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ESTUDO COMPARATIVO DO EFEITO DA FOTOBIOMODULAÇÃO COM LASER INTRAORAL E LASER EXTRAORAL NA MUCOSITE BUCAL QUIMIOINDUZIDA EM RATOS

Porto Alegre
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Dissertação de mestrado apresentada ao programa de Pós-graduação em Odontologia da Universidade Federal do Rio Grande do Sul como requisito à obtenção do título de mestre em Odontologia.

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isso de querer
ser exatamente aquilo
que a gente é
ainda vai
nos levar além

“Incenso fosse música”, Paulo Leminski

RESUMO

PEROTTO, Stéfanie Thieme. **Estudo comparativo do efeito da fotobiomodulação com laser intraoral e laser extraoral na mucosite bucal quimioinduzida em ratos.** 2019. Dissertação (Pós-graduação em Odontologia com ênfase em Patologia Bucal) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2018.

A mucosite bucal (MB) é uma complicação frequente decorrente do tratamento antineoplásico estomatotóxico. As formas mais severas de mucosite estão associadas a ulcerações extensas, sintomatologia dolorosa, comprometimento nutricional, perda de peso, risco de infecções, redução da qualidade de vida, maior uso de recursos de saúde e até interrupção do tratamento oncológico. Consequentemente, esses fatores podem interferir de forma negativa no prognóstico do paciente. O objetivo desse estudo foi avaliar o efeito da fotobiomodulação (FBM) com laser intrabucal e laser extrabucal sobre as manifestações clínicas, histopatológicas e estado redox da mucosite bucal quimioinduzida por 5-Fluorouracil (5-FU) em ratos. Foram utilizados 96 ratos da linhagem Wistar divididos randomicamente em 6 grupos: Controle negativo (CN, sem intervenção, n=6), controle positivo (CP, indução de mucosite e nenhum tratamento, n=18), FBM intrabucal 6 J/cm^2 (IB 6 J/cm^2, indução de mucosite e aplicação de laser intrabucal 6 J/cm^2, n=18), FBM extrabucal 6 J/cm^2 (EB 6 J/cm^2, indução de mucosite e aplicação de laser extrabucal 6 J/cm^2, n=18), FBM extrabucal 12 J/cm^2 (EB 12 J/cm^2, indução de mucosite e aplicação de laser extrabucal 12 J/cm^2, n=18) e FBM extrabucal 24J/cm^2 (EB 24 J/cm^2, indução de mucosite e aplicação de laser extrabucal 24 J/cm^2, n=18). A mucosite foi induzida com a aplicação intraperitoneal de 5-FU nos dias 0 (60 mg/Kg) e 2 (40 mg/Kg) seguida pela escarificação da mucosa jugal bilateral com agulha estéril nos dias 3 e 4. Os animais receberam FBM diária bilateral de acordo com o seu grupo experimental e avaliação nos dias 0, 8, 10 e 14, sendo mortos 6 animais por grupo/dia de eutanásia. A avaliação clínica do grau de mucosite, de 0 a 3, foi realizada através de fotografia por pesquisador cegado e calibrado. Os cortes histológicos da área da lesão foram corados em HE para avaliação do reparo tecidual. Os parâmetros de estado redox foram avaliados através da mensuração fluorimétrica da oxidação de diclorofluorescina (DCFH) e da concentração de enzimas antioxidantes catalase (CAT) e glutationa peroxidase (GPx), além da glutationa (GSH). A análise clínica revelou que no dia 8, o grupo CP apresentou maiores escores de MB. Os grupos FBM apresentaram escores mais baixos de MB, no entanto, apenas o EB 6J/cm^2 apresentou um grau significativamente menor em relação ao CP ($p < 0,05$). Nos dias 10 e 14, todos os grupos de FBM apresentaram melhora na MB em relação ao CP ($p < 0,01$). Histologicamente, no dia 8, o grupo CP apresentou lesões graves, com escores mais elevados (2,8 ± 0,4), diferindo significativamente de todos os grupos irradiados que obtiveram cicatrização acelerada ($p < 0,01$). No dia 8 observamos que a indução de MB utilizando 5-FU (CP) mostrou um aumento do biomarcador de DCFH comparado ao CN (sem mucosite bucal) ($p < 0,001$). Grupos tratados com FBM, em geral,

exibiram nível reduzido deste biomarcador em comparação com o CP. No entanto, EB 6 J/cm^2 e 12 J/cm^2 apresentaram menor dano oxidativo que o CP indicado pelo menor DCFH ($p < 0,001$, $p < 0,01$, respectivamente). Além disso, esses grupos mostraram um nível similar desse biomarcador do que o CN ($p > 0,05$). Em conclusão, a irradiação com laser intra e extrabucal promoveu efeitos clínicos e histológicos positivos sobre a MB, sugerindo uma modulação do estado redox e de enzimas antioxidantes.

PALAVRAS-CHAVE: Lasers Semicondutores. Mucosite bucal. Terapia com Luz de Baixa Intensidade. Estresse Oxidativo.

ABSTRACT

Perotto, Stéfanie Thieme. **Comparative study of the effect of photobiomodulation with intra and extraoral laser in mucosite induced by chemotherapy in rats.**

2018. Dissertação (Pós-graduação em Odontologia com ênfase em Patologia Bucal) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2018.

Oral mucositis (OM) is a frequent complication of stomatotoxic antineoplastic treatment. More severe forms of mucositis are associated with extensive ulcerations, painful symptoms, nutritional impairment, weight loss, risk of infections, reduced quality of life, higher use of health resources and even interruption of cancer treatment. Consequently, these factors may negatively interfere with the patient's prognosis. The purpose of this study was to evaluate the effect of photobiomodulation (PBM) with intraoral and extraoral laser on the clinical, histopathological and redox manifestations of 5-fluorouracil (5-FU) chemo-induced OM in rats. Wistar rats were randomly divided into 6 groups: negative control (NC, no intervention, n=6), positive control (PC, mucositis induction and no treatment, n=18), intraoral PBM 6 J/cm^2 (IO 6 J/cm^2, n=18), extraoral PBM 6 J/cm^2 (EO 6 J/cm^2, induction of mucositis and application of extraoral laser 6 J/cm^2, n=18) , extraoral PBM 12 J/cm^2 (EO 12 J/cm^2, induction of mucositis and application of extraoral laser 12 J/cm^2, n=18) and extraoral PBM 24 J/cm^2 (EO 24J/cm^2, induction of mucositis and extraoral laser application 24 J/cm^2, n=18). OM was induced by intraperitoneal application of 5-FU on days 0 (60 mg/kg) and 2 (40 mg/kg) followed by scarification of the bilateral jugal mucosa with sterile needle on days 3 and 4. The animals received daily bilateral PBM according to their experimental group and were evaluated on days 0, 8, 10 and 14, and six animals per group / day of euthanasia were killed. The clinical evaluation of the degree of mucositis, from 0 to 3, was performed through photography by a blinded and calibrated researcher. Histological sections of the lesion area were stained in HE to evaluate of tissue repair. The redox state parameters were evaluated by fluorimetric measurement of DCFH oxidation and the concentration of antioxidant catalase (CAT) and glutathione peroxidase (GPx), as well as glutathione (GSH). Clinical analysis revealed that on day 8, the PC group presented higher OM scores. The PBM groups had lower OM scores, however, only the EO 6 J/cm^2 presented a significantly lower degree of PC ($p < 0.05$). On days 10 and 14, all PBM groups showed improvement in OM relative to PC ($p < 0.01$). Histologically, on day 8, the PC group presented severe lesions, with higher scores (2.8 ± 0.4), differing significantly from all the irradiated groups that obtained accelerated healing ($p < 0.01$). On day 8 we observed that the induction of OM using 5-FU (PC) showed an increase of the DCFH biomarker compared to NC (without OM) ($p < 0.001$). PBM groups generally exhibited reduced levels of this biomarker compared to PC. However, EO 6 J/cm^2 and 12 J/cm^2 presented lower oxidative damage than the PC indicated by lower DCFH ($p < 0.001$, $p < 0.01$, respectively). In addition, these groups showed a similar level of this biomarker than the NC ($p > 0.05$). In conclusion, irradiation with intra and extraoral laser promoted positive clinical and

histological effects on OM, suggesting a modulation of the redox state and antioxidant enzymes.

KEY WORDS: Diode Lasers, Oral Mucositis, Low-Level Laser Therapy, Oxidative Stress

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LISTA DE ABREVIATURAS E SIGLAS

Lista de abreviaturas e siglas da Introdução

ATP	Adenosina trifosfato
CAT	Catalase
CCO	Citocromo c oxidase
COX-2	Ciclo-oxigenase-2
DCF	Diclorofluoresceína
DCFH	Diclorofluorescina
DCFH-DA	diacetato de diclorofluorescina
DNA	Ácido desoxirribonucleico
EB	Extrabucal
ERN	Espécies reativas de nitrogênio
ERO	Espécies reativas de oxigênio
KGF-1	Fator de crescimento dos queratinócitos humanos-1
FBM	Fotobiomodulação
GCO	Global Cancer Observatory
GPx	Glutationa peroxidase
GSH	Glutationa
Gy	Gray
H_2O_2	Peróxido de hidrogênio
IARC	International Agency for Research on Cancer
IB	Intra bucal
IL-6	Interleucina 6
IL-1β	Interleucina 1β
INOS	óxido nítrico sintase indutível
LED	Diodos emissores de luz
LLLT	Low-level laser therapy
MASCC	Multinational Association of Supportive Care in Cancer
MB	Mucosite bucal

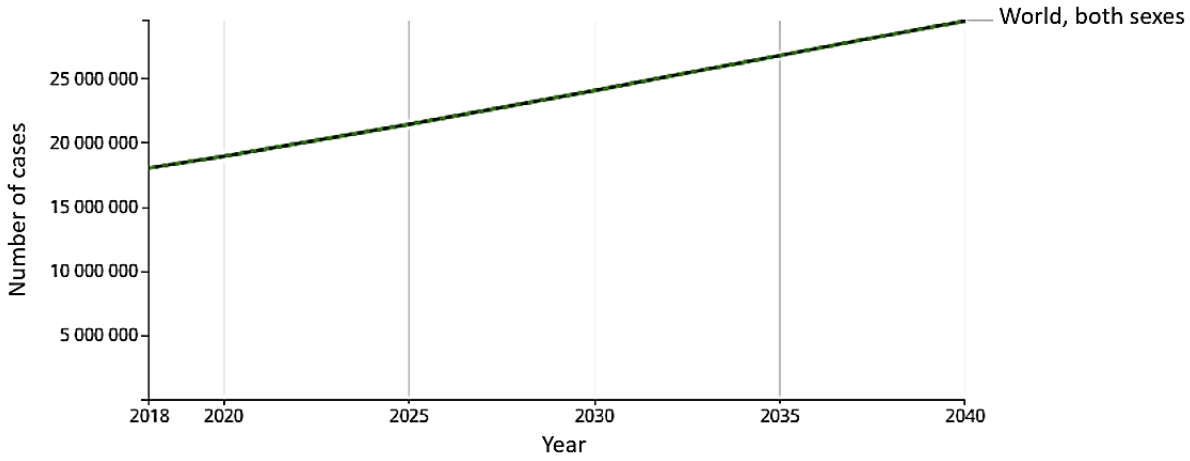
NADPH	Fosfato de dinucleotídeo de adenina e nicotinamida
NCI	National Cancer Institute
NF-Kb	Fator nuclear kappa-B
OMS	Organização Mundial da Saúde
OMAS	Oral Mucositis Assessment Scale
ON	Óxido nítrico
(ONOO-)	Peroxinitrito
PBM	Photobiomodulation
RTOG	Radiation Therapy Oncology Group
SOD	Superóxido dismutase
TCPH	Transplante de células progenitoras hematopoieticas
TNF-α	Fator de necrose tumoral α
5-FU	5-fluorouracil

SUMÁRIO

1 INTRODUÇÃO

O câncer tem um enorme impacto global e os números mais recentes do GLOBOCAN 2018, banco de dados *Global Cancer Observatory* (GCO) da *International Agency for Research on Cancer* (IARC) indicam um aumento da carga de doença, prevendo 18,1 milhões de novos casos em 2018 e 29,5 milhões em 2040 (Figura 1). Isso resulta, em parte, da elevação da expectativa de vida e do envelhecimento da população. Também é importante destacarmos o papel das transformações globais socioeconômicas, de urbanização, industrialização e sobre o estilo de vida, que interferem na incidência e distribuição de determinados tipos de câncer (BRAY et al., 2018; STEWART; WILD, 2014).

Figura 1 - Estimativa da incidência de câncer de 2018 a 2040



Fonte: Globocan 2018. Graph production: Global Cancer Observatory (http://gco.iarc.fr/).
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As modalidades terapêuticas para os diversos tipos de câncer compreendem cirurgia, radioterapia, quimioterapia, terapia biológica ou imunológica, transplante de células progenitoras hematopoiéticas (TCPH), terapia hormonal e terapia gênica (CHAVELI-LÓPEZ, 2014; VIGARIOS; EPSTEIN; SIBAUD, 2017). Os avanços no conhecimento dos mecanismos básicos envolvidos na patobiologia do câncer e o desenvolvimento de novos protocolos terapêuticos têm aumentado as taxas de sobrevida para determinados tipos de câncer. Porém, paralelamente, algumas dessas terapias, em especial a quimioterapia e radioterapia, exibem significativa toxicidade que resultam em diferentes efeitos adversos imediatos e tardios

impactando sobremaneira na qualidade de vida dos pacientes (BUGLIONE et al., 2015; LALLA et al., 2018). Tanto a quimioterapia como a radioterapia afetam, além das células tumorais, as células normais, e seus efeitos em tecidos altamente proliferativos geram danos significativos (KWON, 2016). A cavidade bucal é muito suscetível aos efeitos tóxicos diretos e indiretos da quimioterapia e radioterapia em cabeça e pescoço. Isto se deve à alta taxa de renovação celular da mucosa bucal, a complexa e diversificada microflora e ao microtrauma tecidual que ocorre durante a mastigação (CHAVELI-LÓPEZ, 2014). Dentre as principais complicações bucais associadas ao tratamento antineoplásico encontramos a mucosite, infecções (em especial candidíase, herpes), disgeusia, disfagia, odinofagia, xerostomia, doença periodontal, cárie de radiação, osteorradionecrose, osteonecrose associada a medicamentos e trismo (GERHARD; CARVALHO, 2017; MCCAUL, 2012; MOORE, 2012; SROUSSI et al., 2017).

A mucosite é a complicação mais frequente e grave decorrente do tratamento antineoplásico. Ela se caracteriza por uma reação inflamatória que ocorre no sítio irradiado ou pode afetar toda a mucosa do trato gastrointestinal em caso de uso de quimioterapia (SONIS, 2017). Clinicamente, o aspecto da mucosite bucal (MB) induzida por quimio e radioterapia pode variar de eritema a vários graus de ulceração (EILERS et al., 2014; SHANKAR et al., 2019). As formas mais severas estão associadas à sintomatologia dolorosa intensa, alterações na dieta ou dependência de alimentação parenteral - resultando no comprometimento nutricional e perda de peso -, risco de infecções, redução da qualidade de vida, maior uso de recursos de saúde e até interrupção do tratamento oncológico, podendo, desta forma, interferir negativamente no prognóstico do paciente (LALLA; SAUNDERS; PETERSON, 2014; VILLA; SONIS, 2015).

A frequência e gravidade da MB varia de acordo com fatores de risco complexos e multifatoriais. Dessa forma, além do tipo e dose do tratamento administrado, elementos biológicos individuais dos pacientes como idade, sexo, fatores genéticos, resposta imune inata e microbioma veem sendo estudados (BACHOUR; SONIS, 2018; KWON, 2016). No que diz respeito à quimioterapia, protocolos para TCPH que envolvem o uso de agentes alquilantes, compostos derivados da platina, altas doses de citarabina e 5-Fluorouracil (5-FU) são considerados protocolos de alto risco para o desenvolvimento de MB (CURRA et al.,

2018). Pacientes com câncer de cabeça e pescoço em estadiamentos avançados (III e IV) e com margens cirúrgicas comprometidas são indicados para tratamento com radioterapia. Destes, quase a totalidade desenvolvem algum grau de MB. A incidência de mucosite clinicamente significativa pode variar de 60-100% entre os pacientes em tratamento com altas doses de quimioterapia, radioterapia e transplante de medula óssea (CINAUSERO et al., 2017).

Existem inúmeras classificações que auxiliam na gradação clínica da MB, com o intuito de desenvolver um método confiável e reprodutível de avaliação, permitindo mensurar o impacto da terapia na morbidade, mortalidade e qualidade de vida dos pacientes, especialmente no cenário de doenças incuráveis (MARIA; ELIOPOULOS; MUANZA, 2017; PARULEKAR et al., 1998). Os escores mais comumente utilizados são da Organização Mundial da Saúde (OMS), do *National Cancer Institute* (NCI), do *Radiation Therapy Oncology Group* (RTOG) e *Oral Mucositis Assessment Scale* (OMAS).

Durante o tratamento quimioterápico, os primeiros sintomas da MB surgem, de modo geral, no 4º ou 5º dia após a infusão da droga, atingindo o ponto mais crítico no 10º dia. O acometimento preferencial ocorre nas superfícies bucais não-ceratinizadas (mucosa jugal, palato mole, assoalho, ventre e bordas da língua) e o processo de reparo usualmente ocorre entre o 14° e 21° dia após o início do tratamento (VILLA; SONIS, 2016). No caso da radiação, o efeito celular após a exposição é quase imediato. Embora a patobiologia seja semelhante ao processo resultante do tratamento quimioterápico, o esquema fracionado de dosagem de radiação acarreta danos contínuos e cumulativos. Os sintomas iniciam no final da primeira semana de irradiação, com doses em torno de 10 Gy, surgindo ulcerações dolorosas que coalescem (30 a 70 Gy) e podem persistir por 6 a 7 semanas após a conclusão do tratamento (CINAUSERO et al., 2017; SONIS, 2009).

A patobiologia da MB envolve um modelo composto por cinco fases dinâmicas proposto por Sonis, (1998) descritas como: 1 – iniciação; 2- resposta inicial ao dano; 3 – amplificação da sinalização; 4 – ulceração; 5 – cicatrização. Devido à complexidade envolvida nesse processo, a compartimentalização biológica nessas 5 fases é apenas uma ferramenta para compreensão e investigação, em vez de uma realidade *in vivo.* Os mecanismos envolvidos nesse processo ainda estão

sendo desvendados e, nas últimas décadas, novas contribuições incorporadas. (SONIS; VILLA, 2018).

A mucosite é consequência de uma série de eventos biológicos concatenados que resultam na ulceração tecidual. Embora a perda do epitélio caracterize clinicamente a condição, os eventos que ocorrem na submucosa, antes de manifestações visíveis, desempenham um papel importante na sua patogênese. Na fase de iniciação, os tratamentos quimioterápico e/ou radioterápico são responsáveis por dano direto ao DNA, produção de espécies reativas de oxigênio (ERO) e morte celular clonogênica, levando à apoptose células basais do epitélio e submucosa. Na segunda etapa, chamada de resposta inicial ao dano, esses eventos irão desencadear uma série de mecanismos biológicos e vias como a do fator nuclear kappa-B (NF-kB) que leva, consequentemente, à maior expressão de citocinas pró-inflamatórias tais como a interleucina 6 (IL-6), interleucina 1β (IL-1β) e o fator de necrose tumoral α (TNF-α), moduladores de citocinas, Ciclo-oxigenase-2 (COX-2), óxido nítrico sintase indutível (INOS) e superóxido dismutase (SOD) e moléculas de adesão celular. Na fase de amplificação da sinalização, várias moléculas ativadas regulam positiva ou negativamente a resposta tecidual local, potencializando os sinais biológicos originais e, dessa forma, aumentando e prolongando o dano tecidual. O estágio de ulceração, além de ser o mais crítico para o paciente, quanto à sintomatologia e aspectos clínicos, pode ser agravado pela colonização de bactérias do meio bucal, estimulando ainda mais a liberação de citocinas pró-inflamatórias e recrutamento de macrófagos. Na fase final, que se estabelece após a eliminação do fator agressor, há cicatrização das úlceras pela migração, proliferação e diferenciação de células do epitélio através de sinais da matriz extracelular (AL-DASOOQI et al., 2013; SONIS, 1998, 2007, 2017; VILLA; SONIS, 2015). A Figura 2 ilustra resumidamente os mecanismos envolvidos na patobiologia da mucosite.

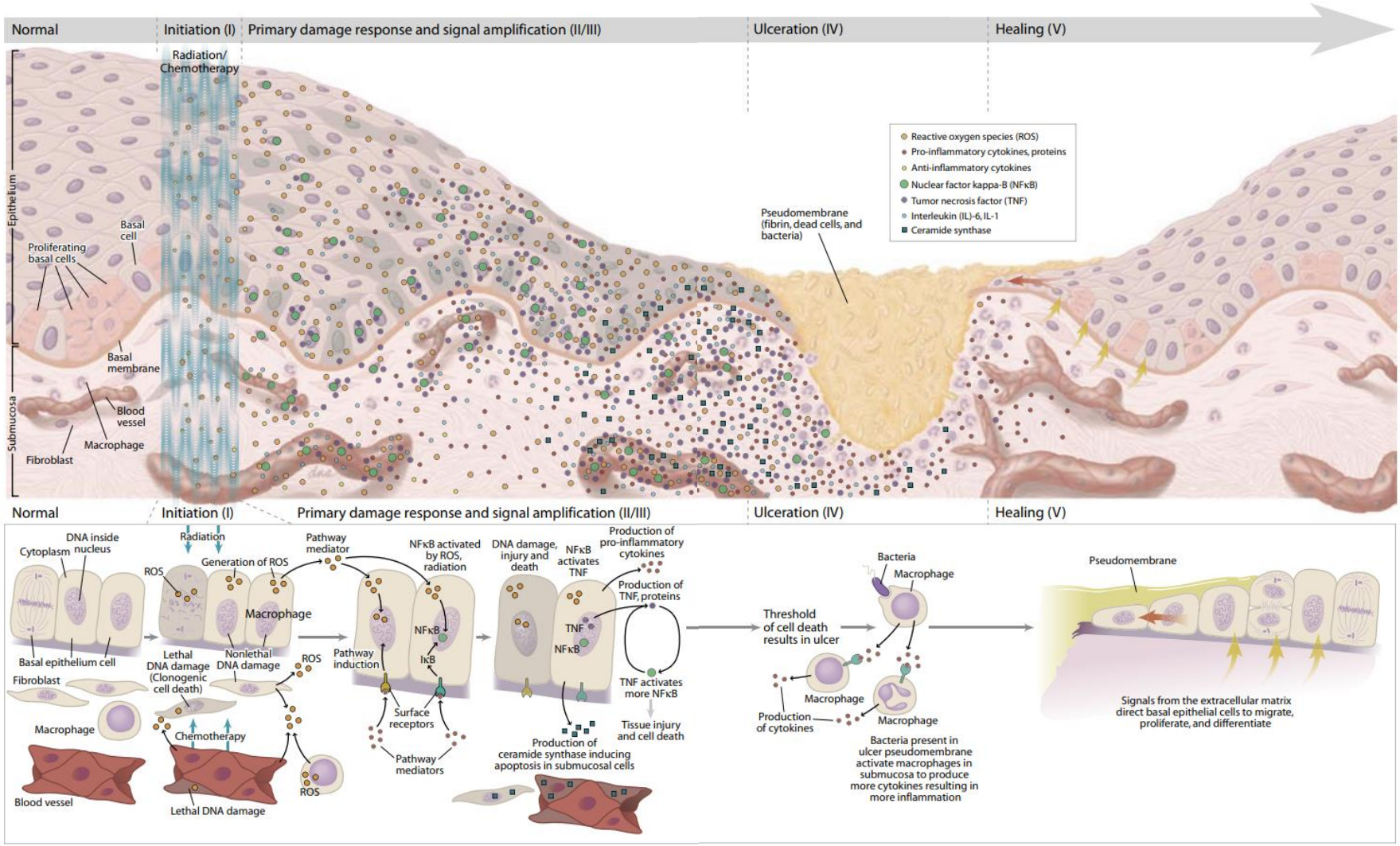
Como abordado acima, o tratamento antineoplásico promove alterações no metabolismo celular e, portanto, torna-se importante a revisão de aspectos relacionados a esses processos. O metabolismo celular normal forma constantemente radicais livres que, quando em excesso, podem ocasionar oxidação de moléculas biológicas. Desta forma, a oxidação é considerada parte fundamental da vida aeróbica e do metabolismo celular, produzindo radicais livres

fisiologicamente ou por disfunção biológica. Esses radicais livres, cujo elétron desemparelhado encontra-se centrado nos átomos de oxigênio ou nitrogênio, são denominados de ERO e espécies reativas de nitrogênio (ERN). No organismo, ambas estão envolvidos na produção de energia, fagocitose, regulação do crescimento celular, sinalização, imunidade, defesa e síntese de substâncias biológicas (CHAPPLE; PUSZYK; MANN, 2015; MIGLIARIO et al., 2018). Em estados patológicos, incluindo a mucosite bucal, esses radicais livres aparecem em níveis mais elevados (SONIS; VILLA, 2018).

Por outro lado, para evitar os danos causados pelos ERO, o organismo possui vários mecanismos de defesa. Os antioxidantes representam um complexo sistema de proteção, podendo ser enzimático ou não enzimático (HALLIWELL; WHITEMAN, 2004). O sistema enzimático é o primeiro a atuar, destacando-se as enzimas: SOD, catalase (CAT) e glutationa peroxidase (GPx), além da glutationa (GSH), um antioxidante não enzimático. A SOD catalisa a dismutação do radical superóxido em peróxido de hidrogênio (H_2O_2). A CAT transforma o peróxido de hidrogênio em água (H_2O) e oxigênio (O_2). A GPx também age sobre o H_2O_2, o reduzindo em álcool e/ou água através da utilização da glutationa. O desequilíbrio entre o desafio oxidativo e a capacidade de defesa antioxidante do organismo é denominado de estresse oxidativo (MACHADO et al., 2009).

A quantificação de ERO e sua relação com dano oxidativo exige uma complexa correlação de fatores e enzimas. A utilização de um composto de permeabilidade celular não fluorescente, o diacetato de diclorofluorescina (DCFH-DA) é um teste relativamente fácil e rotineiramente utilizado para medir a geração intracelular de H_2O_2 e outros oxidantes ou monitorar as alterações de sinalização redox nas células em resposta à ativação intra ou extracelular ao estímulo oxidativo. Uma vez dentro das células, o DCFH-DA é hidrolisado para formar a diclorofluorescina (DCFH), que é retido intracelularmente devido à sua impermeabilidade à membrana. O DCFH reage com ERO intracelular para formar o produto fluorescente diclorofluoresceína (DCF) (GHIZONI et al., 2017; HALLIWELL; WHITEMAN, 2004; KALYANARAMAN et al., 2014).

Figura 2 - Modelo dos múltiplos mecanismos da patobiologia da mucosite.



Fonte: SONIS, 2007

Diversos protocolos têm sido descritos com a finalidade de prevenir e minimizar a severidade da mucosite nos pacientes oncológicos. Neste sentido, a *Multinational Association of Supportive Care in Cancer* (MASCC) desenvolveu uma série de *guidelines* nos últimos anos sobre o tratamento e prevenção da MO e gastrintestinal. Na mais recente atualização, desenvolvida por Lalla et al. (2014) foram descritas diretrizes para manejo da MB na prática clínica, divididas conforme a força das evidências disponíveis relativas à eficácia do tratamento em: recomendações a favor de uma intervenção (com fortes evidências), sugestões a favor de uma intervenção (evidência mais reduzida), recomendações contra uma intervenção (fortes evidências) e sugestões contra uma intervenção (evidência mais reduzida).

Entre as recomendações, com fortes evidências, estão:

- 30 minutos de crioterapia na prevenção da MB em pacientes submetidos à quimioterapia com 5-FU;
- Uso de fator de crescimento dos queratinócitos humanos-1 (KGF-1/Palifermina) na prevenção da MB (60 μg/Kg por dia, nos 3 dias antes e 3 dias pós-transplante) em pacientes com neoplasia hematológica maligna que irão efetuar quimioterapia de altas doses e irradiação corporal total, seguido de TCPH autólogo;
- terapia com laser em baixa intensidade (comprimento de onda até 650nm, potência de 40mW e energia de 2J/cm²) na prevenção da MB em pacientes que efetuaram TCPH após quimioterapia de altas doses e irradiação corporal total;
- analgesia com morfina controlada pelo paciente no tratamento da dor associada à MB em pacientes submetidos a TCPH;
- bochechos com solução de benzidamida na prevenção da mucosite bucal em doentes com cancro/câncer da cabeça e pescoço que efetuaram radioterapia em doses moderadas (até 50Gy) e sem quimioterapia concomitante.

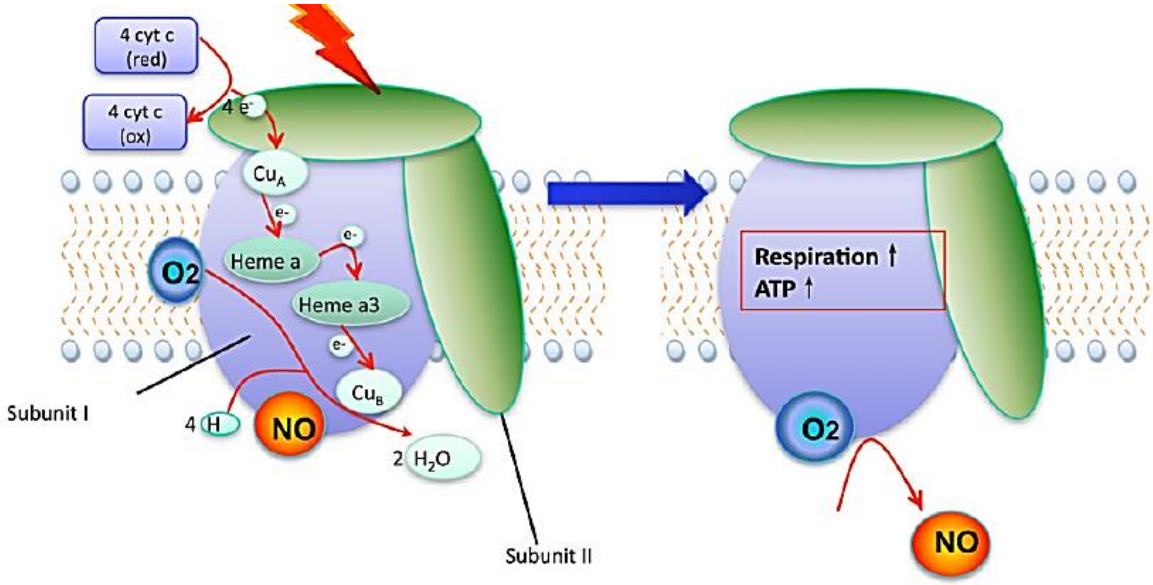
Atualmente a terapia utilizando os chamados lasers em baixa intensidade, lasers de baixa potência, laser frio ou laser de tecidos moles tem sido chamada de fotobiomodulação (FBM). Na verdade o termo FBM surgiu devido à subjetividade da terminologia antiga, além da evidenciação de que os lasers atuam da mesma forma

que outras fontes de luz não coerentes, como os diodos emissores de luz (LED) mas que apresentam comprimentos de onda semelhantes (HAMBLIN, 2017). Desta forma, o conceito de FBM se refere a terapia não invasiva que envolve a exposição de células ou tecidos a baixas potências de luz no espectro visível, azul (λ = 390 a 500nm), verde (λ = 530 a 570nm), vermelho (λ = 600 a 700nm) ou infravermelho (λ = 780 a 1200nm) sem causar elevação da temperatura local. Simplificadamente, esse processo envolve cromóforos endógenos estimulados pela luz provocando eventos fotofísicos e fotoquímicos em diferentes escalas biológicas. Cada cromóforo é sensibilizado por comprimentos de onda específicos. Os resultados são efeitos terapêuticos benéficos, como o alívio da dor, modulação do processo inflamatório e imune, cicatrização tecidual, aumentando a síntese de colágeno, angiogênese, além de estimular a diferenciação miofibroblástica nas fases iniciais da cicatrização, melhorando as características do tecido neoformado (ANDERS; LANZAFAME; ARANY, 2015; HAMBLIN, 2017; ZECHA et al., 2017).

A FBM intrabucal vem sendo amplamente investigada na prevenção e tratamento da MB, tanto em estudos clínicos quanto em modelos experimentais (COTOMACIO et al., 2017; CURRA et al., 2015; FREIRE et al., 2014; MELCHIONDA et al., 2018; RIBEIRO et al., 2017; WEISSHEIMER et al., 2017). O estudo da FBM extrabucal é escasso, mas demonstra resultados bastante promissores (HODGSON, 2012; TREISTER, 2016; ZECHA, 2016; HE, 2017). Dessa forma, ainda são necessários mais estudos que possibilitem a exploração de novas tecnologias e criação de parâmetros reprodutíveis. Uma abordagem extrabucal eficaz para o tratamento da MB poderia permitir um tratamento com o mínimo de desconforto e melhor cooperação do paciente, pois não é preciso manipular os tecidos bucais diretamente já que, muitas vezes eles apresentam limitação de abertura bucal, dor intensa em decorrência das ulcerações e náusea (CAMPOS et al., 2009; KUBOTA, 2004; OTTAVIANI et al., 2013; SUTER; SJÖLUND; BORNSTEIN, 2017; TORRES et al., 2013; ZAND et al., 2012). Outro ponto promissor do uso da FBM extrabucal é o fato de dependendo dos parâmetros utilizados poder atingir estruturas pouco beneficiadas hoje por terapias com pouca profundidade de penetração, tendo efeito em condições como a mucosite de orofaringe (HODGSON et al., 2012)

Os mecanismos de ação da FBM têm sido referidos como efeitos nos níveis molecular, celular e tecidual. Dentro das células, a FBM atua a partir da absorção dos fótons de luz por cromóforos. A citocromo c oxidase (CCO), ou complexo IV, presente nas mitocôndrias é uma enzima terminal da cadeia transportadora de elétrons e tem sido considerada um dos principais cromóforos responsáveis pelos efeitos da FBM, sendo sensibilizada principalmente por comprimentos de onda vermelho e infravermelho. A CCO é responsável por transferir um elétron de cada uma das quatro moléculas do citocromo c para uma única molécula de oxigênio, ao mesmo tempo, quatro prótons são translocados através da membrana mitocondrial formando duas moléculas de água. O gradiente de prótons produzido estimula a atividade da ATP sintase para sintetizar o ATP. O CCO possui dois centros heme-ferro (a e a3) e dois centros de cobre (CuA e CuB), cada um desses centros metálicos pode existir em um estado oxidado ou reduzido, e com diferentes espectros de absorção. A principal hipótese para explicar como a luz aumenta a atividade da enzima CCO, o consumo de oxigênio e a produção de ATP é que o óxido nítrico (NO), presente em concentrações inibitórias em processos patológicos e tecidos lesados inibe a CCO por se ligar aos centros heme-a3 e CuB, bloqueando competitivamente o oxigênio, no entanto, um fóton de energia baixo, como dos dispositivos utilizados para FBM, pode eliminar o NO a partir da fotodissociação e permitir que a respiração celular ocorra (HAMBLIN, 2017, 2018) (Figura 3).

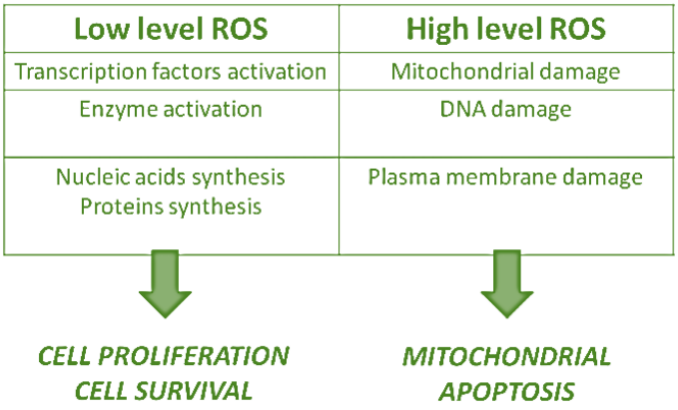
Figura 3 - Fotodissociação do NO a partir da CCO.



Fonte: Hamblin, 2018

Um aspecto importante que deve ser destacado é o fato de a FBM aumentar a produção de ERO que podem, aparentemente, ser tanto benéficas quanto prejudiciais. Dessa forma, sugere-se que baixos níveis de ERO são produzidas em fluências baixas de luz, representadas principalmente por superóxido, enquanto que altos níveis de ERO, responsáveis pelos efeitos mais danosos, são representados principalmente por radicais hidroxila e peroxinitrito, geralmente produzidos por uma fluência mais alta de luz. Baixos níveis de ERO mitocondriais atuam como sinais redox e, assim, produzem um deslocamento do potencial redox total da célula em direção a uma “boa” oxidação que pode resultar na síntese de ácidos nucléicos e proteínas, ativação de enzimas e progressão do ciclo celular, bem como na ativação de fatores de transcrição e a expressão de um grande número de genes que regulam a proliferação e a sobrevivência celular. Por outro lado, a geração de altos níveis de ERO resulta em dano mitocondrial, levando à ativação da via apoptótica mitocondrial, bem como a danos na membrana plasmática e no DNA (Figura 4) (MIGLIARIO et al., 2018).

Figura 4 - Efeitos de altos e baixos níveis de ERO.

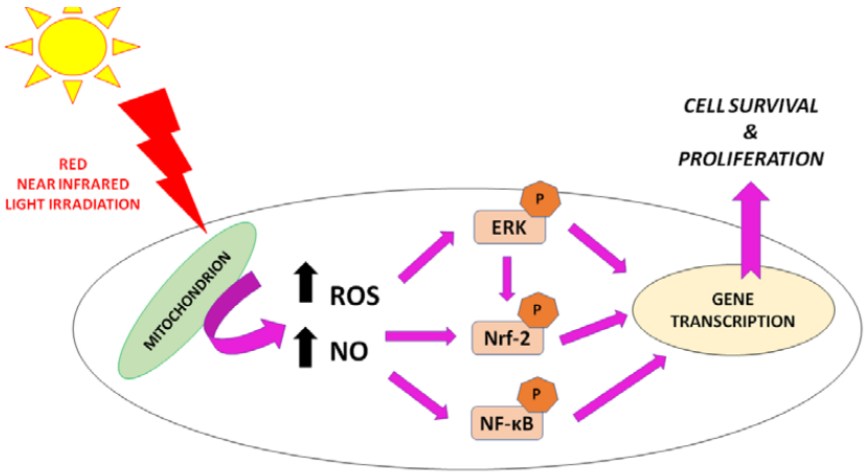


Fonte: Migliario et al., 2018.

Portanto, evidências demonstram que, baixas a moderadas quantidades de ERO podem resultar em efeitos celulares positivos. Os fatores de transcrição NF-kB e Nrf-2 possuem um papel central nesse processo. O NF-kB atua como sensor de estresse oxidativo, sendo ativado em decorrência de um aumento de ERO celular após FBM. Esta explosão inicial na produção de ERO foi associada tanto a uma ativação direta de NF-kB como a uma ativação transitória das vias MAPK/ERK, que

por sua vez poderia ativar Nrf-2, resultando em estimulação da proliferação celular (Figura 5) (CHUNG et al., 2013; GEORGE; HAMBLIN; ABRAHAMSE, 2018; HAMBLIN, 2018).

Figura 5 - FBM estimulando vias redox-sensíveis.



Fonte: Migliario et al., 2018.

Apesar da grande quantidade de informações sobre os efeitos benéficos da FBM em diversas patologias, muitos questionamentos ainda existem acerca dos mecanismos de ação, parâmetros e formas de utilização da FBM em MB. Estudos em animais representam modelos importantes para a melhor compreensão da patobiologia da mucosite tanto quimio como radio induzida, possibilitando investigar a eficácia de novas tecnologias (CAMPOS et al., 2016; SACONO et al., 2008; RTIBI et al., 2017; GERHARD et al. 2017). Estes modelos permitem que seja avaliado, tanto o comportamento clínico, quanto a resposta tecidual, imunológica e molecular envolvidas neste processo. Têm sido descritos, principalmente, o uso de hamsters e ratos para a indução de MB. Além da fácil manipulação, a literatura apresenta estes modelos como padrão para avaliação da resposta tecidual ao tratamento fotobiomodulador (CAMPOS et al., 2016; COTOMACIO et al., 2017; CURRA et al., 2015; FREIRE et al., 2014; SONIS et al., 1990). Dessa forma, o presente estudo visa avaliar o efeito da FBM intra e extrabucal na MB quimioinduzida em ratos por meio de analise clínica, histopatológica e do estado redox.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito da FBM com diferentes fontes emissoras de luz na MB quimioinduzida por 5-FU em ratos.

2.2 OBJETIVOS ESPECÍFICOS

Verificar o efeito clínico da FBM com laser intrabucal e laser extrabucal na prevenção e reparo da MB quimioinduzida por 5-FU em ratos;

Avaliar os parâmetros histopatológicos (reepitelização e inflamação) frente à ação da FBM com laser intra e extrabucal na MB quimioinduzida por 5-FU em ratos;

Verificar o efeito da FBM com laser intrabucal e laser extrabucal sobre parâmetros de estado redox em amostras de MB quimioinduzida por 5-FU em ratos.

3 ARTIGO CIENTÍFICO

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Effect of photobiomodulation with intra and extraoral laser in mucosite induced by chemotherapy in rats.

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Abstract

Purpose: Photobiomodulation (PBM) to prevent and treat of oral mucositis (OM) is very widespread. However, few studies have already demonstrated a reduction in the severity of OM using extraoral appliance. The aim of the present study was to compare the clinical and histopathological effect of intraoral (IO) and extraoral (EO) diode laser irradiation on OM induced by 5-fluorouracil (5-FU) in rats. In addition, we explored the impact of these therapies in mechanisms involved in redox state.

Methods: A total of 96 Wistar rats were randomly divided into 6 groups: negative control (NC) group, positive control (PC) group (only OM induction), IO laser group (IO 6 J/cm^2), EO laser with 6 J/cm^2 (EO 6 J/cm^2), 12 J/cm^2 (EO 12 J/cm^2) and 24 J/cm^2 (EO 24 J/cm^2). OM was induced with intraperitoneal injection of 5-FU on days 0 and 2 followed by scarification of the bilateral buccal mucosa on days 3 and 4. The animals received bilateral daily PBM according to the experimental group. NC was euthanized at day 0 and the other groups at days 8, 10 and 14 (n=6). Clinical evaluation of the degree of mucositis was done through photography by a blinded and calibrated researcher. Histological sections were evaluated through wound healing scores. The parameters of redox state were evaluated through the dichloro-dihydro-fluorescein (DCFH), glutathione-peroxidase (GPx), reduced glutathione (GSH) and catalase (CAT) analysis.

Results: On day 8, PC group presented higher scores of OM. PBM groups showed lower scores of OM, however, only EO 6 J/cm^2 presented a significant lower degree compared to PC ($p<0.05$). On days 10 and 14 all PBM groups showed improvement in OM compared to PC ($p<0.01$). Histologically, on day 8, PC group presented severe lesions exhibiting highest scores (2.8 ± 0.4) differing significantly from all irradiated groups that presented accelerated healing ($p<0.01$). In parallel, on day 8, PC showed an increase oxidative biomarker DCFH compared to NC (without OM) ($p<0.001$). Laser groups exhibited reduced level of this biomarker compared to PC. Also, the antioxidant GPx at the same time was reduced in PC compared to NC ($p>0.001$). All PBM groups demonstrated higher levels of GPx compared to PC.

Conclusions: EO diode laser protocol as well as traditional IO diode laser irradiation showed positive effects in clinical, histopathological and redox state in OM induced by 5-FU in rats. Among the EO protocols, EO 6 J/cm^2 showed the most and encouraging results.

Keywords: diode lasers, oral mucositis, low-level laser therapy, oxidative stress.

Introduction

Cancer incidence and mortality are rapidly growing worldwide with an enormous global impact in the 21st century. GLOBOCAN, in 2018, estimates 18.1 million new cases of cancer and 9.6 million deaths from cancer. The cumulative risk of incidence indicates that 1 in 8 men and 1 in 10 women will develop cancer in a lifetime. This results from the increase in life expectancy and global transformations of urbanization and industrialization on lifestyle, which interferes with the incidence and distribution of certain types of cancer [1, 2]. Whereas there have been many advances in cancer therapies and increased survival rates for many types of tumors, chemotherapy (CT) and radiotherapy are still among the most used treatment regimens isolated or combined with other methods. Several side effects and significant toxicity often accompany the potential benefits of these treatments [3, 4].

OM is a common and burdensome acute complication of the antineoplastic treatment, consisting of an inflammatory response that can affect specially patients undergoing CT, head and neck radiotherapy (HNR) and hematopoietic stem cell transplantation [5]. Its incidence ranges from 15% among patients receiving low-risk treatments to 60–100% among patients being treated with high-dose CT and HNR [6]. Clinically, OM is characterized by erythematous, erosive, and/or ulcerative lesions. Severe forms of OM are associated with painful lesions, impairment of functional status, changes in diet or dependence on parenteral nutrition, infection risk that may interfere in patient's quality of life. Moreover, it has been associated with additional costs resulting in economic impact, treatment interruptions or discontinuation which may negatively interfere with the patient's prognosis [6–8].

The pathogenesis of OM is multifactorial and multi-stage process that involves damage to oral epithelium and submucosa tissues. It has been reported as five-stages: initiation, up-regulation and activation leading to generation of messengers, signal amplification, ulceration and healing. The first moment is called initiation, in which occur oxidative stress, the generation of reactive oxygen species (ROS), direct DNA and non-DNA damage, and activation of the innate immune response. This biological cascade happens within seconds of the stimulating insult. Following, release of endogenous damage associated molecules regulated by transcription factors such as factor nuclear kappa B (NF-Kb) is observed. It causes apoptosis of submucosal and basal epithelial cells resulting in mucosal ulceration (ulcerative

phase). After, the elimination of damage stimuli, healing stage occurs by increase of epithelial proliferation, migration, and differentiation associated to extracellular matrix reorganization [9–11].

Several treatments have been proposed in order to minimize the damage that OM can cause to the oncological patient. Among them, PBM has been recommended in the guideline proposed the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) [12]. PBM is considered a form of light therapy that uses lasers, light emitting diodes (LED), and broadband light, in the visible and infrared spectrum. It is a non-thermal process involving the absorption of light photons by chromosphere eliciting a plethora of biological responses conducted by different biological pathways in dependence of protocol used, cell type and other factors [13].The physiological effects observed following irradiation and light absorption by cytochrome c oxidase are related to the shift in overall cell redox potential in the direction of greater oxidation and increased ROS generation and cell redox activity [14, 15]. These mechanisms promote activation of several transcription factors such NF-kB and synthesis of several protein, growth factors and cytokines. Consequently, the PBM have been associated to decrease pain, modulation of inflammation, decrease edema, acceleration of cellular proliferation and wound healing [16].

Positive effects of different intraoral diode laser protocols in the prevention and treatment of OM have been reported [17–19]. However, few studies have demonstrated a reduction in the severity of OM using extraoral appliance [20–24]. Further studies are needed to enable the exploration of new technologies and the creation of reproducible parameters. An effective extraoral approach can be considered a therapy with better comfort for the patients since it does not require opening the mouth that presents wounds. In addition, it may be used in oropharyngeal mucositis, an area not reached with the intraoral laser [23, 25]. Thus, the aim of the present study was to compare the clinical and histopathological effect of PBM using IO and EO laser on OM induced by 5-fluorouracil (5-FU) in rats. In addition, we explored the effect of PBM in important mechanisms involved in redox state.

Materials and Methods

This is a prospective, randomized, controlled, and blinded animal model study. 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 on Animal Use of the Porto Alegre University Hospital (HCPA, Brazil, n.2018-0096).

Experimental Procedure

Ninety-six male rats (Rattus norvegicus albinus, Rodentia Mammalia Wistar line) were used, with 8-12 weeks of life and body mass of 250-300g each. They were kept in species-specific housing with at least 2 and at most 4 animals, temperature (22 ± 2°C, 20-24°C), relative humidity (40-60%) controlled, air exhaust system and light/dark cycles (12h/12h), with solid chow and water *ad libitum*.
The animals were randomly divided into 6 groups:

- Negative control group (NC, n=6): Without intervention;
- Positive control group (PC, n=18): induction of OM, no treatment, only daily handling;
- Intraoral PBM (IO 6 J/cm^2, n=18): induction of OM and intraoral laser with 6 J/cm^2;
- Extraoral PBM 6 J/cm^2 (EO 6 J/cm^2, n=18): induction of OM and extraoral laser with 6.11J/cm^2;
- Extraoral PBM 12 J/cm^2 (EO 12 J/cm^2, n=18): induction of OM and extraoral laser with 12.22J/cm^2;
- Extraoral PBM 24 J/cm^2 (EO 24 J/cm^2, n=18): induction of OM and extraoral laser 24.45 J/cm^2.

OM was induced in the rats of all study groups, except for NC using intraperitoneal injection of 5-FU chemotherapy at days 0 (60 mg/kg) and 2 (40 mg/kg), according to protocol proposed by Sonis et al. [26], modified by Leitão et al. [27]. On days 3 and 4 the animals were anesthetized with isoflurane diluted in oxygen and the bilateral bucal mucosa was scarified with the tip of a sterile needle twice in a row by the same operator. On Day 5 all animals were evaluated for clinical identification of OM.

Parameters of intraoral PBM

Intraoral PBM was delivered with a continuous indium gallium-aluminum-phosphide (InGaAlP) diode laser (MMOptics Ltda, São Carlos, SP, Brazil) with a spot size of 0.04 cm^2, operating at a wavelength of 660 nm and an output power of 100mW, irradiance of 2500mW/cm^2, during 2.4s in punctual and contact modes. The energy densities were 6J/$cm^2$and energy per point of 0.24 J [28, 29]. Irradiation was performed perpendicularly to the mucosa, in one central point at oral mucosa, once daily, starting at day 0 of induction of mucositis by chemotherapy up to the 14th day (Fig. 1A). The output power of the equipment was checked using a power meter (Laser Check; MMOptics LTDA, São Paulo, Brazil).

Parameters of extraoral laser

Extraoral laser irradiations were performed with diode laser (Gemini® Ultradent Products. Inc.) with pulsed, dual wavelength 810 + 980nm, output area of the laser beam of 4.91cm^2 and with a power of 2 Watts and three distinct protocols: EO 6 J/cm^2, EO 12 J/cm^2 and EO 24 J/cm^2 (Table 1). A daily extraoral application was performed starting at day 0 of OM induction up to the 14th day, perpendicularly and in contact with the skin of the right and left cheek at a central point (no need to remove pelage) (Fig. 1B). Power output was checked using a power meter (Coherent Molectron1, Santa Clara, CA). The laser irradiations were done following biosafety rules.

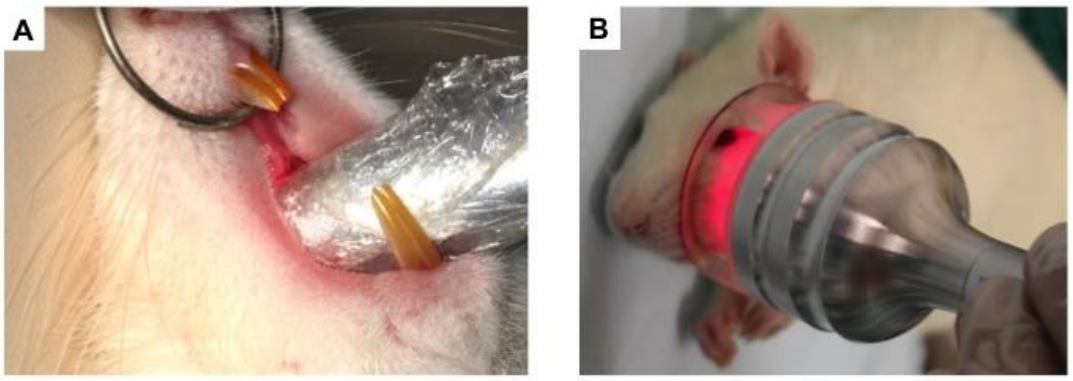

Fig. 1 - Illustrative photographs of intraoral (A) and extraoral (B) diode laser application.

Table 1. Extraoral diode laser parameters.

Protocol	EO6J/cm^2	EO12 J/cm^2	EO24 J/cm^2
Center wavelength (nm)	810nm + 980nm (50%/50%)	810nm + 980nm (50%/50%)	810nm + 980nm (50%/50%)
Operating mode	Pulsed	Pulsed	Pulsed
Frequency (Hz)	50Hz	50Hz	50Hz
Pulse duration (ms)	2ms	2ms	2ms
Duty cycle (%)	10%	10%	10%
Peak power (W)	20W	20W	20W
Average power (mW)	2000Mw	2000mW	2000mW
Polarization	No	No	No
Spot size (cm^2)	4.91	4.91	4.91
Beam shape	Round	Round	Round
Beam profile	Gaussian	Gaussian	Gaussian
Irradiance at target (mW/cm^2)	407mW/cm^2	407mW/cm^2	407mW/cm^2
Exposure duration (s)	15	30	60
Radiant exposure (J/cm^2)	6.11	12.22	24.45
Total radiant energy (J)	20	60	120
Number of points irradiated	1	1	1
Area irradiated (cm^2)	4.91	4.91	4.91
Application technique	Contact	Contact	Contact
Number and frequency of treatment sessions	1x day/ 14days	1x day/ 14days	1x day/ 14days

Euthanasia

Six animals from each group were euthanized using overdose of isoflurane (inhalant anesthetic) at D0 (only animals from NC) and at days 8, 10 and 14, animals from PC, IO 6 J/cm^2, EO 6 J/cm^2, EO 12 J/cm^2 and EO 24 J/cm^2 groups. Left and right buccal mucosa were photographed for clinical evaluation of mucositis grade and then resected. The left buccal mucosa was subsequently removed and fixed in 10% buffered formalin solution to be included in paraffin for histopathological and immunohistochemical study. The right buccal mucosa was conditioned in liquid nitrogen and later in freezer -80^{o}C for evaluation of the redox state.

Weight and Clinical Evaluation

The animals were weighed on Days 0,1,5,8 and 14 and monitored daily to determine morbidity and mortality. For OM clinical analysis, photos of all groups were analyzed by calibrated and blinded oral medicine professional based on the method described by LIMA et al. [30] as follows: 0 - normal oral mucosa, with absence of or slight erythema and hyperemia, and no areas of bleeding, ulceration, or abscesses; 1 - moderate erythema and hyperemia, with no areas of bleeding, ulceration, or abscesses; 2 - severe erythema and hyperemia, presence of areas of bleeding, small ulcers, or eschars, but no abscesses; and 3 - severe erythema and hyperemia, presence of areas of bleeding, extensive ulcers, and abscesses.

Histopathological Evaluation

Left buccal mucosas were fixed in 10% buffered formalin solution for 48 h. After washing with water, the specimens were dehydrated and embedded in paraffin. Slices 5-µm thick were obtained and stained with hematoxylin-eosin. The descriptive analyses of each group and time-point were performed, followed by a semi-quantitative analysis [30]: Score 0 - normal epithelium and conjunctive tissue, with no vasodilatation, absence of or discreet inflammatory infiltrate, absence of bleeding, ulceration, and abscesses; Score 1 - mild vascular hyperemia, areas of re-epithelialization, discreet inflammatory infiltrate with prevalence of mononuclear infiltrates, absence of bleeding, ulceration, and abscesses; Score 2 - moderate vascular hyperemia, areas of hydropic epithelial degeneration, inflammatory infiltrate with prevalence of neutrophils, areas of bleeding, edema and occasional ulceration, and absence of abscesses; Score 3 - severe vascular hyperemia and vasodilatation, inflammatory infiltrate with prevalence of neutrophils, areas of bleeding, edema, and extensive ulcers and abscesses. The analysis and photomicrographs were performed using olympus cx41 microscope.

Redox state analysis

Sample preparation

The specimens stored in freezer -80ºC (right buccal mucosa) were submitted to redox state evaluation protocols. For biochemical analysis, each buccal mucosa was individually homogenized in 10 volumes (1:10 w/v) of 20mM sodium phosphate buffer, pH 7.4 containing 140mM KCl, 1 mM EGAT and 1mM PMSF. Homogenates were centrifuged at 3,000 rpm for 10 min at 4°C., to discard nuclei and cell debris. The pellet was discarded, and the supernatant was taken for biochemical assays.

Oxidant levels measurement

Production of reactive oxygen species was measured fluorimetrically, through the 2′,7′-dichlorofluorescein (DCFH) oxidation method [31]. Briefly, in a 96-well plate, 50µL of the diluted sample was incubated at 37 °C/ 30 min, in the dark, with the addition of 200µL of H2DCF-DA. H2DCF-DA is cleaved by cellular esterases and the DCFH formed is eventually oxidized by reactive oxygen species or reactive nitrogen species presented in samples producing a fluorescent compound, DCF. Fluorescence was measured using excitation and emission wavelengths of 488 nm and 525nm, respectively. A standard curve of DCF (0.25 - 10mM) was performed in parallel with the samples. The results were expressed as nmol/mg protein.

Antioxidant activity

The reduced glutathione (GSH) concentration was determined fluorimetrically [32]. One GSH unit is defined as 1mol of NADPH consumed per minute and specific activity is represented as units per mg protein.

Glutathione peroxidase activity (GPx, EC 1.11.1.9) was determined according to Wendel [35], with modifications. The reaction was carried out at 37°C in 200µL of solution containing 20 mM potassium phosphate buffer (pH 7.7), 2 mM EDTA, 0.8mM sodium azide, 0.5 mM NADPH, 2mM glutathione and 0.4U glutathione reductase. The activity of GPx was measured using tert-butylhydroperoxide as the substrate at 340nm. The contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction ratio. One GPx unit is defined as 1µmol of NADPH consumed per minute and the specific activity was represented as units/mg protein.

Catalase (CAT, EC 1.11.1.6) activity assessment was based upon establishing the rate of hydrogen peroxide (H_2O_2) degradation spectrophotometrically at 240nm at 25°C [36]. CAT activity was calculated in terms of micromoles of H_2O_2 consumed per minute per mg protein.

Statistical analysis

The results were expressed as mean and standard deviation of the mean. Weight analysis was performed using two-way ANOVA test. The data of clinical, histopathological and redox state were analyzed using one-way ANOVA and Tukey's multiple comparison test. The software used for statistical analysis was GraphPad Prism 5 (GraphPad Software Inc., San Diego, California) and level of significance considered was 5% ($p < 0.05$).

Results

Weight analysis

Figure 2 demonstrates the mean weight of each group during the experimental period. There were no statistically significant differences in weight between days in each group or among groups analyzed (p=0.93).

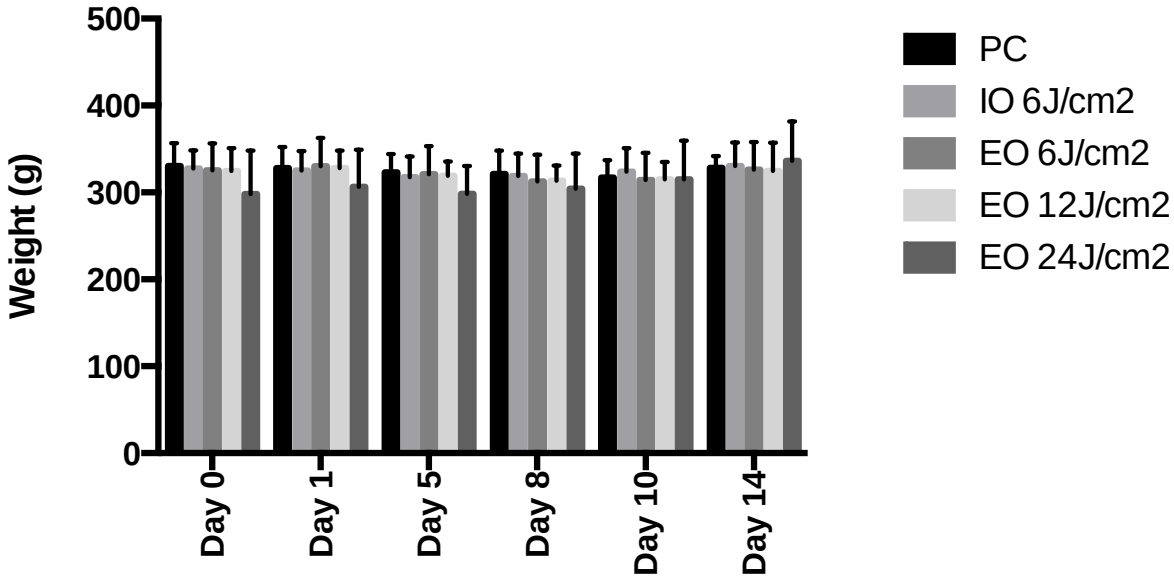

Fig. 2 - Weight analysis during experimental period. Two-Way ANOVA test revealed no significant differences in each group throughout days and among groups in each specific day.

Intra and extraoral PBM promotes clinical reduction of OM

The clinical analysis reveals that all animals exhibited OM on day 5. Figure 3 illustrates the clinical aspects of OM in all groups during the experimental period (8, 10, and 14 days). On day 8, PC group presented higher scores of OM. PBM groups showed lower scores of OM however, only EO6J/cm^2 presented a significant lower degree compared to PC ($p<0.05$) (Fig. 3 A and B). At day 10, all PBM groups showed improvement in OM compared to PC ($p<0.01$) (Fig. 2A and C). At day 14, PC maintained OM while PBM groups showed no more lesions ($p<0.01$) (Fig. 3A and D). No differences were observed among irradiated groups on days 10 and 14 (Fig. 3A, C and D).

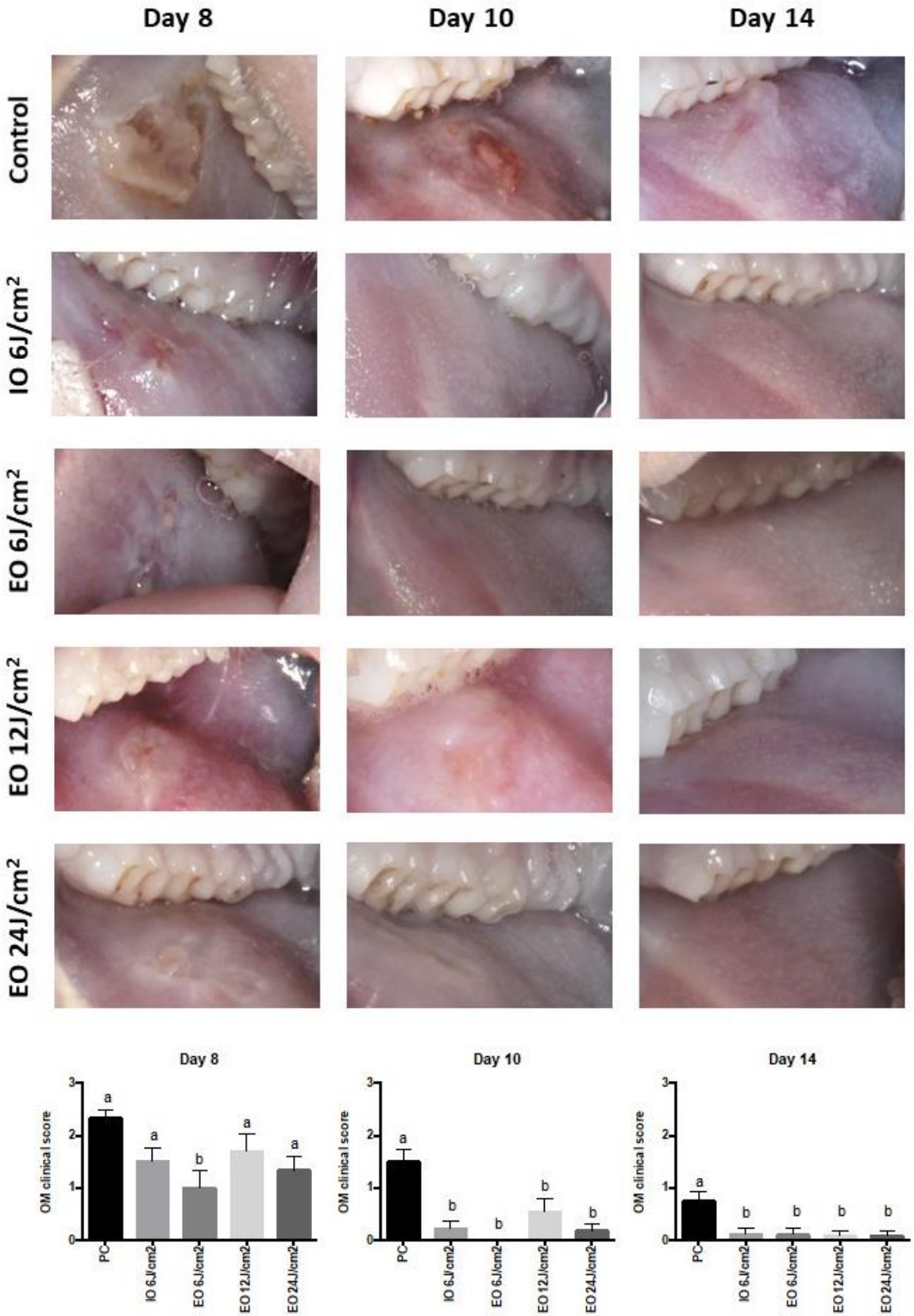

Fig. 3 - Clinical analysis of mean OM scores in control and PBM groups. On day 8, EO 6 J/cm^2 showed lower clinical scores compared to other groups. On days 10 and 14, all irradiated groups presented better clinical response compared to PC.

Intra and extraoral laser protocols accelerates reepithelization and resolution of inflammatory process in 5-FU induced oral mucositis

Histopathological criteria evaluate together the epithelial aspect and severity of inflammation among the experimental groups in different periods of time (Fig. 4). On day 8, PC group presented the highest scores (2.8 ± 0.4) differing significantly from all irradiated groups ($p<0.01$). PC group presented predominance of ulceration with moderate to severe vascular hyperemia, inflammatory infiltrate with neutrophils with areas of abscess, represented by scores 2 and 3. All irradiated groups revealed accelerated OM healing showing similar results to each other ($p>0.05$). They presented preponderance of reepithelialization, slight hyperemia, moderate to slight chronic inflammatory infiltrate, absence of ulceration and abscesses (score 1).

On day 10, some animals of PC still presented small areas of ulceration with chronic inflammatory infiltrate while other animals presented reepithelization and slight inflammatory (mean score 1.3 ±0.5). All irradiated groups revealed total reepithelization with slight to absence of inflammatory infiltrate, predominance of new fibroblasts and appearance of immature skeletal muscle cells. The statistical analysis of mean scores showed no differences among groups ($p>0.05$). At day 14, all groups showed complete OM lesions healing ($p>0.05$) (Figure 4).

A

	PC	IO 6J/cm2	EO 6J/cm2	EO 12J/cm2	EO 24J/cm2
Day 8	2.8 ± 0.4^{A}	1.2 ± 0.5^{B}	1.0 ± 0.6^{B}	1.5 ± 0.5^{B}	1.4 ± 0.5^{B}
Day 10	1.3 ± 0.5^{A}	0.0 ± 0.0^{A}	0.3 ± 0.5^{A}	0.6 ± 0.5^{A}	0.6 ± 0.5^{A}
Day 14	0.6 ± 0.5^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}

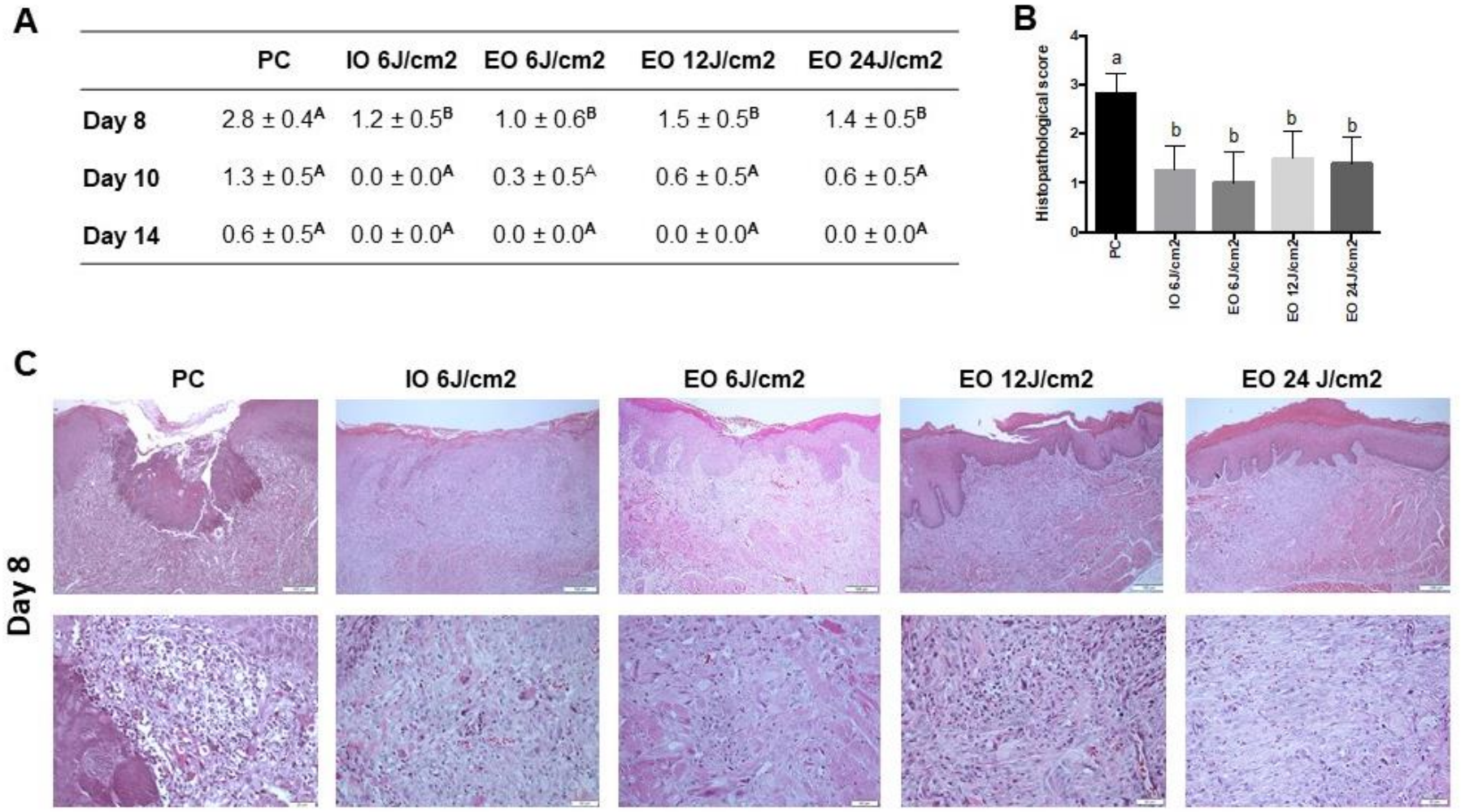

Fig. 4 - Histopathological evaluation of OM healing. (A) Mean and standard deviation observed in all experimental groups on days 8 (B), 10 and 14. On day 8, all irradiated groups revealed accelerated OM healing compared to PC. (C) Photomicrographs of experimental groups on day 8. PC exhibited ulceration and neutrophils inflammatory infiltrate. All irradiated groups showed reepithelization and slight/moderate chronic inflammatory infiltrate (HE, original magnification, x100 and x400).

Intra and extraoral PBM modulate the redox state in 5-FU induced OM

We analyzed the impact of different protocols of PBM in oxidative damage biomarker (DCFH) and antioxidant activities (GSH, GPx and CAT) during 5-FU induced OM (Figure 5). DCFH analyzes the intracellular generation of ROS. On day 8, PC showed an increase of this biomarker compared to NC (without OM) ($p<0.001$). PBM groups, in general, exhibited reduced level of this biomarker compared to PC. However, EO6J/cm^2 and EO12J/cm^2 groups presented significant less oxidative damage than PC indicated by lower level of DCFH ($p <0.001$, $p<0.01$, respectively). In addition, these groups showed similar level of this biomarker when compared to NC ($p>0.05$). On days 10 and 14 no differences of DCFH levels were detected among all groups ($p>0.05$) (Fig. 5A).

GPx is part of the enzymatic defense system that attempts to control the occurrence of oxidative damages in order to balance the production of ROS by converting H_2O_2 to H_2O as a form of cellular adaptation and protection [37]. Our results demonstrated that on day 8 some differences existed among groups. PC showed lower level of this antioxidant enzyme activity compared to NC ($p>0.001$). All PBM groups demonstrated higher levels of GPx compared to PC. Furthermore, IO 6 J/cm^2 and EO 6 J/cm^2 showed similar GPx level to NC ($p>0.05$). On days 10 and 14 no differences were detected ($p>0.05$) (Fig. 5B).

GSH and CAT results are demonstrated on Figure 5C e 5D, respectively. No differences among groups on days 8, 10 and 14 were observed for both antioxidant activities.

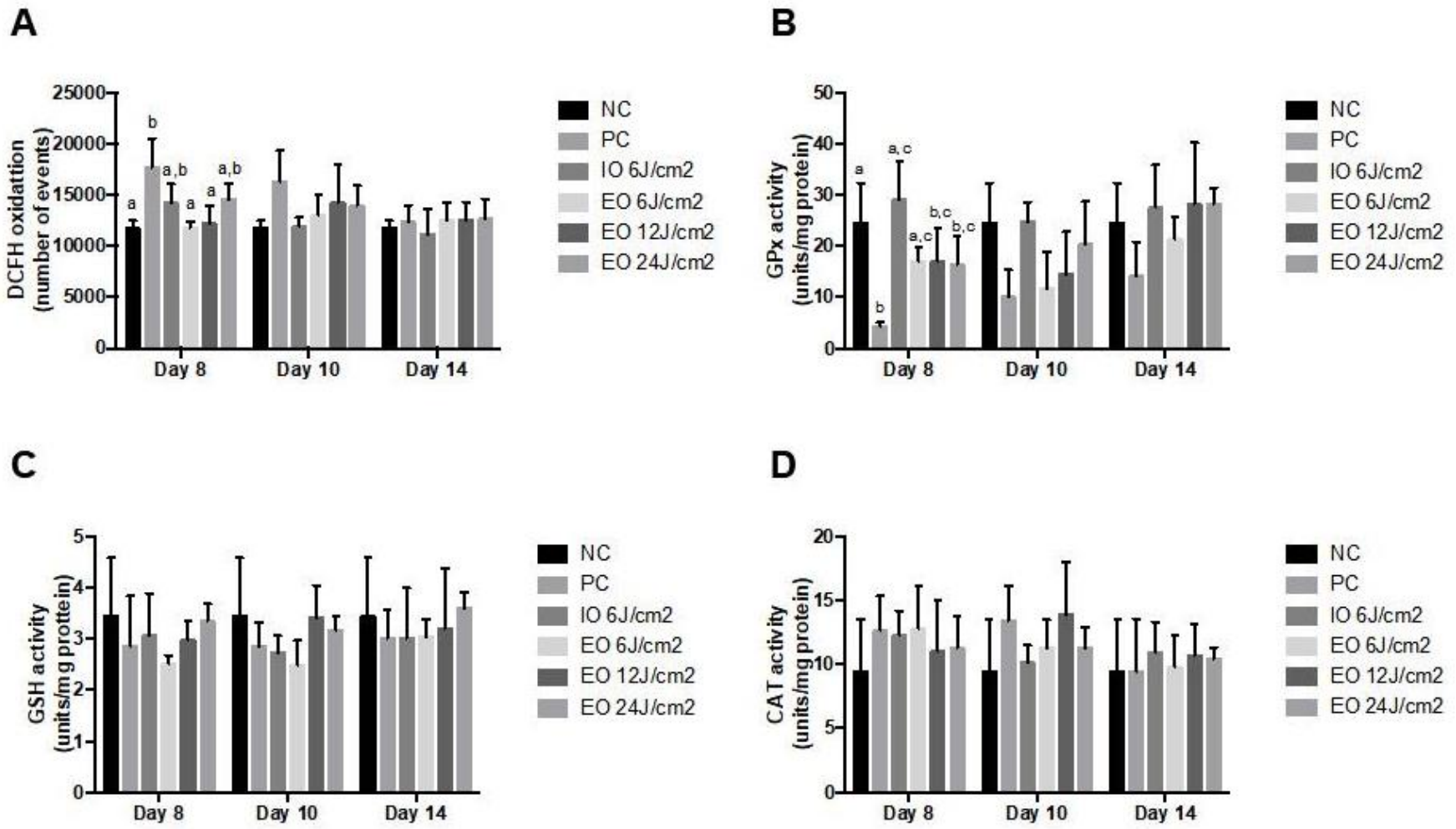

Fig. 5 - Oxidative damage biomarker (DCFH) and antioxidant activities in all experimental groups. (A) DCFH analysis. On day 8, intracellular ROS generation (DCFH level) was increased in PC and reduced in irradiated groups. (B) GPx antioxidant enzyme activity. A reduction of GPx was detected in PC and irradiated groups presented an increase in this protective enzyme. (C) GSH activity showed no variation among groups in each period of time evaluated. (D) CAT antioxidant enzyme level showed no difference among groups

Discussion

PBM has been recommended as an excellent tool in the prevention and treatment of oral mucositis [24, 38]. Most studies were performed with intraoral diode lasers devices applied in contact with the oral mucosa [39, 40]. However, some other forms of phototherapy such as LED extraoral diode laser [23, 25] and intraoral defocused high power diode laser have shown satisfactory results [39, 41, 42]. In the present study, we evaluated the effect of intra e extraoral diode laser irradiation on 5-FU induced OM in rat model. Our results bring interestingly results showing collectively that all protocols of laser promoted reduction of OM. However, the clinical positive effects of EO6J/cm^2 occurred earlier compared to other lasers parameters tested. The clinical improvement of OM lesions with different protocols of irradiation was associated with decrease of inflammatory process and faster reepithelization. In parallel, PBM modulated the redox state regulated by DCFH and GPx antioxidant enzyme activity.

Animal studies represent important models for a better understanding of the chemotherapy and radiotherapy induced OM pathobiology [43]. The first chemotherapy induced OM model was established by Sonis et al [26] that used hamster. At the same time, Wolfgang Dorr developed a mouse radiation model [44]. After that, mice and rabbits models have also been used [17, 28, 39, 40, 45–47]. Most of these studies performed ulceration in the oral mucosa using mechanical or acid irritation in combination with chemo or radio induced damage. Although animal models do not always precisely replicate what happens in humans in a real clinical situation, such studies have proved to be very useful in preclinical situation. They are important for defining events that occur during OM development and for in depth evaluation of new treatment options. Numerous researches have associated the use of these animal models to evaluate the efficacy of intra and extraoral lasers, and LEDs in the treatment of OM [17, 28, 40, 48, 46, 45]. Nevertheless, the present study was the first to test an extraoral oral diode laser in rats for a better understanding of their mechanisms of action in OM pathobiology.

Our results revealed positive effect of all laser protocols tested compared to control. In general, lasers groups promoted accelerated OM healing compared to PC group in clinical (day 8, 10 and 14) and histopathological (day 8) analyzes. These results seem to occur by the stimulation of epithelial migration and proliferation taking

to cover the wounds faster that in control animals. Regarding inflammatory process, laser irradiation promoted a decrease in inflammatory response generating a reduction in the amplification and ulcerative phases of OM pathobiology. Also, the laser group animals showed anticipation of the healing phase characterized by the increase of fibroblast proliferation and collagen deposition. Similar positive effects have been reported in the literature with different diode laser protocols [17, 28, 39, 40, 46, 45, 47]. Unfortunately, wide ranges of laser protocols for OM presenting variation in wavelength, irradiance, power output, energy density, energy and mode of application have been described in the literature. Most evidences from animals and clinical studies recommended wavelength between 633 and 685 nm or 780–830 nm, power output between 10 and 150 mW, and energy density of 2–3 J/cm^2 and no more than 6 J/cm^2 but the literature does not exclude the efficacy of other settings. Few studies were performed using higher energies and all of them were intraoral with controversial results and some important methodological limitations [39, 41]. Ottaviani et al 2016 reported that 970nm diode laser with 2.5 mW in continuous wave and 50% duty cycle at 5 W, 30s, spot size diameter of 0.5 cm, energy density of 375 J/cm^2 presented positive biostimulating and anti-inflammatory effects in mouse model of chemo induce OM. Campos et al [39] compared low level laser, LED and high power laser in a defocused mode with a wavelength of 808 nm, according delivered through a 400 μm optical fiber with 1.0 W output power, applied in continuous- wave mode (irradiance of 1 W/cm2) for 10 seconds in scanning movements. According to the protocols used in the low-level laser and LED therapies presented better results than high power laser.

It has been suggested that new studies should be performed to examine the effect of altering laser parameters in animal models of OM, focusing on elements in the pathobiological process of OM [49]. Based on that, here we decide to use the IO6J/cm^2 protocol, 660nm diode laser, continuous, 100mW, 2.500mW/cm^2, 0.04 spot size, during 6s resulting in 0.24J per point as a “gold standard” PBM protocol for OM [17, 28, 29]. Also, we tested three protocols of extraoral irradiation using 6, 12 ad 24J/cm^2 protocols. In comparison, extraoral diode protocol presented higher pulsed wavelength (810nm+980nm), lower irradiance (407mW/cm2), higher output power (2000mW), spot size (4.91) and energy (20J, 60J and 120J). EO 6 J/cm^2 was the only group, on day 8, that showed better clinical results compared to PC. On day 10,

all laser protocols were similar and superior to PC indicating that even protocols with higher energy with lower irradiance applied extraorally can promote faster healing of OM lesions in animal model. The effects of laser irradiation with different parameters have been explained based on the biphasic dose model (Arndt-Schultz curve) in which low-dose of irradiation stimulates, while higher doses inhibit. Considering that, it is not yet known exactly what is considered a high dose and low dose for OM and we believe that our protocols are among the dose that promotes positive results. According to Huang et al [15] most articles considered energy (J) or fluency (energy density, J/cm^2) as an important descriptor of PBM dose, but neglect other important aspects as wavelength (nm), irradiance (mW/cm^2), pulse structure, coherence, polarization and irradiation time. Thus, there is a consensus that remains a need to identify optimal PBM parameters for OM [50].

The investigations of the mechanisms involved in the pathobiology of OM have evidenced the important role of redox state focused on oxidative stress as triggering factor. Radio and chemotherapy promoted an increase of ROS that will cause an imbalance between the oxidative challenge and the antioxidant defense capacity of the organism, thus generating oxidative stress [7, 9, 51]. 5-FU is a cytotoxic agent that have been associated to generation of ROS playing an important role in cell death mechanisms [52]. In OM model, the initiation phase occurs when animals are exposed to chemotherapeutic drugs such 5-FU resulting in production of large amount of ROS such as superoxide, H_2O_2 and nitric oxide by epithelial and mucosal cells followed by installation of oxidative stress [53]. In the present study we detected on day 8 in PC an increase of DCFH, that is a marker of intracellular generation of ROS, when compared to the NC. It demonstrated that chemotherapy infusion (Days 0 and 2) and scarification (Days 3 and 4) promoted an increase of ROS, which remained until the 8th after the initiation of OM induction protocol. In parallel, at clinical evaluation all PC animals presented ulceration. In contrast, at the same time, all irradiated groups showed lower levels of DCFH with EO 6 J/cm² and EO 12 J/cm² presenting similar levels to the NC, demonstrating less oxidative damage. EO 6 J/cm^2 clinically exhibited biostimulatory results on day 8 with less severe OM compared to PC. In parallel, we examined some the antioxidant mechanisms by GPx, GSH and CAT. On day 8, a decreased activity of GPx was observed in PC, suggesting that there was not enough protective response.

However, in all PBM groups this cellular adaptation occurred evidenced by higher levels of GPx. GSH and CAT did not show alteration between the groups at days 8,10 and 14. These antioxidants can be produced and consumed at an early stage of oxidative and at 8 days they may no longer be active. Collectively our results indicated that laser irradiation protocols modulate redox state in OM promoting better clinical response.

Recent studies have suggested that ROS is a key molecular circuitry activated during laser irradiation promoting tissue stimulation by increase the "good" ROS, able to activate redox sensitive signal transduction pathways such as Nrf-2, NFkB, and ERK which act as key redox checkpoints and several signaling pathways [14, 54–56]. In addition, Rupel et al [57] demonstrated that ROS production is regulated by the wavelength used. They showed that 660nm laser light promoted and increase ROS production when applied either before or after an oxidative stimulus. Instead, near infrared 970 nm lasers presented a moderate antioxidant activity. The 800 nm or the combination of the three wavelengths exhibited the most marked reduction in the levels of ROS suggesting that multiwavelength PBM protocol could represent a promising therapeutic tool to be introduced into the clinics. In the present study we used dual wavelength (810nm+980nm) in EO protocols and our results evidenced that lower energy group (EO6J/cm^2) showed acceleration of clinical healing of OM lesions associated to lower DCFH and higher GPx levels. In addition, potential benefits of oral extraoral PBM with double and higher wavelengths include: rapid, simple, non-intraoral administration that may be more feasible in young children, patients with limited oral opening, patients with severe pain, as well as accomplishing structures that are poorly benefited today by therapies with lower depth of penetration, such as in oropharyngeal mucositis [22, 23]

In conclusion, EO diode laser protocol as well as traditional IO diode laser irradiation showed positive effects in clinical, histopathological and redox state in OM induced by 5-FU in rats. In this preclinical study, among the EO protocols, EO6J/cm^2 showed the most encouraging results. Since dosimetry is highly complex and EO with diode laser was rarely studied until now, new studies involving different parameters and their effects in other cellular mechanisms should be performed.

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4 CONSIDERAÇÕES FINAIS

A MB é uma complicação grave e frequente decorrente do tratamento antineoplásico estomatotóxico que pode afetar negativamente o prognóstico do paciente. Em razão disso, a patobiologia da MB é alvo de muitos estudos, assim como formas eficazes de tratamento que consigam reduzir essa comorbidade e prover uma maior qualidade de vida ao paciente durante a terapia antineoplásica.

Dentre os tratamentos mais utilizados está a FBM e, mesmo com o grande número de evidências demonstrando seus efeitos benéficos, os complexos mecanismos envolvidos na sua atuação na MB ainda necessitam ser mais aprofundados.

Os efeitos positivos dos diferentes protocolos de lasers de diodo intrabucais na prevenção e tratamento da MB são conhecidos. No entanto, poucos estudos já demonstraram a ação de dispositivos extrabucais. Nossos resultados demonstram que a irradiação com laser intra e extrabucal promoveu efeitos clínicos e histológicos positivos sobre a MB quimioinduzida em ratos, através da modulação do estado redox favorecendo a ação de enzimas antioxidantes, sugerindo uma redução dos danos oxidativos. Dessa forma, mais estudos são necessários para permitir a exploração de novas tecnologias e a criação de parâmetros reprodutíveis.

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