MÔNICA DE OLIVEIRA BELÉM

EFEITO DA FOTOTERAPIA SOBRE COLITE EXPERIMENTAL INDUZIDA POR ÁCIDO ACÉTICO EM CAMUNDONGOS

Londrina
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Dissertação apresentada ao Programa de Pós Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para obtenção do título de Mestre.

Orientação: Prof. Dr. Eduardo José de Almeida Araújo.
Co-orientação: Prof. Dr. Waldiceu Aparecido Verri Júnio.r

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“Há uma força motriz mais poderosa que o vapor, a eletricidade e a energia atômica: A Vontade.”

(Albert Einstein)

BELÉM, Mônica de Oliveira. **Efeito da fototerapia sobre colite experimental induzida por ácido acético em camundongos.** 2016. 125 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

As doenças inflamatórias intestinais (DII) apresentam intenso infiltrado inflamatório, abscesso nas criptas, ulcerações, com perda da arquitetura tecidual, depleção de células caliciformes, alteração do sistema nervoso entérico e até perda de função intestinal. As DIIs têm fisiopatologia complexa, sem tratamentos completamente eficazes. O uso de luz no comprimento de onda do infravermelho apresenta potencial terapêutico sobre inflamações. Objetivou-se avaliar se a fototerapia (940 nm) é capaz de atenuar o processo inflamatório e proteger o tecido lesado do cólon de camundongos com colite experimental. Os experimentos foram divididos em duas etapas. A primeira visou avaliar a existência de potencial anti-inflamatório da fototerapia sobre a colite experimental. Havendo essa ação, a segunda etapa visou avaliar como as estruturas que compõe a parede colônica eram protegidas das lesões causadas pela colite experimental. Cada etapa experimental resultou em um artigo científico original. Foi realizada indução de colite experimental com ácido acético 7,5% (pH 2,5) em camundongos Swiss machos. Os camundongos foram tratados com luz proveniente de lâmpadas de LED no comprimento de onda de 940 nm, 45 nm largura de banda, intensidade de 4,05 J/cm2 e potência total de 270 mW e dose total de 64,8 J por 4 minutos; ou tratados com Prednisolona (5mg/kg/5dias, via oral). Os resultados da primeira etapa mostraram que houve um aumento de 35,71% do número de camundongos com colite que apresentaram trânsito intestinal retardado quando comparados aos animais com colite tratados com a LED terapia. A colite aumentou a área e o peso do cólon, o que foi controlado pela LED terapia. Quanto às lesões macroscópicas, animais com colite tiveram um escore médio de 9,5, com inflamação e ulcerações características da colite, e a fototerapia reduziu esse escore em 66,66%, ficando o cólon desses animais somente com hiperemia ulcerações leves. O tratamento com luz reduziu a atividade da enzima mieloperoxidase (MPO) nos camundongos com colite em cerca de 53%, reduzindo também a quantidade de infiltrado inflamatório em todas as camadas intestinais. A colite provocou aumento nos níveis de IL-1β, TNF-α, IL-6 no tecido colônico e essas citocinas estavam reduzidas nos camundongos tratados com luz. O nível de IL1-10 não foi alterado pela LED terapia. Os resultados da segunda etapa mostraram que a fototerapia reduziu lesões microscópicas provocadas pela colite, havendo manutenção da arquitetura colônica normal, redução do infiltrado celular e manutenção do número de células caliciformes. A colite experimental não alterou o número de neurônios mientéricos gerais (beta-tubulina III positivos), porém causou aumento no número de neurônios produtores de óxido nítrico, peptídeo vasoativo intestinal e substância P, o que foi atenuado pela fototerapia. Na população geral de neurônios mientéricos observou-se que a colite provocou hipertrofia do corpo celular, o que foi controlado pela fototerapia. Nas subpopulações neuronais avaliadas, a colite provou atrofia do corpo celular. A fototerapia também controlou essa atrofia, exceto para os neurônios produtores de subtância P. Varicosidades VIPérgicas mientéricas estavam reduzidas nos camundongos com colite e a fototerapia não modificou essa condição. A colite e a fototerapia não causaram alteração no número

de células de glia entérica (S100 positivas). Conclui-se que a fototerapia a 940 nm apresentou efeito anti-inflamatório no quadro de colite experimental, já que reduziu edema e alterações no tamanho do cólon, diminuiu o escore de lesão macroscópica, a atividade de MPO e o escore de infiltrado inflamatório. Além disso, a fototerapia reduziu níveis de citocinas pró-inflamatórias (IL-1β, TNF-α e IL-6). Em função disso, observou-se que a fototerapia teve efeito protetor sobre as lesões microscópicas provocadas pela colite, além de atenuar as alterações sofridas por neurônios mientéricos nitrérgicos, VIPérgicos e produtores de substância P desencadeadas pela inflamação.

Palavras-chave Colite Microscópica. Sistema Nervoso Entérico. Fototerapia. Colite Experimental. Lesão Macroscópica.

BELÉM, Mônica de Oliveira. **Phototherapy efects in acid acetic-induced experimental colitis in mice.** 2016. 125 p. Dissertation (Master's degree in Experimental Pathology)– Universidade Estadual de Londrina, Londrina, 2015.

ABSTRACT

Inflammatory bowel diseases (IBD) present intense inflammatory infiltration, abscesses in crypts, ulcerations, loss of tissue architecture, goblet cells depletion, changes in the enteric nervous system and even loss of function. IBD presents complex pathophysiology and there is no effective treatments. The use of infrared light has therapeutic potential on inflammation. Our objective was to analyze whether light-emitting diodes (LED) therapy at 940 nm is able to attenuate the inflammation process and to protect the injured colon tissue of colitis-induced mice. The experiments were divided into two steps. First, we evaluated the anti-inflammatory potential of LED on an experimental colitis model. Second, we assessed how the colonic wall could be protected from experimental colitis injury if treated with LED at 940 nm. Each experiment resulted in an original scientific paper. Experimental colitis was induced administrating 7.5% acetic acid(pH 2.5) rectal via in male Swiss mice. Mice were treated with LED with wavelength 940 nm, 45 nm bandwidth, intensity 4.05 J/cm² total power 270 mW and total dose of 64.8 J for 4 minutes; or treated with Prednisolone (5mg/kg/5days, orally). Results from the first experiment showed increase of 35.71% colitis-induced mice with no treatment presenting slowed intestinal transit compared to mice submitted to LED therapy. Colitis provoked increase in the area and weight of the colon, which was controlled by LED therapy. As for gross lesions, colitis-induced mice had a mean score of 9.5, presenting inflammation and ulceration colitis characteristics, and the phototherapy has reduced this score in 66,66%, getting the animas colon only with light hyperemia ulcerations. The LED therapy reduced 53% of MPO activity in colitis-induced mice, and decreased the inflammatory infiltrate in all intestinal layers. Colitis caused increase in IL-1β, TNF-α and IL-6 levels inside the colonic tissue and they were reduced by LED therapy. The light treatment had no influence on IL-10 levels. Data from the second experiment demonstrated that LED therapy reduced microscopic injury, showing maintenance of the normal colonic architecture, reduction of cellular infiltration and conservancy of the goblet cells number. Colitis did not change the general myenteric neurons (beta-tubulin III positive) number. However, the inflammation caused increase of number of nitric oxide, vasoactive intestinal peptide and substance P producing-neurons, which was attenuated by LED therapy. Colitis caused cell body hypertrophy in general myenteric neuron, which was controlled by LED therapy. Colitis provoked cell body atrophy on all evaluated neuronal myenteric subpopulation. The LED therapy also controlled this atrophy, except for substance P producing-neurons. VIPergic myenteric varicosities were reduced in colitis-induced mice and the LED therapy did not change this condition. Colitis and LED therapy did not cause any change in enteric glial cell (S100 positive) number. We conclude that LED therapy at 940 nm showed anti-inflammatory effect in experimental colitis, characterized by reduction of edema, grossinjury score, MPO activity and inflammatory infiltrate score. Furthermore, LED therapy reduced pro-inflammatory cytokines levels (IL-1β, TNF-α and IL-6). As the LED therapy had effective antiinflammatory effect, it was observed protective effect on colitis-induced

microscopic injury, including mitigation of changes in nitrergic, VIPergic and substance P producing-myenteric neurons triggered by inflammation.

Keywords: Microscopic Colitis. Enteric Nervous System. Phototherapy. Experimental Colitis. Macroscopic Injury.

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LISTA DE ABREVIATURAS E SIGLAS

AB pH 0,5	Alcian Blue pH 0,5
AB pH 2,5	Alcian Blue pH 2,5
ATP	Adenina Trifosfato
BSA	Albumina Bovina Sérica
°C	Graus Celcius
cAMP	Adenosina 3',5'-monofosfato Cíclico
CAPES	Coordenação de Aperfeiçamento de Pessoal de Nível Superior
CEUA/UEL	Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina
CGE	Célula da Glia Entérica
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
COX-1	Cicloxigenase 1
COX-2	Cicloxigenase 2
DC	Doença de Crohn
DII	Doença Inflamatória Intestinal
DSS	Sulfato Sódico de Dextrana
ELISA	Enzyme-linked Immuno Sorbent Assay
ERK	Quinase Extracelular Regulada por Sinal
ERO	Espécie Reativa de Oxigênio
FGF	Fator de Crescimento de Fibroblastos
GSNO	s-Nitroglutationa
HE	Hematoxilina e Eosina
HTAB	Brometo de Hexadeciltrimetil-amônio
H_2O_2	Dióxido de Hidrogênio
IgY	Imunoglobulina Y
IL-10	Interleucina 10
IL-1R	Receptor de Interleucina 1
IL-1β	Interleucina 1 beta
IL-33	Interleucina 33
IL-6	Interleucina 6
IL-6R	Receptor de Interleucina 6
IL-8	Interleucina 8
IM	Intramuscular
iNOS	Oxido Nítrico Sintase Induzida
J/cm²	Joule/centímetro²
KGF	Fator de Crescimento de Queratinócitos
LASER	Amplificação de Luz por Emissão Estimulada de Radiação
LED	Diodos Emissores de Luz
µL	Microlitro
µm	Micrômetro
MPO	Mieloperoxidase
mW	Milliwatt
NaCl	Cloreto de sódio
NASA	Administração Nacional Aeronáutica e Espacialnm – Nanômetro
mM	Milimolar
nNOS	Óxido Nítrico Sintase Neuronal
NO	Óxido Nítrico
PAS	Ácido Periódico Reativo de Schiff

PBS	Tampão Fosfato-salino
PDGF	Fator de Crescimento Derivado de Plaquetas
PGE2	Prostaglandina E2
pH	Potencial hidrogeniônico
PRED	Prednisolona
RCU	Retocolite Ulcerativa
RNAm	Acido Ribonucléico Menssageiro
RPM	Rotação por Minuto
SNE	Sistema Nervoso Entérico
SP	Substância P
TGF	Fator de Crescimento Transformante
TNBS	Ácido Trinitrobenzóico
TNF-α	Fator alfa de Necrose Tumoral
VIP	Peptídeo Vasoativo Intestinal

SUMÁRIO

1 INTRODUÇÃO

Doenças Inflamatórias Intestinais (DII) é um termo genérico usado para identificar um grupo de doenças inflamatórias crônicas que ocorrem no intestino. As duas formas mais comuns de DII são a Doença de Crohn (DC) e a Retocolite Ulcerativa (RCU). Até o momento não se sabe, com precisão, quais são os fatores que desencadeiam o surgimento destas doenças, mas há evidencias de que fatores genéticos, microbianos e a imunorregulação da mucosa estejam envolvidos na patogênese das DIIs (FIOCCHI, 2005; STEFANELLI *et al*., 2008; BAUMGART, 2009; BRAZILIAN STUDY GROUP OF IBD, 2010). Sabe-se também que a DC é mais regulada geneticamente do que a RCU (HALME *et al*., 2006) e que nenhum fator ou agente sozinho é responsável pelo desenvolvimento dessas doenças.

Atualmente, as DII representam uma das principais preocupações dentro da gastroenterologia, pois o número de indivíduos com diagnóstico confirmado tem aumentado progressivamente e, além disso, atingem indivíduos de diferentes faixas etárias (sobretudo dos 20 aos 40 anos), ambos os sexos, cursam com recidivas frequentes e podem manifestar formas clínicas de alta gravidade (HANAUER, 1990; KWON *et al*., 2005; ZHU; LI, 2012).

Dados epidemiológicos disponíveis demonstram que as DII têm maior prevalência nos países ocidentais, sendo o Canadá o país com maiores taxas (MOLODECKY *et al*., 2012), além dos Estados Unidos e países da Europa (HENDRICKSON; GOKHALE; CHO, 2002; MARGOLIS; KIRCHGESSNER, 2009; BAUMGART, 2009; LAKHAN; KIRCHGESSNER, 2010; ZHU; LI, 2012). No Brasil os dados epidemiológicos são esparsos com estudos regionalizados como por exemplo GABURRI et al., (1998) em Juiz de Fora (MG), SOUZA et al., (2002) em Ribeirão Preto (SP), DE SOUZA; BELASCO; AGUILAR-NASCIMENTO, (2008) em diferentes municípios do Mato Grosso, OLIVEIRA; EMERICK; SOARES, (2010) na região leste do estado de Minas Gerais, KLEINUBING-JÚNIOR *et al*., (2011) em Joinville (SC). A Federação Brasileira de Gastroenterologia admite que haja uma subnotificação dos casos e, para isso, organizaram um Grupo de Estudos que está tentando criar uma estratégia mais eficaz de registro.

Entre os grandes desafios para esse registro está a falta de padronização dos critérios diagnósticos e de profissionais especializados (OLIVEIRA; EMERICK; SOARES, 2010). Apesar de poucos dados epidemiológicos de países em

desenvolvimento, estudos mostram que as taxas de incidência das DII têm aumentado em diferentes regiões do mundo, indicando sua emergência como doença global (VICTORIA; SASSAK; NUNES, 2009; MOLODECKY *et al*., 2012; ZHU; LI, 2012).

Do ponto-de-vista morfofisiológico, a RCU pode comprometer o reto e o colo, especialmente o cólon descendente, de maneira difusa, enquanto que a DC pode ocorrer em qualquer segmento do tubo digestório, da boca ao ânus, embora o íleo e a região íleo-cecal sejam mais frequentemente afetadas (STEFANELLI *et al*., 2008; ZHU; LI, 2012). Além disso, na RCU, as regiões inflamadas são contínuas e podem atingir toda a extensão do colo e reto, enquanto que na DC as lesões apresentam-se de maneira descontínua quando se considera a extensão do tubo digestório, ou seja, há regiões inflamadas intercaladas com regiões não inflamadas (ZHU; LI, 2012). Outra diferença é observada quando se analisa o comprometimento das camadas da parede intestinal das regiões inflamadas, pois na RCU, geralmente, a mucosa apresenta-se alterada devido a presença de abscessos nas criptas intestinais e infiltrado de células inflamatórias na lâmina própria, os quais provocam distorções nas glândulas mucosas e depleção de células caliciformes, e também não está associada a presença de granulomas, estreitamento e fístulas; enquanto que na DC todas as camadas da parede intestinal podem estar alteradas (inflamação transmural) e pode estar associada a presença de granulomas, estreitamento e fístulas (ABRAHAM; CHO, 2009).

Do ponto-de-vista clínico, a RCU é marcada por períodos de exacerbação e remissão, e os sintomas mais frequentes são: diarréia, enterorragia, possibilidade de muco nas fezes, dor abdominal com cólicas, urgência em evacuar e tenesmo (ISKANDAR; CIORBA, 2012). Além disso, pacientes com RCU apresentam maior risco de desenvolvimento de carcinoma colo-retal (BAUMGART, 2009; ZHU; LI, 2012). Os pacientes de DC geralmente apresentam os seguintes sintomas: dor abdominal, diarréia, perda de peso, mal-estar e, em alguns casos, pode ocorrer obstrução intestinal (CARTER; LOBO; TRAVIS, 2004). Cerca de 5% dos pacientes com DII, com comprometimento da região do colo, apresentam características clínicas, radiológicas, endoscópicas e/ou histopatológicas de ambas as condições (DC e RCU). Nestes casos, a doença é denominada colite “não classificada” (MOWAT *et al*., 2011).

Vários modelos experimentais de DII têm sido descritos com o objetivo de elucidar os mecanismos envolvidos na patogênese destas doenças e também de avaliar a eficácia de novas propostas terapêuticas (GREENWOOD-VAN MEERVELD; PRUSATOR; JOHNSON, 2015). Embora os modelos não reproduzam exatamente a doença em humanos, eles têm sido úteis para estudar muitos aspectos importantes destas condições. Os modelos mais comumente utilizados são aqueles induzidos pela administração de um agente químico exógeno, como ácido trinitrobenzóico (TNBS), sulfato sódico de dextrana (DSS) e o ácido acético. A administração do ácido acético pela via retal induz colite de forma aguda e restrita a porção distal do cólon devido ao dano epitelial causado por seu caráter ácido. Além das alterações na porção distal, também são registradas alterações motoras nas porções superiores do trato gastrointestinal na vigência da inflamação crônica (LIMA JÚNIOR, 2013). O modelo de indução de RCU utilizando-se o ácido acético tem sido utilizado para testes de tratamentos farmacológicos, pois caracteriza os episódios de recidiva da RCU de forma típica (GUAZELLI *et al*., 2013; LIMA JÚNIOR, 2013).

Em 1978, MacPherson e Pfeiffer mostraram que a administração de solução de ácido acético por via retal era capaz de induzir de maneira dose-dependente uma inflamação aguda, difusa e reprodutível na porção distal do cólon de ratos. A lesão colônica induzida pela administração via retal de ácido acético em murinos apresenta características similares a RCU em humanos, já que ambas são caracterizadas por inflamação difusa da mucosa, ulcerações superficiais e redução das células caliciformes (RANI *et al*., 2011). Além disso, também é possível verificar infiltração de polimorfonucleares no tecido, liberação de citocinas pró-inflamatórias, como interleucina-1β (IL-1β) e o fator de necrose tumoral-alfa (TNF-α) (LUO *et al*., 2010; AMIRSHAHROKHI; BOHLOOLI; CHINIFROUSH, 2011).

Parte dos sintomas observados nas DII é decorrente de alterações do funcionamento de células pertencentes ao Sistema Nervoso Entérico (SNE), o principal componente do Sistema Nervoso Autônomo que regula o funcionamento do tubo digestório (FURNESS, 2012). O SNE é composto por plexos interconectados que interagem com células posicionadas desde o revestimento da mucosa até à serosa. Seus componentes celulares são neurônios e células gliais entéricas, os quais são considerados como os principais reguladores da função motora e secretora do tubo digestório (FURNESS, 2006). A literatura apresenta-se heterogênea quanto às descrições de lesões provocadas pelas DII sobre essas

células. Moynes *et al*. (2014) apresenta uma consistente revisão da literatura que destaca as seguintes alterações do SNE em decorrência da RCU: a) perda neuronal; b) aumento de densidade axonal; c) alteração do conteúdo neuroquímicos de neuritos: redução de peptídeo vasoativo intestinal (VIP) e aumento de substância P; d) alterações morfométricas no corpo celular dos neurônios. Villanacci *et al*. (2008) defendem que, nos caso de perda neuronal, não se sabe ainda se ela ocorre de maneira generalizada ou se afeta alguma(s) subpopulação(ões) específica(s). Porém o comportamento neuronal é dependente do modelo de indução da DII (WINSTON; LI; SARNA, 2013).

A inflamação de tecidos tem início principalmente diante de uma resposta imune exacerbada que leva a sinalização de moléculas pró-inflamatórias como prostaglandina E2 (PGE_2), ciclooxigenase-1 (COX-1), ciclooxigenase-2 (COX-2), interleucina-1β (IL-1β), interleucina-6 (IL-6), interleucina-8 (IL-8) e fator de necrose tumoral- α (TNF-α) (ABBAS et al., 2010), o mesmo acontece no tecido intestinal durante as DII. Esse quadro inflamatório intestinal acarreta o início de alterações no padrão de contratilidade muscular intestinal (DANESE; GASBARRINI, 2005; AKIHO *et al*., 2011), sobretudo no cólon de indivíduos com RCU, como resultado da estreita interação entre células imunes entéricas e do SNE, dentre as quais as células gliais entéricas têm papel importante na permeabilidade intestinal, pois em casos extremos de inflamação ocorre ausência da função dessas células (NEZAMI; SRINIVASAN, 2010). Assim, de uma forma geral, considera-se que, durante as crises de indivíduos com DII, desordens inflamatórias desencadeiam diferentes formas de dismotilidade intestinal, que no caso da RCU manifesta-se principalmente como diarréia (BERNARDINI *et al*., 2011).

As desordens inflamatórias são resultantes da infiltração transmural de neutrófilos, macrófagos e linfócitos na mucosa e submucosa do cólon (ROBERTS-THOMSON *et al*., 2011). Os neutrófilos e macrófagos são considerados as células responsáveis pela destruição da integridade epitelial e desenvolvimento da lesão colônica observada em pacientes com colite ulcerativa (GRISHAM; YAMADA, 1992; LATELLA; PAPI, 2012; SANEI *et al*., 2014). Inúmeros mediadores, incluindo leucotrienos e várias citocinas inflamatórias, contribuem na quimiotaxia de neutrófilos na RCU (VILLEGAS *et al*., 2003; DANESE; GASBARRINI, 2005). Os níveis de IL-1β são aumentados na mucosa de pacientes com colite ulcerativa (BAMIAS; KALTSA; LADAS, 2011; AKIHO *et al*., 2011). A interleucina-33 (IL-33) também contribui para a

migração de neutrófilos através da ativação de macrófagos e consequente produção de quimiocinas e citocinas (como a própria IL-1β), e também agindo diretamente sobre o rolamento e migração dos neutrófilos (VERRI *et al*., 2010). Estudos recentes mostraram que os níveis de IL-33 estão aumentados no cólon de pacientes com colite ulcerativa ativa (BELTRAN *et al*., 2010; SEIDELIN *et al*., 2010; KOBORI *et al*., 2010), sugerindo o envolvimento desta citocina na patogênese da RCU. Sendo assim, o bloqueio de IL-1β e da IL-33 durante a RCU, poderia ajudar na redução da severidade da doença. Por outro lado, a interleucina-10 (IL-10) é considerada uma citocina importante na manutenção da homeostase intestinal (PAUL; KHARE; GASCHE, 2012). Camundongos geneticamente deficientes para IL-10 desenvolvem RCU espontaneamente, sendo este um modelo experimental genético para doenças inflamatórias intestinais (KÜHN *et al*., 1993). Além disso, alterações genéticas no locus da IL-10 também contribuem para o desenvolvimento da RCU (PAUL; KHARE; GASCHE, 2012). Neste sentido, fica clara a participação da IL-10 como um mediador que previne o desenvolvimento da RCU. Desta maneira, tratamentos que evitem a queda dos níveis de IL-10 no cólon durante a RCU podem ser uma boa alternativa para redução do processo inflamatório nestes casos.

Os tratamentos convencionais para as DII incluem os aminosalicilatos, corticosteróides, imunossupressores não esteroidais e terapia anti-TNF (BURGER; TRAVIS, 2011), porém nenhuma estratégia terapêutica já estabelecida é completamente eficaz no tratamento das DII (BRAZILIAN STUDY GROUP OF IBD, 2010; BELÉM; ODA, 2015). Por isso, se faz necessário o desenvolvimento de novas alternativas terapêuticas. Os objetivos terapêuticos na RCU são: a indução e manutenção da remissão e o restabelecimento da integridade da mucosa, a fim de evitar a intervenção cirúrgica e diminuir a probabilidade do desenvolvimento do câncer (NG; KAMM, 2009), além de melhorar a qualidade de vida do paciente (BRAZILIAN STUDY GROUP OF IBD, 2010).

Terapia com luz de baixa intensidade ou fotobiomodulação por luz em comprimento de onda do vermelho distante ao infravermelho próximo (630-1000nm), modula numerosas funções celulares (DESMET, et al., 2006). O uso da luz é considerado uma das possibilidades de intervenção terapêuticas de sistemas biológicos de boa repercussão dentro da área médica (GREENWELL; WYMAN; ROGERS, 2001; MASUMOTO *et al*, 2003; LIMA *et al*., 2011; KIRKBY *et al*., 2012; LIMA *et al*., 2013a; LIMA *et al*., 2013b). Na verdade, o potencial terapêutico da luz

depende do comprimento de onda utilizado, da intensidade, das condições do tecido lesado e do sistema que ele está inserido (KARU, 1987). A ausência de cuidado com essas variáveis por alguns autores causou certo ceticismo por parte da comunidade científica sobre a aplicação da luz na prática clínica. Karu na década de 1980 (KARU *et al*., 1982; KARU *et al*., 1983a; KARU *et al*., 1983b) estabeleceu as bases para a compreensão dos mecanismos moleculares associados aos efeitos da luz sobre as células. Esses autores também demonstraram que a resposta celular à fotoestimulação não está associada às propriedades específicas da luz LASER (Amplificação de luz por emissão estimulada de radiação) (KARU; KOLYAKOV, 2005). A utilização da luz com comprimentos de onda dentro da vermelho distante até a faixa do infravermelho próximo (630-1000nm), associado a uma densidade de energia mínima de 4 J/cm2 apresentam eficácia para estimulação de processos biológicos (DESMET, et al., 2006).

Fontes de luz como os LASERs apresentam a desvantagem de fornecer luz apenas monocromática, ou seja, não há a possibilidade de combinar diferentes comprimentos de onda. Alem disso, por ter o feixe de incidência de luz pontual, não consegue atingir áreas grandes, o que também contribui para a emissão, junto da luz, de uma quantidade razoável de calor que pode resultar em dano tecidual (DESMET, et al., 2006). Como alternativa ao uso de LASERs outras fontes emissoras de luz, como os Diodos Emissores de Luz (LED), foram desenvolvidos pela Administração Nacional Aeronáutica e Espacial (NASA) (DESMET, et al., 2006). Esses dispositivos são mais baratos, de maior facilidade de manuseio, e operam com correntes elétricas relativamente baixas em comparação ao LASER (SCHUBERT, 2006). Além disso, o LED emite luz de comprimentos de onda do vermelho distante ao infravermelho combinadas, com feixe de incidência de luz cônico o que compreende a ação sobre grandes áreas, podendo ser construído de vários tamanhos e sem a emissão de calor, o que evita o dano tecidual, alem de contar com um poder de penetração tecidual de até 23 centímetros (DESMET, et al., 2006).

O uso de LED como alternativa terapêutica (LED terapia) é relatado pela literatura da última década (VINCK *et al*., 2003; EELLS *et al*., 2004; WONG-RILEY *et al*., 2005; KOMINE *et al*., 2010; SERAFIM *et al*., 2011; FONSECA *et al*., 2012; CHOI *et al*., 2011; CAMARGO *et al*., 2012; SANTOS *et al*., 2014; SIQUEIRA *et al*., 2015),

demonstrando seu potencial anti-inflamatório e cicatrizante, sobretudo para doenças dermatológicas e danos neurológicos (KOMINE *et al*., 2010).

Em modelos animais, a LED terapia com comprimentos de onda do vermelho próximo e infravermelho atua no metabolismo energético celular levando a uma ativação de cascatas de sinalização intracelular que reduz a expressão de citocinas e moléculas pró-inflamatórias (TNF-α Il-1β, IL-6, leucotrienos, PGE2, COX-1 e COX-2), diminuindo a infiltração e ativação de células pró-inflamatórias (VINCK *et al*., 2003; EELLS *et al*., 2004; WONG-RILEY *et al*., 2005; SERAFIM *et al*., 2011; CHOI *et al*., 2011; CAMARGO *et al*., 2012; FONSECA *et al*., 2012). Além disso, acelera a liberação de restos teciduais, induz a diferenciação e proliferação celular, estimula a angiogênese e acelera a síntese de colágeno e outros componentes da matriz extracelular, além de inibir a dor e a nocicepção (EELLS *et al*., 2004; DESMET, et al., 2006; LIM *et al*., 2007; KOMINE *et al*., 2010; FONSECA *et al*., 2012). A LED terapia ainda atua na redução de espécies reativas de oxigênio (EROs) intracelular, o que reduz os danos à membrana e inibe a ativação da fosfolipase A2 (EELLS *et al*., 2004; DESMET, et al., 2006; CHOI *et al*., 2011).

Bioensaios mostram que a fotobiomodulação ativa a cadeia respiratória mitocondrial, o que leva a ativação de várias cascatas de sinalização intracelular e proliferação celular (DESMET, et al., 2006). O citocromo C oxidase, presente nas cristas mitocondriais, é o receptor dos fótons irradiados pelo LED, esse é estimulado, culminando no aumento do metabolismo e maior produção de energia (ATP), estimulando a produção e substituição de componentes celulares danificados (EELLS *et al*., 2004; DESMET, et al., 2006) e também de fatores de crescimento, tais como: fator de crescimento transformante (TGF) e fator de crescimento de fibroblastos (FGF). Esses fatores de crescimento podem ativar a via de sinalização extracelular regulado por quinases (ERK) em várias linhagens celulares (DESMET, et al., 2006; KOMINE *et al*., 2010).

A LED terapia com comprimento de onda de 635 nm em culturas de amostra de gengiva humana inflamada estimula redução da inflamação por inibição da produção de PGE_2, COX-1 e COX-2, reduziu a expressão de IL-6 e IL-8, alivia o stress celular reduzindo as ERO, sendo assim compatível com o tratamento por anti-inflamatórios (CHOI *et al*., 2011).

Sobre feridas na pele, a LED terapia, com comprimento de onda de 670 nm de intensidade, demonstrou que sua principal atuação se dá principalmente na fase

II do processo de cicatrização (proliferação celular), possivelmente devido ao aumento do metabolismo respiratório energético, com proliferação de fibroblastos, síntese de colágeno e pró-colageno, fator de crescimento de queratinócitos (KGF), fator de crescimento transformante (TGF) e fator de crescimento derivado de plaquetas (PDGF), e produção de matriz extracelular (EELLS *et al*., 2004).

Após o exercício físico fatigante, a LED terapia com comprimento de onda na faixa 940 nm reduz creatino quinase e proteína C reativa, indicando assim um efeito protetivo à inflamação do músculo estriado esquelético estudado. Além disso, sabe-se que o tecido muscular lesado de animais não tratados com LED dentro das propriedades descritas apresenta infiltrado inflamatório contendo neutrófilos, eosinófilos, macrófagos e linfócitos, que estavam removendo as fibras musculares danificadas. Porém, com a utilização da LED terapia, observa-se redução nas fibras musculares danificadas, bem como das áreas de necrose e do infiltrado de células inflamatórias, sobretudo leucócitos e neutrófilos circulantes (CAMARGO *et al*., 2012). A LED terapia nessas condições atua positivamente também no exercício de exaustão, sendo mais eficaz para a prevenção da lesão muscular pós exercício do que a crioterapia (SANTOS *et al*., 2014).

Estudos em animais com o esmagamento do nervo ciático e tratado com LED terapia com comprimento de onda de 940 nm demonstram que há uma regeneração tecidual e redução das áreas de edema, diminui a migração de células mononucleadas e reduz a degeneração tecidual após o tratamento (SERAFIM *et al*., 2011).

Há ainda estudos que relatam que a LED terapia com comprimento de onda de 940nm atua beneficamente sobre a reabsorção radicular induzida em ratos, demonstrando que esse tipo de tratamento reduziu o número de osteoclastos, diminuiu o infiltrado de células inflamatórias, aumentou a quantidade de fibroblastos, bem como a densidade capilar (FONSECA *et al*., 2012).

Porém na literatura não encontramos trabalhos que avaliem o possível potencial terapêutico da terapia de luz emitida por LED sobre a colite, nem experimental nem clínica.

Assim sendo, o objetivo dessa dissertação é avaliar qual o efeito da LED terapia, com comprimento de onda de 940 nm, largura de banda de 45 nm, potência total de 270 mW, dose total de 64.8 J e intensidade de 4.05 J/cm^{2,} sobre a inflamação da parede intestinal e sobre os componentes da parede colônica durante

a colite experimental aguda induzida pela administração de ácido acético por via retal em camundongos.

2 OBJETIVO

2.1 OBJETIVO GERAL

Avaliar o potencial terapêutico da luz no comprimento de onda no infravermelho (940 nm) emitido por diodos emissores de luz (LED) sobre as alterações funcionais, morfológicas e moleculares da parede colônica de camundongos com colite experimental.

2.1.1 Objetivos Específicos

- Avaliar o efeito da fototerapia com comprimento de onda na faixa do infravermelho (940 nm) sobre o tempo de trânsito intestinal e a formação de edema no cólon de camundongos com colite experimental induzida por ácido acético.
- Examinar o efeito da fototerapia com comprimento de onda na faixa do infravermelho (940 nm) sobre lesões macroscópicas e microscópicas (histologia das camadas da parede intestinal, análise do plexo mioentérico e de células caliciformes) no cólon de camundongos com colite experimental induzida por ácido acético.
- Investigar o efeito da fototerapia com comprimento de onda na faixa do infravermelho (940 nm) sobre alterações moleculares (concentração de mieloperoxidase e citocinas IL-1β, TNF-α, IL-6 e IL-10) no cólon de camundongos com colite experimental induzida por ácido acético.

ARTIGO CIENTÍFICO 1

LIGHT-EMITTING DIODES AT 940 NM ATTENUATE COLITIS-INDUCED INFLAMMATORY PROCESS IN MICE

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Abstract:

Inflammatory bowel diseases (IBD) feature intense inflammatory infiltrate, abscesses in crypts, ulceration and even loss of function. IBD present complex pathophysiology and no effective treatment. Infrared wavelength phototherapy shows therapeutic potential on inflammation. Our goal was to evaluate whether light-emitting diodes (LED) at 940 nm are capable of mitigating the colitis-induced inflammatory process in mice. Forty male Swiss mice were assigned into five groups: control; control treated with LED therapy; colitis without treatment; colitis treated with LED therapy; colitis treated with Prednisolone. Experimental colitis was induced administrating acetic acid 7.5% (pH 2.5) via rectum. LED therapy was performed with light chacterized by wavelength of 940 nm, 45 nm bandwidth, intensity of 4.05 J/cm², total power of 270 mW and total dose of 64.8 J for 4 minutes in a single application. Treatment with Prednisolone used dose of 5 mg/kg/5days before colitis induction. Part of induced-colitis mice with no treatment showed retarded intestinal transit compared to those treated with LED therapy. Colitis caused increase in total area and weight colon, which was controlled by LED therapy. Colitis-induced mice had an average score of

9.5 for gross lesion, characterized by typical colitis ulceration. LED therapy reduced the average score to 2, characterized by mild ulcerations. LED therapy decreased myeloperoxidase activity in colitis-induced mice and reduced the presence of inflammatory infiltrate in all intestinal layers. Colitis increased the levels of IL-1 β, TNF-α, IL-6, which was controlled by LED therapy. We conclude LED therapy at 940 nm showed anti-inflammatory effect on experimental colitis in mice.

Key words: inflamatory bowel diseases; phototherapy; myeloperoxidase; pro-inflammatory citokine; Macroscopic injury; inflammatory infiltrate.

1. INTRODUCTION

Cronh disease (CD) and ulcerative colitis (UC) are the main forms of inflammatory bowel disease (IBD) [1-3]. IBD remains with no known etiology [4]. UC is a complex disease that possibly comes from an inadequate immune response in genetically susceptible individuals [1]. This is a result of an intimate interaction between environmental factors and enteric immune system, which contribute to inflammatory acute crises, which become chronic when they are not controlled [1-3].

UC is characterized by continuous inflammation in the large intestine wall, reaching the mucosa and submucosa specifically [2, 5]. It is common to observe ulceration, edema and colonic bleeding [6], polymorphonuclear infiltrate in the mucosa and increase in pro-inflammatory cytokines, particularly IL-1β, TNF-α and IL-6 [3].

Epidemiological data show that IBD have higher prevalence in Western countries. Canada is the country with the highest rate [7], in addition to the United States and countries in Europe [2, 3, 6]. UC patients complain about quality of life due to diarrhea persistent, rectal bleed, abdominal pain, fever and weight loss [2, 5]. In addition, UC is considered a risk factor for colorectal carcinoma [3].

All colitis-induced injuries come from lymphocyte, neutrophil and monocyte recruitment to the lesion [1, 3]. Local chemokines and citokines, such as IL-1β, IL-6 and TNF-α, stimulate this recruitment [4, 8]. Anti-inflammatory citokines, such as IL-10, are secreted in order to balance the inflammatory process [9].

UC does not have gold standard for diagnosis and treatment, nor cure [8]. The current treatment indicates four drug classes: aminosalicylates, steroids, immunomodulators [9], and biological therapies that act on inflammatory mediators using antibodies [10, 11]. It worths noting that all these treatments have many side effects [9]. Thus, it is very important to investigate others effective treatments with less side effect.

Discoveries in the last decade have revealed the potential of phototherapy for treatment of inflammatory diseases [12, 13] and tissue regeneration [14]. However, there are no studies evaluating the effectiveness of phototherapy as a possible treatment of UC.

The therapeutic potential of light depends on the wavelength used, bandwidth, the intensity of energy, the condition of the injured tissue and the system where the light will be applied [15-17]. Therefore, these variables should be controlled to achieve the desired effect. Studies show that 600-1000 nm wavelengths have anti-inflammatory and regenerative effects [18-22].

Considering the anti-inflammatory potential of light at red and near infrared spectral regions (600-1000 nm) in different types injuries [18-20], this study was performed to evaluate whether light-emitting diodes (LED) at 940 nm are capable of mitigating the colitis-induced inflammatory process in mice.

2. METHODOLOGY

All procedures were approved by the Ethics Committee on use of Animals at State University of Londrina, Protocol n. 113/2014, process n° 14276.2014.60.

2.1. Experimental groups

Male Swiss mice (*Mus muscullus*) of 60 days of age (25 g) were kept in an animal house with light-dark cycle of 12 hours and controlled temperature of 23 °C. Water and chow (Nuvilab CR1, Nutrient Nuvitl Ltda.®, Curitiba, Brazil) were provided *ad libitum*. The mice were randomly assigned into five groups (n = 8/group): Control: healthy animals; Control_LED: healthy animals treated with LED; Colitis: colitis induced-mice with no treatment; Colitis_LED: colitis induced-mice treated with LED; Colitis_PRED: colitis induced-mice treated with Predinisolone.

2.2. Colitis induction model and treatments

We followed the protocol for induction of colitis in mice using acetic acid as described before [23]. After 12 hours of fasting, the animals were anesthetized with ketamine hydrochloride (20 mg/kg, Dopalen® 11.6%, Sespo Industry and Commerce Ltda, Paulinia, Brazil, IM) and xylasine hydrochloride (2.5 mg/kg, Anasedan® 2.3%, Sespo Industry and Commerce Ltda, Paulinia, Brazil, IM). It was used a 3 cm-polyethylene cannula for rectal solution administration. Previously, all animals received 100 µL of saline rectal colon cleanse. After one hour, the mice of Control and Control_LED groups received 200 µL of rectal saline. The Colitis, Colitis_LED and Colitis_PRED groups received 200 µL of 7.5% acetic acid (pH 2.5) for induction of colitis. The Control_LED and Colitis_LED groups were kept in ventral decubitus. The equipment used to apply light was positioned one cm above the skin on the dorsal abdominal region. LED therapy was performed the apparatus 16 cm² and 36 LED lamp with 7.5 mW power unitary, was positioned 1 cm above the dorsal skin of the mice in the abdominal region to treatment with LED light with wavelength of 940 nm, 45 nm bandwidth, intensity of 4.05 J / cm², 270 mW total power and total dose of 64.8 J for 4 minutes in a single dose. The treatment was carried out always by the same operator. The Colitis_PRED group received treatment with Prednisolone (5 mg/kg body weight, orally, Prednisolone, Pharma Nostra, Campinas, Brazil), as a control drug of this experiment, during five days prior to induction of colitis.

2.3. Euthanasia and tissue collection

After 18 hours of induction of colitis, the mice were anesthetized with ketamine hydrochloride (80 mg/kg, Dopalen® 11.6%, Sespo Industry and Commerce Ltda, Paulinia, Brazil, IM) and xylasine hydrochloride (10 mg/kg, Anasedan® 2.3%, Sespo Industry and Commerce Ltda, Paulinia, Brazil, IM) and they were euthanized by decapitation. The large intestine from the the cecum to the rectum was removed.

2.4. Evaluation of intestinal transit, dimensions, edema and gross lesion

Ten hours after colitis induction, it was administered 100 µL of dye solution containing Water-soluble Carmine WS 52% (6%, Cochonilha Carmin, Corantec Natural Dyes Ltda, São Paulo, Brazil) and Methylcellulose (0.5%, Methycellulose, Galena Chemical and Pharmaceutical, Campinas, Brazil) via oral to evaluate the

intestinal transit [24]. Then, the animals were kept in individual boxes with chow and water *ad libitum* for eight hours. It was measured the time required for expulsion of first red faeces pelet (non-absorbable dye). This time was divided into three classes: up to 2 hours (normal transit), 2:01-7 hours (traffic slightly retarded) and over 7:01 hours (traffic slowed). Each collected colon was measured for its length and width.

One centimetre-fragment from the distal portion was weight for edema evaluation. The weight of the fragment (g) was expressed as percentage compared to the control group [25].

The colon was open following the mesocolic edge for evaluation of gross lesions. The gross injury scores were determined according to Morris *et al*. [26]. It was considered this score range: 0 (no damage), 1 (hyperemia with no ulceration), 2 (linear ulcerations with no significant inflammation), 3 (linear ulcerations with inflammation in only one area), 4 (two or more areas of inflammation and ulceration), 5 (injury area bigger than 1 cm along the colon), 6 - 10 (injury area bigger than 2 cm along the the colon; each additional centimeter of injury add one point to the score).

2.5. Determination of myeloperoxidase (MPO) activity and inflammatory infiltrate

The MPO activity was assayed by colorimetric method, as described by Bradley *et al*. [27]. One centimetre-samples from the distal portion of the colon were placed in 50 mM potassium phosphate buffer (pH 6.0) containing 13.72 mM HTAB (hexadeciltrimetil-ammonium bromide). The samples were frozen in liquid nitrogen and stored to -80 °C. Then, the samples were homogenized, centrifuged (14000 rpm, 2 min, 4 °C) and the supernatant was used for the colorimetric reaction in 96 wells plate. It was added 200 µL reaction solution to each aliquot of 15 µL of sample. The reaction solution contained 52.64 mM O-dianisidine dihydrochloride, 0.015% H_2O_2 and 30% of 50 mM potassium phosphate buffer (pH 6.0). The reading was performed in spectrophotometer (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) at 450 nm. The number of neutrophils per mg of tissue was determined using a standard curve of neutrophils.

One centimetre-fragments of the colon were fixed in 4% buffered formalin in order to analyse inflammatory infiltrate. These samples were included in paraffin, following routine histological processing. Five micrometers thickness-sections were stained in hematoxylin and eosin (HE). Qualitative analysis was performed on 400x magnified images, considering the following parameters: 0-3 score to intensity of

inflammatory foci on the intestinal layers (mucosa, submucosa and muscle); feature of the cellular infitrate in the layers as non-existent, circumscribed, diffuse, both; presence or absence of ganglionitis in the submucosal and myenteric plexi [28, 29].

2.6. Determination of cytokines (IL-1 β, TNF-α, IL-6 and IL-10)

One centimetre-fragments of the colon were placed in sterile saline, frozen in liquid nitrogen and stored at -80°C. The samples were homogenized and a part was used for dosage of proteins by Spectrophotometric method as described by Lowry *et al*. [30] and modified by Miller [31]. Bovine serum albumin was used as stantard. The results were expressed as mg of protein per mg of tissue. A second aliquot of tissue was centrifuged (3000 rpm, 4°C, 10 minutes) and the supernatant was used to assess the concentration of the cytokines IL-1β, TNF-α, IL-6 and IL-10 by ELISA (*enzyme-linked immunosorbent assay*) using eBioscience kits (Affymetrix eBioscience, San Diego, USA). The test was performed according to the manufacturer's guidelines. The results were obtained by comparing the densities of optical density curves and standards expressed in pg of cytokines per mg of protein.

2.7. Statistical analysis

Data were expressed as mean ± standard error of the mean. The data were analyzed to identify the type of distribution using Shapiro-Wilk test. One-way ANOVA test followed Newman-Keuls test was used to compare the groups for most of the evaluated parameters. Analysis of gross lesion scores was compared between the groups using non-parametric Kruskall-Wallis test followed by Dunn’s test. Analysis of intestinal transit was compared between the groups using two-way ANOVA followed by Newman-Keuls test. All experiments were conducted in duplicate. All statistical analyses were carried out using the GraphPad Prism6 software (GraphPad Software Inc., San Diego, USA). The differences were considered statistically significant for p values < 0.05.

3. RESULTS

3.1 LED therapy minimized changes of intestinal transit and dimensions, edema and gross lesions in inflamed colon

All mice from the Control and Control_LED groups showed normal intestinal transit (up to 2 hours). This shows that the LED therapy in non-inflamed intestine does not interfere in the intestinal transit. Seven mice (87.5%) of the Colitis group had intestinal transit intensely retarded (7:01 > hours). All mice treated with Prednisolone also had intestinal transit intensely retard. Part (37.5%) of mice submitted to LED therapy showed less colitis-induced impairment of intestinal transit (Figure 1).

The analysis of edema (Figure 2A) revealed that LED therapy did not cause any change in healthy tissue. Inflamed colon with no treatment had significant edema (increase of 89.89% intestinal weight). LED therapy and the treatment with Prednisolone contributed similarly to minimize the development of colitis-induced edema.

It was observed LED therapy in healthy tissue did not cause any change in intestinal dimensions ($p > 0.05$). On the other hand, inflamed colon with no treatment had reduced length (Figure 2B), increased diameter (Figure 2 C) and increased intestinal area (Figure 2 D; $p < 0.05$). The treatment with Prednisolone tended to recover the intestinal dimensions in comparison to control. LED therapy showed effective result, keeping the intestinal dimensions similar to colons from healthy mice ($p < 0.05$).

There were no gross lesions in healtht tissue submitted to LED therapy (Figure 3). Inflamed colon with no treatment showed average score 9.5 for gross lesions, with typical purulent ulcerations that extended to the most of the organ (Figures 3 C and F). The score for gross lesion reduced to 2 in mice submitted to LED therapy, with ulceration and inflammation more regionalized (Figures 3 D and F). The Prednisolone treatment reduced the score to 8.5, with typical purulent ulcerations that extended up to 3 cm of the colonic length (Figures 3 E and F).

3.2 LED therapy reduced myeloperoxidase (MPO) activity and inflammatory infiltrate in inflamed colon

The LED therapy in healthy tissue did not change the MPO activity. On the other hand, colitis with no treatment caused intense increased MPO activity in relation to control group ($p < 0.05$). The LED therapy and the treatment with Prednisolone had expressive reduction of MPO activity in inflamed colon when compared to those with no treatment (Figure 4A).

Inflamed colon with no treatment showed increased score of inflammatory infiltrate in the mucosa, submucosa and muscle compared to control group (Figure 4B; $p < 0.05$). LED therapy has reduced the inflammatory infiltrate in the three intestinal layers (Figure 4B).

3.3 LED therapy decreased levels of pro-inflammatory cytokines (IL-1 β, TNF-α, IL-6) in inflamed colon

LED therapy did not change any evaluated cytokine level in healthy tissue ($p > 0.05$). Colitis provoked significantly increased in IL-1β, TNF-α and IL-6 levels ($p < 0.05$). LED therapy reduced the level of IL-1β, TNF-α and IL-6 in inflamed colons ($p < 0.05$). Mice treated with Prednisolone had reduction only of IL-1β ($p < 0.05$). LED therapy did not change the level of IL-10. All these results are in Figure 5.

3. DISCUSSION

There are two types of phototreatment: that uses LASER (light amplification by stimulated emission of radiation) and that uses LED (light-emitting diodes). The LASER presents the disadvantage of providing light monochrome only, i.e., there is no possibility to combine different wavelengths. In addition, LASER cannot reach wider areas because this light has punctual indicidence, which also contributes to increase the heat causing tissue injury [32]. The light-emitting diodes (LED) were developed by the National Aeronautics and Space Administration (NASA) [33]. These devices are cheaper, easier to handle, and operate with relatively low electrical currents in comparison to the LASER [33]. In addition, the LED emit light of wavelengths from the red to infrared with conical incidence, allowing action on large

areas. LED can be built in several sizes and without emission of heat, which avoids tissue lesion. The light emitted from LED can penetrate up to 23 cm of tissue [32].

In this study, we demonstrate that LED therapy at 940 nm reduced the inflammatory response triggered by the action of acetic acid on the colon (experimental colitis). Mice submitted to LED therapy produced less pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), had less recruitment of immune cells to the intestinal wall, less edema, less gross lesions and less functional impairment (intestinal transit).

The acetic acid administration via rectum causes disrupture of the intestinal epithelium and, consequently, triggers a cascade of events that generates a local inflammatory process [34]. The colitis is marked by an increase in inflammatory infiltrate, MPO activity, production of pro-inflammatory cytokines and loss of function [23, 34]. These characteristics were observed in mice with colitis with no treatment.

Our results showed evidences that the control of acetic acid-induced inflammation ocorred by decreasing the production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), since the level of the anti-inflammatory cytokines IL-10 remained unchanged by the treatments. We have also verified less activity of MPO, which suggests a decrease in recruitment of neutrophils and lower production of pro-inflammatory cytokines as consequence [23]. These data suggest that mice from the Colitis_LED group had lower recruitment of polymorphonuclear to the inflamed colon. This recruitment is a typical characteristic of colitis [29].

The reduction of the inflammatory infiltrate observed in inflamed colon submettied to LED therapy, as well as the reduction of pro-inflammatory cytokines, also contributed to the normalization of intestinal dimensions, including reduction of edema. Changes in intestinal dimensions are very comom in colitis [35] and it can be related to alterations of the intestinal transit [36], since colitis-induced mice had decrease in intestinal length, increase in intestinal diameter and delayed intestinal transit. This fact can be associated with alterations of the enteric nervous system, and it can be related to impairment of colonic movements [37].

In addition, the molecular changes caused by colitis are also associated with functional loss of bowel motility [36]. The intestinal motility control is done by the autonomic nervous system, especially by its intrinsic component of the gastrointestinal tract: the enteric nervous system [36]. Enteric neurons have receptors for several types of cytokines, including pro-inflammatory cytokines, such

as IL-6R, IL-1R [38-40]. Studies show that these cytokines alter intestinal motility pattern [41, 42].

Finally, the intense reduction of gross lesions observed in mice submitted to LED therapy highlights the therapeutic, anti-inflammatory and protective potential of the treatment with light at 940 nm, as shown in other tissues [18-21].

This is the first study that presents evidence of the anti-inflammatory effect of the light on experimental colitis in mice. It should be noted that, in this study and others in the literature [18-22], light with wavelengths close to the near-infrared spectrum (particularly close to 1000 nm) presents efficient anti-inflammatory effect. Unlike the use of anti-inflammatory drugs, no side effects have been described for the use of the light emitted by LED [43], as also observed in this study.

Translational studies are required to evaluate the therapeutic potential of the light in colitis patients. It is supported by evidences of clinical efficacy of LED therapy showing decrease in mucosite oral pain in patients with bone marrow transplantation [44], prevention of muscle fatigue [45], and also in patients with chronic ulcer [22].

4. CONCLUSION

We conclude that the LED therapy at 940 nm was capable of mitigating the colitis-induced inflammatory process in mice, since we observed partial recovery of the intestinal transit, reduction of edema colonic, normalization of intestinal dimensions, reduction of myeloperoxidase activity and inflammatory infiltrate and reduction of colonic pro-inflammatory cytokines IL-1 β, TNF-α and IL-6.

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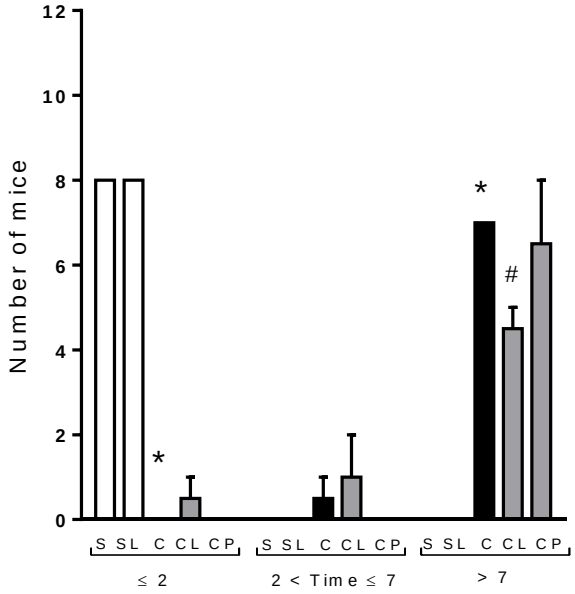


Figure 1. Digestive tract transit time in colitis induced-mice submitted to LED therapy at 940nm or treated with predinisolone. ≤2h normal transit; 2 <tempo≤7h slightly delayed transit and > 7h intensely slowed transit. Groups: Control (S), Control_LED (SL), Colitis (C), Colitis_LED (CL), Colitis_PRED (CP). * p <0.05 compared to Control group. # p <0.05 compared to Colitis group. Two-way ANOVA followed by Newman-Keuls test.

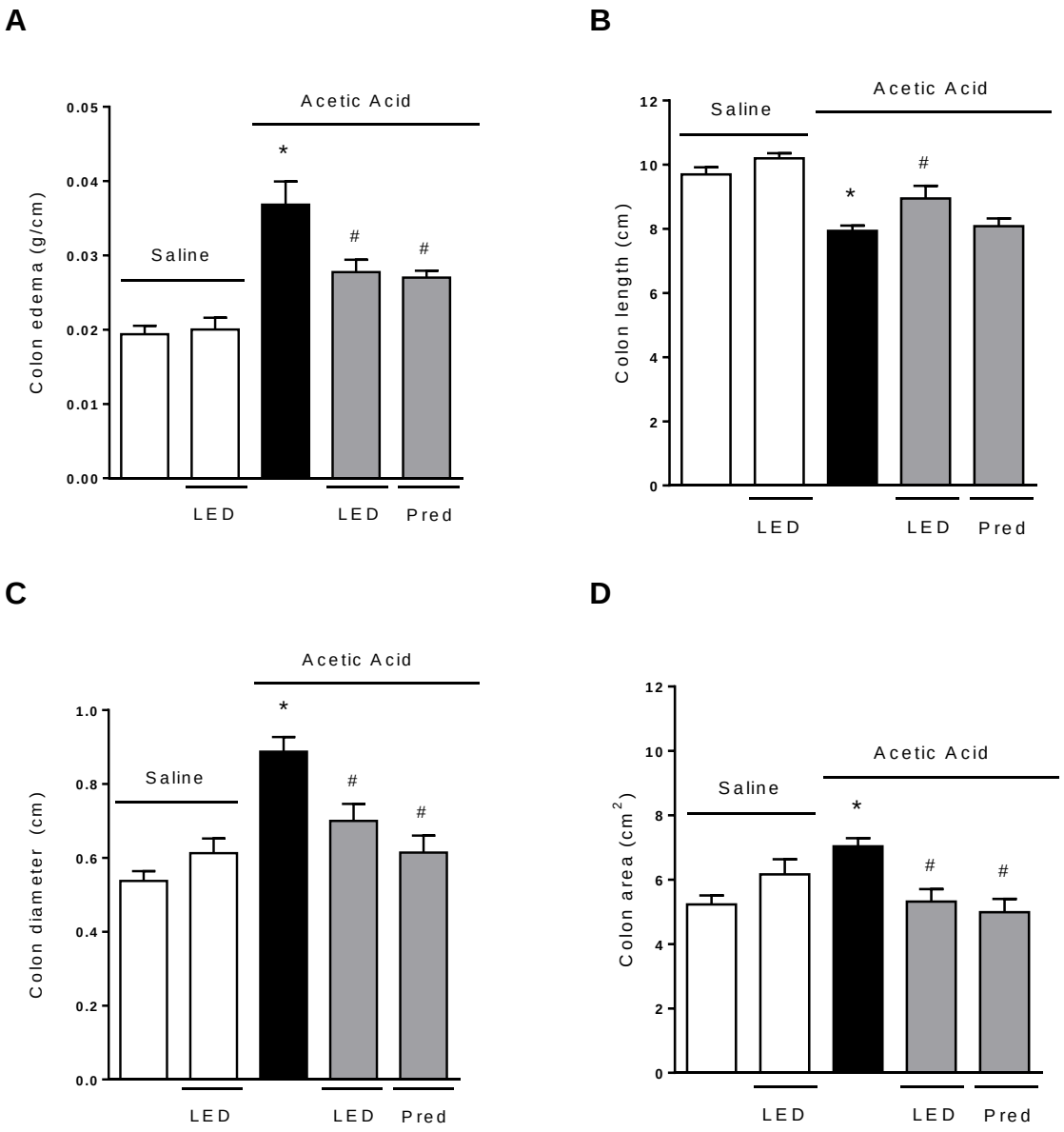
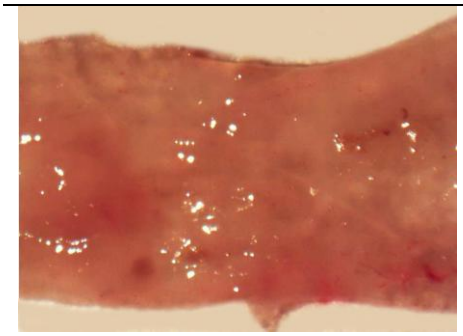


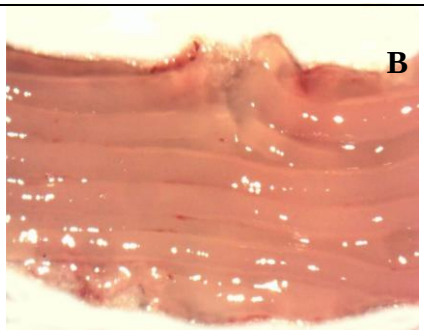
Figure 2. Edema (A), length (B), diameter (C) and area (D) of total colon of colitis induced-mice submitted to LED therapy at 940nm or treated with predinisolone (Pred). * $p < 0.05$ compared to Control group. # $p < 0.05$ compared to Colitis group. One-way ANOVA followed by Newman-Keuls test.

CONTROL



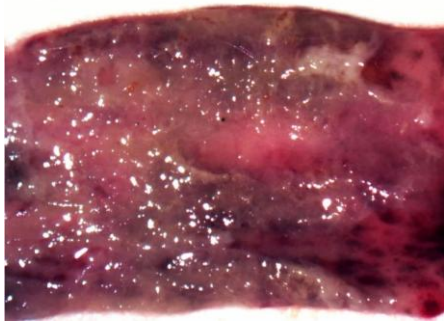
A

CONTROL_LED



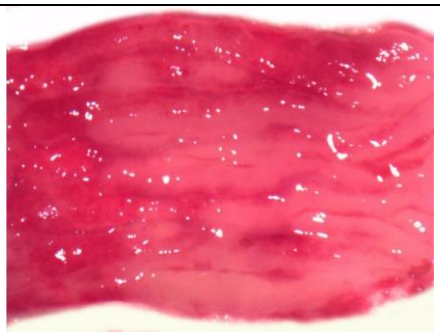
B

COLITIS



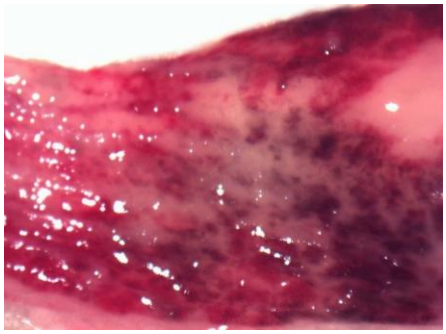
C

COLITIS_ LED



D

COLITIS_PRED



E

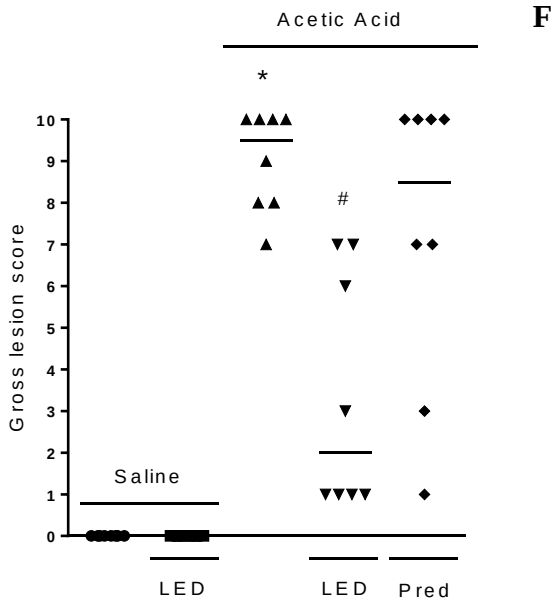


Figure 3. Photomicrography in stereomicroscope of colon of colitis induced-mice submitted to LED therapy at 940nm or treated with Predinisolone (Pred). (A) Control: healthy animals; (B) Control_LED: healthy animals treated with LED; (C) Colitis: colitis induced-mice with no treatment; (D) Colitis_LED: colitis induced-mice treated

with LED; (E) Colitis_PRED: colitis induced-mice treated with predinisolone; (F) gross lesion score (bar indicates median). * $p < 0.05$ compared to Control group. # $p < 0.05$ compared to Colitis group. Kruskal-Wallis test followed by Dunn's test.

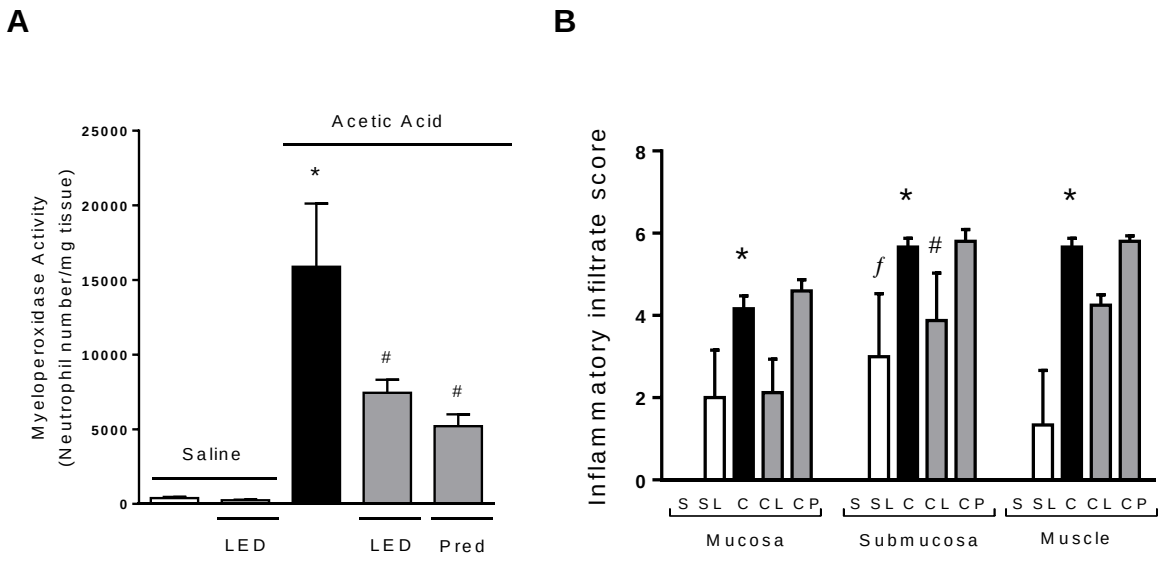


Figure 4. Myeloperoxidase enzyme activity (A) and inflammatory infiltration score (B) in colonic mucosa, submucosa and muscle of colitis induced-mice submitted to LED therapy at 940nm or treated with predinisolone (Pred). S: Control; SL: Control_LED; C: Colitis; CL: Colitis_LED; CP: Colitis_PRED. * $p < 0.05$ compared to Control group. # $p < 0.05$ compared to Colitis group. $^{f}p < 0.05$ compared to Control group. (A) one-way ANOVA followed by Newman-Keuls test. (B) two-way ANOVA followed by Newman-Keuls test.

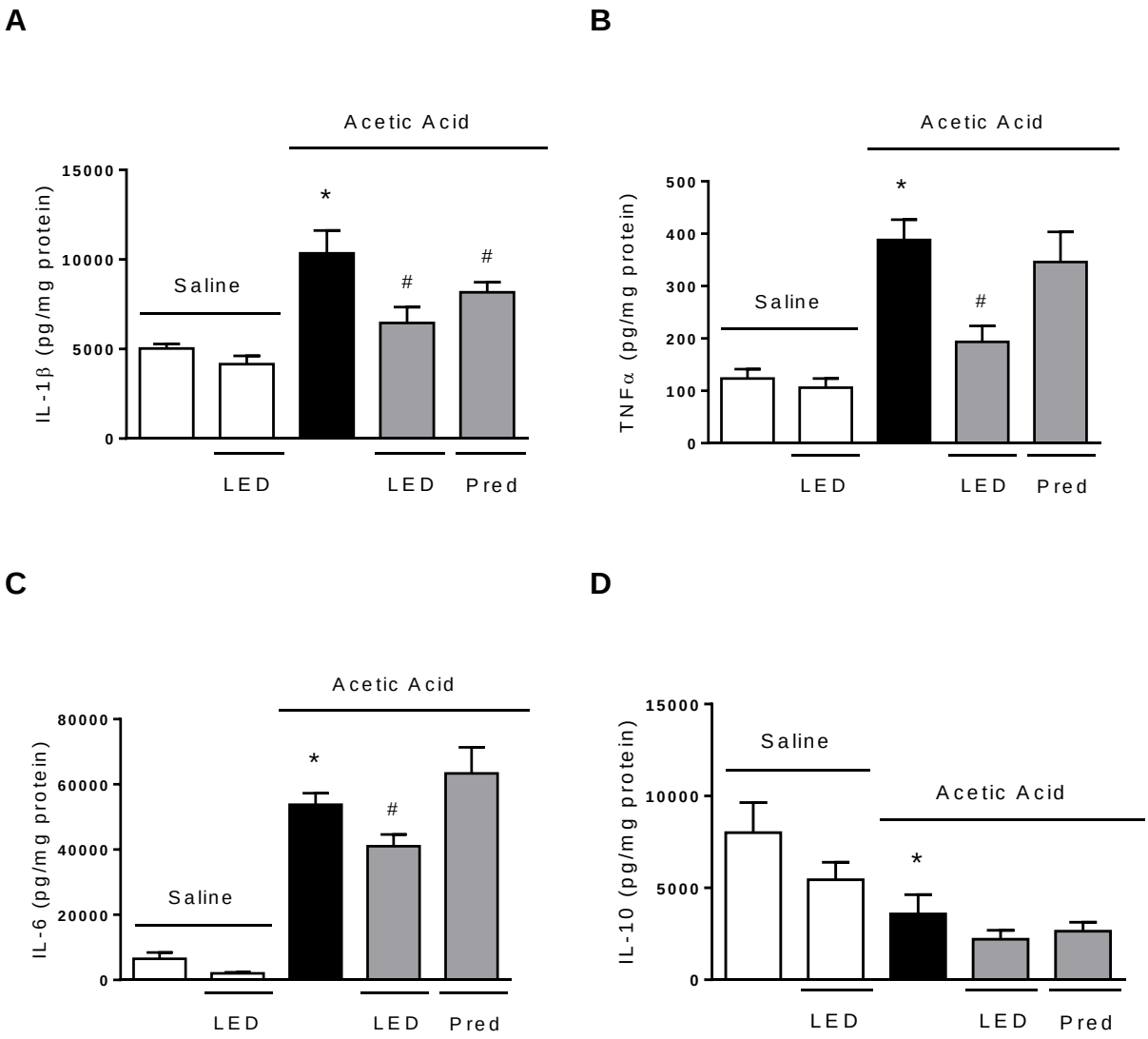


Figure 5. IL-1β (A), TGF-α (B), IL-6 (C) and IL-10 (D) levels in colonic tissue of colitis induced-mice submitted to LED therapy at 940nm or treated with predinisolone (Pred). * p <0.05 compared to Control group. # p <0.05 compared to Colitis group. one-way ANOVA followed by Newman-Keuls test.

ARTIGO CIENTÍFICO 2

DIODOS EMISSORES DE LUZ A 940 nm REDUZEM LESÕES TECIDUAIS PROVOCADAS POR COLITE EXPERIMENTAL EM CAMUNDONGOS

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Resumo

A retocolite ulcerativa induz aumento de infiltrado inflamatório, perda da arquitetura tecidual, depleção de células caliciformes e alteração do sistema nervoso entérico. Na área fotobiomédica utiliza-se diodos emissores de luz (LED) no infravermelho para o controle da inflamação. Objetivou-se avaliar o efeito da LED terapia (940 nm) sobre a morfologia do cólon de camundongos com colite experimental. Utilizou-se camundongos Swiss machos para indução de colite experimental por ácido acético 7,5% (pH 2,5). Aplicou-se LED terapia com comprimento de onda de 940 nm, 45 nm largura de banda, intensidade de 4,05 J/cm^2 e potência total de 270 mW, dose total de 64,8 J por 4 minutos em aplicação única; ou tratamento com Prednisolona (5mg/kg, via oral) durante os cinco dias pré-indução. Avaliou-se lesão microscópica, contagem de células caliciformes e o plexo mientérico. A LED terapia reduziu as lesões microscópicas provocadas pela colite, com manutenção da arquitetura colônica normal, redução do infiltrado celular e manutenção do número de células caliciformes. A colite experimental causou aumento no número e atrofia de neurônios produtores de óxido nítrico, peptídeo vasoativo intestinal e substância P, o que foi atenuado pela fototerapia. A colite provocou hipertrofia do corpo celular da população geral de neurônios mientéricos, o que foi controlado pela fototerapia. Varicosidades VIPérgicas mientéricas estavam menores no cólon com colite e a fototerapia não modificou essa condição. A colite e a fototerapia não causaram alteração no número de células de glia entérica. Conclui-se que a fototerapia a 940 nm apresentou efeito protetor sobre as lesões microscópicas provocadas pela colite, além de atenuar as alterações sofridas por neurônios mientéricos nitrérgicos, VIPérgicos e produtores de substância P desencadeadas pela inflamação.

Palavra-chave: Lesão Microscópica. Fototerapia. Colite Experimental. Infravermelho. Sistema Nervoso Entérico

1. INTRODUÇÃO

As doenças inflamatórias intestinais (DII) são representadas principalmente pela doença de Cronh (DC) e a retocolite ulcerativa (RCU).[1-3] Sem etiologia completamente conhecida, sofrem influência de fatores ambientais, da microbiota e do sistema imune entérico.[2,4,5] Além disso, DIIs parecem estar relacionadas a uma resposta imune inadequada de indivíduos geneticamente susceptíveis.[1]

A RCU é caracterizada por inflamação contínua que se estende do cólon proximal ao reto,[3] com danos das camadas mucosa e submucosa.[2,6] As lesões microscópicas comumente encontradas na colite são caracterizadas pela formação de abscessos nas criptas intestinais e infiltrado de células inflamatórias na lâmina própria.[7,8]

Neutrófilos e macrófagos são considerados as células responsáveis pela destruição da integridade epitelial colônica e a lesão tecidual.[910] É ainda comum observar distorção das glândulas mucosas e a depleção de células caliciformes.[11] Esses fatores contribuem para uma maior suscetibilidade à entrada de patógenos da microbiota, em função do rompimento da barreira epitelial colônica.[12]

A colite causa também alterações no sistema nervoso entérico (SNE).[13] O SNE é uma subdivisão do sistema nervoso autônomo que funciona de forma quase independente, dividido em dois plexos ganglionados interconectados. O plexo submucoso é responsável pela estimulação de secreção e controle vascular, e o plexo mientérico é responsável pelo controle da contração muscular esofáfica e gastrointestinal.[14,15]

A resposta entérica é dependente do modelo de indução experimental de colite, da espécie em estudo, região intestinal e tempo pós indução.[16-18] Existe relato de perda neuronal, aumento de densidade axonal, alteração do conteúdo neuroquímicos de neuritos, redução de VIPérgicos e aumento de neuritos positivos para substância P, além de alterações morfométricas no corpo celular dos neurônios.[19] Não se sabe, com certeza, se a possível perda neuronal é generalizada ou se afeta subpopulações neuronais específicas.[20] Essa alteração neuronal entérica pode culminar com perda de função.[18]

Por não apresentar etiologia completamente conhecida, a RCU também não apresenta tratamento completamente eficaz ou cura.[21] As terapias medicamentosas incluem os aminosalicilatos, os corticosteróides, os imunomoduladores[5,22] e terapias biológicas com anticorpos.[23-25] Porém todos esses tratamentos apresentam muitos efeitos colaterais,[26] o que reduz ainda mais a qualidade de vida desses pacientes.[27]

Diante desse cenário, a busca por novas terapias com reduzidos efeitos colaterais se faz necessária. Nesse sentido, a utilização de luz é considerada uma possibilidade de intervenção em sistemas biológicos com bons efeitos dentro da área médica.[28-31]

O potencial terapêutico da luz é dependente do comprimento de onda, intensidade e condições do tecido lesado, bem como o sistema no qual será utilizado.[32-35] O comprimento de onda na faixa do vermelho e infravermelho próximo (600 – 1000 nm) têm demonstrado efeitos anti-inflamatórios, reduzindo lesões teciduais, de remodelamento do tecido lesado e regenerativos.[36-40]

Sendo assim, esse estudo teve por objetivo avaliar o efeito da LED terapia (940 nm) sobre a morfologia do cólon de camundongos com colite experimental induzida por ácido acético.

2. MATERIAIS E MÉTODOS

Todos os procedimentos foram aprovados pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) protocolo 113/2014, processo n°14276.2014.60.

2.1. Grupos Experimentais

Foram utilizados camundongos Swiss (*Mus muscullus)* machos de 60 dias de idade e peso médio de 25 g, mantidos em caixas de polipropileno com quatro animais/caixa, sob ciclo claro-escuro de 12 horas e temperatura de 23°C com água e ração comercial para roedores (Nuvilab CR1, Nuvitl Nutrientes Ltda.®, Curitiba,

Brasil) fornecidos *ad libitum*. Os animais foram distribuídos aleatoriamente nos grupos (n=8/grupo): Controle, Controle_LED, Colite_LED, Colite, Colite_PRED.

2.2. Modelo de Indução de Colite e Tratamentos

Para indução da colite seguiu-se protocolo de estudo prévio.[41] Após 12 horas de jejum sólido, os animais foram anestesiados cloridrato de ketamina (20 mg/kg, Dopalen® 11,6%, Sespo Indústria e Comercio Ltda, Paulínia, Brasil, IM) e cloridrato de xilasina (2,5 mg/kg, Anasedan® 2,3%, Sespo Indústria e Comercio Ltda, Paulínia, Brasil, IM). Foi utilizada uma cânula de polietileno de 3 cm de comprimento para administração das soluções via retal. Previamente, todos os animais receberam 100 µL de salina via retal para limpeza do cólon. Após uma hora, os camundongos dos grupos Controle e Controle_LED receberam 200 µL de salina via retal. Os dos grupos Colite_LED, Colite e Colite_PRED receberam 200 µL de ácido acético 7,5% pH 2,5, via retal, para indução da colite. Os grupos Controle_LED e Coilte_LED foram mantidos em decúbito ventral. O equipamento de 16 cm² e com 36 lâmpadas de LED, com potência unitária de 7,5 mW foi posicionado a 1 cm acima da pele do dorso dos camundongos na região abdominal, para tratamento com luz de LED com comprimento de onda de 940 nm, 45 nm largura de banda, intensidade de 4,05 J/cm^2 e potência total de 270 mW, dose total de 64,8 J por 4 minutos em aplicação única. O tratamento foi realizado sempre pelo mesmo operador. Os animais do grupo Colite_PRED receberam tratamento com Prednisolona (5 mg/kg de peso de animal, via oral, Prednisolone, Pharma Nostra, Campinas, Brazil), droga controle do modelo experimental, durante os cinco dias prévios à indução da colite.

2.3. Eutanásia e coleta de amostras

Após 18 horas da indução da colite os camundongos foram novamente anestesiados com cloridrato de ketamina (80 mg/kg, Dopalen® 11,6%, Sespo Indústria e Comercio Ltda, Paulínia, Brasil, IM) e cloridrato de xilasina (10 mg/kg, Anasedan® 2,3%, Sespo Indústria e Comercio Ltda, Paulínia, Brasil, IM) para serem

submetidos à eutanásia por decaptação. Por laparotomia ventral, o intestino grosso, nos limites do ceco e reto, de todos os camundongos foi removido.

2.4. Avaliação de Lesões Microscópicas e Histologia da Parede Colônica

2.4.1. Processamento

Os segmentos intestinais foram cuidadosamente lavados com solução de NaCl 0,9% e imersos em solução de formalina tamponada a 4% em tampão fosfato de sódio 0,1M pH 7,4, durante 24 horas. Na sequência, foram submetidos à rotina de processamento histológica para confecções lâminas contendo cinco cortes histológicos transversais, na região mediana do seguimento colônico, com 5 µm de espessuras e corados com: Hematoxilina e Eosina (HE), Ácido Periódico Reativo de Schiff (PAS), Alcian Blue pH 2,5 (AB pH 2,5) e Alcian Blue pH 0,5 (AB pH 0,5).

2.4.2. Obtenção de imagens

Oito imagens do melhor corte foram capturadas por câmera digital de alta resolução (Moticam 2500, 5.0 M pixel USB 2.0) acoplada ao microscópio de luz (Axiophot Zeiss Axiophot). De cada técnica foram capturadas 8 imagens/animal/grupo, conferindo uma volta completa na circunferência colônica. Para cortes corados em HE foi utilizada a objetiva de 20x. E para imagens coradas em PAS, AB pH 2,5 e AB pH 0,5 foi utilizada a objetiva de 10x.

2.4.3. Análise

- Avaliação das lesões microscópicas

As imagens dos cortes corados com HE foram avaliadas de lesão histopatológicas, de acordo com os critérios descritos por Appleyard e Wallace (1995).[42] Para essa avaliação considerou-se alterações da estrutura histológica da parede colônica, infiltrado celular, espessamento das camadas submucosa e muscular, formação de abcesso nas criptas e depleção das células caliciformes. O escore final de lesão microscópica de cada amostra foi obtido pela soma dos escores individuais determinados para cada um dos achados. Para cada parâmetro avaliado os escores variaram entre 0-3, sendo considerado 0, tecido normal, 1, alterações leves, 2, alterações severas, e 3 completamente alterado.

- *Análise quantitativa de células caliciformes*

As células caliciformes foram quantificadas utilizando-se imagens de mucosa dos cortes corados com PAS, AB pH 2,5 e AB pH 0,5. Dessas imagens foi determina a área de mucosa colônica a partir do software Image-Pro Plus 4.5. Dentro dessa área determinada foi contado o número total de células caliciformes. Foi realizada projeção para 1cm² quadrado de mucosa.

2.5. Avaliação do sistema nervoso entérico

2.5.1. Imunofluorescência

Após retirada do cólon nos limites do ceco e reto, o mesmo teve suas dimensões medidas para determinação da área total do órgão. O cólon foi lavado cuidadosamente com tampão fosfato salino (PBS) 0,1M pH 7,4 com auxílio de uma seringa. Amarrou-se uma das extremidades do segmento, o qual foi preenchido com solução fixadora de Zamboni, e amarrou-se a outra extremidade formando uma bexiga completamente preenchida de fixador. O segmento foi armazenado na solução fixadora por 18 horas a 4°C. Em seguida, a bexiga foi desfeita e o segmento foi aberto na borda mesocólica para ser desidratado com álcool etílico em concentrações crescentes, diafanizado com xilol e reidratado com álcool etílico em concentrações decrescentes. Os segmentos foram armazenados em solução de PBS 0,1M pH 7,4 + azida sódica 0,08% à 4ºC. Anéis de 1 cm de espessura foram microdissecados com auxílio de um estereomicroscópico para obtenção de preparados totais do cólon contendo apenas o estrato muscular longitudinal e plexo mioentérico. Os preparados totais foram lavados em solução de PBS 0,1M pH 7,4 com Triton a 0,5%, incubados em solução de bloqueio antigênico (2% albumina soro bovina - BSA, 10% soro de burro e PBS 0,1M pH 7,4 com Triton a 0,5%) e, em seguida, foram incubados em solução contendo os anticorpos de interesse (Tabela 1) por 48h. Foram novamente lavados em solução de PBS 0,1M pH 7,4 com Triton a 0,5%, e incubados em solução contendo anticorpo secundário associado a fluorocromo (Tabela 1) por 2 horas. Por fim, os preparados totais foram lavados em solução de PBS 0,1M pH 7,4 e montados com meio de montagem anti-fade. As lâminas prontas foram armazenadas a 4°C protegidas da luz até o momento da captura das imagens.

2.5.2. Aquisição de imagens

Para essa análise foram utilizadas imagens capturadas por câmera digital de alta resolução (AxioCam, Carl Zeiss, Jena, Germany) acoplada ao microscópio de fluorescência (axioscop Plus light microscope, Carl Zeiss, Jena, Germany) usando o software AxioVisionRel 4.1. Para análise da imunomarcação de corpo celular (β-tubulina III, nNOS, VIP e Substância P) foram capturadas 20 imagens/animal/grupo, utilizando objetiva de 10x. Para imunomrcação de varicosidades VIPérgicas foram capturadas 15 imagens/animal/grupo, utilizando objetiva de 40x. Para imunomarcação de células da glia entérica (CGE) S100 foram capturadas 30 imagens/animal/grupo, utilizando objetiva de 20x.

2.5.3. Análise quantitativa

Neste estudo contou-se o número total de corpos celulares de neurônios mientéricos imunomarcados para β-tubulina III, nNOS, VIP e Substância P (SP). Em seguida, foi realizada projeção do número de neurônios/cm² e para a área total do cólon. Por fim, contou-se o número total de células da glia entérica imunomarcados para S100. Foi realizada projeção do número de neurônios/cm² e pela área total do cólon. Para todas as análises foi utilizado o software Image-Pro Plus 4.5.

2.5.4. Análise morfométrica

Neste estudo, também, mediu-se a área (µm²) de 200 do corpo celular de neurônios mientéricos imunomarcados para β-tubulina III, nNOS, VIP e Substância P (SP) de cada animal. Foi mensurada também a área de 500 varicosidades mientéricas VIPérgicas de cada animal/grupo.

3. Análise estatística

Os dados foram expressos como média ± erro padrão da média. Os dados foram analisados quanto ao tipo de distribuição pelo teste de normalidade de Shapiro-Wilk. As diferenças estatisticamente significativas entre os grupos foram identificadas através do teste ANOVA one-way seguida do pós-teste de Newman-

Keuls. Para a análise de dados não paramétricos foi utilizado o teste de Kruskall-Wallis seguido pelo teste de Dunn. Essas análises foram realizadas usando-se o software GraphPad Prism6 (GraphPad Software Inc., San Diego, EUA). As diferenças foram consideradas estatisticamente significativas para valores correspondentes a $p < 0,05$.

4. RESULTADOS

4.1. LED terapia reduziu escore de lesão microscópica e atenuou depleção de células caliciformes no cólon de camundongo com colite experimental

A LED terapia no comprimento de onda de 940 nm mostrou-se eficaz para atenuar as lesões microscópicas provocadas pela colite experimental. Observou-se que o escore médio foi reduzido de 16 ± 0,8 nos camundongos com colite sem nenhum tipo de tratamento (Figura 1 C e E) para 10 ± 1,7 nos tratados com LED ($p < 0,05$, Figura 1 D e F) e para 12 ± 0,5 nos tratados com Prednisolona ($p > 0,05$, Figura 1 E e F). A LED terapia em tecido sadio não causou alteração significativa (Figura 1A-C).

De uma forma geral, observou-se que a colite provocou depleção de células caliciformes, independentemente da natureza química do muco produzido por essas células. O tratamento com LED a 940nm promoveu a proteção completa de células caliciformes produtoras de sialomucinas (AB^+ pH 2,5, Figura 2B) e sulfomucinas (AB^+ pH 0,5, Figura 2C) ($p < 0,05$); e proteção parcial de células caliciformes produtoras de mucinas neutras (PAS^+, Figura 2A). A LED terapia não provou nenhuma alteração em células caliciformes de tecido sadio (Figura 2).

4.2. LED terapia a 940 nm controlou o desvio fenotípico de neurônios mientéricos nitrérgicos, VIPérgicos e produtores de substância P do cólon de camundongos com colite experimental

A colite experimental no modelo utilizado neste estudo não alterou o número de neurônios mientéricos gerais (beta-tubulina III positivos) (Figura 3), porém causou aumento no número de neurônios produtores de óxido nítrico (Figuras 4), peptídeo vasoativo intestinal (Figuras 5) e substância P (Figuras 6), o que foi atenuado pela fototerapia. Considerando que a área colônica estava aumenta pela colite sem nenhum tipo de tratamento (Controle: 5,23 ± 0,27 cm^2; Colite: 7,03 ± 0,25 cm^2; $p < 0,05$), observou-se que o modelo de colite experimental induzida por ácido acético provoca um desvio fenotípico das subpopulações dos neurônios mientéricos colônicos, uma vez que se observou uma maior quantidade de neurônios em cólons com ampliação de área. Esse efeito foi atenuado pela fototerapia. Vale destacar que a LED terapia a 940 nm foi eficaz para conter o aumento de área colônica induzido pela colite (Colite: 7,03 ± 0,25 cm^2; Colite_LED: 5,32 ± 0,38 cm^2; $p < 0,05$) e o desvio fenotípico de neurônios mientéricos nitrérgicos, VIPérgicos e produtores de SP.

Na população geral de neurônios mientéricos observou-se que a colite provocou hipertrofia do corpo celular, o que foi controlado pela fototerapia (Figura 3F). Já nas subpopulações avaliadas, a colite provocou atrofia do corpo celular (Figuras 4-6F). A fototerapia também controlou essa atrofia, exceto para os neurônios produtores de subtância P. Varicosidades VIPérgicas mientéricas estavam reduzidas nos camundongos com colite e a fototerapia não modificou essa condição (Figura 7). A colite e a fototerapia não causaram alteração no número de células de glia entérica (S100 positivas) (Figura 8).

Vale ainda destacar que a LED terapia provocou em tecidos sadios uma redução no número e hipertrofia de neurônios VIPérgicos (Figura 4F), além de atrofia no corpo celular de neurônios nitrérgicos e de produtores de substância P (Figura 5-6F).

5. DISCUSSÃO

Neste estudo, demonstramos que o tratamento com luz de comprimento de onda na faixa do infravermelho próximo (940 nm) reduziu as lesões teciduais desencadeadas pela ação do ácido acético sobre o epitélio colônico, uma vez que, camundongos com colite submetidos à LED terapia tiveram menor escore de lesão

microscópica, menor depleção de células caliciformes intestinais e melhor preservação da morfologia do plexo mientérico.

A administração de ácido acético via retal provoca ruptura do epitélio de revestimento do cólon e, consequentemente, desencadeia uma cascata de eventos que gera um processo inflamatório local.[18] A colite é caracterizada por lesão da camada mucosa e submucosa que se estende do cólon proximal ao reto.[3] Essa lesão se deve ao aumento de infiltrado inflamatório[7] devido a maior produção de citocinas pró-inflamatórias,[41] desencadeando alterações microscópicas,[10] incluindo alterações do sistema nervoso entérico[13] que podem levar à perda de função.[18] A maior parte dessas características foram observadas neste estudo em camundongos com colite sem nenhum tratamento.

A redução do escore de lesões microscópicas em animais com colite submetidos à LED terapia evidencia o efeito protetor que esse tratamento pode promover no tecido colônico lesionado pela administração de ácido acético. Os nossos resultados corroboram com o efeito positivo da LED terapia encontrado também em estudos que avaliaram outros tipos de tecidos em processo inflamatório desencadeados por diferentes formas de lesão e também com diferentes protocolos de avaliação.[36-39]

Sabe-se que o muco produzido pelas células caliciformes protege o epitélio intestinal contra a invasão de microorganismos, os quais ficam aderidos à superfície de carboidratos que recobre o epitélio.[43] A intensa depleção de células caliciformes ocorrida nos animais com colite sem tratamento aumenta a suscetibilidade da barreira epitelial intestinal à microbiota. Essa vulnerabilidade do epitélio pode resultar em aumento da expressão de proteínas que regulam a permeabilidade paracelular visando aumentar a efetividade da barreria epitelial.[44] A LED terapia se mostrou eficaz em proteger o tecido colônico contra essa depleção de células caliciformes, o que deve ter contribuído para uma menor invasão da microbiota. Assim sugerimos, que houve menos disruptura do epitélio intestinal e, portanto, menor invasão de microrganismos. Esse achado pode estar relacionado ao menor infiltrado inflamatório e preservação da arquitetura tecidual observados neste estudo no cólon de camundongos com colite submetidos à LED terapia.

Alterações no SNE decorrentes das DIIs são heterogêneas, com mudanças estruturais em neurônios e glias.[13,49] Em humanos, a colite pode levar a hipertrofia do corpo celular e hiperplasia de neurônios entéricos.[49] Além disso, relata-se dano axonal e do corpo celular de neurônios, além de hiperplasia de glias entéricas e ganglioneurite.[49] Na colite experimental, os resultados disponíveis na literatura são bastante variados. Observa-se que o comportamento neuronal entérico na colite experimental se difere de acordo com o modelo de indução adotado, podendo ocorrer redução ou aumento de neurônios de uma maneira geral, bem como de subpopulações. Além disso, os resultados variam de acordo com a espécie animal estudada, da região intestinal avaliada e do tempo pós indução considerado.[16-18]

Estudos demonstram que processos inflamatórios induzem neurônios entéricos a alterar seu fenótipo estrutural, funcional ou químico no intuito de manter a homeostasia das funções intestinais.[45] O aumento de citocinas inflamatórias está diretamente relacionado com a alteração da expressão de VIP e substância P.[16,48,49] Dentre os mediadores inflamatórios que influenciam o funcionamento de neurônios entéricos, destaca-se a citocina pró-inflamatória IL-1β.[51] Esta citocina está aumentada em quadros de colite experimental.[41] As alterações neuronais entéricas decorrentes do quadro de colite culminam em alterações funcionais como falhas no controle neuronal da secreção epitelial, alteração na transmissão sináptica, variabilidade na expressão de fatores derivados de neurônios e glias, como NOS, VIP e S100, alteração contrátil do músculo liso, alteração das células secretoras da mucosa, células endócrinas, inflamatórias e imunes, bem como na microvasculatura da mucosa.[49,52] Portanto, o processo inflamatório observado neste estudo no cólon de camundongos é provavelmente o principal fator responsável pelas alterações que foram observadas em neurônios do plexo mientérico.

Nós observamos que a colite experimental não provocou perda de neurônios mientéricos. Embora os nossos resultados mostrem que a administração do ácido acético tenha provocado um intenso processo inflamatório na parede do cólon, sugere-se que o tempo pós-indução adotado neste estudo (18 horas) não foi suficiente para provocar perda de neurônios e glias. Por outro lado, observou-se aumento no número e atrofia no corpo celular de neurônios produtores de NO, VIP e SP. A atrofia neuronal observada nos animais com colite sem tratamento pode indicar que o maquinário de metabolismo celular dessas células estava reduzido ou

talvez menos ativo. Isso pode ser entendido como um mecanismo de compensação em função do aumento no número de neurônios nitrérgicos, VIPérgicos e produtores de SP. Além disso, observou-se que a colite provocou atrofia de variosidades contendo VIP. Varicosidades menores podem representar uma menor produção do neurotransmissor ou uma produção intensa com liberação rápida.[46,47] Sugerimos que no modelo de colite avaliado neste estudo tenha havido maior liberação de VIP, pois o VIP é um neurotransmissor com efeito anti-inflamatório.[13] Além disso, nossos dados mostraram aumento do número de neurônios produtores de VIP, o que pode ser entendido como uma necessidade de maior produção e secreção de VIP.

A LED terapia contribuiu para que o corpo celular de neurônios produtores de NO, VIP e SP se mantivesse dentro de seu tamanho natural. Além disso, o tratamento foi eficaz para conter o desvio fenotípico das subpopulações neuronais VIPérgicas e produtoras de SP observado em camundongos com colite sem tratamento. Nós hipotetizamos que esse efeito positivo da LED terapia sobre os neurnônios mientéricos avaliados neste estudo seja uma consequência do controle do processo inflamatório neste modelo de indução de colite experimental. Esses restulados indicam que a LED terapia deve ter contribuído para a manutenção do funcionamento desses neurônios e, portanto, do controle nervoso das atividades do cólon desses animais. Neurônios nitrérgicos e VIPérgicos são funcionalmente do tipo motores inibitórios para a musculatura lisa. Portanto, esses neurotransmissores influenciam fortemente o padrão de movimentos colônicos. Além disso, regulam o movimento do fluido luminal pela barreira epitelial, alteram o fluxo sanguíneo e interagem com os sistemas imune e endócrino.[14,15] Já os neurônios produtores de substância P são células excitatórias que ativam outros neurônios e enzimas sinterizadoras de acetilcolina, levando a um aumento da contração muscular.[14] Neurônios produtores de substância P são conhecidos ainda pelo seu papel pró-inflamatório e neurônios VIPérgicos como anti-inflamatórios,[13] sendo as duas subpopulações mais afetadas na colite.[48] Na colite experimental, sabe-se que aumento no número de neurônios produtores de substância P é maior em áreas inflamadas do que em áreas não inflamadas, já os VIPérgicos mostram-se aumentados em igual proporção em áreas inflamadas ou não.[48] Pacientes com colite apresentam neurônios mientéricos produtores de substância P em maior proporção, em comparação com as outras subpopulações neuronais entéricas.[49] Neurônios

produtores de substância P são caracterizados marcadores da patologia de colite.[48,49]

Tanto a colite como a LED terapia não alteraram a densidade populacional de CGE. Essas células envolvem os corpos celulares e axônios de neurônios e vasos sanguíneos, e tem prolongamentos que podem chegar até a mucosa. Fazem a regulação da homeostasia e resposta inflamatória, sendo o tipo celular mais abundando no SNE.[54] Funcionalmente fazem o suporte mecânico dos neurônios com liberação de fatores responsáveis pelo desenvolvimento, sobrevivência e diferenciação neuronal.[15,54] Regulam a função da barreira epitelial como permeabilidade,[55] lançamento de s-nitroglutationa (GSNO) e regulam a expressão de zonulina 1 e ocludina.[49] São ativadas por estímulos inflamatórios com a apresentação de antígenos e promoção do lançamento de citocinas pró-inflamatórias, já que podem produzir IL-1β, IL-6 e TNF-α[45] e expressam seus receptores,[49] também expressa MHC de classe II, participando ativamente da resposta imune.[45,49]

6. CONCLUSÃO

Conclui-se que a LED terapia a 940 nm apresentou efeito anti-inflamatório para a colite experimental induzida por ácido acético em camundongos, preservando a morfologia do cólon desses animais. Houve proteção da arquitetura intestinal, incluindo redução da depleção de células caliciformes. Além disso, o tramento com luz preservou o plexo mientérico evitando o desvio fenotípico e atrofia do corpo celular de neurônios nitrérgicos, VIPérgicos e produtores de substância P. Células da glia entérica não sofreram alteração pela colite e pela LED terapia.

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Tabela 1. Anticorpos utilizados para imunofluorescência. nNOS (óxido nítrico sintetase neuronal); VIP (peptídeo vasoativo dilatador); SP (substância P), β-tubulina III; S100; Alexa Fluor 568 e FITC IgY.

Antígeno tecidual	Espécie produtora	Código	Diluição	Fabricante
nNOS	Coelho	SC8309	1:1000	Santa Cruz
VIP	Coelho	AB8556	1:200	ABCam
SP	Coelho	AB1566	1:1000	Millipore
β-tubulina III	Ave	TUJ	1:750	AvesLab
S100	Coelho	S2644	1:500	Sigma
Alexa Fluor 568	Burro	A100042	1:500	Invitogen
FITC IgY	Cabra	A16055	1:500	Novex

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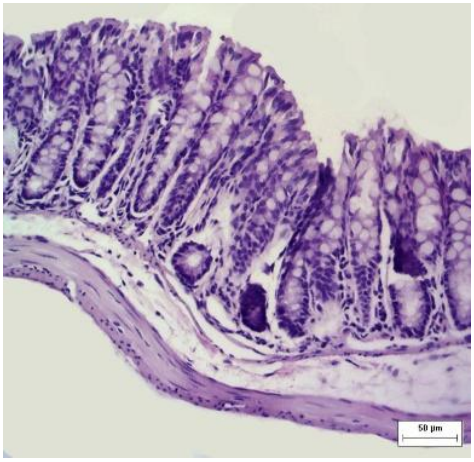

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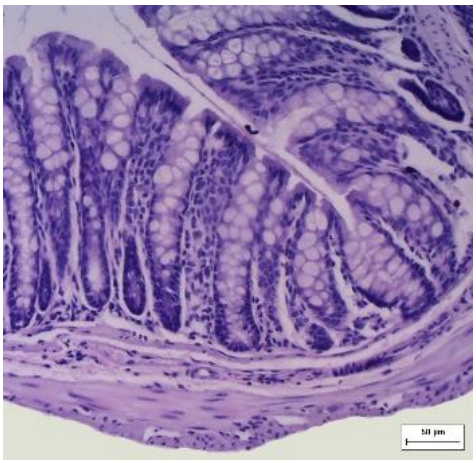

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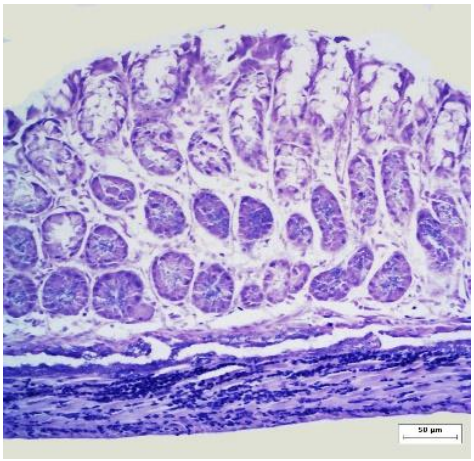

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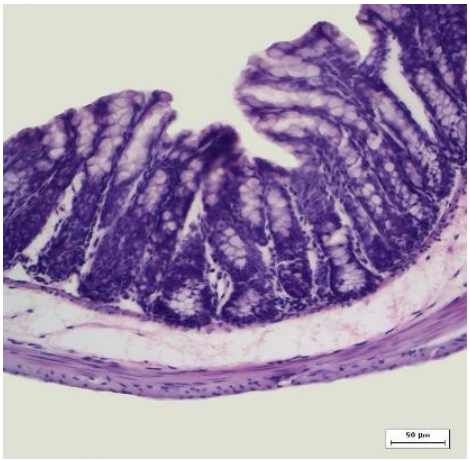

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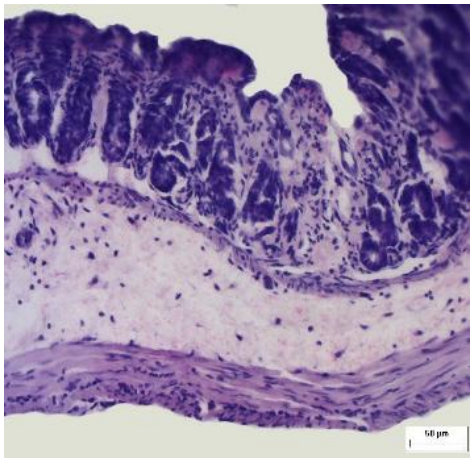

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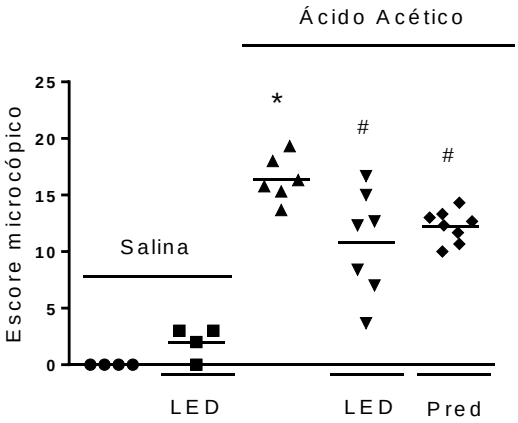
Ácido Acético
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LED
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Figura 1. Análise de lesão microscópica do tecido colônico de camundongos com colite experimental induzida por ácido acético tratados com luz de comprimento de onda de 940nm. Fotomicrografia colônica de camundongos: (A) Controle; (B) Controle_LED: saudáveis tratados com LED terapia; (C) Colite: indução por ácido acético 7,5% (v/v), pH2,5 sem tratamento; (D) Colite_LED: colite induzida por ácido acético 7,5% (v/v), pH2,5 tratados com LED terapia; (E) Colite_PRED: colite induzida por ácido acético 7,5% (v/v), pH2,5 tratados com Prednisolona (5mg/kg/5dias, v.o.); (F) Escore de lesão microscópica por grupo experimental apresentando o escore individual de cada animal. *$p < 0,05$ em relação ao grupo Controle. $^{\#}p < 0,05$ em relação ao grupo Colite. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

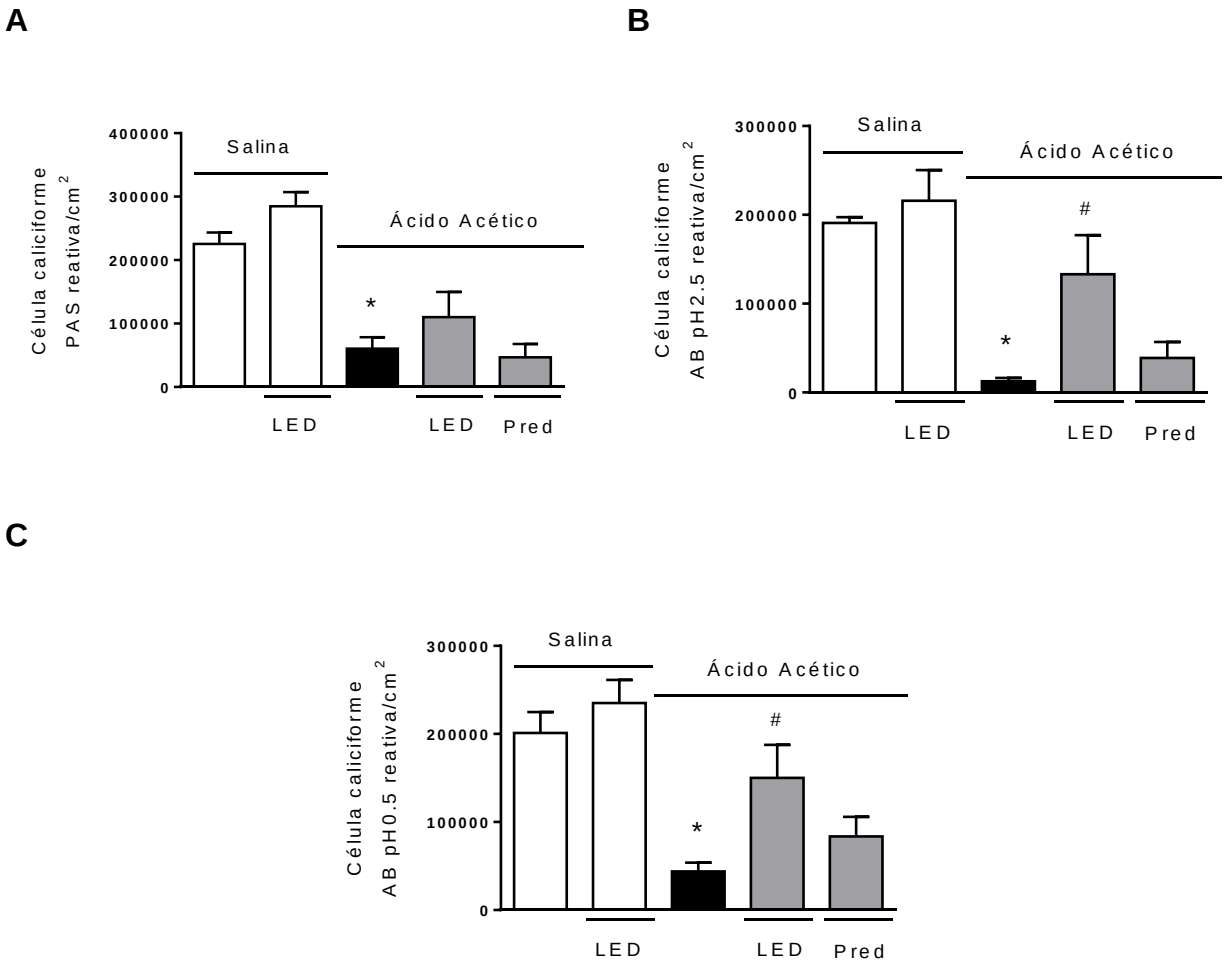

Figura 2. Contagem de células caliciformes colônicas a partir de histoquímica por ácido periódico de Schiff (A); Acian Blue pH 2,5 (B); e Acian Blue pH 0,5 (C) do tecido colônico de camundongos com colite experimental induzida por ácido acético tratados com luz de comprimento de onda de 940nm. *$p < 0,05$ em relação ao grupo Controle. $^{\#}p < 0,05$ em relação ao grupo Colite. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

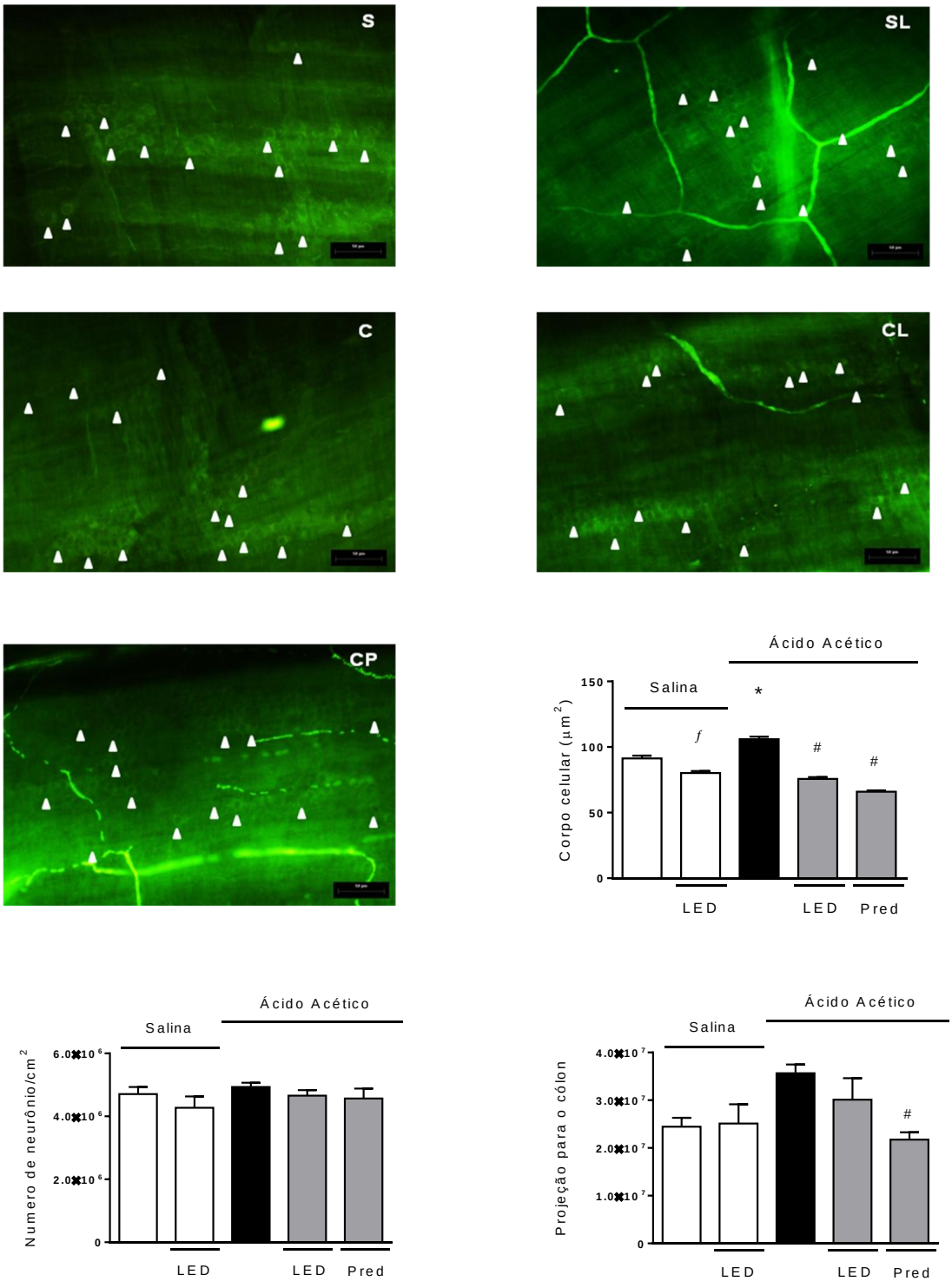

Figura 3. Fotomicrografia de imunofluorescência mostrando população mientérica geral de neurônios (beta tubulna III) nos grupos experimentais (ponta da seta). Barra 50 µm. Controle (S); Controle_LED (SL); Colite (C); Colite_LED (CL); Colite _PRED (CP). Avaliação morfométrica e quantitativa do plexo mioentérico de camundongos

com colite experimental submetidos à LED terapia ou tratados com predinisolona. *p < 0,05 em relação ao grupo Controle. $^{\#}$p < 0,05 em relação ao grupo Colite. ^{f}p < 0,05 em relação ao grupo Colite_LED. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

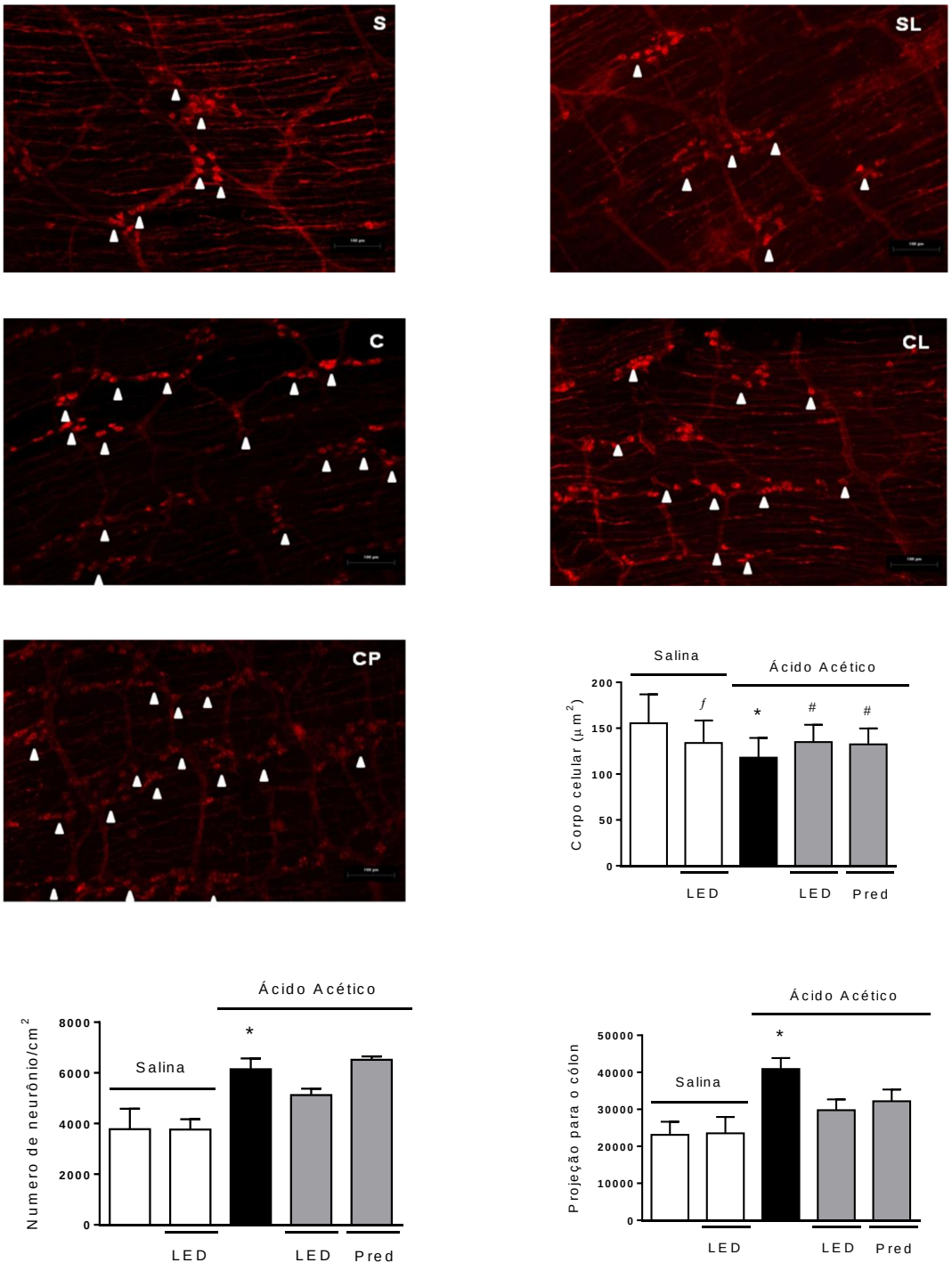

Figura 4. Fotomicrografia de imunofluorescência mostrando população mientérica nitrérgica de neurônios (neurônio motor inibitório) nos grupos experimentais (ponta da seta). Barra 100 µm. Controle (S); Controle_LED (SL); Colite (C); Colite_LED (CL); Colite _PRED (CP). Avaliação morfométrica e quantitativa do plexo mioentérico de camundongos com colite experimental submetidos à LED terapia ou tratados com

predinisolona. *p < 0,05 em relação ao grupo Controle. #p < 0,05 em relação ao grupo Colite. ƒp < 0,05 em relação ao grupo Colite_LED. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

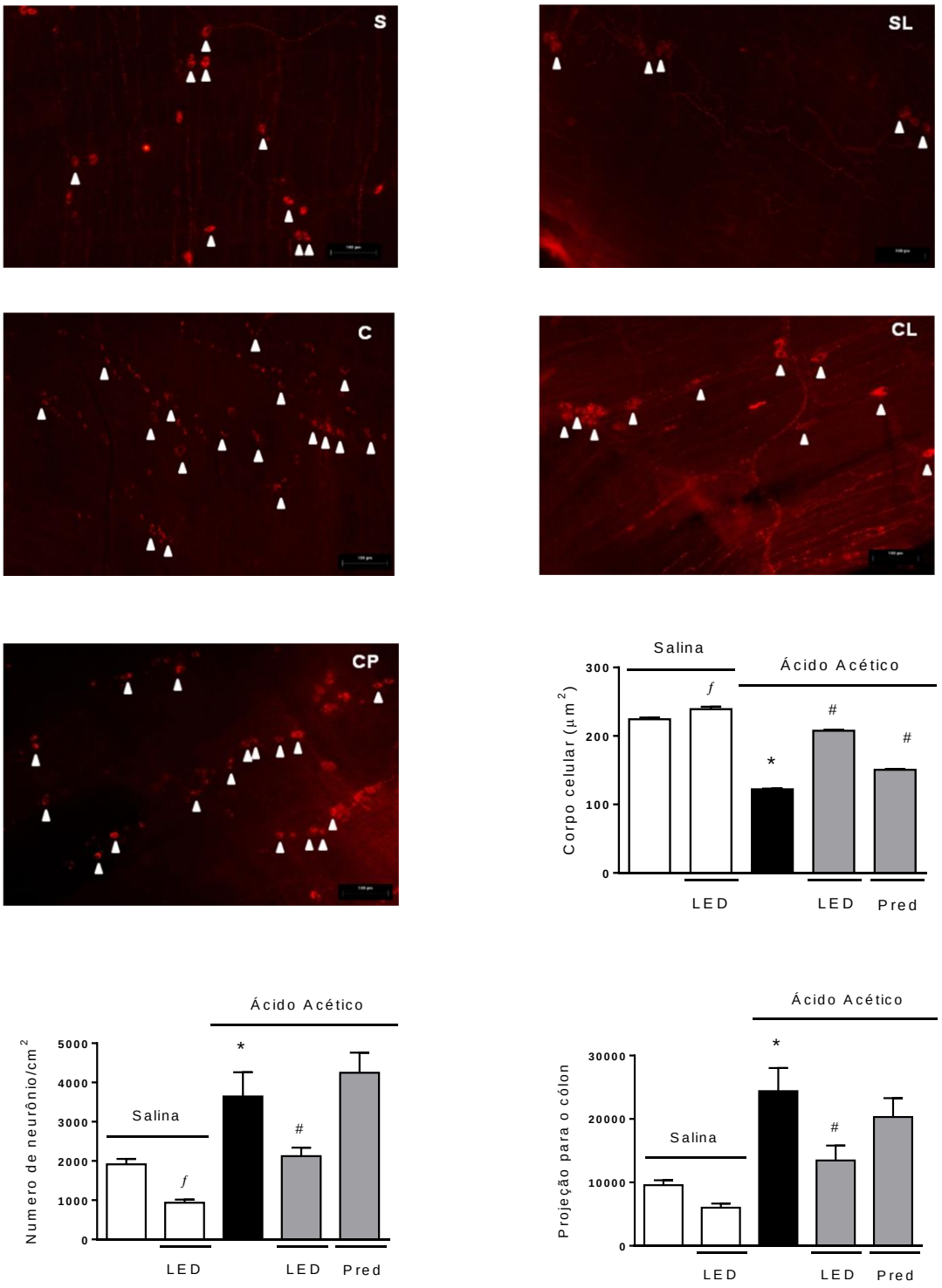

Figura 5. Fotomicrografia de imunofluorescência mostrando população mientérica VIPérgica de neurônios (neurônio motor inibitório) nos grupos experimentais (ponta da seta). Barra 100 µm. Controle (S); Controle_LED (SL); Colite (C); Colite_LED (CL); Colite _PRED (CP). Avaliação morfométrica e quantitativa do plexo mioentérico

de camundongos com colite experimental submetidos à LED terapia ou tratados com predinisolona. *p < 0,05 em relação ao grupo Controle. #p < 0,05 em relação ao grupo Colite. ƒp < 0,05 em relação ao grupo Colite_LED. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

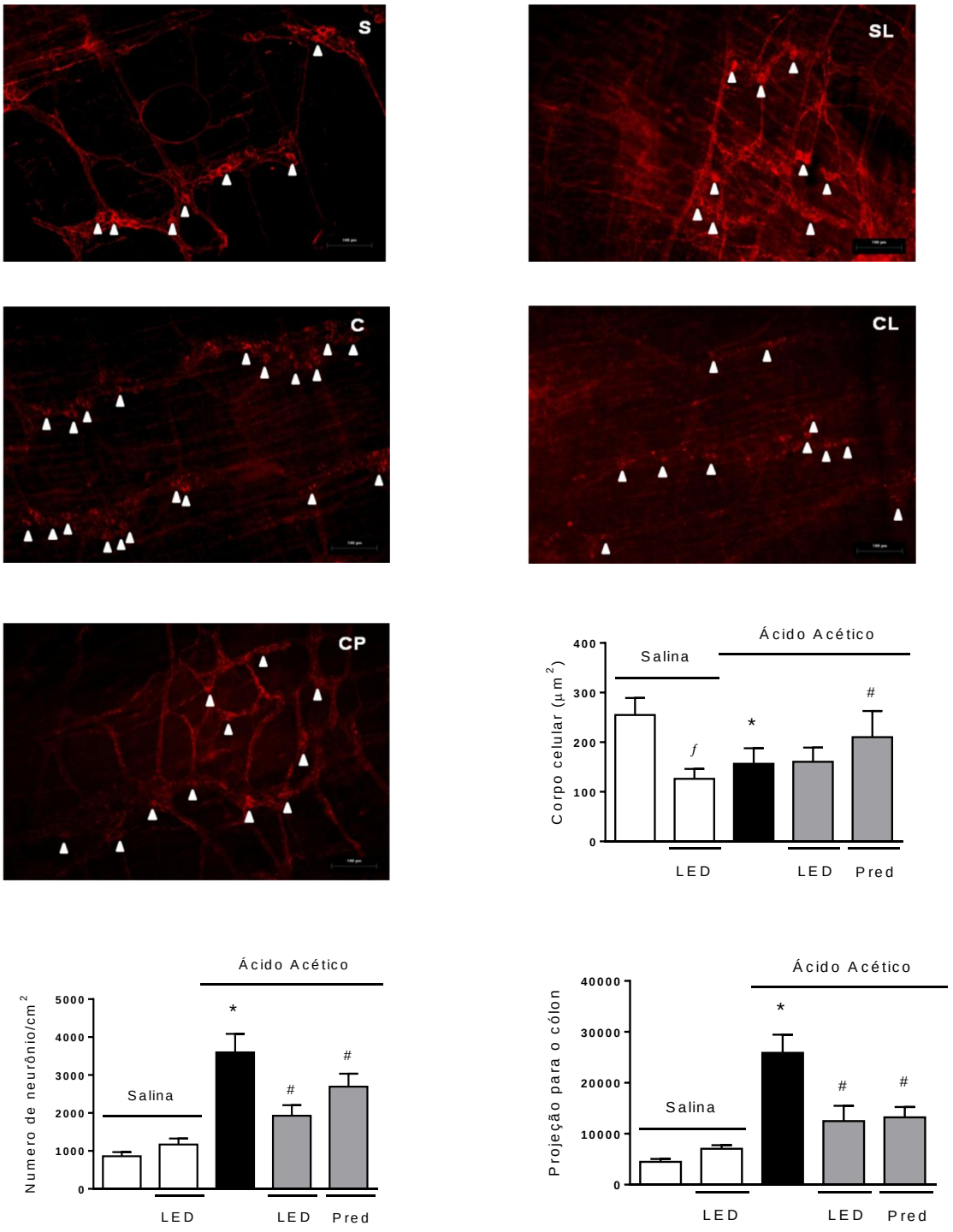

Figura 6. Fotomicrografia de imunofluorescência mostrando população mientérica de neurônios produtores de substância P (neurônio motor excitatório) nos grupos experimentais (ponta da seta). Barra 100 µm. Controle (S); Controle_LED (SL); Colite (C); Colite_LED (CL); Colite _PRED (CP). Avaliação morfométrica e

quantitativa do plexo mioentérico de camundongos com colite experimental submetidos à LED terapia ou tratados com predinisolona. *p < 0,05 em relação ao grupo Controle. #p < 0,05 em relação ao grupo Colite. ƒp < 0,05 em relação ao grupo Colite_LED. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

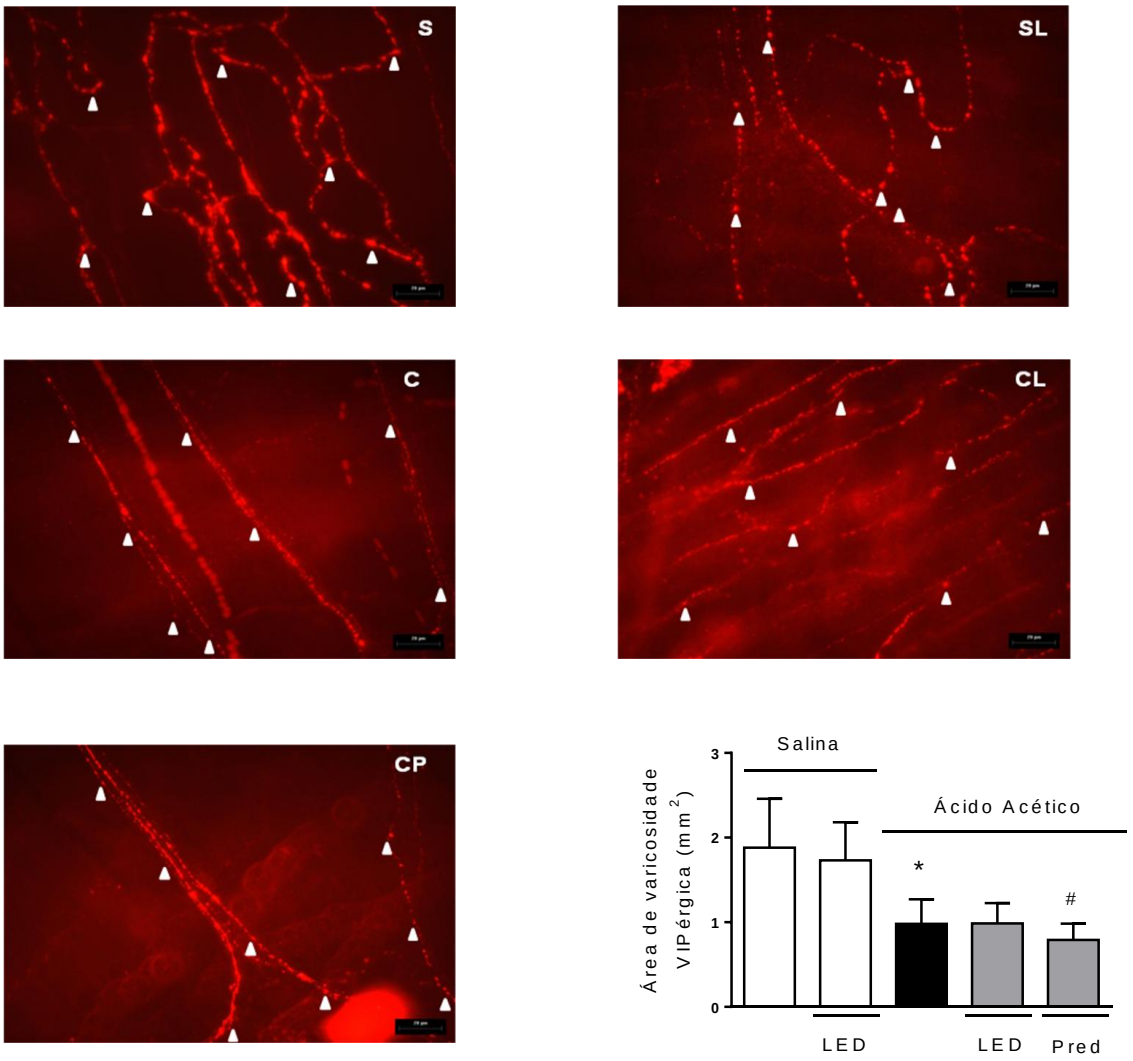

Figura 7. Fotomicrografia de imunofluorescência mostrando varicosidades mientéricos VIPérgicos (neurotransmissor inibitório) nos grupos experimentais (ponta da seta). Barra 20 µm. Controle (S); Controle_LED (SL); Colite (C); Colite_LED (CL); Colite _PRED (CP). Análise morfométrica da área de varicosidades mientéricas VIPérgicas do colón de camundongos com colite experimental induzida por ácido acético com tratamento de LED a 940 nm. *p < 0,05 em relação ao grupo Controle. $^{\#}$p < 0,05 em relação ao grupo Colite. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

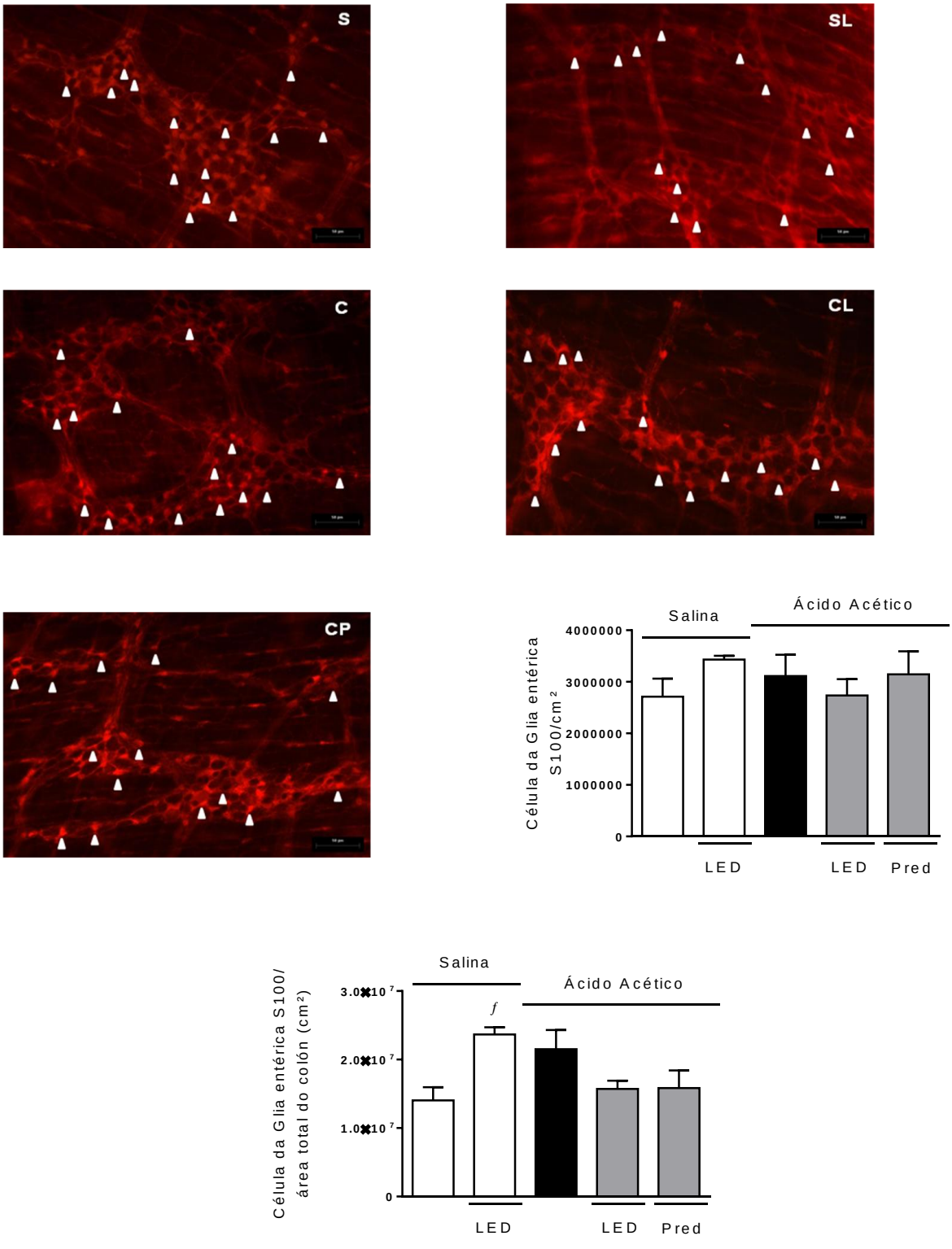

Figura 8. Fotomicrografia de imunofluorescência mostrando células da glia entérica (S100) nos grupos experimentais (ponta da seta). Barra 50 µm. Controle (S); Controle_LED (SL); Colite (C); Colite_LED (CL); Colite _PRED (CP). Análise de células da glia entérica ($S100^{+}$) do colón de camundongos com colite experimental

induzida por ácido acético com tratamento de LED a 940 nm. (A) Número de células da glia entérica/cm²; (B) Número de células da glia entérica projetado para a área total do cólon. $^{f}p < 0,05$ em relação ao grupo Colite_LED. ANOVA one-way seguido de pós-teste de Tukey. Pred: Prednisolona (droga controle do modelo experimental).

CONCLUSÃO

Baseada na hipótese de que o tratamento com LED a 940 nm seria eficaz como anti-inflamatório na colite experimental induzida por ácido acético e que esse efeito anti-inflamatório protegeria o tecido colônico das lesões causadas pelo modelo experimental que motivou o desenvolvimento desse trabalho, podemos concluir que a nossas hipóteses levantadas são verdadeiras.

O tratamento com LED a 940 nm foi efetivo como anti-inflamatório na colite experimental induzida por ácido acético demonstrando por parâmetros macroscópicos, microscópicos e moleculares de que o tratamento proposto protege o tecido colônico das lesões decorrentes da instilação de ácido acético, uma vez que atuou sobre as variações dimensionais colônicas, conteve o edema local, reduziu o escore médio de lesão macroscópica, retomando o tempo de trânsito intestinal normal. Além disso, a LED terapia foi efetiva na contenção da degranulação de neutrófilos, uma vez que houve uma menor concentração de mieloperoxidase, nos tecidos de camundongos com colite e tratados com LED, reduzindo consequentemente o infiltrado de células inflamatórias em todas as camadas intestinais. A nível molecular a LED terapia foi capaz de reduzir os níveis das citocinas anti-inflamatórias, contendo a progressão do quadro.

Uma vez que o tratamento com LED a 940nm se mostrou efetivo como anti-inflamatório, ele também apresentou eficácia na proteção do tecido colônico, e das estruturas que o formam, pois foi capaz de reduzir o escore médio de lesão microscópica, atuando sobretudo nas células caliciformes, garantindo que o número normal dessas células estivesse presente no colo de camundongos com colite e submetidos a esse tratamento. Alem do que a LED terapia também conseguiu atuar sobre componentes do sistema nervoso entérico, contendo o desvio fenotípico dos neurônios mientéricos produtores de óxido nítrico, peptídeo intestinal vasoativo e substância P, garantindo que o numero normal de neurônios estivesse produzindo esses neurotransmissores.

Sendo assim, é possível afirmar que o tratamento com LED a 940 nm, largura de banda de 45 nm, intensidade de 4 J/cm² e potência óptica de 9,5mW, aplicado sobre o colo de camungondos submetidos ao modelo experimental de colite induzida por ácido acético, é um potencial tratamento anti-inflamatório capaz de

conter e controlar as alterações macroscópicas, microscópicas, celulares e moleculares geradas no tecido colônico decorrentes do modelo. Por isso é pertinente pensar no desenvolvimento de um trabalho translacional, que avalie a efetividade desse tratamento em pacientes em crise aguda de colite.

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ANEXOS

ANEXO A

Aprovação da Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) protocolo 113/2014, processo n°14276.2014.60.

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 113/2014 **Londrina, 06 de Agosto de 2014.**

Prezado Pesquisador,

A CEUA/UEL reunida em 15 de julho de 2014 avaliou o projeto de pesquisa intitulado "**Potencial terapêutico do emprego de diodos emissores de luz sobre modelo de retocolite ulcerativa induzida em camundongos.**", registrado sob o processo CEUA n°**14276.2014.60**, pesquisa do Centro de Ciências Biológicas, de sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados o projeto esta **aprovado**, entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizados 440 camundongos Swiss machos com idade de 60 dias, com peso aproximado 20-25 g, provenientes do Biotério do Centro de Ciências Biológicas – UEL. O projeto tem como objetivo avaliar o potencial terapêutico da LEDterapia (luz com feixes com comprimento de onda predominantemente de 940m,) por meio da análise de alterações moleculares e teciduais da parede intestinal de camundongos com retocolite ulcerativa (RCU) induzida por ácido acético. Os animais serão anestesiados com cetamina (80mg/kg, i.m.) e xilazina (10mg/kg, i.m), e a indução da RCU será realizada através da administração intra retal de 200µl de solução de ácido acético 7,5%(v/v) utilizando uma cânula de polietileno. Os animais controle receberão 200µl de salina. Os animais serão tratados com aparelho de LED com comprimento de onda predominante de 940nm ou desligado (controle). Outro grupo experimental será tratado com 5mg/kg de prednisolona por cinco dias antes da indução da RCU. Os animais serão submetidos à eutanásia 4h ou 18h após a indução da RCU e serão retiradas amostras do cólon. A parede intestinal será analisada quanto às lesões microscópicas, morfometria, quantificação de células califormes e infiltrado neutrofílico. A partir do plexo mioentérico, serão avaliados o corpo celular dos neurônios mioentéricos (análise quantitativa e morfométrica), os neuritos de neurônios mioentéricos (análise morfométrica e de intensidade de brilho) e a ganglionite. Será realizada ainda análise das células enteroendócrinas serotoninérgicas, do trânsito intestinal, de lesões macroscópicas, edema, dosagem de proteínas e citocinas, estresse oxidativo e atividade da mieloperoxidase. Os protocolos experimentais estão aprovados para execução em 36 meses.

Cumpre orientar que caso pretendam-se quaisquer alterações no projeto de pesquisa, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo-me, cordialmente,

Prof. Dr. João Waine Pinheiro
Vice-Coordenador da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Eduardo Jose de Almeida Araújo
Coordenador do Projeto
Departamento de Histologia
Centro de Ciências Biológicas
Com cópia para Sra Égle Maria de Sousa (Chefe da DCA/PROPPG), Diretor(a) do Centro de Ciências Biológicas e Prof. Luiz Carlos Juliana (Diretor do Biotério Central da UEL).

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone (043) 3371-4000 PABX - Fax 3328-4440 - Caixa Postal 6.001 - CEP 86057-990 – Internet http://www.uel.br
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ANEXO B

Normas da revista Laser in Medical Science

Instructions for Authors

TYPES OF PAPERS

- Original Article – limited to 4000 words, 45 references, no more than 5 figures
- Review Article – limited to 5000 words, 50 references, no more than 5 figures
- Brief Report - limited to 2000 words, 25 references, no more than 4 figures - Case Reports will not be accepted!
- Letter to the Editor – up to 600 words

MANUSCRIPT SUBMISSION

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

TITLE PAGE

Title Page

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

TEXT

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

- LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

SCIENTIFIC STYLE

Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

Units and abbreviations

- Please adhere to internationally agreed standards such as those adopted by the commission of the International Union of Pure and Applied Physics (IUPAP) or defined by the International Organization of Standardization (ISO). Metric SI units should be used throughout except where non-SI units are more common [e.g. litre (l) for volume].
- Abbreviations (not standardized) should be defined at first mention in the abstract and again in the main body of the text and used consistently thereafter.

Drugs

- When drugs are mentioned, the international (generic) name should be used. The proprietary name, chemical composition, and manufacturer should be stated in full in Materials and methods.

REFERENCES

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- ISSN.org LTWA

If you are unsure, please use the full journal title.
For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

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- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
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ARTWORK AND ILLUSTRATIONS GUIDELINES

Electronic Figure Submission

- Supply all figures electronically.
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- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
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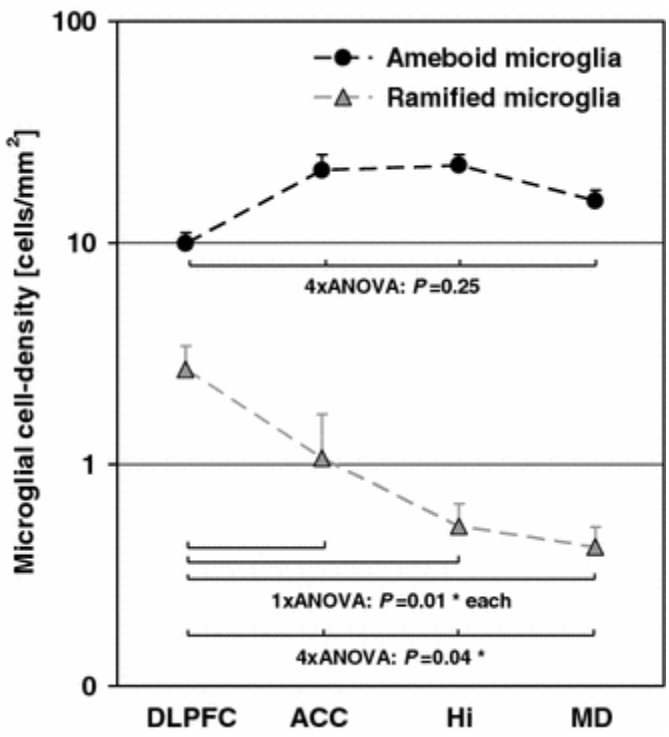
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



- Definition: Black and white graphic with no shading.
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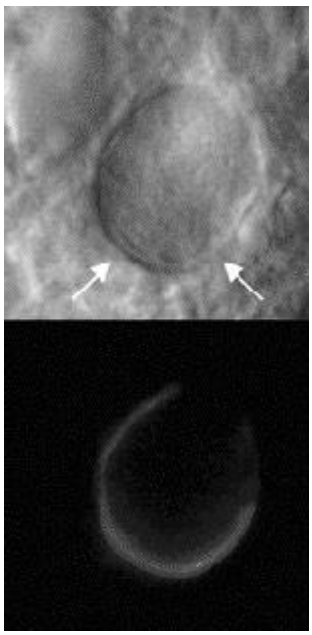

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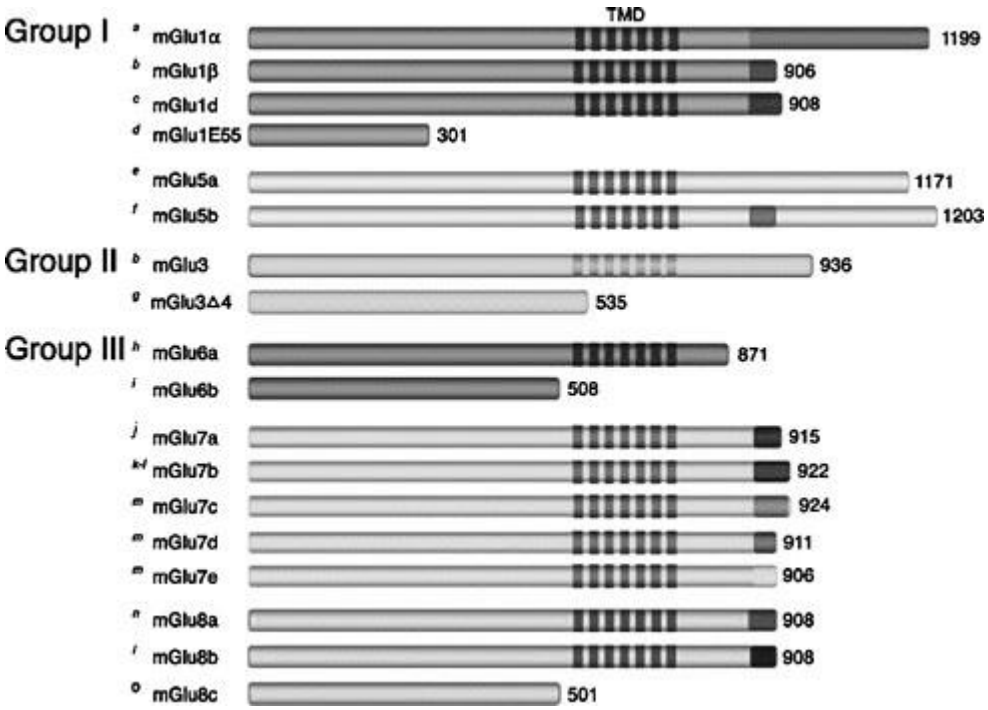

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- All figures are to be numbered using Arabic numerals.
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ANEXO C

Normas da revista Inflammatory Bowel Disease

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Sample references are given below:

Journal Article

1. Gudlaugsdottir S, van Dekken H, Stijnen T, et al. Prolonged use of proton pump inhibitors, CagA
status, and the outcome of Helicobacter pylori gastritis. *J Clin Gastroenterol.* 2002;34:536-540.

Book Chapter

2. Tobin RW, Kimmey MB. Painful diseases of the gastrointestinal tract. In: Loeser JD, ed. *Bonica's*
Management of Pain. 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2001:1269-1292.

Entire Book

3. Rohen JW, Yokochi C, Lütjen-Drecoll E. *Color Atlas of Anatomy: A Photographic Study of the Human*
Body. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2002.

Software

4. Epi Info [computer program]. Version 6. Atlanta: Centers for Disease Control and Prevention; 1994.

Online Journals

5. Friedman SA. Preeclampsia: a review of the role of prostaglandins. ObstetGynecol [serial online].
January 1988;71:22-37. Available from: BRS Information Technologies, McLean, VA. Accessed
December 15, 1990.

Database

6. CANCERNET-PDQ [database online]. Bethesda, MD: National Cancer Institute; 2014. Updated
March 29, 2014.

World Wide Web

7. Gostin LO. Drug use and HIV/AIDS [JAMA HIV/AIDS Web site]. June 1, 2015. Available at:
http://www.ama-assn.org/special/hiv/ethics. Accessed July 26, 2015.

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