Universidade Estadual de Londrina

PAULO DA SILVA WATANABE

ALTERAÇÕES MORFOLÓGICAS E FISIOLÓGICAS NO CÓLON DE CAMUNDONGOS COM RETOCOLITE ULCERATIVA AGUDA E CRÔNICA INDUZIDA POR DEXTRAN SULFATO DE SÓDIO

Londrina
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Orientador: Prof. Dr. Eduardo José de Almeida Araújo

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RESUMO

A doença inflamatória intestinal é uma inflamação recorrente que afeta o trato gastrointestinal, causando alterações na motilidade colônica. A evolução dessas alterações não é completamente compreendida e possivelmente está relacionada a sintomas que aparecem em diferentes graus da inflamação intestinal. Os estudos realizados em modelos animais para avaliar motilidade colônica usualmente utilizam apenas uma porção do cólon, e logo, os resultados geralmente não refletem as alterações que ocorrem em todo o órgão. Mais estudos são necessários para avaliar o desenvolvimento da colite, investigando as alterações causadas no epitélio intestinal e na parede do cólon, no modelo agudo e crônico da doença e investigar se essas alterações morfológicas interferem na motilidade de diferentes porções do cólon. O objetivo deste estudo foi avaliar as alterações estruturais e de motilidade do cólon durante o desenvolvimento da retocolite ulcerativa (RCU) aguda e crônica induzida por DSS em camundongos C57Bl/6. Os animais foram distribuídos em 5 grupos, sendo um grupo controle e os demais grupos expostos ao DSS 3% por 2, 5, 7 dias (RCU aguda) ou 2 semanas (RCU crônica). Durante a indução da RCU, os animais foram monitorados diariamente para obter o índice de atividade da doença (IAD). Após eutanásia, os cólons foram removidos e processados para análises histológicas e para as técnicas de imunofluorecência para avaliação de células *tuft* e neurônios mioentéricos. A motilidade do cólon foi determinada usando um aparelho de manometria convencional de perfusão multilúmen. De acordo com o IAD, o modelo foi eficaz em induzir a RCU tanto de forma aguda como crônica. Houve um aumento na espessura das camadas mucosa, submucosa, dos estratos da camada muscular, na parede total e redução no número de células *tuft*. Além disso, observou-se depleção de células caliciformes e um aumento na densidade de fibras de colágeno, bem como aumento numérico de neurônios colinérgicos no plexo mioentérico dos animais com RCU crônica. Tanto a população total como as subpopulações avaliadas de neurônios mioentéricos estavam atrofiadas nos grupos com RCU. A exposição progressiva ao DSS foi acompanhada de acúmulo de ajustes no padrão de motilidade colônica. O avanço da RCU provocou, dentre outros aspectos característicos de dismotilidade, redução no número de contrações, no número de complexos motores de migração colônica e no tempo total de trânsito gastrointestinal. De uma forma geral, a inflamação progressiva fez com que as contrações se tornaram mais vigorosas nas extremidades e mais longas na região medial do cólon. Concluímos que durante o progresso da RCU induzida por DSS ocorrem alterações estruturais no cólon, incluindo aumento no número de neurônios mioentéricos colinérgicos, seguidas de variações no padrão de motilidade de diferentes regiões do cólon que, em conjunto, caracterizam dismotilidade colônica.

Palavras-chave: Células *tuft*; dextran sulfato de sódio; doença inflamatória intestinal; retocolite ulcerativa; sistema nervoso entérico.

WATANABE, Paulo da Silva. **Morphological and physiological changes in the colon of mice with acute and chronic sodium dextran sulfate-induced ulcerative colitis**. 2021. 71 p. Thesis - State University of Londrina, Londrina, 2021.

ABSTRACT

Inflammatory bowel disease is a recurrent inflammation that affects the gastrointestinal tract, causing changes in colonic motility. The evolution of these changes is not completely understood and is possibly related to symptoms that appear in different degrees of intestinal inflammation. Studies carried out on animal models to assess colonic motility usually use only a portion of the colon, and therefore, the results generally do not reflect the changes that occur in the entire organ. Further studies are needed to assess the development of colitis, investigating the changes caused in the intestinal epithelium and in the colon wall, in the acute and chronic model of the disease and to investigate whether these morphological changes interfere with the motility of different portions of the colon. The aim of this study was to evaluate the structural and motility changes of the colon during the development of acute and chronic DSS-induced ulcerative colitis (UC) in C57Bl/6 mice. The animals were assigned into 5 groups, one control group and the other groups exposed to the DSS 3% for 2, 5, 7 days (acute UC) or 2 weeks (chronic UC). During the RCU induction, the animals were monitored daily to obtain the disease activity index (IAD). After euthanasia, the colons were removed and processed for histological analysis and for immunofluorecence techniques to evaluate tuft cells and myenteric neurons. Colon motility was determined using a conventional multi-lumen perfusion manometry device. According to the IAD, the model was effective in inducing UC at both acute (up to 7 days DSS) and chronic (two weeks DSS). There was an increase in the thickness of the mucous, submucosal, muscular, in the total wall and a reduction in the number of tuft cells. In addition, depletion of goblet cells and an increase in the density of collagen fibers was observed, as well as a numerical increase of cholinergic neurons in the myenteric plexus of animals with chronic UC. Both the total population and the assessed subpopulations of myenteric neurons were stunted in the groups with UC. Progressive exposure to DSS was accompanied by an accumulation of adjustments in the colonic motility pattern. The advancement of the RCU caused, among other characteristic features of dysmotility, a reduction in the number of contractions, in the number of colonic migrating motor complex and in the total gastrointestinal transit time. In general, progressive inflammation has caused contractions to become more vigorous in the extremities and longer in the medial region of the colon. We conclude that during the progress of the DSS-induced UC, structural changes occur in the colon, including an increase in the number of cholinergic myenteric neurons, followed by variations in the motility pattern of different regions of the colon that, together, characterize colonic dysmotility.

Keywords: Inflammatory bowel; ulcerative colitis; enteric nervous system; tuft cells; dextran sodium sulfate.

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LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celcius
ACh	Acetilcolina
AUC	*Area under the curve*
ChAT	Colina acetiltransferase
CMMC	*Colonic Migrating Motor Complex*
DC	Doença de Crohn
DII	Doença inflamatória intestinal
DSS	Dextrano sulfato de sódio
Fig	Figura
DAI	*Disease activity index*
IAD	índice de atividade da doença
IBD	*Inflammatory Bowel Disease*
IFNs	Interferons
IL	Interleucina
MC	Muscular circular
ML	Muscular Longitudinal
NF-κB	Fator nuclear κB
NO	Oxido Nítrico
PAS	Ácido periódico de Schiff
PBS	Tampão fosfato-salina
PFA 4%	Paraformaldeído 4%
PRRs	Receptor de reconhecimento de padrões
RCU	Retocolite ulcerativa
SNE	Sistema nervoso entérico
TGI	Trato gastrointestinal
TLRs	Receptores do tipo Toll
TNBS	ácido trinitrobenzeno sulfônico
TNF	Fatore de Necrose Tumoral

SUMÁRIO

1. INTRODUÇÃO

1.1 Doenças Inflamatórias Intestinais

Atualmente na literatura são descritas diferentes doenças inflamatórias que acometem o intestino delgado e grosso. A doença inflamatória intestinal (DII), do inglês *Inflammatory Bowel Disease* (IBD), refere-se às doenças inflamatórias crônicas do trato gastrointestinal, incluindo a retocolite ulcerativa (RCU) e a doença de Crohn (DC) (ACTIS et al., 2019).

A DII ocorre em todo o mundo, embora seja mais comum em países ocidentais, a europa, américa do norte e a oceania são os locais onde se encontra o maior número de casos diagnosticados, com taxas de incidência de 4 a 10 casos para cada 100.000 habitantes por ano e taxas de prevalência entre 40 a 100 para cada 100.000 habitantes (Figura 1) (ALATAB et al., 2020; KAPLAN, 2015).

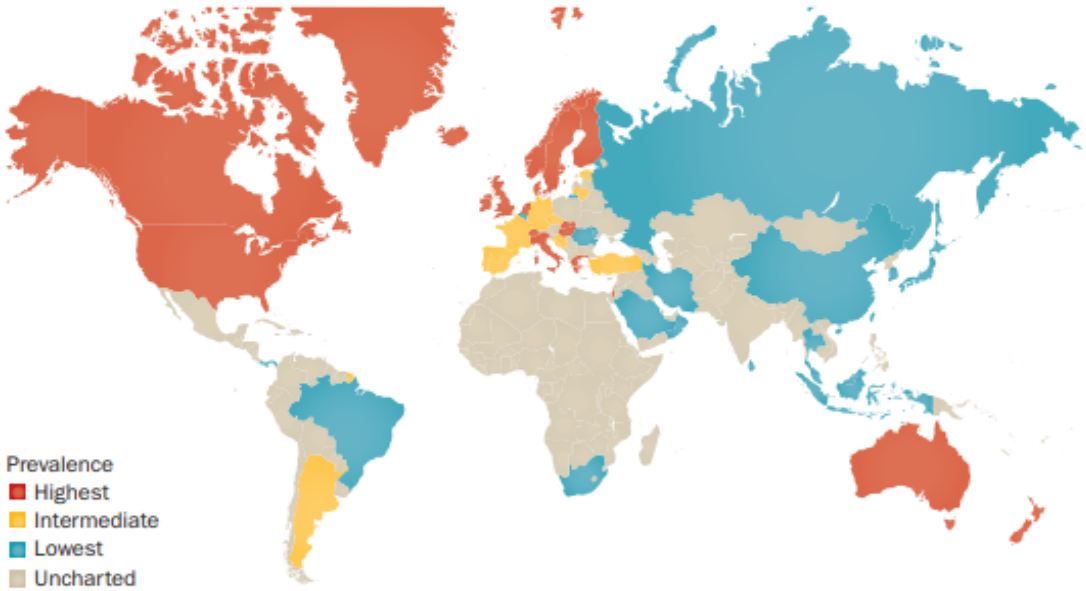

Figura 1. Prevalência global das doenças inflamatórias intestinais destacando os locais de menor e maior prevalência no ano de 2015. Fonte: adaptado de Kaplan et al. (2015).

Acredita-se que a menor incidência em outras partes do mundo esteja relacionada a hábitos alimentares, estilo de vida ou problemas técnicos de diagnóstico da doença (HENDRICKSON; GOKHALE; CHO, 2002). No Brasil, entre o ano de 1986 e 1990, foi registrado um caso para cada 100.000 mil habitantes, já entre 2001 e 2005 esse número subiu para 4,5 casos para cada 100.000 habitantes (KAPLAN, 2015).

Em São Paulo, um estudo mostrou que o número de novos casos dobrou de 4,5 por 100.000 habitantes em 1995 para 9,5 casos em 2001-2005. Ambos os sexos são afetados (VICTORIA; SASSAK; NUNES, 2009). Um levantamento realizado em 2017 mostrou que todo dia no Brasil uma pessoa é diagnosticada com DII (CURY; OLIVEIRA; CURY, 2019).

O impacto econômico destas doenças é sem dúvidas um fato preocupante. Estima-se que os gastos diretos e indiretos possam chegar até 8 bilhões nos EUA e 12 bilhões na Europa de dólares por ano (GOYAL et al., 2014).

A DII é mais comumente diagnosticada entre a terceira e a quarta década de vida, sem diferença observada entre homens e mulheres. Aproximadamente 20% de todos os pacientes com DII desenvolvem sintomas durante a infância (ROGERS; CLARK; KIRSNER, 1971), com cerca de 5% sendo diagnosticados antes dos 10 anos de idade (MIR-MADJLESSI; MICHENER; FARMER, 1986). Cerca de 25% das crianças afetadas têm um histórico familiar positivo de DII (GILAT et al., 1987). Ainda em relação à idade, a RCU apresenta padrão de incidência bimodal, com o pico de início entre 20 e 30 anos e um segundo pico menor entre 60 e 70 anos de idade (MALIK, 2015).

Em pacientes com DII pode ocorrer manifestações extra intestinais, como em: a) articulações periféricas e axiais: artralgia, artrite periférica, sacroiliite e espondilite anquilosante; b) pele e mucosas: eritema nodoso, pioderma gangrenoso e aftas orais; c) fígado: hepatite autoimune e colangite esclerosante primaria; e d) olhos: episclerite e uveíte (NIKOLAUS; SCHREIBER, 2007).

A fisiopatologia da DII é complexa e ainda não está totalmente elucidada. A literatura aponta que em indivíduos geneticamente susceptíveis vários fatores ambientais (DANESE; SANS; FIOCCHI, 2004) e alterações na composição da microbiota intestinal (NAGAO-KITAMOTO et al., 2016), ingesta alimentar inadequada, estresse psicológico e a utilização de fármacos anti-inflamatórios podem ser os gatilhos de distúrbios nas respostas imunológicas inatas e adaptativas do trato gastrointestinal (TGI), os quais provocam o surgimento e a modulação dessas doenças (KNIGHTS; LASSEN; XAVIER, 2013).

A DC, ao contrário da RCU, pode envolver qualquer parte do trato gastrointestinal desde a orofaringe até a área perianal. Os segmentos acometidos frequentemente são intercalados por porções de tecidos normais. A inflamação pode ser transmural, muitas vezes se estendendo até a serosa, resultando em vias sinusais ou formação

de fístula. Os achados histológicos incluem pequenas ulcerações superficiais e inflamação crônica focal que se estende até a submucosa, às vezes acompanhada por formação de granuloma não causador de câncer. A localização mais comum é a região ileocecal, seguida apenas pelo íleo terminal, intestino delgado difuso ou doença colônica isolada em ordem decrescente de frequência (HENDRICKSON; GOKHALE; CHO, 2002).

A DC e a RCU diferem entre si por possuírem aspectos fisiopatológicos, clínicos, e terapêuticos inerentes a cada uma delas (BETTERIDGE et al., 2013; DIGNASS et al., 2012; VAN ASSCHE et al., 2010). Em alguns casos não é possível diferenciar a DC e a RCU, como por exemplo casos de colite que apresentam elementos clínicos, endoscópicos e anatomopatológicos das duas doenças. Estes casos são classificados como colite indeterminada ou colite não classificada (BAUMGART; SANDBORN, 2007).

A DC e a RCU podem também se desenvolver por herança genética (SPEHLMANN et al., 2008). Há descrito na literatura que ao menos 110 lócus genéticos são relacionados a susceptibilidade tanto para DC quanto para RCU; 30 lócus são específicos para DC e 23 para RCU (VERMEIRE; RUTGEERTS, 2013).

Em média os pacientes com RCU possuem de 5,7 a 15,5% parentes de primeiro grau com a mesma doença (ORDÁS et al., 2012), também a ocorrência em gêmeos monozigóticos é extremamente alta, sendo aproximadamente 800 vezes maior que na população geral (CHILDERS et al., 2014).

A RCU é uma condição na qual a resposta inflamatória e as alterações morfológicas permanecem confinadas ao cólon e reto. O reto está envolvido em 95% dos pacientes, com graus variáveis de extensão proximal. A inflamação é limitada principalmente à mucosa e consiste no envolvimento contínuo de gravidade variável com ulceração, edema e hemorragia ao longo do cólon e reto. Os achados histológicos característicos são inflamação aguda e crônica da mucosa por leucócitos polimorfonucleares e células mononucleares, abscessos de cripta, distorção das glândulas mucosas e depleção de células caliciformes (HENDRICKSON; GOKHALE; CHO, 2002).

Para o diagnóstico da RCU deve-se realizar uma colonoscopia com análise anatomopatológica de biópsias do reto e cólons. Os achados endoscópicos incluem: perda do padrão vascular submucoso, eritema, granulosidade, friabilidade erosões, ulcerações e sangramento espontâneo (FORD; MOAYYEDI; HANAUER, 2013).

1.2 Resposta imunológica

As respostas imunes inata e adaptativa são os dois tipos de classes efetoras do sistema imunológico. A resposta imune inata atua como uma primeira linha de defesa e fornece proteção inespecífica, enquanto a resposta imune adaptativa é altamente específica por natureza e é ativada pela imunidade inata (ABUL ABBAS ANDREW H. LICHTMAN SHIV PILLAI, 2018).

São componentes da imunidade inata a barreira epitelial, neutrófilos, macrófagos, células dendríticas, células assassinas naturais (células NK) etc. Essa forma de imunidade é ativada pelos agentes microbianos reconhecidos pelos receptores de reconhecimento de padrões, receptores do tipo Toll (TLRs) e receptores do tipo NOD (PASTORELLI et al., 2013). Os neutrófilos, juntamente com outras células, iniciam a inflamação liberando citocinas pró-inflamatórias, como TNF-α, IL-a, IL-6 e IL-8, que ativam ainda mais a resposta imune adaptativa (WALLACE, 2014).

O sistema imunológico adaptativo compreende linfócitos T e células B que na ativação liberam citocinas e anticorpos (ABUL ABBAS et al., 2018). Pensa-se que as células T sejam o principal contribuinte da DII devido ao aumento da liberação de mediadores pró-inflamatórios. As células Th-1 produzem uma grande quantidade de interferons (IFNs), induzidas pela interleucina 12 (IL-12), e causam a patogênese em pacientes com DC. Por outro lado, as células Th-2 estão envolvidas na produção de IL-4, IL-5 e IL-13 e causam inflamação em pacientes com RCU (ZHANG, 2014).

Há ainda um terceiro tipo de célula T-auxiliar que também está envolvido na patogênese da DII, isto é, as células Th-17 (SIEGMUND; ZEITZ, 2011). Essas células produzem IL-17 e IL-22, ambas citocinas pró-inflamatórias capazes de promover a destruição local do tecido (SIEGMUND; ZEITZ, 2011). Essas citocinas aumentam ainda mais a permeabilidade intestinal, rompendo a barreira epitelial e resultando em inflamação descontrolada (GOYAL et al., 2014).

Existem certas diferenças no perfil mediador inflamatório da CD e RCU, como IL-2 e IFN-γ são responsáveis pela inflamação na CD, enquanto IL-4, IL-5 e IL-10 estão envolvidas na patogênese da RCU (MONTELEONE et al., 2002). A concentração desses mediadores é altamente elevada no sangue, fezes e mucosa intestinal de pacientes com DII (GOYAL et al., 2014). A liberação dessas células inflamatórias é regulada por diferentes vias envolvidas na inflamação, ou seja, via fator nuclear κB (NF-κB) e MAPK e via JAK/STAT (PEDERSEN, 2014).

O NF-κB e as MAPK (*Mitogen Activated Protein Kinases* - Proteíno-quinases ativadas por mitógenos) são ativadas pelos TLR-4 que estimulam ainda mais a liberação de TNF-α, IL-6 e IL- 12 por meio de macrófagos, exacerbando a resposta inflamatória (COSKUN et al., 2011). Além disso, as citocinas exercem sua sinalização ativando a via JAK/STAT, que está envolvida na produção de várias interleucinas. JAK, quando fosforilam proteínas STAT, translocam-se do citoplasma para o núcleo para alterar a expressão gênica dos genes alvo (COSKUN et al., 2013). A liberação de mediadores pró-inflamatórios por essas vias resulta na progressão da doença (WU et al., 2016).

Naturalmente, em condições saudáveis o sistema imune do TGI mantém estável equilíbrio com a microbiota, respondendo apenas de modo controlado aos antígenos provenientes da dieta, condição denominada tolerância imunológica. Desta forma, a RCU pode ocorrer devido a quebra desta tolerância, mencionado anteriormente, levando assim a resposta imune inadequada, demasiada, contra bactérias comensais não patogênicas (GOYAL et al., 2014).

Os antígenos oriundos do lúmen intestinal ativam a resposta imune inata por meio da interação com macrófagos e células dendríticas residentes na lâmina própria. Estes, por sua vez, apresentam os antígenos para os linfócitos B e T, desencadeando assim a ativação da reposta imune adaptativa (ORDÁS et al., 2012). Como pode ser observado na figura 2, as células dendríticas podem ainda abrir as junções oclusivas presentes entre as células epiteliais e projetar seus prolongamentos para a luz do órgão e entrar em contato direto com fragmentos bacterianos do lúmen (NIESS, 2005).

Os macrófagos residentes exercem papel fundamental na manutenção da homeostasia do intestino. São células importantes para atividade bactericida e fagocitose, e também produzem e liberam mediadores anti-inflamatórios (SWIRSKI et al., 2009).

Os macrófagos humanos possuem características fenotípicas singulares e vias de sinalização distintas das encontradas nos monócitos do sangue periférico (SMYTHIES et al., 2010). Ou seja, após a estimulação de PRR, como os TLR, alguns macrófagos intestinais não secretam citocinas pró-inflamatórias, isto é, secretam seletivamente

citocinas anti-inflamatórias (IL-10), que ajudam na resolução do processo inflamatório intestinal (DENNING et al., 2007).

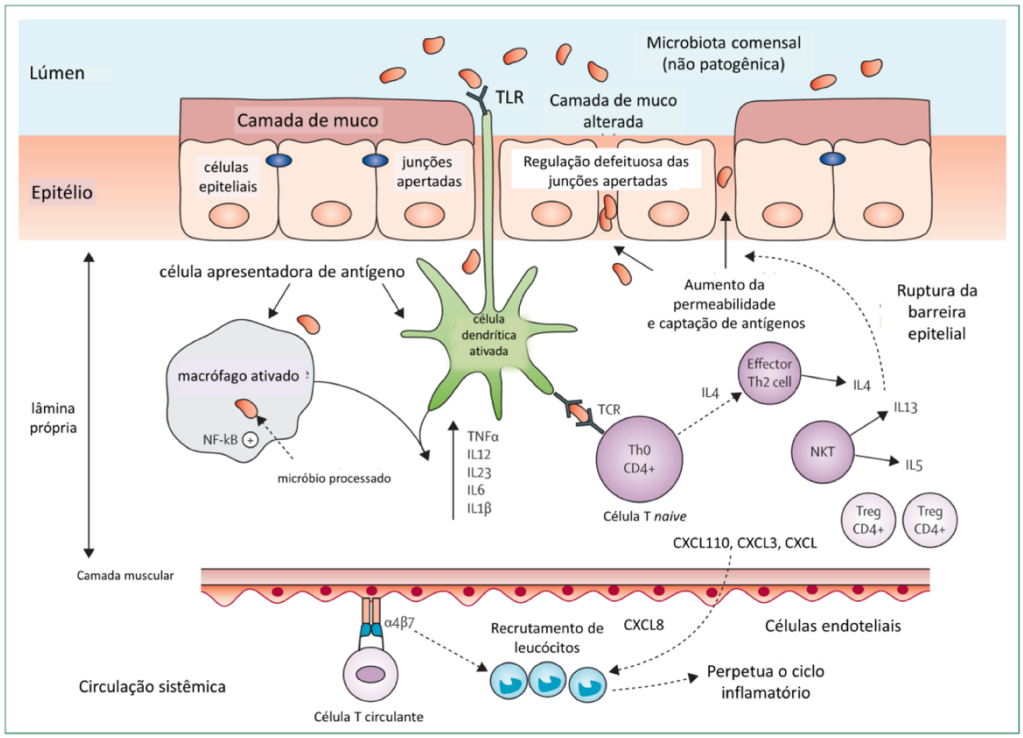

Figura 2. Fisiopatologia da retocolite ulcerativa - A ruptura das junções oclusivas e a camada de muco que cobre o epitélio causa o aumento da permeabilidade intestinal, resultando em aumento da captação de antígenos luminais. Macrófagos e células dendríticas no reconhecimento de bactérias não patogênicas por meio de receptores de reconhecimento de padrões, alteram seu status funcional de tolerogênico para um fenótipo ativado. A ativação das vias NF-κB estimula a transcrição de genes pró-inflamatórios, resultando no aumento da produção de citocinas pró-inflamatórias. Após o processamento de antígenos, macrófagos e células dendríticas as apresentam para células T CD4 naive, promovendo diferenciação em células efetoras Th2, caracterizadas pela produção de IL-4. As células T natural-killers são a principal fonte de interleucina 13, que tem sido associada a ruptura da barreira celular epitelial. As células T circulantes portadoras de integrina-α4β7 se ligam às células endoteliais que estão expressando muitas moléculas de adesão endotelial, levando ao aumento da entrada de células T específicas do intestino na lâmina própria. Fonte: adaptado de Ordás (2012).

Na mucosa de pacientes com RCU, o equilíbrio homeostático entre as células T reguladoras e efetoras (T-*helper* [Th] 1, Th2 e Th17) está alterado. A RCU está associada a uma resposta Th2 (células do tipo T *helper*, do inglês T auxiliares), atípica mediada por células NK não clássicas que produzem IL-5 e IL-13 (WU et al., 2016).

A IL-13, possui papel primordial, pois exerce função citotóxica contra as células epiteliais, incluindo indução de apoptose e alteração na composição das proteínas de junção epitelial amplificando a lesão tecidual. Haja visto que com estudos que fizeram o bloqueio da IL-13 e a depleção de células T foi possível prevenir o desenvolvimento da colite (HELLER et al., 2005, 2008).

1.3 Sistema digestório

O sistema digestório é constituído pelo tubo digestório e glândulas anexas, que realizam a ingestão, mastigação, deglutição e digestão do alimento e absorção dos nutrientes, além da eliminação dos restos não digeridos. O tubo digestório é a porção responsável por macerar, encaminhar, digerir, selecionar e absorver os elementos nutritivos e a água, sendo subdividido em esôfago, estômago, intestino delgado e intestino grosso. No humano o intestino delgado é formado pelo duodeno, jejuno e íleo que possuem as funções de digestão e absorção dos nutrientes, enquanto o intestino grosso é formado pelo ceco, colos ascendente, transverso, descendente e sigmoide seguido do reto e do canal anal que fazem a absorção de eletrólitos e água, além de formarem o bolo fecal (SANT'ANA; MIRANDA-NETO; OLIVEIRA, 2006). Em ratos o sistema digestório é muito semelhante ao humano, possuindo a mesma nômina anatômica do esôfago ao íleo, sendo o intestino grosso dividido em ceco, cólon proximal, cólon distal, reto e canal anal (JOAN, 1979).

O intestino delgado é formado histologicamente por: túnica mucosa, tela submucosa, túnica muscular e túnica serosa. A túnica mucosa é altamente vascularizada e composta pelo epitélio, lâmina própria e muscular da mucosa. Na mucosa são encontrados os vilos, que são projeções da lâmina própria em direção ao lúmen intestinal, que estão recobertas por um epitélio de revestimento simples prismático; além de invaginações epiteliais nos espaços intervilares que formam numerosas glândulas, denominadas de criptas intestinais (JUNQUEIRA; CARNEIRO, 2008). As criptas são glândulas simples e tubulares que circundam a base dos vilos e se abrem como perfurações do revestimento interno do intestino (JUNQUEIRA LC, 2008).

No epitélio intestinal as células mais numerosas são os enterócitos, de formato colunar, com aproximadamente 25 µm de altura, com núcleo oval localizado no polo basal e com uma borda em escova (microvilosidades) no polo apical. Essas células são responsáveis pela digestão terminal de macromoléculas e absorção de água e nutrientes, além de modificar ácidos graxos em triglicerídeos, formando quilomícrons, e transportar nutrientes para vasos sanguíneos e linfáticos da lâmina própria, de onde serão distribuídos para todo o corpo (JUNQUEIRA LC, 2008).

Células caliciformes também estão presentes no epitélio intestinal, são glândulas unicelulares com formato de cálice, com a base apoiada na lâmina basal e polo apical

repleto de gotículas secretoras revestidas por membranas voltadas para o lúmen intestinal, de modo a deslocar o citoplasma para a periferia da célula e o núcleo para o polo basal. Essas células possuem a função de produzir e secretar o mucinogênio, cuja forma hidratada é a mucina, componente fundamental do muco que reveste o lúmen intestinal. O muco lubrifica e protege o epitélio contra a abrasão e ação da microbiota intestinal, embora também sirva como fonte de nutrientes para esses microrganismos (GARTNER LP, 2003).

Ainda no epitélio intestinal está presente um tipo celular muito singular, as células tuft (GERBE; JAY, 2016). Em camundongos adultos, as células Tuft representam 0,4% de todas as células epiteliais (GERBE et al., 2011). Este tipo celular apresenta características morfológicas bastante singulares como demostrado na figura 3, possui uma volumosa quantidade de microvilosidade apicais mais altas do que as dos enterócitos e que se organizam para formar uma borda em escova, e esta característica propicia uma aparência de um “tufo” (GERBE; JAY, 2016). A morfologia deste tipo celular sofre variações dependendo de sua localização: no trato respiratório assumem um formato achatado (MEYRICK; REID, 1968), na vesícula biliar uma forma cuboide (LUCIANO; GROOS; REALE, 2003) e no intestino apresentam um corpo cilindro que se estreita nas extremidades apicais e basais (BANERJEE et al., 2018).

Os cytospinule das células Tuft são estruturas morfológicas únicas e inerentes a este tipo celular, são 3-4 projeções laterais que perfuram a membrana da célula vizinha e podem fazer contato direto com a membrana nuclear desta célula (figura 3). Acredita-se que os cytospinule funcionem como um meio direto de comunicação e transporte de carga (HOOVER et al., 2017).

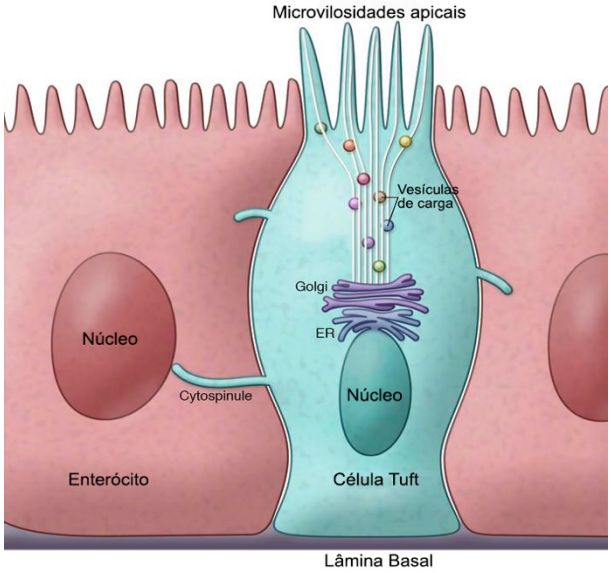

Figura 3. Morfologia da célula Tuft - As células tuft intestinal são facilmente distinguíveis dos enterócitos vizinhos por sua borda apical única e corpo da célula em forma oval. Os microvilos apicais conectam o ambiente extracelular do lúmen ao citoplasma intracelular através de um feixe filamentoso. Vesículas que transportam carga desconhecida são intercaladas dentro do feixe filamentoso, que termina em uma rede tubular no ápice do núcleo. Projeções laterais da membrana ou cytospinule emanam da célula Tuft e perfuram a membrana dos enterócitos adjacentes. Fonte: adaptado de Banerjee et al. (2018).

As células Tuft desempenham papel imunológico importante nas infecções parasitárias, alguns trabalhos demonstraram que diferentes parasitos induziram hiperplasia de células Tuft (GERBE; JAY, 2016; VON MOLTKE et al., 2016). A citocina IL-25 desempenha papel fundamental na imunidade Th2 (BANERJEE et al., 2018). Por meio de imunohistoquímica foi observado que as células Tuft expressam IL-25 de maneira constitutiva, citocina inexistente nas demais célula do epitélio intestinal (GERBE; JAY, 2016). A citocina IL-13 desempenha papel direto na hiperplasia das células Tuft, uma vez que animais nocautes para IL-13 não desenvolveram hiperplasia dessas células após infecções parasitárias, por outro lado quando a IL-13 foi administrada de maneira exógena, houve indução de hiperplasia tanto in vitro como in vivo das células Tuft (VON MOLTKE et al., 2016).

As células Tuft também possuem importante papel na neuromodulação, uma vez que tem sido demonstrando que esse tipo celular é fonte não neural de acetilcolina (Ach) e estas por sua vez se encontram extremamente perto dos neurônios do sistema nervoso entérico (SNE) (MIDDELHOFF et al., 2017). Desde a década de 1990 a ocorrência de células imunorreativas para colina acetiltransferase (ChAT) no epitélio da mucosa do intestino grosso humano já era relatada (COSTA et al., 1996).

A lâmina própria situa-se abaixo do epitélio intestinal e é constituída de tecido conjuntivo frouxo, abrigando diversos vasos sanguíneos e linfáticos de pequeno calibre, além de envolver externamente glândulas e nódulos linfáticos ocasionais. A muscular da mucosa circunda a lâmina própria, delimitando a mucosa e a submucosa. Ela é composta por células musculares lisas reunidas em uma camada circular interna e uma longitudinal externa que são responsáveis por desenvolverem contrações rítmicas que movimentam os vilos de forma intensa durante a digestão (GARTNER LP, 2003).

A tela submucosa é formada por tecido conjuntivo denso fibroelástico não-modelado com a presença de numerosos vasos sanguíneos e linfáticos de maior calibre (arteríolas e vênulas). Nesta tela ainda se encontra o plexo submucoso, componente nervoso que controla a motilidade da mucosa, a atividade secretora das glândulas e a vasomotricidade (JUNQUEIRA LC, 2008).

A túnica muscular é formada por dois estratos de músculo liso: o estrato circular da túnica muscular (MC) disposta mais próxima do lúmen, e o estrato longitudinal da túnica muscular (ML) disposta mais periférico do lúmen. Essas fibras musculares são responsáveis pela atividade motora intestinal. Entre os dois estratos da túnica muscular situa-se o plexo mioentérico, outro componente nervoso que regula a atividade peristáltica, bem como outros tipos de movimentos exercidos pela túnica muscular. A túnica serosa é formada por tecido conjuntivo frouxo que recobre externamente todo o tubo intestinal. Essa túnica é chamada de serosa quando o segmento do tubo digestório é revestido por peritônio, e conhecida como adventícia quando o segmento é retroperitoneal, como é o caso do esôfago (JUNQUEIRA LC, 2008).

O sistema digestório possui um sistema nervoso intrínseco, denominado Sistema Nervoso Entérico (SNE), que controla todas as funções digestórias. O SNE é a maior e mais complexa divisão do sistema nervoso autônomo em vertebrados. Distribuído por todo o TGI, vesícula biliar e o pâncreas, é organizado como uma rede interconectando neurônios e células da glia agrupados no interior de gânglios, localizados nos dois maiores plexos: mioentérico e o plexo submucoso, demonstrados na figura 4 (FURNESS; COSTA, 1987).

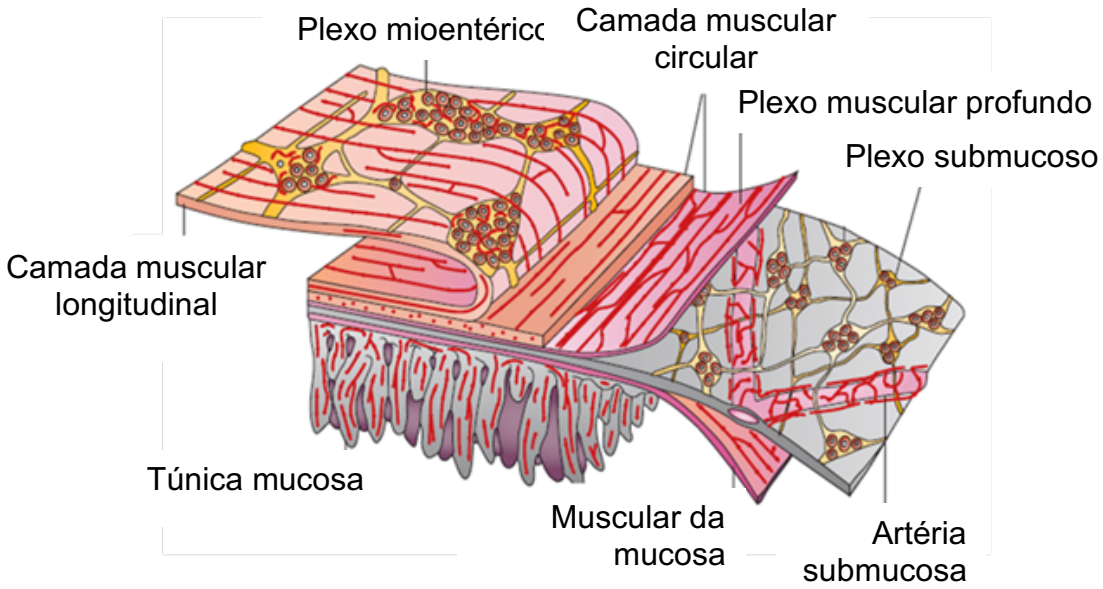

Figura 4. Esquema explicando a disposição dos neurônios em gânglios e plexos na parede do trato gastrintestinal (adaptado de FURNESS, 2012).

Os plexos ganglionados são o mioentérico e o submucoso, dos quais emergem feixes de fibras nervosas que formam os plexos não-ganglionados, presentes em toda a parede intestinal (FURNESS JB, 2006).

Além de neurônios, no SNE existem também as Células da Glia Entérica, que possuem diversas funções ligadas a sobrevivência neuronal, como apoio metabólico, estrutural, trófico e de proteção (RUHL; NASSER; SHARKEY, 2004).

O SNE é formado por vários tipos de neurônios comparáveis em número ao da medula espinal (80-100 milhões) e com uma série de neurotransmissores e neuromoduladores semelhantes aos encontrados no sistema nervoso central. Com base nas propriedades eletrofisiológicas e histoquímicas podem ser classificados em subpopulações funcionalmente distintas, incluindo neurônios aferentes primários intrínsecos, interneurônios, neurônios motores, neurônios intestinofugais, neurônios secretores e vasomotores (FURNESS, 2012).

A maior parte dos neurônios mioentéricos humanos são colinérgicos ou nitrérgicos. Os neurônios nitrérgicos humanos parecem ser interneurônios descendentes ou neurônios motores inibitórios. Por outro lado, os neurônios mioentéricos colinérgicos são neurônios motores excitatórios (FURNESS JB, 2006).

A motilidade colônica depende do SNE para propulsão do conteúdo. A motilidade colônica funciona inadequadamente quando o SNE do colón distal e do reto está ausente congenitamente em pacientes com doença de Hirschsprung, por exemplo (SWENSON, 2002), ou quando o SNE sofre alguma degeneração mais tarde na vida, como na doença de Chagas (MATSUDA; MILLER; EVORA, 2009). Em indivíduos saudáveis, os reflexos propulsivos do cólon distal e reto são regulados por centros de controle central que mantêm a continência fecal e, quando apropriado, desencadeiam a defecação por meio de comandos centrais retransmitidos pelo centro de defecação na medula espinal lombossacral (DE GROAT et al., 1981; LYNCH et al., 2001).

Para haver a peristalse intestinal, uma série de eventos, e comunicação inter neuronal deve ocorrer. Quando ocorre um estímulo no lúmen intestinal, células enteroendócrinas podem sinalizar, secretando seus hormônios na lâmina própria, estes hormônios por sua vez irão ativar os neurônios aferentes primários que então ativarão um interneurônio. Estímulos de estiramento também podem ativar os interneurônios mecanossensoriais ascendentes e descendentes por si só, não dependendo então de outra célula para sinalizar (SPENCER et al., 2016). Os interneurônios ascendentes ativam neurônios motores excitatórios na ML e na MC. Os interneurônios descendentes ativam os neurônios motores inibitórios na ML e no MC (SMITH et al., 2007).

Os neurônios motores excitatórios da ML e MC liberam ACh para evocar contração no músculo. Já os neurônios motores inibitórios da ML e da MC liberam NO e NO/ATP, respectivamente, para promover um relaxamento do músculo (FURNESS, 2012).

1.4 Modelos experimentais para RCU

Como a etiologia exata da DII ainda é incerta, vários modelos experimentais utilizando animais foram desenvolvidos nas últimas décadas para estudar o possível mecanismo envolvido na fisiopatologia da doença e novos alvos terapêuticos. Os diferentes tipos de modelos animais são desenvolvidos para o estudo da DII (Tabela 1).

Categoria	Especificidade	
	RCU	DC
Modelos quimicamente induzidos	DSS	TNBS/DNBS
	TNBS/DNBS	Indometacina
	Oxazolona	
	Ácido acético	
	Carragenina	
	Iodoacetamida	
Modelo imunológico	2, 4-Dinitroclorobenzeno (DNCB)	
Modelos induzidos por bactérias	Induzida por *Salmonella*	
	E. coli invasiva aderente	
Modelos espontâneos		Modelo de camundongos C3H/HejBir
		Modelo de camundongos SAMP1/4it
Modelos geneticamente modificados	TGF-b -/-	IL-10 -/-
	IL-2 -/-	NOD2 -/-
	MDR1A -/-	A20 -/-
	Gai2 -/-	IL-23 -/-
	TCRa -/-	NEMO -/-
	XBP1 -/-	
	NEMO -/-	
Modelos de camundongos transgênicos	camundongo IL-7 Tg	STAT 4
	HLA-B27	HLA-B27
		Camundongos DNN Caderina Tg
Modelos knock-in de mutação		TNFΔARE
Modelos de transferência adotiva	Modelo de alta transferência CD45RB	Modelo de alta transferência CD45RB

Tabela 1. Classificação dos modelos experimentais de doença inflamatória intestinal de acordo com a especificação, para retocolite ulcerativa (RCU) ou doença de Chron (DC). Fonte: Adaptado de Goyal et al., 2014.

O modelo mais utilizado para indução de RCU em roedores utiliza dextran sulfato de sódio (DSS), um polissacarídeo sulfatado sintético composto de dextrano e unidade de anidroglucose sulfatada (VAUGHN; MOSS, 2013).

Esse modelo foi primeiramente descrito em 1985 no Japão e os primeiros experimentos foram desenvolvidos em hamsters e posteriormente em camundongos (OHKUSA, 1985). Por meio deste modelo, pode ser induzida a RCU aguda ou crônica a depender do peso molecular do DSS, da concentração, da duração e da frequência de exposição. No entanto, dentro do mesmo modelo, os animais apresentam susceptibilidade diferente à colite devido a variações genéticas e microbiológicas (GOYAL et al., 2014).

A suplementação da água de roedores com DSS com baixo peso molecular resulta em características sintomáticas semelhantes à RCU (LAROUI et al., 2012). O DSS carrega uma carga altamente negativa por conta de seus grupos sulfatos, sendo assim muito tóxico para o epitélio do cólon pois induz erosões que comprometem a integridade da barreira epitelial, resultando em aumento da permeabilidade epitelial.

Além disso, sua propriedade anticoagulante agrava o sangramento intestinal. Por razões desconhecidas, a extensa patologia induzida por DSS está confinada ao intestino grosso, especificamente ao cólon distal, onde há número bastante expressivo de bactérias (CHASSAING et al., 2014).

O mecanismo pelo qual o DSS passa pelas células epiteliais da mucosa permanece incerto, mas Laroui *et al.* (2012) sugere o DSS induzir colite em camundongos por meio da formação de nano-lipocomplexos com ácidos graxos de cadeia média no cólon. A especificidade do DSS para o cólon pode decorrer da absorção de água e eletrólitos na presença de numerosas bactérias. Teoricamente, o DSS com carga negativa pode danificar as membranas celulares com carga negativa por forças repulsivas, mas a falta de inflamação induzida por DSS com pesos moleculares inferiores ou superiores argumenta contra isso (CHASSAING et al., 2014).

O DSS provoca além de lesões, aumento na permeabilidade intestinal e em análises histoquímicas da distribuição tecidual de DSS com peso molecular de 40 kDa demonstraram que o DSS penetra na membrana mucosa do intestino e um dia após o tratamento com DSS já é possível encontrar pequenas quantidades de DSS em macrófagos no intestino grosso, nos linfonodos mesentéricos e nas células de Kupffer no fígado, com três dias já é possível encontrar o DSS em alguns macrófagos no baço e 7 dias no rim. DSS foi observado nas células de Kupffer do fígado mesmo 8 semanas após a remoção do DSS (KITAJIMA; TAKUMA; MORIMOTO, 1999). Durante a fase crônica da colite por DSS, quantidades consideráveis de DSS também foram encontradas no baço (COOPER et al., 1993). As principais vias de excreção do DSS são a urina e as fezes. Assim, a presença de DSS nas células epiteliais dos túbulos renais proximais após 7 dias de tratamento com DSS é uma indicação do processo de excreção do DSS absorvido (KITAJIMA; TAKUMA; MORIMOTO, 1999).

A eficácia da RCU induzida por DSS depende de vários fatores, incluindo peso molecular, 5 kDa para colite leve e 40 kDa para colite grave. A dosagem geralmente é entre 1 e 5%, quando a duração pode ser aguda ou crônica, o modelo pode variar conforme a espécie de roedores também, o C57Bl/6 por exemplo é uma espécie mais susceptível a indução (MELGAR; KARLSSON; MICHAËLSSON, 2005), o sexo dos animais, os machos são mais suscetíveis. A microbiota também apresenta um fator fundamental na indução, em animais livres de microbiota por exemplo, a indução de RCU por DSS não é efetiva (NAGAO-KITAMOTO et al., 2016).

Para a indução, o DSS é geralmente administrado por 7 a 10 dias para induzir uma inflamação aguda. Ao prolongar a administração, a RCU aguda pode avançar para a RCU crônica, usualmente administrando-se DSS em ciclos que pode ser de três a cinco, com um período de descanso de uma a duas semanas entre os ciclos (GOYAL et al., 2014).

Na fase aguda da RCU causada por DSS podemos observar algumas características clínicas como perda de peso, diarreia, teste de sangue oculto nas fezes positivo, piloereção, anemia e eventualmente, morte, enquanto na fase crônica da colite geralmente não refletem a gravidade da inflamação ou características histológicas encontradas no intestino grosso (PERŠE; CERAR, 2012).

Na RCU induzida por DSS, semelhante a RCU que ocorre em humano, há um aumento nos diferentes níveis de citocinas, quimiocinas, óxido nítrico e óxido nítrico sintase indutível (iNOS) que estão implicados na patogênese da DII (GOYAL et al., 2014). O DSS causa colite progressiva e grave com aumento da ativação de NF-κB, o que piora ainda mais a colite, também ocorre um aumento da expressão de genes de citocinas inflamatórias (IL-1, TNF-α, IL-6 e IL-8) e alguns genes de moléculas de adesão (DOU et al., 2013).

2. OBJETIVO

2.1 Objetivo Geral

- Avaliar as alterações estruturais e de motilidade do cólon durante o desenvolvimento da retocolite ulcerativa aguda e crônica induzida por DSS em camundongos C57Bl/6.

2.2 Objetivos específicos

- Padronizar o modelo experimental de RCU aguda e crônica, utilizando exposição ao DSS, no Laboratório de Neurociência Entérica da Universidade Estadual de Londrina.

- Verificar a evolução de sinais clínicos durante o desenvolvimento da retocolite ulcerativa aguda e crônica induzida por DSS, relacionando com o número de células *tuft*.

- Caracterizar as alterações anatomopatológicas durante o desenvolvimento da retocolite ulcerativa aguda e crônica induzida por DSS.

- Avaliar a densidade populacional e morfometria do corpo celular de neurônios mioentéricos gerais, assim como as subpopulações nitrérgica e colinérgica, durante o desenvolvimento da retocolite ulcerativa aguda e crônica induzida por DSS.

- Elucidar as alterações no padrão de motilidade colônica durante o desenvolvimento da retocolite ulcerativa aguda e crônica induzida por DSS.

- Relacionar alterações estruturais do plexo mioentérico com variações no padrão de motilidade colônica durante o desenvolvimento da retocolite ulcerativa aguda e crônica induzida por DSS.

COLONIC MOTILITY ADJUSTMENTS IN ACUTE AND CHRONIC DSS-INDUCED COLITIS

Running Title: Colitis-induced motility changes

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Abstract Inflammatory bowel disease is a recurrent inflammation that affects the gastrointestinal tract, causing changes in intestinal motility. The evolution of these changes is not completely understood. The aim of this study was to evaluate the structural and motility changes of the colon during the development of acute and chronic DSS-induced ulcerative colitis (UC) in C57Bl/6 mice. Mice were assigned into 5 groups: control and groups exposed to DSS 3% for 2, 5, 7 days (acute UC) or 3 cycles (chronic UC). Mice were monitored daily. After euthanasia, colon was removed for histological, immunofluorescence and colon motility analysis. There was an increase in the thickness of the mucosa, submucosa, muscle layers and the total wall and reduction in the number of tuft cells. We observed a depletion of goblet cells and an increase in collagen fibers as well as an increase in the positive ChAT myenteric neurons in chronic UC mice. Both UC models caused atrophy of myenteric neurons, reduction in number of colonic contractions, colonic migration motor complex, total time of gastrointestinal transit. Besides, contractions became more vigorous in the ends and longer in the medial region of inflamed colon, especially in chronic UC. We conclude that during the progress of the DSS-induced UC, structural changes occur in the colon, including increase of cholinergic myenteric neurons, followed by variations in the motility pattern of different regions of the colon that taking together characterize colonic dysmotility.

Keywords: dextran sodium sulfate; enteric nervous system; inflammatory bowel; tuft cells; ulcerative colitis.

1 INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic, chronic and recurrent inflammatory bowel disorder, characterized by abdominal pain and diarrhea [1]. It can result in significant morbidity and mortality, compromising the quality and life expectancy of patients [2].

IBD occurs worldwide, although it is more common in Western countries. Europe, North America and Oceania are the places with the highest number of diagnosed cases, with incidence rates of 4 to 10 cases per 100,000 inhabitants per year and prevalence rates between 40 to 100 per 100,000 inhabitants, and these numbers are increasing every year [3], which shows us the importance of further studying this disease.

There are two major forms of chronic intestinal disorders: Crohn's disease (CD) and ulcerative colitis (UC) [4]. Some clinical and pathological characteristics of CD and UC are the same, but they also have different characteristics, such as in relation to the regions of gastrointestinal tract that can be affected, as well as in the distribution and depth of inflammation [5]. CD, unlike UC, can involve any part of the gastrointestinal tract from the oropharynx to the perianal area [6]. On the other hand, UC is a condition in which the inflammatory response and morphological changes remain confined to the colon and rectum[7].

The aetiology of UC is not yet fully elucidated. There are several factors that can contribute to the pathophysiology of this disease such as imbalance in the tolerance of the innate and adaptive immune system, commensal bacteria, oxidative stress, inflammatory mediators and changes in intestinal permeability [3,5].

One of the most used protocols for experimental induction of UC is by using sodium dextran sulfate (DSS) [8,9]. The DSS-induced UC is very efficient due the high reproducibility of the lesions mainly in the distal colon, similarly to the UC in humans[10]. DSS is a sulphated polysaccharide, heparin like, which has a highly negative charge due to its sulphate groups, being toxic to the colon epithelium inducing erosions that compromise the integrity of the epithelial barrier, resulting in increased epithelial permeability. In addition, its anticoagulant property worsens intestinal bleeding [11].

The correlation between inflammation that occurs in the UC and smooth muscle function has been reported in *ex vivo* studies [12]. Since few studies assess *in vivo*

information on colonic motility, the studies in the literature are still controversial, some report that UC can cause an increase [13] or decrease [14] in colonic motility. Studies carried out on animal models usually analyse only a small portion of the colon [14,15], therefore, the results usually do not reflect the changes that occur in the whole organ.

Although there are several studies investigating the pathophysiology of UC [2,8,9], as its aetiology is still unclear [3,5], further studies are needed to evaluate its developments in animal models, investigating the changes caused by this disease in the intestinal epithelium and in the wall of the colon, investigating the acute and chronic model of the disease and also verifying whether these morphological changes interfere with colonic motility. A better understanding of these nuances can point out possible pharmacological targets for treatment of UC.

This study aimed to investigate how the development of acute and chronic DSS-induced colitis alters the colonic wall, including myenteric neurons, and how it affects the types of contractions and Colonic Migrating Motor Complex (CMMC) in different portions of the colon in mice.

2 Methods

2.1 Ethical approval

The experimental protocols used previously were approved by the Ethics Committee on the Use of Animals (CEUA) of the State University of Londrina (no 48/2017)

Fifty-five C57BL/6 mice aged 12 weeks (CEMIB/Unicamp) were used during the experiment. The mice were kept in the animal house, in standard polyethylene boxes (29.0 x 18.0 x 12.0 cm) with a metal grid lid, temperature of 22±2ºC with exhaustion and alternation of light-dark cycles of 12 hours. All animals receive feed for rodents marketed by NUVITAL® (recommended by the National Research Council and National Health Institute - USA) and liquid *ad libitum*.

2.2 Induction to DSS colitis

The animals were distributed into five experimental groups. The control group received only pure autoclaved water and the other groups received water autoclaved

supplemented with 3% DSS (Molecular weight: 40000 Da, TdB Consultancy) for different times, as described in Table 1.

2.3 Monitoring of clinical signs and number of tufts cells

Throughout the induction, all mice were monitored daily and the loss of body mass, stool consistency and presence of fecal blood was scored to determine the disease activity index (IAD), as previously described by Bento (2011).

After the colitis induction, the mice were euthanized by deepening anesthetic with halothane vapor (1%), oxygen (14%) and nitrogen (86%). The total colon was removed, and subsequently processed according as described below for each technique.

Aiming immunolabeling tuft cells, colon was divided into two parts, proximal and distal, and then fixed in 4% paraformaldehyde (PFA) for 3 hours at room temperature. Afterwards, it was washed in phosphate-saline buffer (PBS) pH 7.4, 0.1M and kept in saturated sucrose solution (30%) for 24 hours at 4ºC, and then frozen in OCT. Two semi-serial 10 µm sections from the proximal region and two from the distal region were performed per mice. Then, the sections were submitted to the immunofluorescence to label the enzyme choline acetyltransferase - ChAT (Table 2). Sections were washed 3 x 5 min in 0.1 M PBS; antigenic block using 2% serum bovine albumin, 10% donkey serum, 0.5% Triton X-100 in PBS 0.1 M pH 7.4; incubation with primary anti-ChAT antibody for 24 hours at room temperature; incubation with secondary antibody (table 2) for 2 hours; slides were then mounted using a coverslip and antifade conjugated with DAPI (INVITROGEN, P36931) and left to dry before viewing under the microscope at 40x. Images of the entire mucosa were captured, where the presence of tuft cells was quantified in each section.

2.4 Histopathological analysis

The total length and diameter of the distal colon were measured, in addition, 1 cm of the colon was weighed to assess the presence of edema.

One-centimeter rings of the colon were collected from all mice and opened by the mesocolic border, fixed in 4% PFA for 3 hours at room temperature. Colons were embedded in paraffin to obtain 4µm semi-serial transversal sections.

The sections stained with hematoxylin and eosin (HE) were used for intestinal wall morphometry. Images captured under light microscope at 20x were used to

measure the depth of the crypts, thickness of submucosa and muscle layers and total wall. Four images were obtained from each of the four histological sections and, thus, 42 measurements were performed per mice using the software Image Pro Plus® [17].

For Periodic acid Shiff (PAS) staining, four sections were stained, and six images of each section were captured at 20x. The number of goblet cells present in 0.96 mm^2 of mucosa of each mice was quantified [17].

To quantify the collagen, using Sirius Red staining, four sections were stained, 24 images were captured per mice, using optical polarization at 40x. The collagen was then quantified by using the software Image Pro Plus® [18]. All analyses were performed in a blinded manner.

2.5 Morphological evaluation of the myenteric plexus

Colons were fixed in 4% PFA for 3 hours at room temperature and then whole mounts were prepared.

Whole mounts were washed in PBS 0.1M pH 7.4 solution and then incubated in antigenic blocking solution and then they were incubated with the primary antibodies shown in table 2. Whole mount preparations were then incubated for 2 h at room temperature with AlexaFluor conjugated secondary antibody (table 2). Then, they were mounted using a coverslip and anti-fade conjugated with DAPI and left to dry before viewing under the microscope at 20x.

For neuronal counting, 32 images were captured in the intermediate regions considering the intestinal circumference and all neurons present in these fields were counted. For the morphometric analysis, the area of 100 neuronal body cell per mice was measured using software Image Pro Plus! ®. All analyses were performed in a blinded manner.

2.6 TGI motility assessment

Gastrointestinal transit was assessed following the method previously described by Welch (2014). A volume of 100 μL of a non-absorbable marker (6% Carmine Red; 0.5% methylcellulose; Sigma-Aldrich, St. Louis, USA) was administered by gavage. Mice were immediately placed in individual cages with free access to food and water. Gastrointestinal transit was expressed as the time between the administration of the biomarker and the appearance of the first red faecal pellet.

For the colon manometry we used the experimental procedure similar to described by Fraser (1997). After colitis induction and euthanasia as previously described, the colons were collected and carefully washed and then kept in an organ bath with perfusion of oxygenated Krebs solution at 35°C ± 1. Colonic motility was determined by conventional multi-lumen perfusion manometry. Spontaneous contractions were recorded as pressure waves at four points 1 cm apart from each other, taking place in the regions: proximal, medial proximal, medial distal and distal. The recordings were analysed manually using the software Spike2®.

Intracolonic pressure data analysis.

The area under the curve (AUC) of the phasic component of the intraluminal pressure trace for every contraction was analysed. In addition, frequency, amplitude, duration of contractions and duration of quiescence between contractions were determined during 30 min of analysis. Data were obtained from 10 mice from each group.

Colonic contractile pressure changes were quantified by measuring the propagation of CMMCs. The propagative nature of contractions was evaluated by determining the direction of appearance of the next peak of the intraluminal pressure wave as described previously[21]. Three different types of propagative and nonpropagative contractions were identified. Contractions that propagate from proximal to distal region were classified as oral-aboral propagating contractions whereas contractions that propagate from distal to proximal were classified as aboral-oral propagating contractions. Lastly, contractions that happen simultaneously at two or more portions of the colon are considered as static.

The speed of propagation in mm/s was obtained by dividing the distance between the first (proximal) and the last (distal) pressure sensors (40mm) by the time (s) elapsed between the peaks of the contractions at proximal and distal sites.

The amplitude and duration of the contractions were also categorized and quantified according to the number of events that occurred, as previously demonstrated by Gourcerol (2009).

2.7 Statistical analysis

Statistical tests were performed using Graphpad Prism© 7 software. The data distribution was checked using D'Agostino-Pearson normality test. As all data presented normal distribution, groups were compared using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The level of significance was 5%.

3 RESULTS

Experimental acute and chronic colitis was successfully induced by DSS

Clinical signs of DSS-induced colitis were observed after the third day by means of piloerection, prostration, progressive body weight loss (Fig. 1A), diarrhea and bleeding from 5th day, which contributed for DAI reached values much greater in colitis mice vs control (Fig. 1B) from the 7th day of exposure to DSS ($P < 0.05$). Edema was observed only in DSS 3C group ($P < 0.05$; Fig. 1C). In addition, we observed the typical shortening of colon size in colitis mice ($P < 0.05$; Figs. 1D and E). Based on these results, DSS7d group was considered the representative for acute UC and the DSS3C group for chronic UC during the next experiments.

The histopathological analysis showed an architectural irregularity in the crypts, a massive leukocyte infiltrate in the submucosa and lamina propria, irregular epithelial surface, rupture of the intestinal epithelium and presence of inflammatory cells infiltrated in myenteric plexus ganglia of the colitis groups vs control (fig 2A-E). The leukocyte infiltrate caused an increase in the thickness of the colonic mucosa and total wall for all groups, and submucosa, circular muscle and total muscle of the DSS3C groups ($P < 0.05$; Fig. 2F). Goblet cells were depleted only in DSS3C group ($P < 0.05$; Fig. 2G-J). Fibrosis was observed in both colitis groups characterized by the increasing in the quantity of collagen types I and III ($P < 0.05$; Fig. 2L and M). In addition, we observed that the number of tuft cells in the total colon decreased progressively during the course of the disease ($P < 0.05$; Fig. 3A), reflected by the impact of DSS-induced colitis on the proximal colon rarther than distal colon (Fig. 3B and C).

DSS-induced colitis promoted atrophy of myenteric neurons and increase in the number of cholinergic neurons

The number of general myenteric neurons (PGP9.5$^+$) did not change between the experimental groups (Fig. 4A) but these cells were atrophied in both UC groups (DSS7d and DSS3C) when compared to the control group ($P < 0.05$; fig 4B). Similar result was observed for the nitrergic neurons (nNOS$^+$), however the atrophy was observed only in the DSS7d group ($P < 0.05$; Fig 4B). Contrarily, the number of cholinergic neurons (ChAT$^+$) increased in the DSS3C group ($P < 0.05$; Fig. 4A), which provoked a significant increase in the ratio of ChAT/PGP9.5 myenteric neurons ($P < 0.05$; Fig. 4D). However, cholinergic neurons were also atrophied as also observed for the other types of myenteric neurons ($P < 0.05$; Fig. 4B). All these results are illustrated on the Fig. 5.

Colonic motility adjustments during course of colitis

Chronic DSS-induced UC caused increase in the area under the curve (AUC) of contractions in almost the entire colon and, on the other hand, acute colitis provoked reduction only in the middle distal portion ($P < 0.05$; Fig. 6A). Chronic UC caused increase in the contraction amplitude of the proximal and distal colon, where it was observed reduction of the number of contractions < 25 mmHg ($P < 0.05$; Figs. 6B-C). Regarding the number of overall contractions, it has reduced progressively over the entire colon during the course of the disease, but significant changes were observed only in the chronic phase (Figs. 6C-F).

Although the proportion of time that the colon has kept in contraction during the manometric observation (30 min) was not changed in almost its whole extension (Fig. 7B), the duration of the contractions increased in the middle portion of the colon of chronic UC mice ($P < 0.05$; Fig. 7A). On the other hand, the duration of colonic contractions in acute UC mice was not changed (Fig. 7A), but the proportion of contraction tended to reduce in the entire organ, becoming significant in the distal region ($P < 0.05$; Fig. 7B). The relaxation time between contractions (Fig. 7C) and the proportion of quiescence time (Fig. 7D) was unchanged in both UC groups. Besides, we observed fewer contractions lasting less than < 100 seconds in the entire colon in both UC groups and greater number of contractions lasting between 100 and 150 seconds in the middle distal colon of chronic UC mice ($P < 0.05$; Figs. 7E-H).

All these adjustments implied in changes on CMMCs. Acute and chronic DSS induced-colitis caused reduction in the number and increase in the duration of CMMCs ($P < 0.05$; Figs. 8A and B), but no changing the interval of time between CMMCs was

699 observed (Fig. 8C). The speed propagation was reduced and time of gastrointestinal
700 transit was slower in UC mice when compared to control group ($P < 0.05$; Figs. 8D
701 and E). The number of aboral-oral and static contractions did not change, but the
702 frequency of oral-aboral contractions was reduced in both UC groups ($P < 0.05$; Figs.
703 8E and F).
704
705 **4. DISCUSSION**
706
707 The incidence of IBD is progressively growing in many countries, however there
708 are many aspects of the disease that remain unclear. Among the several experimental
709 models developed in order to research aetiology, pathophysiology and treatment of
710 the IBD, the DSS-induced experimental colitis is one of the mostly used in the world
711 [23]. Independently of the model, there are consistent evidences of IBD alters colonic
712 motility, however we do not understand yet how DSS-induced colitis impacts the
713 manometric parameters of contractions in different portions of the colon.
714 In this report, we showed that acute and chronic DSS-induced colitis
715 experimental models imply in different adjustments of colonic motility. Although the
716 typical inflammatory parameters were noticed in the acute colitis model, salient
717 changes were not observed on the colonic motility during the first week of exposition
718 to DSS. However, after three cycles of exposition, the colon was less mobile and
719 slower especially in its middle portion in spite of the contractions on the proximal and
720 distal ends were more vigorous.
721 It is known that DSS causes a toxic effect directly to the colonic epithelium
722 provoking intestinal morphophysiological changes similar to several
723 pathophysiological aspects of UC, characterized by shortening of the colon, bloody
724 diarrhoea and loss of sustained body mass [18] exactly as we observed in our study.
725 Besides, the typical histopathology observed in UC [2,11] such as goblet cell depletion,
726 epithelial degeneration and necrosis, inflammatory cell infiltration in the lamina propria
727 and submucosa were also noticed in mice acutely and chronically exposed to DSS.
728 The colonic epithelial rupture is the pitch to trigger the inflammatory process [24],
729 which make the intestinal barrier crucial to prevent UC. Recently, some researches
730 highlighted the role of tuft cells as important modulators of the integrity of the intestinal
731 mucosa [25,26] and others showed DSS-induced structural changes were more severe
732 when the number of tuft cells is reduced [25,27]. In this study we showed that tuft cells

733 did not reduce right after the exposition to DSS. It was necessary at least five days of
734 exposition to observe reduction in the number of tuft cells. Besides, the proximal colon
735 showed more susceptible to deplete tuft cells in comparison to distal colon, which is
736 on the opposite way to the description of DSS affects more the distal colon in
737 comparison to the proximal region [11]. Thus, our results indicate that the number of tuft
738 cells is not altered directly by the exposition to DSS but it is a consequence of the Th1
739 response usually observed in DSS-induced colitis in C57BL6 [11,28].

740 During the DSS-induced immune response, many cells migrate to the colonic
741 wall especially to the mucosa and submucosa [8]. Besides, it is very common to report
742 plexitis in UC, which consists of an inflammatory infiltrate within the submucosal or
743 myenteric plexus [29]. This inflammatory environment certainly contributed to modulate
744 myenteric neurons and colonic motility as we discussed below.

745 The structure and neurochemical composition of the SNE are usually altered
746 during active IBD. The number of mucosal and muscular nerve fibers increases,
747 together with the immunoreactivity of neurochemicals, including tyrosine hydroxylase,
748 substance P, peptide related to the calcitonin peptide, vasoactive intestinal
749 polypeptide, 5-hydroxytryptamine and nitric oxide [30]. Hypertrophy and hyperplasia of
750 nerve fibers and architectural changes in neuronal cell bodies and enteric glial cells
751 are usually found in colitis tissue [31,32]

752 The severity of intestinal inflammation is associated with the density of enteric
753 innervation. Thus, enteric neurons are likely to be able to influence and to be
754 influenced by the severity of inflammation by releasing neurotransmitters /
755 neuromodulators. Inflammation is more severe when the ENS is hyperplastic and less
756 severe when the ENS is hypoplastic [33].

757 Although it is suggested that the inflammation observed in UC patients is
758 restricted to colonic mucosa [5], some studies indicate that changes in myenteric
759 neurons can happen [32,34], but there are several controversies. There are findings
760 showing increase in the number of neurons, suggestive of neurogenesis [35], however
761 in some cases there is no change or decrease in the number of myenteric neurons [36].

762 In DSS-induced colitis, it was observed that the quantity of ChAT in the colon
763 was higher compared to the control group, but not reflecting on increase in the amount
764 of $ChAT^+$ neuronal bodies [34]. Changes in the neuronal chemical code, such as the
765 increase in the number of $ChAT^+$ neurons observed in our study, are considered as
766 mechanism of adaptation to stimuli [37]. Thereby, it may be related to a possible

767 mechanism of adaptation in order to normalize the pattern of colonic contractions in
768 response to the inflammatory environment. It could help to explain the general
769 reduction in the number of contractions (probably involving increased interneuronal
770 activity) and the increase of strength of contractions (probably involving increased
771 excitatory motor neuronal activity) in the colonic ends of DSS-induced colitis mice. Our
772 results support the involvement of interneuronal activity as we will explain below.

773 DSS-induced colitis can upregulate the expression of ChAT which has the
774 potential to increase rhythmic phasic contractions [34]. On the other hand, the DSS-
775 induced inflammation inhibits the reactivity of smooth muscle to ACh resulting into
776 two opposite changes which suppress the rhythmic phasic contractions in DSS
777 inflammation [38,39].

778 During intestinal inflammatory processes, dysmotility may occur depending on
779 the experimental model used [40]. Colonic motility has been shown to decrease during
780 chemically induced colitis [41], an effect attributed to the suppression of excitatory and
781 inhibitory signalling in the colon [34] and loss of myenteric neurons [36]. However, the
782 impact of DSS-induced colitis on profile of contractions and CMMC activity remain to
783 be definitively determined.

784 Considering only the DSS-induced colitis model, the intestinal immune
785 response can be different depending on the specie, strain and gender of the
786 experimental animal [23]. Here, we have chosen to use male C57Bl/6 mice because
787 they show an immune response more intense [28]. This type of immune response
788 causes hypocontractability, reduced motility [42] and we showed these alterations on
789 colonic motility are progressive during the exposition to DSS. After three weeks,
790 contractions were stronger in the colonic ends due reduction in the number of less
791 strength contractions. Besides, they have become longer in the middle region due
792 reduction in the number of short duration contractions. For these reasons, the number
793 of overall contractions was reduced in the chronic DSS-induced colitis. Interestingly, it
794 would be expected to observe a greater number of contraction when we consider that
795 the proportion of cholinergic neurons was increased in the inflamed colon. This
796 contradiction indicates that the extended number of cholinergic neurons plays as
797 interneurons in order to coordinate the movements of the inflamed colon.

798 Our results demonstrate that reduction in the number of contractions reflected
799 on the reduced number of CMMCs, especially in oral-aboral direction, in DSS-induced
800 colitis. Similarly, the increase in the duration of contractions also impacted the duration

of CMMCs once they became double longer in colitis mice with chronic inflammation. This dysmotility may have contributed to the development of diarrhea in colitis mice, but it is important to consider that this clinical sign can be also related to bleeding, deficiency in the absorption of water and electrolytes and efflux of fluids promoted by inflammation [43].

Considering other chemically induced colitis models, it was showed less CMMCs and lower amplitude of contractions in TNBS-induced UC, but the duration of contractions was also increased [14]. TNBS also demonstrated that the speed of propulsive motility was slower in inflamed colon [44] being completely obstructed or temporarily interrupted [41,45]. Unfortunately, there are no other manometric studies evaluating the whole colon in animals exposed to colitis-inducing chemical agents.

Few information is available regarding the mechanisms involved in adjustments of colonic motility trigged by inflammation. The inhibition of CMMC in TNBS-induced UC is not due direct effects of IL-1B (Hofma et al., 2018). Studies suggest the increased prostaglandin synthesis due upregulation of cyclooxygenase 2 in myenteric neurons during UC may contribute to the reduction in colonic motility [46]. Other suggest that about 50% of primary afferent neurons, or interneurons, exhibit spontaneous action potentials in the colonic inflamed regions. This can promote the activation of upward and downward contracting inhibitory signals. This uncontrolled neuronal behaviour can result in mixed excitatory and inhibitory messages converging on smooth muscle throughout the inflamed region causing a kind of intestinal fibrillation [47].

We concluded DSS was efficient to induce acute and chronic colitis, causing increasing in the number of cholinergic neurons and also progressive dysmotility pattern during the progress of the disease. In acute DSS-induced colitis the colonic motility was little changed, but during the choric colitis the colon has become less mobile and slower in its middle portion and stronger on its proximal and distal ends.

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TABLES

Table 1. Organization of groups to induce UC with DSS in drinking water.

Groups	Treatment
Control (GC)	Pure water for 7 days
DSS 2 Days (DSS2d)	DSS 3% for 2 consecutive days
DSS 5 Days (DSS5d)	DSS 3% for 5 consecutive days
DSS 7 Days (DSS7d)	DSS 3% for 7 consecutive days
Chronic (DSS3C)	Three cycles of DSS 3% for 5 days alternated with 7 days of pure water.

Table 2. Primary and secondary antibodies used for the immunofluorescence technique.

ANTIBODY	SPECIES	DILUTION	MANUFACTURER	CODE
anti-PGP9.5	Rabbit	1:500	Abcam	AB27053
anti-nNOS	Goat	1:300	Abcam	AB1376
anti-ChAT	Goat	1:100	Merck	AB144P
Alexa Fluor® 488 anti-rabbit	Donkey	1:500	Invitrogen	A-21206
Alexa Fluor® 568 Anti-goat	Donkey	1:500	Invitrogen	A-11057

FIGURES AND LEGENDS

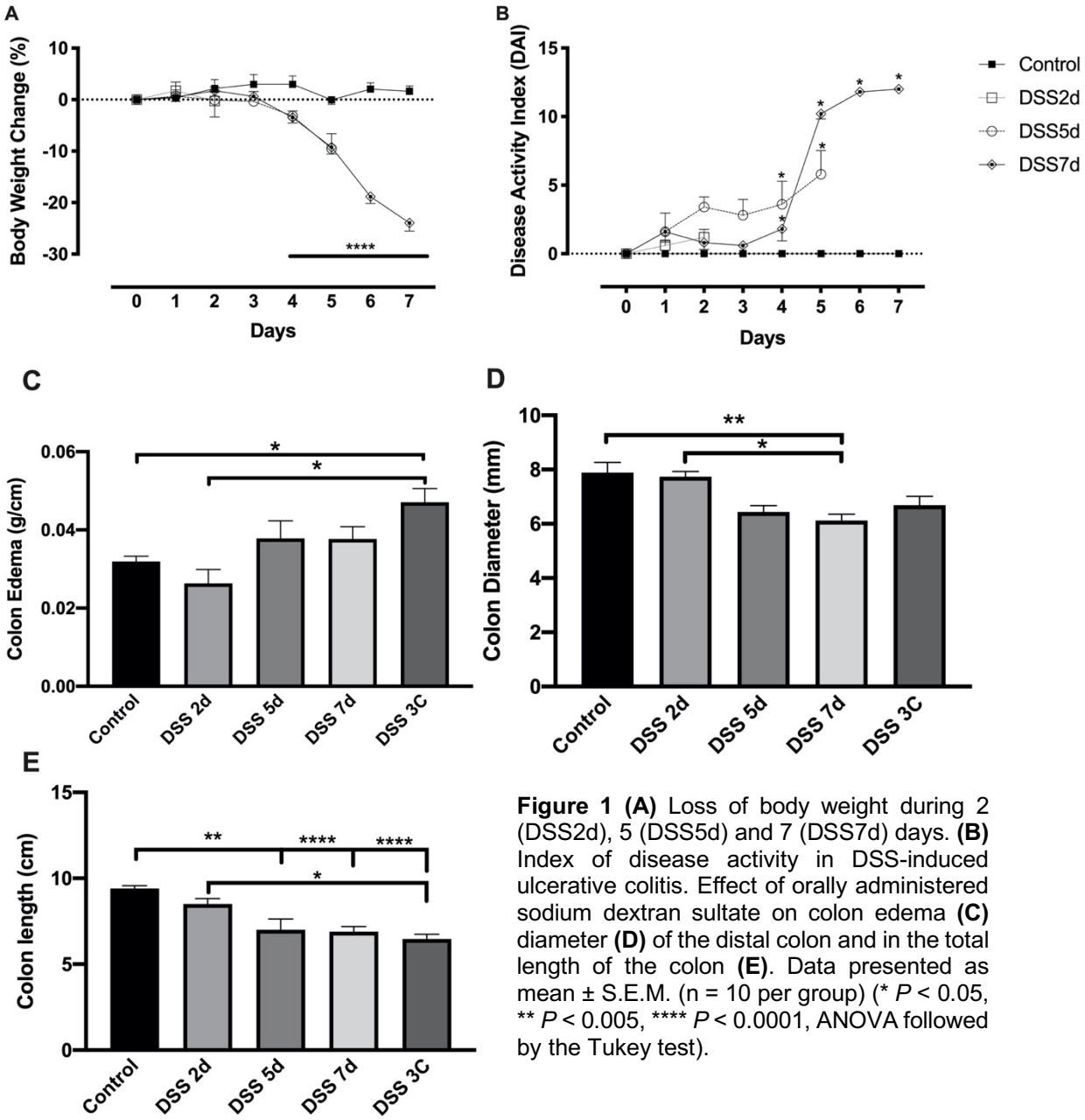

Figure 1 (A) Loss of body weight during 2 (DSS2d), 5 (DSS5d) and 7 (DSS7d) days. **(B)** Index of disease activity in DSS-induced ulcerative colitis. Effect of orally administered sodium dextran sultate on colon edema **(C)** diameter **(D)** of the distal colon and in the total length of the colon **(E)**. Data presented as mean ± S.E.M. (n = 10 per group) (* $P < 0.05$, ** $P < 0.005$, **** $P < 0.0001$, ANOVA followed by the Tukey test).

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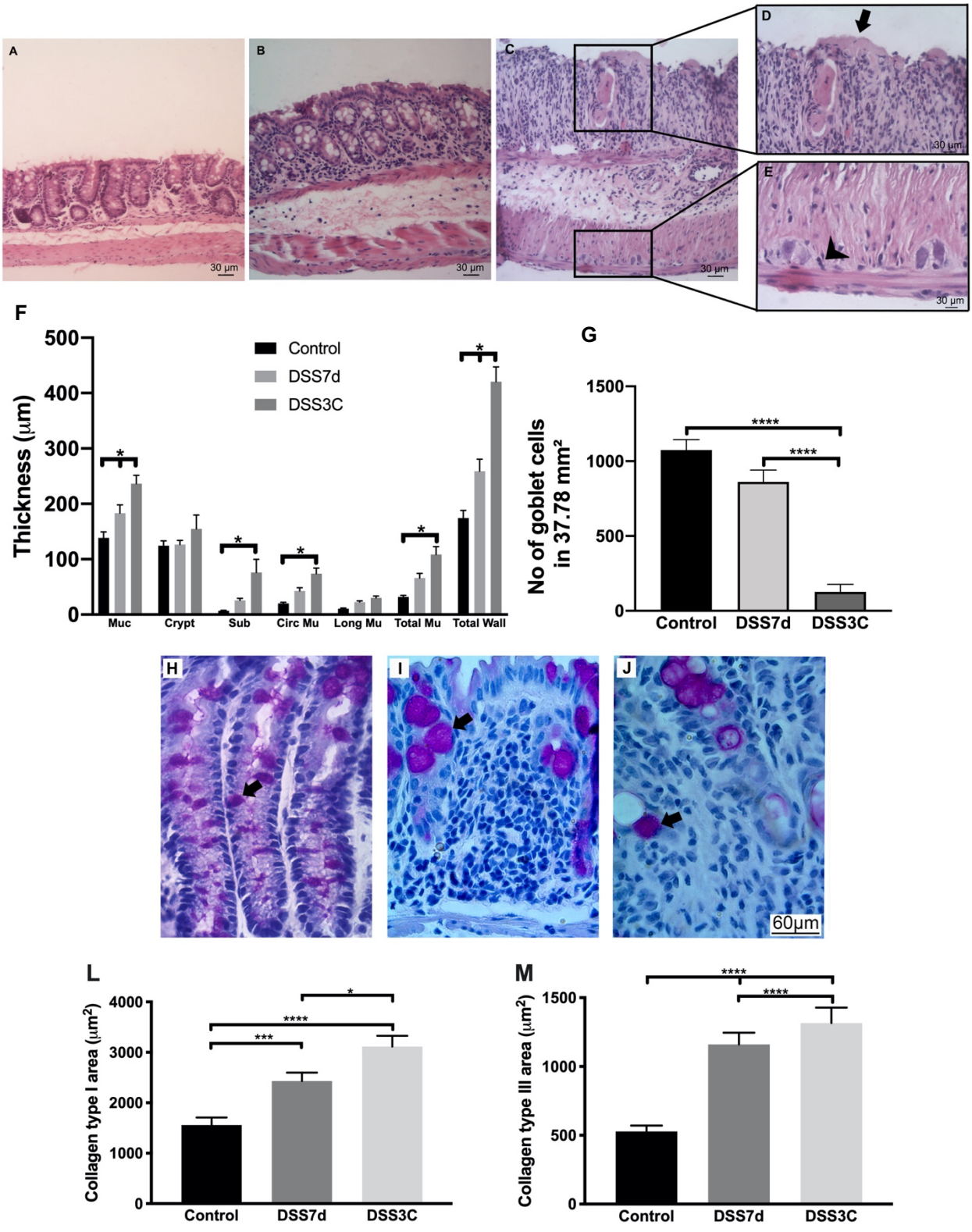

Figure 5 Photomicrograph of histological sections stained by H&E. Groups: Control **(A)** DSS7d **(B)** and DSS3C **(C)**. **(D)** The arrow shows a rupture of the intestinal epithelium. **(E)** arrowhead - Inflammatory infiltrate within the ganglion. **(F)** Graph representing the distal colon's analysis of wall. Mucosa thickness (Muc), crypt depth (crypt), submucosa (sub), circular muscle (Circ. Mu.), Longitudinal muscle (Long. Mu.), Total muscle (Total Mu.) were analyzed. Histological sections of the distal colon stained with PAS from mice in the control group (**H)** and exposed to the DSS for 7 (DSS7d) days (**I)** and the group with chronic colitis (DSS3C) (**J)** and in (**G)** Graph representative. Arrow points to goblet cells. Type I **(L)** and III (**M)** collagen quantification using Sirius Red staining. Data presented as mean ± S.E.M. (n = 5 per group) (* $P < 0.05$, *** $P < 0.005$, **** $P < 0.0001$, ANOVA followed by the Tukey test).

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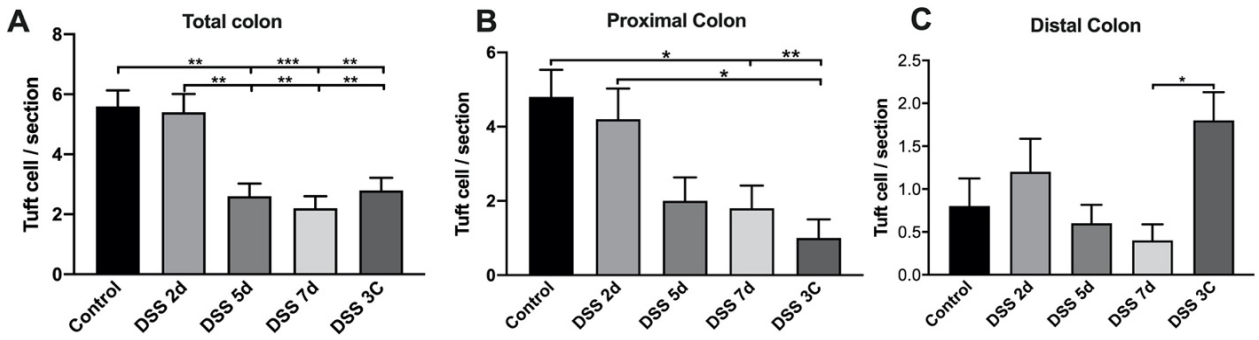

Figure 6 (A) Number of tuft cells in mice's total colon exposed to DSS for 2 (DSS2d), 5 (DSS5d), 7 (DSS7d) days and also in the group induced to chronic ulcerative colitis (DSS3C) and number of tuft cells in the proximal **(B)** and distal **(C)** colon. Data presented as mean ± S.E.M. (n = 5 per group) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. ANOVA followed by the Tukey test).

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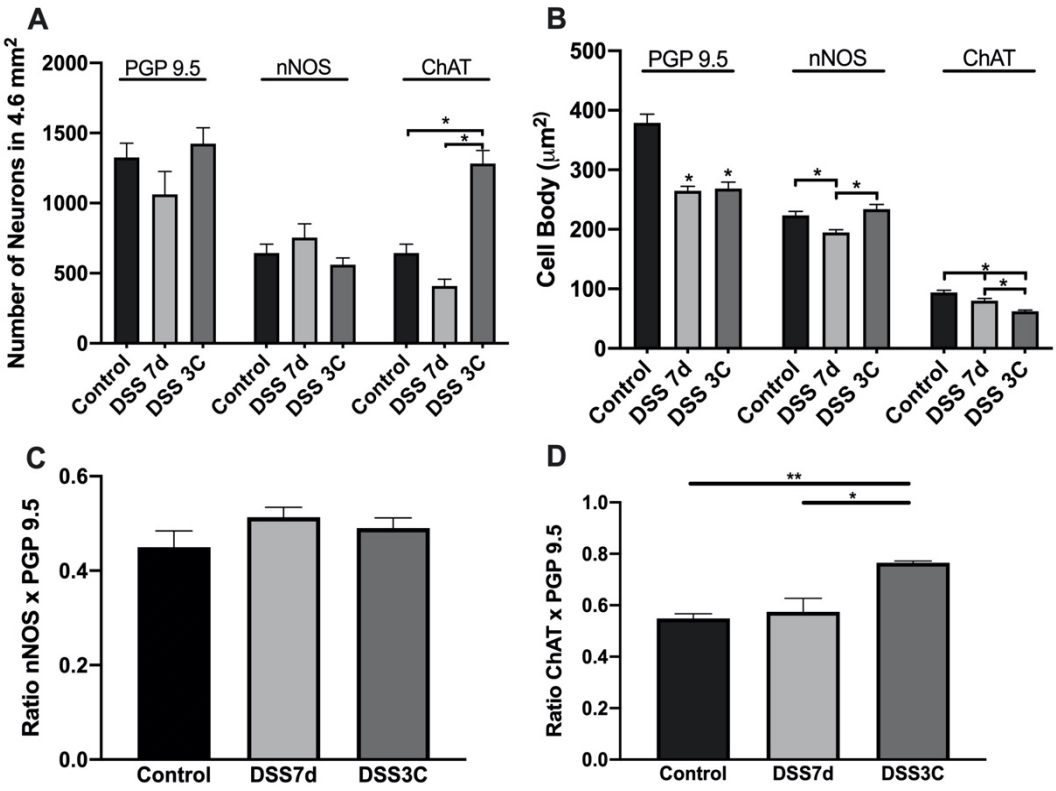

Figure 9 Representative graph of neuronal distribution **(A)** and morphometry **(B)** of neurons of general population (PGP 9.5), nitrergic (nNOS) and cholinergic (ChAT) positive. **(C)** ChAT x PGP 9.5 ratio and **(D)** nNOS x PGP 9.5 ratio of control mice and exposed to DSS for 7 (DSS7d) days and group with chronic colitis (DSS3C). Data presented as mean ± S.E.M. (n = 5 per group) (* $P < 0.05$, ** $P < 0.01$, ANOVA followed by the Tukey test).

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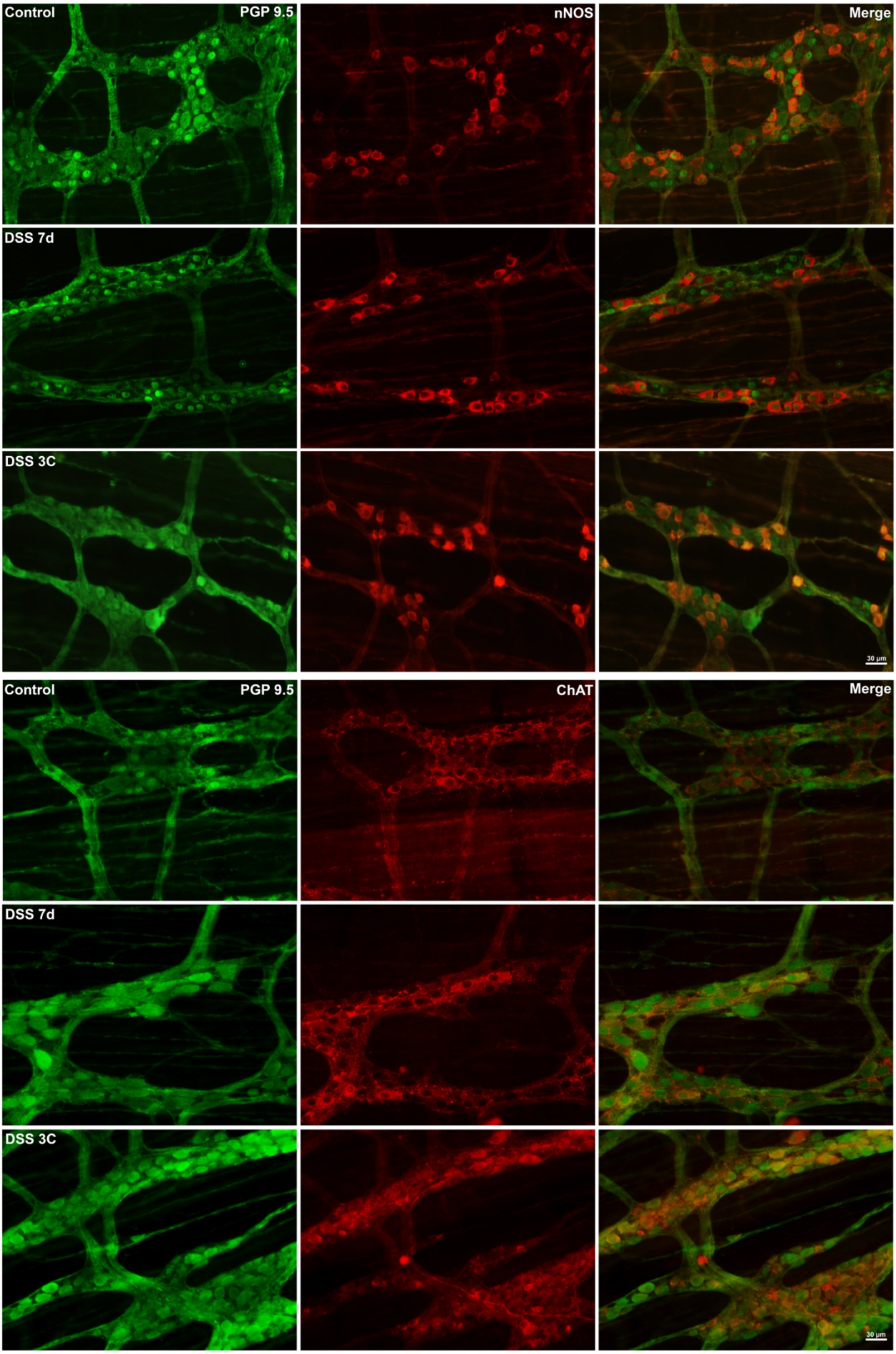

Figure 10 Representative photomicrograph showing the PGP 9.5 +, nNOS + and ChAT + neurons of control group and groups exposed to DSS for 7 days (DSS7d) and chronic colitis (DSS3C).

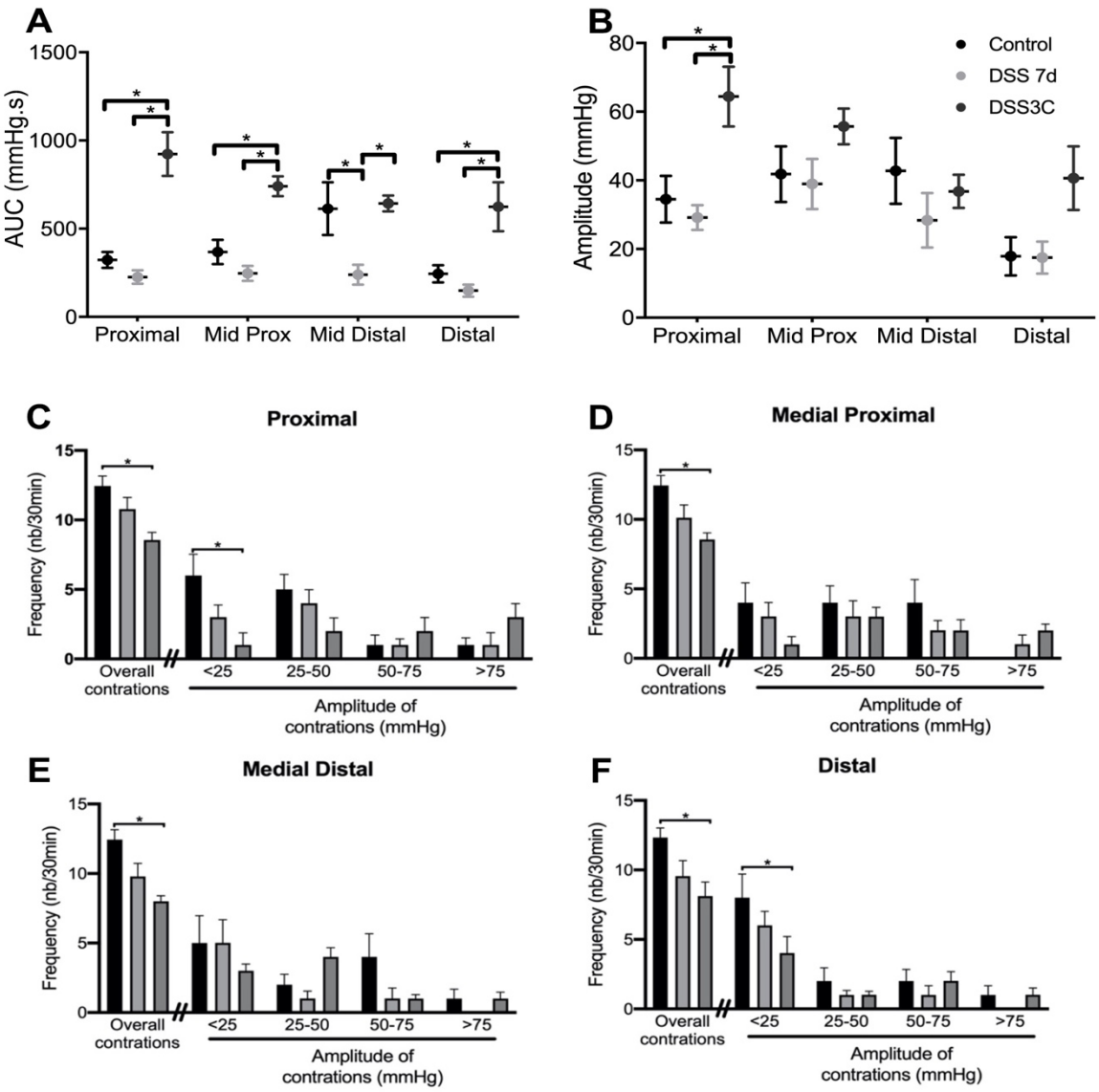

Figure 12 Representative graph of the colonic manometry, comparing control group and groups exposed to the DSS for 7 (DSS7d) days and with chronic colitis (DSS3C). Analyzed parameters: Amplitude of contractions **(A)**, area under the curve (AUC) **(B)** and frequency [number (Nb)] that spontaneous contractions occurred, quantified in four categories in the proximal colon **(C)**, Proximal Medium **(D)**, Medial Distal **(E)** and Distal **(F)**. Data presented as mean ± S.E.M. (n = 10 per group) (* $P < 0.05$, ANOVA followed by the Tukey test).

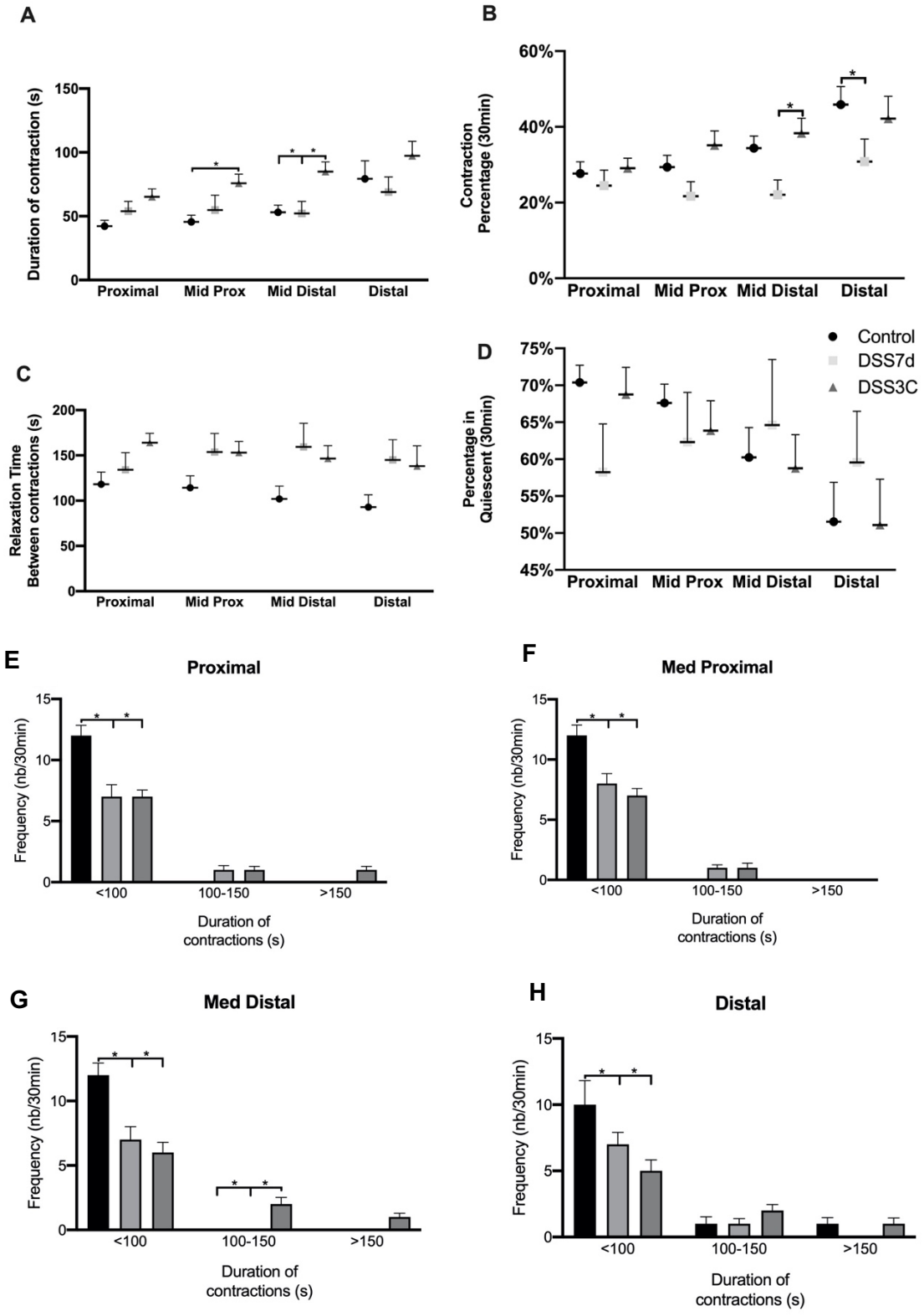

Figure 14 Representative graph of the colonic manometry, comparing control group and groups exposed to the DSS for 7 (DSS7d) days and with chronic colitis (DSS3C). The duration of spontaneous contractions **(A)**. The percentage of time the colon remained contracted **(B)**. **(C)** the relaxation time between contractions and the percentage of time the colon remained quiescent **(D)** was calculated. The duration of contractions were categorized and quantified [number (Nb) in the proximal **(E)**, middle proximal **(F)**, distal **(G)** and distal **(H)** colon. Data presented as mean ± S.E.M. (n = 10 per group) (* $P < 0.05$, ANOVA followed by the Tukey test).

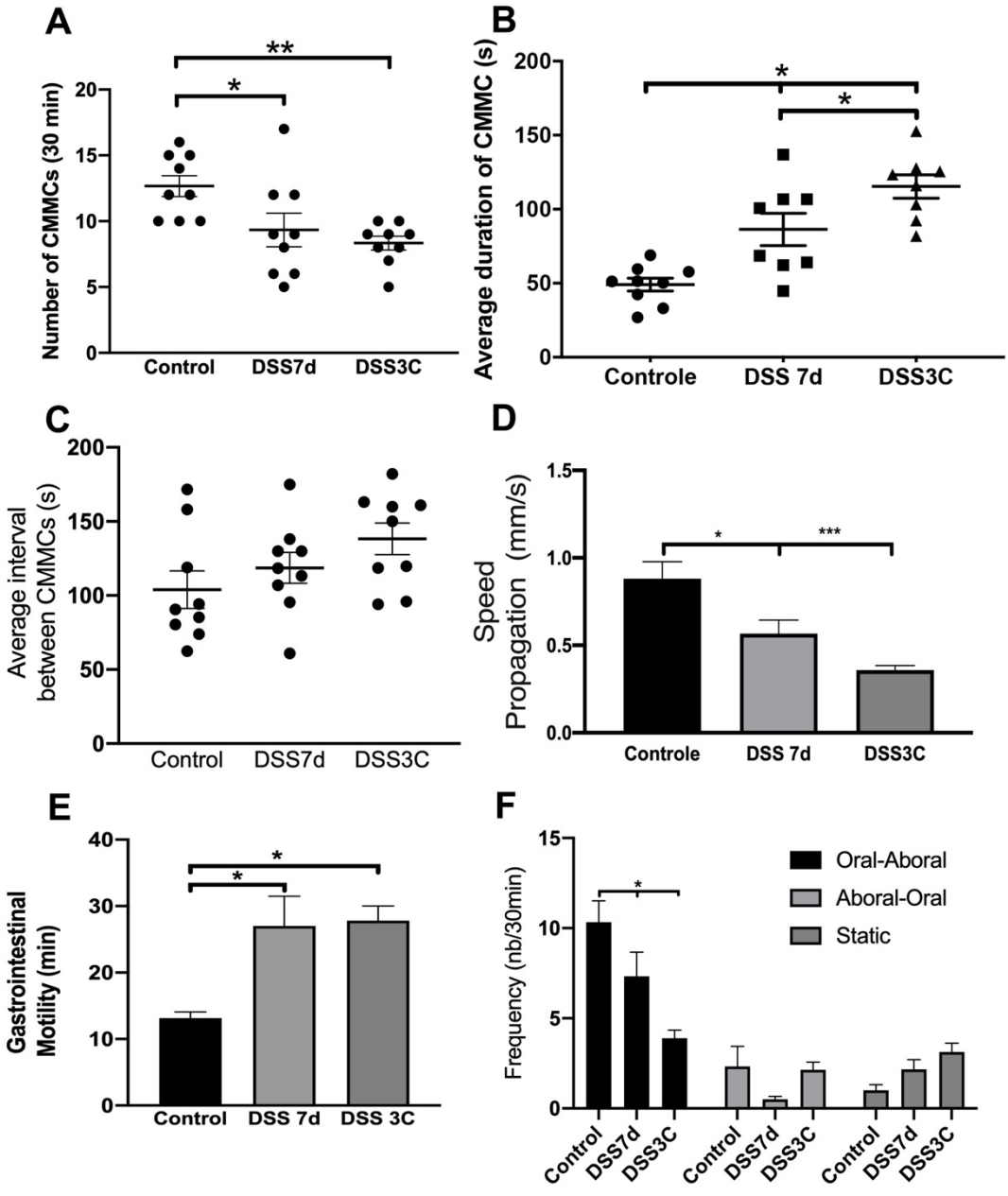

Figure 15 Colonic migratory motor complex (CMMC) profile in mice with acute (DSS7d) and Chronic (DSS3C) colitis. **A:** Plot showing the CMMCs number in 30min. **B:** Average time duration of each CMMC. **C:** Average quiescent time (s) between CMMCs. **D:** Classification of direction and Number (Nb) of CMMCs in 30min. Data presented as mean ± S.E.M. (n = 10 per group) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$, ANOVA followed by the Tukey test).

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4. CONSIDERAÇÕES FINAIS

O uso de DSS foi eficiente para indução química da RCU, tanto no modelo agudo quanto crônico. A inflamação foi responsável pela diminuição proporcional no número de células tuft, além der promove alterações histológicas na parede do cólon e depleção no número de células caliciformes e intensa fibrose. Também foi observado que não ocorre perda neuronal neste modelo, no entanto há uma mudança no código químico dos neurônios mioentéricos, aumentando os neurônios ChAT+ nos animais com colite crônica. Todavia os neurônios mioentéricos estavam atrofiados.

No que tange os estudos de motilidade colônica, encontramos algumas alterações promovidas pela inflamação como: redução no número de contrações; aumento na força das contrações, especialmente na porção proximal e distal; redução no número e aumento na duração dos CMMCs. Sugerimos que essas alterações no padrão de motilidade fazem parte da adaptação do órgão, sendo evidente que na colite crônica estas alterações de motilidade são mais intensas.

Diante de todos os resultados aqui obtidos, acreditamos que futuros experimentos devam ser realizados de modo a entender melhor o papel de outras células neste modelo de colite induzida por DSS na fase aguda e cônica, como por exemplo analisar as alterações neuronais no plexo submucoso e correlacionando com possíveis alterações fisiológicas na secreção do epitélio por meio da utilização de sistemas de Ussing,

Também seria importante testar alguma estratégia, sendo ela farmacológica ou biológica, de modo a aumentar a expressão de células tufts tanto antes quanto depois da indução da colite, estimulando assim uma melhora da barreira epitelial, amenizando inflamação intestinal, podendo então reduzir os efeitos deletérios sobre a motilidade colônica. Nesta mesma questão, estudos de motilidade colônica seriam de extrema importância utilizando fármacos no banho de órgãos e assim investigar fármacos com potencial de reestabelecer a motilidade colônica.

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ANEXOS

A

Aprovação da Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) processo 4023.2017.31.

Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 48/2017 **Londrina, 05 Maio de 2017.**

Prezado Profº.

Certificamos que o projeto intitulado: **" Efeitos da fototerapia durante a colite experimental crônica induzida por sulfato sódico de dextrana (DSS) em camundongos"** protocolo CEUA **nº4023.2017.31**, sob a responsabilidade de **Eduardo José de Almeida Araujo**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **02/05/2017**

O Objetivo do projeto é avaliar o potencial anti-inflamatório da luz com comprimento de onda na faixa do infravermelho (próximo a 940 nm) sobre a colite crônica induzida pela administração do DSS em camundongos. Grau de invasividade =2

Vigência do Projeto	01/06/2017 a 28/02/2020
Espécie/linhagem	Camundongos Isogênico (C57Bl/6)
Nº de animais	156
Peso/Idade	20-25 g / 2 meses
Sexo	Machos
Origem	Biotério da UEL/ CCB
Amostras a serem coletadas	Cólon

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, 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 necessária. Sem mais para o momento, subscrevo, cordialmente,

Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coordenadora da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Eduardo José de Almeida Araujo
Responsável pelo projeto
Departamento de Histologia/CCB.

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