral mucositis. The understanding of the mechanisms involved in the pathobiology of mucositis has contributed to the development of preventive strategies and treatment of oral mucositis during oncological treatment.

Over the last several years, appropriate laboratory and clinical evidence have been accumulating progressively to also support the use of Laser phototherapy (LPT) for prevention and treatment of oral mucositis.[7,8] The MASCC/ISOO (Mucositis

Study Group of the Multinational Association of Supportive Care in Cance/International Society of Oral Oncology) mucositis guidelines proposed that LPT seems to have beneficial effects in the prevention of oral mucositis in hematopoietic stem cell transplant (HSCT) patients.[9] It has been proposed that LPT have anti-inflammatory effect and are effective in controlling mucositis-associated pain.[10,11] Besides that, LPT improves cell proliferation, migration, and transcription of genes involved in wound healing.[12-15] Few studies analysed the cellular and molecular effects of LPT in oral mucositis pathobiology.

The objectives of the current study were to evaluate the tissue levels of NF-kB activated during the development of oral mucositis and determine whether preventive and/or treatment LPT modality influence this transcription factors in 5-FU induced oral mucositis in hamster.

Materials and Methods

Animal model

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and received approval from the ethics committee of the Porto Alegre University Hospital (Brazil) under process number 11-0134. Ninety-six male golden Syrian hamsters (eight weeks of life; body mass approximately 150 g) were kept under standard conditions of temperature (20 to 24°C) and light/dark cycle, with solid chow and water *ad libitum*. The animals were randomly divided into four cohorts of 24 animals each: Control group (without treatment); Therapeutic Group (LPT from D+5 to D+15); Conjugate Group (LPT from D-7 to D+15) and Preventive Group (LPT application 7 days before (D-7) 5-FU induction until day +5 (D+5). (Figure 1)

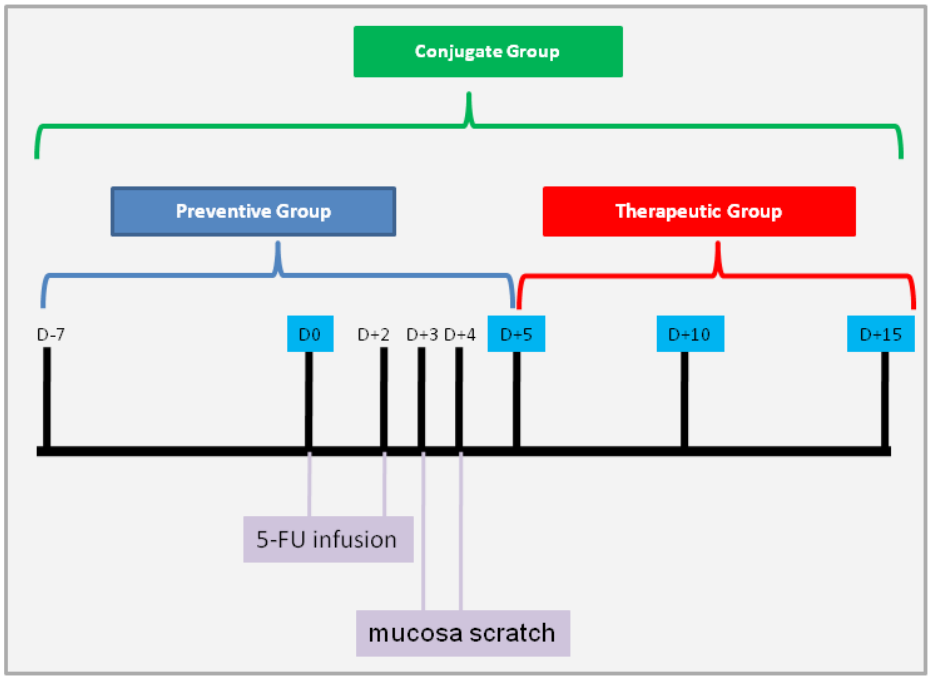


Figure 1. Diagram of experimental protocol adopted along the study.

Oral mucosite induction

On day 0 (D0) was administered 60 mg/Kg of 5-fluoracil (5-FU) to each animal intraperitoneally and on day 2 (D+2) 40 mg/Kg of same drug was infused as protocol proposed by Sonis et al. (2007)[2] and modified by Leitão et al. (2007).[16] The midle of the right buccal mucosa was scratched twice with the tip of a sterile needle by the same operator on days 3 (D+3) and 4 (D+4). Six animals in each group were euthanized using a CO_2 chamber on D0, D+5, D+10 and D+14 .The right buccal mucosa was photograped, removed and immediately frozen in liquid nitrogen and stored in a freezer at −80°C.

Laser irradiation

LPT was applied by a single professional (CSD) using a continuous wave diode laser (InGaAlP; MM Optics, São Carlos, SP, Brazil) with a wavelength of 660 nm (visible-red). The irradiation was done in the midlle of the right buccal mucosa in six points (Figure 2) in punctual and contact mode technique. Irradiation parameters were, as follows: spot size of 0.04cm^2, power output of 40 mW, energy density of 6 J/cm^2, for 6 seconds and 0.24J per point, totalizing 1.44J per day of application. LPT was applied daily during the period establish for each group (Figure 1) and the total

dose of each group according to experimental time (D0, D+5, D+10 and D+15) are demonstrated on Table 1. The control group was treated under identical conditions but with the laser equipment switched off. The output power of the equipment was checked using a power meter (Laser Check; MMOptics LTDA, São Paulo, Brazil).

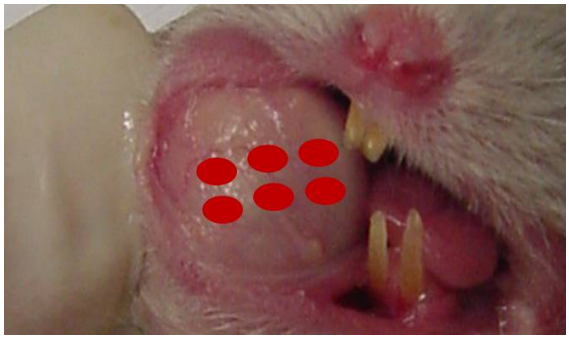


Figure 2. Laser irradiation procedure. Circles represent the point of laser delivery.

Table 1. Total dose of energy (J) in each group according to experimental time.

Group/Experimental time	D0	D5	D10	D15
Control	0 J	0 J	0 J	0 J
Preventive	11.52 J	18.72 J	18.72 J	18.72 J
Therapeutic	0 J	1.44J	8.64 J	15.84 J
Conjugate	11.52 J	18.72 J	25.92 J	33.12 J

Clinical Analysis

After euthanasia the right buccal mucosa was phographed for the characterization of the mucositis severity. For the macroscopic analysis, inflammatory aspects such as erythema, hyperemia, bleeding, epithelial ulcers, and abscesses were assessed by a blind evaluator, receiving scores from 0 to 3 based on the method described by Lima et al. (2005),[17] as follows: Score 0—normal buccal mucosa, with absence of or discreet erythema and hyperemia, with no areas of bleeding, ulceration, or abscesses; Score 1—moderate erythema and hyperemia, with no areas of bleeding, ulceration or abscesses; Score 2—severe erythema and hyperemia, presence of areas of bleeding, small ulcers or eschars, but no abscesses; and Score 3—severe erythema and hyperemia, presence of areas of bleeding, extensive ulcers, and abscesses.

NF-kB Analysis

The proteins of the buccal mucosa were extracted by phosphatoglicerol buffer with the protease inhibitor and separeted nucleous fraction and citosol fraction. Protein concentration was measured by Bradford assay. 15ug of nuclear extracts were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% milk TBS tween, then incubated with primary anti-NF-kB (1:1,000; Cell Signaling Technology) antibody overnight at 4°C, p65 protein (65kDa). The membranes were then washed, blocked with 5% milk and incubated with goat anti-rabbit secondary antibodies (1:5000;Cell Signaling Technology) for 1h at room temperature. The proteins were detected by enhanced chemiluminescence detection system (ECL kit, Millipore). The density of the specific bands was quantified with an imaging densitometer (Image J). β-actina was used as the relative control. For the semi-quantitative analysis were calculated arbitrary numbers by multiplying the area x intensity of bands. The resulting number of NF-kB band was divided by resulting number of β-actina.

Statistical Analysis

The clinical data were expressed as mean and standard deviation values. The SPSS version 18.0 was employed for the statistical analysis. Groups, evaluation times and the interaction between group and evaluation time were compared using the generalized estimating equation followed by a post-hoc Bonferroni correction, when necessary. The significance levelwas set at 5% ($p < 0.05$).

Results

Clinical Analysis

The analysis of clinical mean scores fixing each group during the experimental times are demonstrated on Table 2. The control group showed a gradual increase on clinical scores since D0 to D+10. A peak of mucositis was observed on D+10 and represented a severe erythema and hyperemia, hemorrhagic areas and extensive ulcers and/or abscess (score 3). On D+15 a decrease of clinical score was observed and indicated the presence of normal buccal mucosa (score 0). All LPT groups showed similiar score on D+5 (score 2). On D+10 all the LPT groups exhibited lower

mean scores (score 1). These groups on D+15 showed a decrease of clinical score (score 0).

Table 2. Comparison between experimental times fixing each group.

	Experimental groups				
	D0	**D+5**	**D+10**	**D+15**	***p***
Control	0,00 (0,00-0,00)[a]	2,00 (2,00-2,00)[bc]	2,80 (2,45-3,15)[bd]	0,83 (0,54-1,13)[be]	0,00
Therapeutic	0,00 (0,00-0,00)[ae]	1,67 (1,29-2,04)[bcd]	1,40 (0,70-2,10)[bcde]	0,40 (-0,30-1,10)[abde]	≥0,011
Conjugated	0,00 (0,00-0,00)[ae]	2,00 (2,00-2,00)[bcd]	1,50 (0,89-2,11)[bcd]	0,20 (-0,15-0,55)[abe]	≥0,002
Preventive	0,00 (0,00-0,00)[ae]	2,00 (2,00-2,00)[bc]	0,60 (0,17-1,03)[bde]	0,20 (-0,15-0,55)[abde]	≥0,037

*** Different lower case letters indicate statistical difference within experimental group

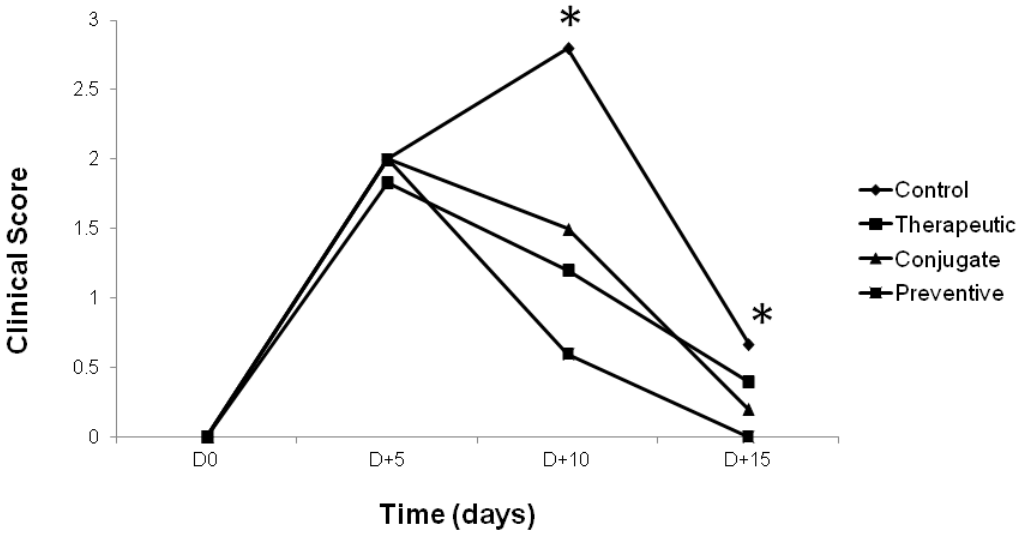
The analysis of clinical mean scores between groups fixing experimental times are illustrated on Figure 3a and b. On D+5 all groups showed similiar clinical aspect with score 2 feature. On D+10 the control group showed significant higher score than all LPT groups (Figure 3a,b and c). On D+15 all the groups showed low scores. Preventive group exhibited lower mucositis score than control group($p<0,01$). Comparing treatments in general analysis of experimental times, the control group differed from all LPT groups ($p<0,01$). Analyzing different LPT protocols no significant difference was observed ($p>0,05$).

a

	Experimental Groups				
	Control	Therapeutic	Conjugate	Preventive	*p*
Day 0	0,00 (0,00-0,00)^{A}	0,00 (0,00-0,00)^{A}	0,00 (0,00-0,00)^{A}	0,00 (0,00-0,00)^{A}	1,00
Day 5	2,00 (2,00-2,00)^{A}	1,67 (1,29-2,04)^{A}	2,00 (2,00-2,00)^{A}	2,00 (2,00-2,00)^{A}	1,00
Day 10	2,80 (2,45-3,15)^{A}	1,40 (0,70-2,10)^{B}	1,50 (0,89-2,11)^{B}	0,60 (0,17-1,03)^{B}	≥0,003
Day 15	0,83 (0,54-1,13)^{A}	0,40 (-0,30-1,10)^{AB}	0,20 (-0,15-0,55)^{B}	0,20 (-0,15-0,55)^{B}	≥0,042

*** Different upper case letters indicate statistical difference within experimental group.

b



c

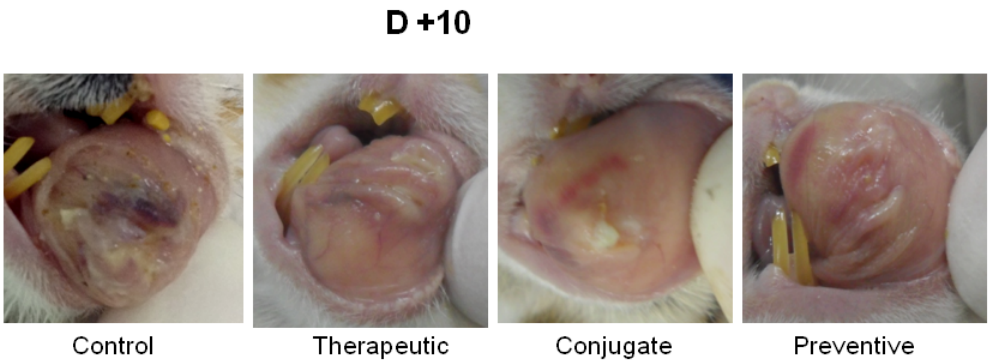


Figure 3. (a and b) Comparison of clinical scores between groups fixing experimental times. (c) Clinical aspect of hamster buccal mucosa at D+10 in control, therapeutic, conjugated and preventive group.

NF-kB Activation

The level of NF-kB activated was analyzed by bands appearance and the relative band density compared with the loading in each lane (β-actin). On D+5 the band density showed a similar arbitrary numbers indicating no difference between protein level of activated NF-kB in all groups (Figure 4). On D+10 the LPT groups showed higher levels of activated NF-kB protein than control group (Figure 5). As shown on Figure 6, therapeutic group exhibited the higher level of activated NF-kB compared to other groups.

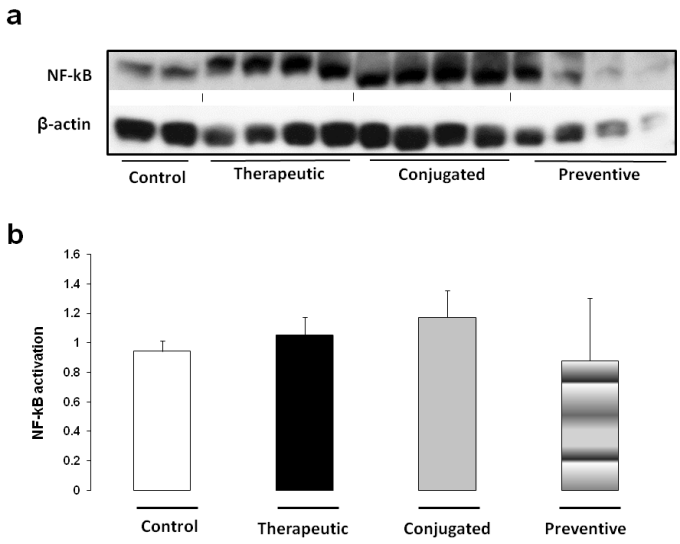


Figure 4: Western blot analysis of NF-kB protein level on D+5 in all experimental groups. (a) Shows representative Western blot photographs. Equal loading of proteins is illustrated by β-actin bands. (b) Mean values of densitometric analysis.

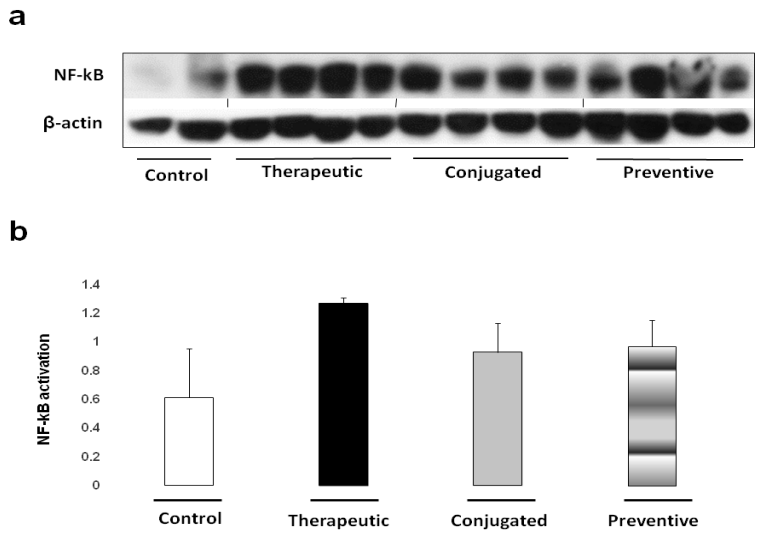


Figure 5: Western blot analysis of NF-kB protein level on D+10 in all experimental groups. (a) Shows representative Western blot photographs. Equal loading of proteins is illustrated by β-actin bands. (b) Mean values of densitometric analysis.

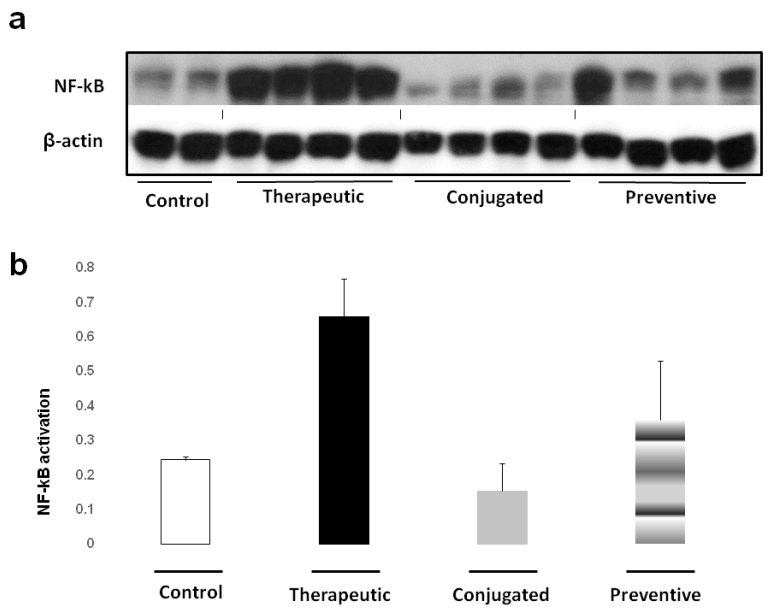


Figure 6: Western blot analysis of NF-kB protein level on D+15 in all experimental groups. (a) Shows representative Western blot photographs. Equal loading of proteins is illustrated by β-actin bands. (b) Mean values of densitometric analysis.

Discussion

Oral mucositis is an important side effect of oncological treatment like chemotherapy and/or head and neck radiotherapy. It pathobiology involves the activation of NF-kB and subsequent upregulation of proinflammatory cytokines resulting in tissue damage and clinical manifestation of inflammatory reaction.[2,4] Among all the therapeutic options available LPT have been studied revealing positive effects in oral mucositis, especially reducing pain and preventing lesions appearance[7,10,15] The mechanisms by which the LPT influence the oral mucositis still unknown as well their role in NF-kB level. The aim of the present study were to evaluate the tissue levels of NF-kB activated during the development of oral mucositis and determine whether preventive and/or treatment LPT modality influence NF-kB in 5-FU induced oral mucositis in hamster.

The animal model used in this study followed the protocol of 5-FU-induced developed by Sonis et al.1990[18] and modified by Leitão et al. 2007[16] is a usefull model to study the five overlapping phases of oral mucositis pathobiology: initiation, upregulation and message generation, signalling and amplification, ulceration and healing.[1,2,4] It is also important to help the comprehension of molecular and cellular aspects after different therapies. NF-kB and several cytokines have been suggested to play a key role in mucositis development. Changes in the levels of NF-kB have been demonstrated in alimentary tract mucositis with different chemotherapy agents.[5,6] It is also well recognized that NF-kB modifications occur prior to clinical evidence of mucositis manifesting. Such findings have led to a better understanding of the pathobiology of mucositis and have assisted in the search for more effective therapies for this complication of cancer treatment.[19]

Our clinical results showed a mucositis peak in control group on D+10 indicating the effectiveness of the experimental model, as described by Sonis et al. (1990).[18] At this period the oral mucosa exhibited a ulcerative pattern that is concomitant with advanced stage of leukopenia and disruption of the integrity of the mucosa. The LPT groups, independing of the protocol, showed a reduction in oral mucositis manifestation. It was observed by significant lower score than control group on D+10. Despite the positive effects in all LPT groups a more efficient lesions repair on D+15 was observed in preventive and conjugated group. The animals that received laser since D-7 had lower scores than others groups in this period of time. Animals[20] and humans[10] studies have demonstrated the effective of LPT in reducing the degree and severity

of mucositis. Based on the accumulating evidence, the MASCC/ISOO group[9] suggested that LPT has the potential to become a routine practice in the prevention and treatment of oral mucositis and its associated pain.

LPT has been widely used in inflammatory pathologies and it main effects are accelerate the wound healing associated to modulation of inflammatory process, as well as its analgesic and biomodulatory effects.[14] Such effects may be related to the action of LPT preventing cell death,[14,21,22] and restoring the cellular metabolism.[14,15,23] Thus, our study hypothesis that clinical benefits of LPT should be associated to NF-kB modulation.

NF-kB is a dimeric transcription factor formed by members of a family of proteins that share a conserved N-terminal dimerization/DNA-binding region designated the Rel homology domain.[24] Oxidative stress and inflammatory stimulus activate of IkB kinase (IKK), which in turn phosphorylates the IkB causing the release of the NF-kB dimers and their nuclear translocation. In the nucleus, the NF-kB dimers bind to a kappaB site in the promoter or enhancer region of target genes thereby controlling gene expression involved in the inflammatory responses. Activated NF-kB can induce the transcription of many genes such as cytokines, growth factors, adhesion molecules and mitochondrial anti-apoptotic genes. While the crucial role of NF-kB in the immune response is well established, cumulative evidence has shown that it is a key mediator in inflammation as well as in tumor development, progression, and neovascularization.[24,25]

Our results showed that 5 days (D+5) after the 5-FU infusion a similar activated NF-kB level was detected between control and LPT groups. It is in accordance with the clinial observations demonstrating that all groups are in same stage of mucositis induction. The experimental model used include a mucosa scrating on D+3 and D+4 to permit the mucositis instalation. So, indepedently of the group, all animals received a traumatic ulceration and similar tissue reaction.

On D+10 the control group developed a severe mucositis as demonstrated by Sonis et al. (2007)[2] and LPT group showed lower score of mucositis. On this period, LPT groups showed higher levels of activated NF-kB protein than control group. These results were surprising based in fact that high levels of NF-kB have been associated with severity of several inflammatory disease.[26,27] However, Logan et al (2009 e 2008)[5,6] that studied the tissue expression of NF-kB and other proinflammatory cytokines in oral and intestinal mucositis induced by irinotecan, 5-FU and

methotrexate (MTX) described that the type of drug administered influence the level of this protein. The mucositis induced by 5-FU showed no significant increase in tissue satining for NF-kB or IL-6. The 5-FU could lead to oral mucositis using different tissue events inducing apoptosis and inhibiting cell proliferation.[28] Further investigation are necessary to explain the oral mucosa toxicity caused by 5-FU. By the other hand, on D+10 the LPT stimulate NF-kB activation and promote clinical improvement of oral lesions. To the best of our knowledge, the results of this study are the first to indicate that LPT are usefull for prevent and treat oral mucositis stimulating the NF-kB pathway. These aspects could be explaned by the fact that LPT induce angiogenesis, cell proliferation and migration and prevent apotosis.[12,13,15] As well as, NF-kB activation have been involved in survival responses of epithelial cells and increase angiogenesis mechanisms that involves the VEGF expression.[29]

In conclusion, the LPT in preventive or treatment protocol reduced the severity of oral mucositis activating the NF-kB pathway.

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4 CONSIDERAÇÕES FINAIS

Tendo em vista todas as complicações que a mucosite bucal pode causar interferindo na qualidade e na sobrevida do paciente sabemos que preveni-la ou tratá-la é essencial. Baseado no resultado do artigo 1 podemos concluir que a camomila apresenta efeito antiinflamatório na mucosite. Este estudo demonstrou que os animais que receberam camomila como tratamento apresentaram menos citocinas pró-inflamatórias (IL-1b e TNF-a) no dia 10 quando comparados aos animais que receberam corticóide.

Baseando-se nos resultados do artigo 2, percebemos que o LBP mostrou-se eficaz no controle clínico da mucosite quimioinduzida em hamsters. No dia 10, quando a mucosite apresenta seu pico clínico, todos os grupos que receberam protocolo de laser de alguma forma (preventivo, terapêutico ou conjugado) apresentaram menor severidade de mucosite quando comparados ao grupo controle. Os protocolos de laser preventivo e conjugado mostraram que o laser preventivo está relacionado a um reparo acelerado das lesões. Com base no artigo 2 também pudemos concluir que o LBP está relacionado a um aumento dos níveis de de NF-kB ativado. Foi percebido que níveis mais altos de NF-kB ativado estão relacionados ao reparo acelerado das lesões, uma vez que este fator de transcrição está relacionado a angiogênese e a proliferação celular.

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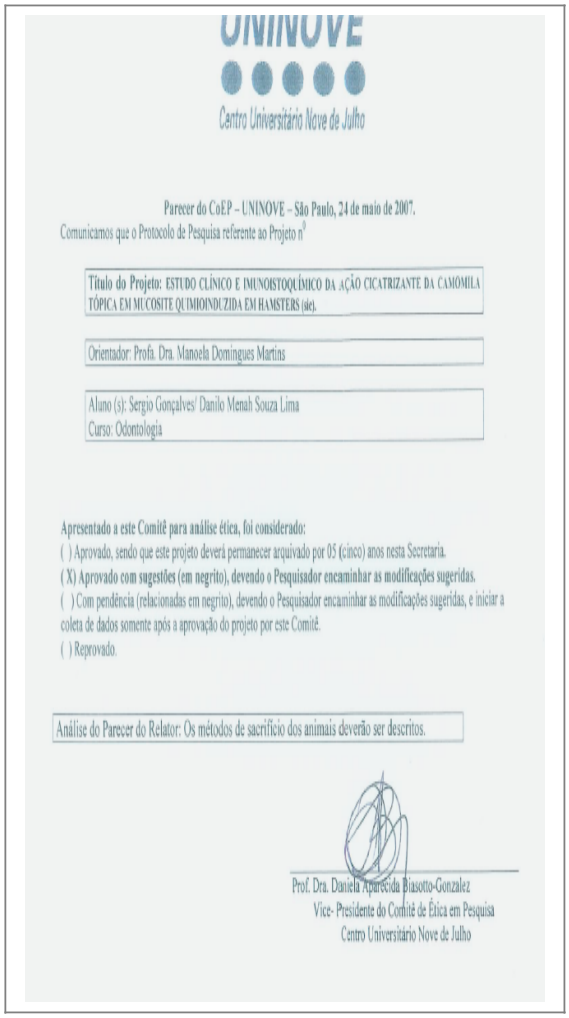
ANEXO A – CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA ARTIGO 1



UNINOVE

Centro Universitário Nove de Julho

Parecer do CoEP – UNINOVE – São Paulo, 24 de maio de 2007.

Comunicamos que o Protocolo de Pesquisa referente ao Projeto nº

Título do Projeto: ESTUDO CLÍNICO E IMUNOISTOQUÍMICO DA AÇÃO CICATRIZANTE DA CAMOMILA TÓPICA EM MUCOSITE QUIMIOINDUZIDA EM HAMSTERS (sic).

Orientador: Profa. Dra. Manoela Domingues Martins

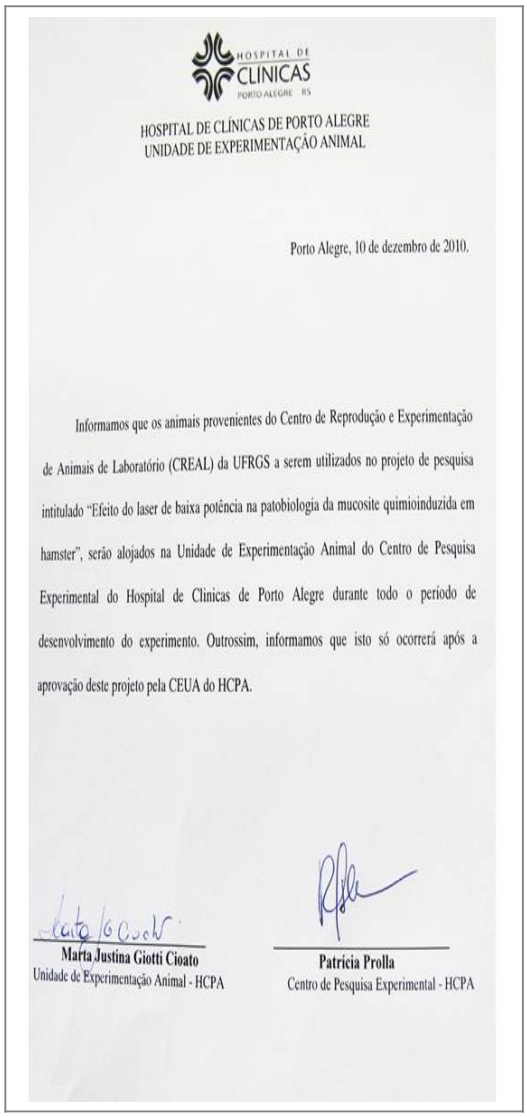
Aluno (s): Sergio Gonçalves/ Danilo Menah Souza Lima
Curso: Odontologia

Apresentado a este Comitê para análise ética, foi considerado:
( ) Aprovado, sendo que este projeto deverá permanecer arquivado por 05 (cinco) anos nesta Secretaria.
(X) Aprovado com sugestões (em negrito), devendo o Pesquisador encaminhar as modificações sugeridas.
( ) Com pendência (relacionadas em negrito), devendo o Pesquisador encaminhar as modificações sugeridas, e iniciar a coleta de dados somente após a aprovação do projeto por este Comitê.
( ) Reprovado.

Análise do Parecer do Relator: Os métodos de sacrifício dos animais deverão ser descritos.

Prof. Dra. Daniela Aparecida Biasotto-Gonzalez
Vice- Presidente do Comitê de Ética em Pesquisa
Centro Universitário Nove de Julho

ANEXO B – CARTA DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA. ARTIGO 2



HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
UNIDADE DE EXPERIMENTAÇÃO ANIMAL

Porto Alegre, 10 de dezembro de 2010.

Informamos que os animais provenientes do Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL) da UFRGS a serem utilizados no projeto de pesquisa intitulado "Efeito do laser de baixa potência na patobiologia da mucosite quimioinduzida em hamster", serão alojados na Unidade de Experimentação Animal do Centro de Pesquisa Experimental do Hospital de Clinicas de Porto Alegre durante todo o período de desenvolvimento do experimento. Outrossim, informamos que isto só ocorrerá após a aprovação deste projeto pela CEUA do HCPA.

Marta Justina Giotti Cioato
Unidade de Experimentação Animal - HCPA

Patrícia Prolla
Centro de Pesquisa Experimental - HCPA