UNIVERSIDADE SÃO FRANCISCO
Programa de Pós-Graduação *Stricto Sensu* em Ciências da Saúde

JOSÉ AIRES PEREIRA

AVALIAÇÃO DOS EFEITOS PROTETORES DO SUCRALFATO NO CONTEÚDO TECIDUAL DAS PROTEÍNAS E-CADERINA, β-CATENINA, CLAUDINA-3, OCLUDINA, ATIVIDADE ANTI-INFLAMATÓRIA E ANTIOXIDANTE NA MUCOSA CÓLICA DESPROVIDA DE TRÂNSITO INTESTINAL

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Tese apresentada ao Programa de Pós-graduação *Stricto Sensu* em Ciências da Saúde da Universidade São Francisco, como requisito parcial para obtenção do Título de Doutor em Ciências da Saúde.

Área de Concentração: Biologia Celular e Molecular

Orientador: Prof. Dr. Carlos Augusto Real Martinez

Bragança Paulista
2016

WI 522 Pereira, José Aires.

P492a Avaliação dos efeitos protetores do sucralfato no conteúdo tecidual das proteínas E-caderina, β-catenina, claudina-3, ocludina, atividade anti-inflamatória e antioxidante na mucosa cólica desprovida de trânsito intestinal /José Aires Pereira . -- Bragança Paulista, 2016.
68 p.

Tese (doutorado) – Programa de Pós-Graduação Stricto Sensu em Ciências da Saúde da Universidade São Francisco.

Orientação de: Carlos Augusto Real Martinez.

Ficha catalográfica elaborada pelas bibliotecárias do Setor de Processamento Técnico da Universidade São Francisco.

PEREIRA, A., José, "Avaliação dos efeitos protetores do sucralfato no conteúdo tecidual das proteínas E-caderina, β- catenina, claudina-3, ocludina, atividade anti-inflamatória e antioxidante na mucosa cólica desprovida de trânsito intestinal ". Tese defendida e aprovada no programa de Pós Graduação *Stricto Sensu* em Ciências da Saúde da Universidade São Francisco em 18 de fevereiro de 2016 pela Banca examinadora constituída pelos professores:

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DEDICATÓRIA

Ao meu pai, **EROTIDES PEREIRA** (*in memoriam*) aquele me ensinou o significado da palavra gratidão e honestidade e à minha mãe **PERGENTINA PEREIRA** pelo amor e dedicação em todos os momentos da minha vida.

Aos meus **irmãos**, pelos incentivos em todos os momentos dessa caminhada.

À minha esposa **Gladis** pela compreensão e ajuda nas horas difíceis.

Às minhas filhas, **Geovanna e Poliana**, a razão de tudo.

AGRADECIMENTOS

Primeiramente agradeço á **DEUS** pela minha vida e pela possibilidade da realização dessa pós-graduação.

Ao **PROFESSOR DOUTOR CARLOS AUGUSTO REAL MARTINEZ**, a minha eterna gratidão pela orientação nesse trabalho e pelos outros feitos.

Aos professores do **PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE DA UNIVERSIDADE SÃO FRANCISCO, BRAGANÇA PAULISTA,** pelas orientações para a realização deste.

Aos acadêmicos do Curso de Medicina, em especial aos acadêmicos **MURILO ROCHA RODRIGUES** e **DANIELA TIEMI SATO** pela dedicação em todos os momentos desse experimento.

Aos funcionários do **BIOTÉRIO CENTRAL DA UNIVERSIDADE SÃO FRANCISCO**, pela dedicação e os cuidados que tiveram com os animais durante todo o período do experimento.

À Pós-graduanda **CAMILA DE MORAIS GONÇALVES SILVA**, pela disponibilidade e cooperação fundamental nos ensaios bioquímicos do presente estudo.

Meu especial agradecimento para **VILMA APARECIDA TESCKE** e **TATINE MARTINS DE OLIVEIRA** secretárias da Pós-graduação pelas orientações dadas durante o desenvolvimento deste trabalho.

À **UNIVERSIDADE SÃO FRANCISCO**, os meus agradecimentos pela oportunidade de permitir mais uma realização profissional dentre outras.

À **FUNDAÇÃO DE AMPARO A PESQUISA DO ESTADO DE SÃO PAULO (FAPESP)**, pelo apoio financeiro para a realização do presente estudo.

Enfim, a todos aqueles que de alguma forma se fizeram presentes e me ajudaram nessa caminhada, os meus sinceros agradecimentos.

‘‘A mente que se abre a uma nova ideia jamais voltara ao seu tamanho original’’.

Albert Einstein

RESUMO

As junções intercelulares representam um dos principais constituintes da barreira epitelial cólica e podem estar danificadas na colite experimental. No epitélio do cólon são descritas três tipos principais de junções intercelulares, representadas pelas junções de oclusão, aderência e comunicantes. As junções de oclusão e aderências são as mais importantes para assegurar a manutenção da barreira epitelial. As junções de oclusão do epitélio cólico são formadas pelas proteínas da família das claudinas (principalmente a claudina-3) e ocludina, enquanto as junções aderentes pelas proteínas E-caderina e β-catenina. Estudos mostraram que existe redução no conteúdo dessas proteínas em modelo experimental de colite de exclusão (CE). O sucralfato (SCF) vem sendo utilizado há várias décadas para o tratamento de diferentes formas de colite. A substância possui um efeito protetor sobre a mucosa cólica, melhora a cicatrização epitelial, além de apresentar propriedades anti-inflamatórias e antioxidantes. Os efeitos do SCF na manutenção do conteúdo tecidual das proteínas existentes nas junções de oclusão e de aderência do epitélio intestinal nunca foram avaliados em modelos de CE. **Objetivo:** Avaliar os efeitos protetores da aplicação tópica do sucralfato no conteúdo tecidual das proteínas claudina-3, ocludina, E-caderina, β-catenina, atividade anti-inflamatória e antioxidante na mucosa cólica sem trânsito intestinal. **Método:** Trinta e seis ratos foram submetidos ao desvio do trânsito intestinal por colostomia proximal e fístula na mucosa distal. Os animais foram divididos em três grupos após terem recebido enemas diários com solução fisiológica (SF), SCF 1,0 g/kg/dia ou 2,0 g/kg/dia. Cada um desses grupos experimentais foram divididos em dois subgrupos, a eutanásia foi realizada após 2 e 4 semanas. A presença de colite foi diagnosticada após análise histológica. A intensidade do processo inflamatório tecidual foi avaliada utilizando escala de inflamação previamente validada. O infiltrado neutrofílico foi avaliado pelo conteúdo tecidual de mieloperoxidase (MPO) identificada por imunohistoquímica. As proteínas claudina-3, ocludina, E-caderina, β-catenina e MPO foram identificadas por técnica imunohistoquímica utilizando-se anticorpos monoclonais específicos para cada uma. O conteúdo tecidual de todas as proteínas foi quantificado por análise de imagem assistida por computador, e o valor final atribuído para cada animal foi representado pelos valores médios após a análise de três campos histológicos distintos e expressos em porcentagem/campo (%/campo). Para comparar resultados obtidos entre os grupos utilizou-se o teste de Mann-Whitney e, para análise de variância com relação ao tempo de intervenção, o teste de Kruskal-Wallis, adotando-se nível de significância de 5% ($p<0,05$). **Resultados:** A intervenção do cólon excluso de trânsito intestinal com SCF, na concentração de 2,0 g/kg/dia por 4 semanas, diminuiu o escore inflamatório e mostrou-se relacionada a concentração utilizada e ao tempo de intervenção. A intervenção com SCF, em ambas concentrações utilizadas aumentou o conteúdo das proteínas E-caderina, β-catenina, claudina-3 e ocludina e reduziu o conteúdo de MPO e MDA independentemente da concentração utilizada. A redução do escore inflamatório variou com o tempo de intervenção e com a concentração de SCF utilizada. Não houve variação no conteúdo tecidual das demais proteínas estudadas com o tempo de intervenção. **Conclusões:** A aplicação de enemas com SCF reduz o escore inflamatório e o infiltrado neutrofílico, e preserva o conteúdo tecidual das proteínas E-caderina, β-catenina, claudina-3 e ocludina sugerindo que a substância possua efeito protetor das junções intercelulares em modelo de colite de exclusão.

Palavras Chaves: Cólon; Colite Experimental; Junções Intercelulares; Moléculas de Adesão Celular; Junções oclusivas; Junções aderentes; Mieloperoxidase; Malondialdeído; Análise de Imagem Assistida por Computador.

ABSTRACT

The intercellular junctions represent one of the major constituents of the colonic epithelial barrier and may be damaged in experimental colitis. In the colonic epithelium there are three main types of intercellular junctions, represented by tight junctions, adherents junctions and gap junctions. Tight junctions and adherents junctions are the most important to ensure the selective permeability of the epithelial barrier. Tight junctions of the colonic epithelium are formed by protein's of the claudin family (mainly claudin-3) and occludin, while the adherents junctions by E-cadherin and β-catenin proteins. Studies have shown that there is a reduction in the tissue content of protein in experimental model's of diversion colitis. Sucralfate (SCF) has been used for several decades for the treatment of different forms of colitis. The substance has protective effect on the colonic mucosa, improves epithelial healing, besides presenting anti-inflammatory and antioxidant properties. The effects of the SCF in maintaining the tissue contents of the proteins existing in the tight and adherents junctions of the intestinal epithelium was not assessed yet in experimental models of diversion colitis. Objective: To evaluate the effects protectors of topical application of sucralfate in the tissue content of claudin-3, occludin, E-cadherin, β-catenin, anti-inflammatory and antioxidant activity in the colonic mucosa without fecal stream. Method: Thirty-six rats were under went to deviation of the fecal stream transit through proximal colostomy and distal mucous fistula. The animals were divided into three experimental groups according having received daily enemas with 0.9% saline, 1.0 SCF / kg / day or 2.0 g / kg / day. Each of the experimental group was divided into two subgroups, according to the euthanasia be performed after 2 or 4 weeks. The presence of colitis was diagnosed after histological analysis. The intensity of tissue inflammation was measured using previously validated scale of inflammation. The neutrophilic infiltrate was evaluated by the tissue content of myeloperoxidase (MPO) identified by immunohistochemistry technique. Claudin-3, occludin, E-cadherin, β-catenin and MPO proteins' were identified by immunohistochemistry technique using primary specific monoclonal antibodies for each one. Tissue contents' of all proteins were quantified by image assisted computer-analysis, and the final value assigned to each animal was represented by the mean values after analysis of three distinct histological fields and expressed in percentage/field (% / field). To compare the results among the experimental groups we used the Mann-Whitney test and for analysis of variance with respect to time of intervention the Kruskal-Wallis test, adopting for both tests a significance level of 5% ($p < 0.05$). Results: The intervention with SCF of the colon without fecal stream at a concentration of 2.0 g / kg / day for 4 weeks, decreased the inflammatory score and was related to the concentration used and time of intervention. The intervention with SCR, used in both concentrations increased content of the protein E-cadherin, β-catenin, claudin and occludin-3 and reduced MPO content irrespective of the concentration and MDA used. The inflammatory score showed reduction of the values with the time of intervention and with the concentration of the SCF used. There was no change in the tissue content of all other proteins studied with the intervention time. Conclusions: Enemas with SCF reduces the inflammatory score and neutrophilic infiltrate and preserves tissue content of the proteins E-cadherin, β-catenin, claudin-3 and occludin suggesting that the substance has protective effect of intercellular junctions in experimental model of diversion colitis.

Key words: Colon; Experimental colitis; Intercellular Junctions; Cell Adhesion Molecules; Tight Junctions; Adherens Junctions; Peroxidase; Image Processing Computer-Assisted.

LISTA DE SÍMBOLOS E ABREVIATURAS

β	Beta
Ca	Cálcio
cm	Centímetro
F	French (medida que se usa para expressar calibre de cateter)
ºC	Graus centígrados
µL	Microlitro
µm	Micrômetro
mL	Mililitro
nº	Número
%	Por cento
V	Volume
5-ASA	Ácido 5-aminosalicílico
AGCC	Ácidos Graxos de Cadeia Curta
APC	*Adenomatous polyposis coli*
BHT	Hidroxitolueno butilado
CE	Colite de exclusão
CEUA	Comitê de Ética no Uso de Animal em Pesquisa
COBEA	Colégio Brasileiro de Experimentação Animal

COX-1	Ciclo-oxigenase 1
COX-2	Ciclo oxigenase 2
DAB	Diaminobenzidina
DII	Doença inflamatória intestinal
DSS	Sulfato de dextrano
EDTA	Etileno-diamino-tetra-acético
EGF	Fator de crescimento epitelial
Et al	E colaboradores
G	Grama
H	Hora
HCL	Ácido clorídrico
H.E	Hematoxilina e Eosina
H2O2	Peróxido de hidrogênio
iNOS	Ácido nítrico sintase (induzível)
Kg	Quilograma
LIM	Laboratório de Investigação Médica
LIM - USF	Laboratório de Investigação Médica da Universidade São Francisco
MDA	Malondialdeído
mm	Milímetro

MPO	Mieloperoxidase
NF-kB	Fator nuclear Kappa B
OH	Radical de Hidroxila
PBS	Tampão fosfato de sódio
PGDF	Fator de crescimento derivado de plaquetas
PGE	Prostaglandina
PGE-2	Prostaglandina E2
P.M.	Peso molecular
RA	Retite actínica
RCUI	Retocolite ulcerativa inespecífica
RLO	Radicais livres de oxigênio
SCF	Sucralfato
SF	Solução fisiológica
TBARS	Ácido tiobarbitúrico
TGF	Fator de crescimento transformador
TNBS	Ácido trinitrobenzeno sulfônico
TRIS HCL	(hydroxymethyl)aminomethane·hydrochloride
Wnt	Gene Wnt (Via de sinalização)

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SUMÁRIO

1. INTRODUÇÃO

Glotzer et al. (1981) chamaram a atenção, pela primeira vez, que o processo inflamatório que se desenvolve nos segmentos cólicos exclusos de trânsito intestinal denominado pelos autores de colite de exclusão (CE) – surge em decorrência da falta do suprimento regular de ácidos graxos de cadeia curta (AGCC) para as células epiteliais da mucosa cólica (GLOTZER et al., 1981). Estudos clínicos e experimentais mostraram que os AGCC representam o principal substrato energético utilizado pelas células especializadas do epitélio intestinal (BOSSHARDT e ABEL, 1984; AGARWAL e SCHIMMEL, 1989; GERAGHTY e TALBOT, 1991; MORTENSEN e CLAUSEN, 1996; COOK e SELLIN, 1998). Esses resultados foram confirmados quando demonstrou-se que o fornecimento de soluções nutricionais ricas em AGCC ao cólon excluso, o restabelecimento do trânsito fecal e o transplante autólogo de fezes associado a oferta conjunta de butirato revelaram-se estratégias capazes de regredir o processo inflamatório intestinal e cicatrizar o epitélio cólico danificado (SCHEPPACH et al., 1997; OLIVEIRA NETO e AGUILAR-NASCIMENTO, 2004; NASRRI et al., 2008; PACHECO et al., 2012; GUNDLING et al., 2015). Todavia, os mecanismos moleculares pelos quais a deficiência no suprimento de AGCC leva ao surgimento da CE permanecem controversos.

O epitélio do intestino grosso é uma das mais perfeitas barreiras funcionais do ser vivo, separando o interior do lúmen intestinal, que concentra grande população bacteriana, do meio interno estéril (PEREIRA et al., 2013; CHAIM et al., 2014; BONASSA et al., 2015). Essa barreira epitelial é formada por uma série de estruturas celulares que conferem proteção mecânica e por células de defesa do sistema imune que constituem uma barreira funcional imunológica. Esses dois sistemas atuando em conjunto são responsáveis pela separação entre os meios interno e externo do corpo humano impedindo que agentes bacterianos e outras toxinas presentes no lúmen intestinal migrem para o meio interno ocasionando bacteremia e, até mesmo, septicemia (COSKUN, 2014). Hoje em dia admite-se que o processo inflamatório que ocorre na parede intestinal em portadores de doenças inflamatórias intestinais (DII) surge a partir da quebra desse sistema de defesa epitelial.

A barreira de proteção mecânica é formada por diferentes linhas de defesa, principalmente representadas pela camada de muco que recobre o epitélio cólico, membranas citoplasmáticas das células epiteliais da mucosa cólica, o complexo sistema de junções intercelulares formados pelas junções de oclusão, adesão e comunicação e, finalmente, pela membrana basal (MARTINEZ et al., 2010). Por outro lado, a proteção funcional é conferida por células do sistema imune representadas por neutrófilos, eosinófilos, basófilos, linfócitos e macrófagos, bem como, por citocinas anti-inflamatórias e

anticorpos abundantes na parede cólica. Esse exuberante sistema imunológico de defesa faz com que o cólon seja considerado o maior órgão linfóide do ser humano (Fig. 1).

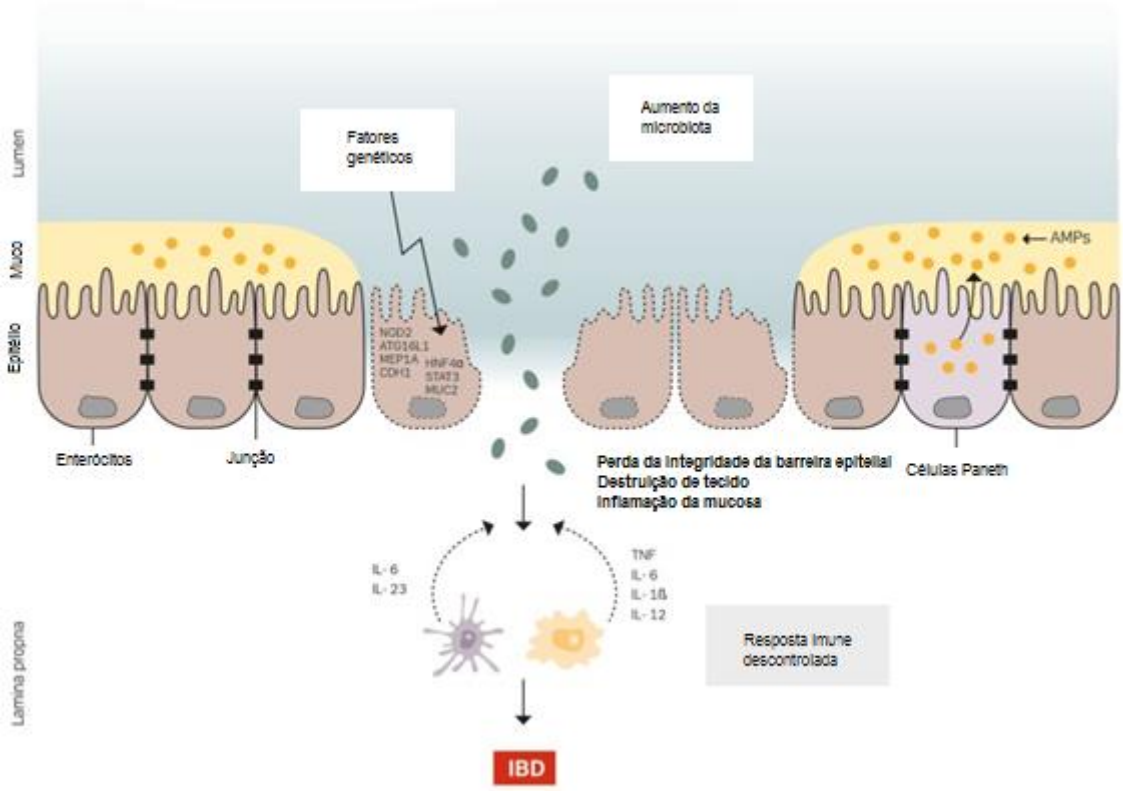

Figura 1 – Quebra da barreira epitelial na fase de iniciação da inflamação da mucosa intestinal. Fonte: Figura retirada de: COSKUN (2014, pag.2)

Uma série de estudos experimentais realizados ao longo dos últimos 10 anos pelo nosso grupo no Laboratório de Investigação Médica da Universidade São Francisco (LIM-USF) mostrou que esses mecanismos de defesa mecânica se encontram danificados na mucosa cólica exclusa de trânsito intestinal (SOUSA et al., 2008; NONOSE et al., 2009; MARQUES et al., 2010; MARTINEZ et al., 2010a; MARTINEZ et al., 2010b; CALTABIANO et al., 2011; CUNHA et al., 2011; LAMEIRO et al., 2011; ALMEIDA et al., 2012; MARTINEZ et al., 2012; KADRI et al., 2013; CHAIM et al., 2014; BONASSA et al. 2015; MARTINEZ et al., 2015).

Com o objetivo de contribuir para a elucidação dos mecanismos moleculares que ocasionam a quebra da barreira epitelial cólica na CE nosso grupo, numa sequência de estudos, verificou-se que as células da mucosa cólica desprovidas do suprimento normal de AGCC sofrem alterações metabólicas na cadeia respiratória tendo como consequência a

maior produção de radicais livres de oxigênio (RLO) (MARTINEZ et al., 2010b). Quando a produção desses RLO excede a capacidade dos sistemas antioxidantes em neutralizá-los existe o desenvolvimento de uma condição patológica conhecida como estresse oxidativo (MARTINEZ, 2010). O excessivo contingente de RLO produzidos a partir de células da mucosa cólica exclusa de trânsito oxidam diferentes substâncias constituintes células epiteliais, danificando-as e ocasionando quebra dos diferentes mecanismos estruturais de defesa (PRAVDA, 2005; NONOSE et al., 2009; MARTINEZ et al., 2010b; MARTINEZ et al., 2010b; MARTINEZ et al., 2012; KADRI et al., 2013). Resumidamente, os resultados de todos esses estudos mostram que o estresse oxidativo reduz e modifica a produção de mucinas que recobrem o epitélio cólico (NONOSE et al., 2009; MARTINEZ et al., 2010b), danificam as membranas citoplasmáticas das células epiteliais (MARQUES et al., 2010), rompem as junções aderentes intercelulares formadas pelas proteínas E-caderina (KADRI et al., 2013) e β-catenina (MARTINEZ et al., 2012), e as proteínas constituintes das junções de oclusão representadas por proteínas da família das claudinas, particularmente a claudina-3, e a ocludina (MARTINEZ et al., 2015a). Recentemente demonstrou-se que a ruptura desses mecanismos de adesão intercelular do epitélio intestinal relaciona-se ao estresse oxidativo e a maior presença de infiltrado neutrofílico (MARTINEZ et al., 2015b).

As evidências de que os RLO são as moléculas responsáveis pelo dano ao epitélio cólico e pelo desenvolvimento da CE foram reforçadas por outra série de estudos onde nosso grupo mostrou que a aplicação tópica no cólon excluso de trânsito intestinal de substâncias com atividades antioxidantes, sintéticas como a n-acetilcisteína e o ácido 5-ASA, ou naturais como o extrato aquoso de *Ilex paraguariensis*, além de reduzirem a produção de RLO mostraram-se relacionadas à melhor cicatrização do epitélio cólico ulcerado e a redução do infiltrado inflamatório tecidual (CALTABIANO et al., 2011; CUNHA et al., 2011; MARTINEZ et al., 2013). Do mesmo modo, foram confirmados achados de outros autores mostrando que a aplicação de enemas com AGCC ou glutamina, restabelecendo o suprimento do substrato energético habitual, diminui a produção de RLO pelas células epiteliais melhorando as alterações inflamatórias características da CE (LAMEIRO et al., 2011; OLIVEIRA et al., 2010; PACHECO et al., 2012). Esses resultados foram recentemente contemplados com um editorial publicado em periódico internacional, chamando a atenção para a importância da manutenção do adequado suprimento energético à mucosa cólica, como estratégia válida para evitar o estresse oxidativo e prevenir o surgimento de diferentes formas de colite (HARTY, 2013).

Vários princípios ativos vêm sendo testados para o tratamento da CE. Essas substâncias geralmente apresentam efeitos anti-inflamatórios e antioxidantes que melhoram a inflamação epitelial (CALTABIANO et al., 2011; CUNHA et al., 2011; LAMEIRO et al., 2011; MARTINEZ et al., 2013). Todavia, a manutenção dessa atividade terapêutica, também

se encontra relacionada ao tempo de permanência em que a substância se mantém em contato com a superfície epitelial cólica inflamada. Assim sendo, a substância ideal para o tratamento da CE, além de possuir atividades anti-inflamatória e antioxidante deve ter como propriedade adicional a capacidade aderir-se firmemente à superfície epitelial ulcerada. Se possível essa substância ideal deveria ainda reduzir a apoptose celular no local inflamado e estimular fatores relacionados à reparação tecidual (PEREIRA et al., 2013).

Na procura por uma substância que apresentasse essas propriedades terapêuticas nos lembramos que há mais de duas décadas a aplicação de enemas contendo sucralfato (SCF), vem sendo utilizada como opção terapêutica para o tratamento tópico de diferentes tipos inflamações do trato digestivo (CARLING; KAGEBI; BORVALL,1986, SINGAL; ANAND, 1998, PIENKOWSKI et al., 1989). O SCF é o sal formado pelo dissacarídeo octosufalto de sacarose associado ao hidróxido de polialumínio (VOLKIN et al., 1993). A substância é considerada um complexo citoprotetor sendo inicialmente utilizada para prevenir ou tratar doenças do trato digestivo superior, principalmente representadas pelas doenças ulcerosas péptica, úlceras de estresse e lesões agudas da mucosa gástrica (SZABO,1991). Apesar do mecanismo de ação do SCF estar relacionado à sua grande capacidade de adesão às úlceras epiteliais decorrentes da agressão inflamatória, nos últimos anos estudos vem mostrando que essa ação citoprotetora do SCF no epitélio do tubo digestivo é mais complexa do que se supunha e está relacionada a diferentes mecanismos de ação (ROBERT et al., 1979; REES,1992). Constatou-se que o SCF aumenta a produção de prostaglandina E2 (PGE-2), estimula a produção de mucinas pelas células caliciformes, induz a maior produção local do fator do crescimento epitelial (EGF), incrementa a cicatrização epitelial, além de possuir atividade antibacteriana, anti-inflamatória e antioxidante (SLOMIANY et al., 1994; MASUELLI et al., 2010).

Kochhar et al. (1990) foram os primeiros autores a comprovar a eficácia da aplicação de clisteres contendo SCF no controle do sangramento retal decorrente da inflamação do intestino grosso após a aplicação de radioterapia para o tratamento de tumores localizados na próstata, colo do útero e reto (retite actínica). A partir de então, uma série de estudos confirmou os efeitos benéficos do SCF na melhora clínica, endoscópica e histológica nos portadores deste tipo de retite, passando-se a preconizar o uso da substância em outras formas de doenças inflamatórias intestinais que cursam com a formação de úlceras epiteliais (WRIGHT et al., 1999; DENTON et al., 2002; MATSUU-MATSUYAMA et al., 2006; HENSON, 2010; DEHGHANI et al., 2012; MENDENHALL et al., 2013). De modo análogo outra série de estudos avaliou a eficácia terapêutica do SCF em modelos de colite química ou fisicamente induzida (RILEY; GUPTA e MANI,1989; BJÖRCK et al., 1997; DEL VAL ANTOÑANA et al., 2002; MATSUU-MATSUYAMA et al., 2006). Entretanto, do melhor do nosso conhecimento, apenas três estudos, sendo tais, desenvolvidos no LIM-USF pelo

nosso grupo, mostraram que a aplicação de enemas contendo SCF reduz o processo inflamatório na mucosa cólica exclusa de trânsito fecal, bem como aumenta a produção dos diferentes subtipos de mucinas que recobrem a mucosa do cólon (PEREIRA et al., 2013; CHAIM et al., 2014; BONASSA et al., 2015). Esses achados sugerem que o SCF tenha efeito terapêutico benéfico para o tratamento da CE, pois, a substância além de reduzir a inflamação tecidual é capaz de aumentar a proteção conferida pela camada de muco que representa a primeira linha de defesa do epitélio cólico contra a agressão proveniente do lume intestinal.

Contudo, os efeitos do SCF na manutenção da integridade de outras estruturas componentes da barreira de defesa da mucosa cólica, ainda não foram estudados em modelos experimentais de CE. Não se conhece os efeitos do SCF sobre os sistemas de junções intercelulares, um dos principais componentes da barreira mecânica do epitélio cólico que podem ser danificadas pelos maiores níveis de estresse oxidativo existentes na mucosa cólica exclusa de trânsito intestinal. Da mesma forma, não se conhecem os efeitos anti-inflamatórios do SCF na mucosa cólica em modelos experimentais ou em portadores de CE. Quando se considera que o SCF possui boa capacidade adesiva sobre a superfície epitelial erodida, apresenta importante atividade antioxidante e é capaz de estimular a reparação tecidual, torna-se interessante avaliar a capacidade da substância na preservação da integridade das junções intercelulares do epitélio intestinal num modelo de CE. Caso a aplicação tópica de SCF seja capaz de preservar a integridade dessas junções, seu baixo custo e grande disponibilidade poderiam torná-lo alternativa interessante para o tratamento da CE.

2. REVISÃO DA LITERATURA

2.1 Colite e Estresse Oxidativo

A literatura atual vem dando importância crescente ao papel desempenhado pelos RLO na etiopatogenia de diversas doenças inflamatórias e neoplásicas (CADENAS e DAVIES, 2000; CETINKAYA et al., 2005; BERRA e MENK, 2006). Nos últimos anos pesquisas vem demonstrando, de maneira inequívoca, que os RLO encontram-se relacionadas à etiopatogenia das DII representadas, principalmente, pela colite ulcerativa (HENDRICKSON; GOKHALE; CHO, 2002; PRAVDA, 2005; BERRA e MENK, 2006). A relação entre RLO e DII vem despertando cada vez mais a curiosidade, fazendo com que novos estudos avaliem o papel representado pelos RLO nas etapas iniciais da agressão a mucosa intestinal em portadores das DII (MILLAR et al., 1996; SHERIDAN et al., 1996; SERIL et al., 2003; CETINKAYA et al., 2005; JENA; TRIVEDI; SANDALA, 2012; PARMAR; TRIVEDI; JENA, 2014; FATANI et al., 2015; PEREIRA et al., 2015).

Os RLO são constantemente formados durante o metabolismo energético das células vivas. Habitualmente, a célula possui defesas antioxidantes naturais, enzimáticas e não-enzimáticas, que atuam contra a toxicidade desses radicais sendo responsáveis pela manutenção do equilíbrio entre a produção e eliminação destes agentes oxidantes (SERIL et al., 2003; MARTINEZ, 2010). Todavia, em certas condições, quer pela diminuição da capacidade antioxidante do organismo, quer pelo aumento exagerado na produção de RLO, ocorre desequilíbrio determinando o aparecimento de fenômeno conhecido como estresse oxidativo (BERRA e MENK, 2006). Já se demonstrou que a capacidade dos sistemas antioxidantes do cólon são deficientes, quando comparada a outros órgãos e tecidos, determinando maior vulnerabilidade da mucosa cólica ao estresse oxidativo (THAM; WITHIN; COHEN, 2002). A possibilidade de colite ocasionada por RLO já é conhecida há vários anos, quando verificou-se que a infusão de peróxido de hidrogênio (H_2O_2), um potente formador de RLO, no interior do cólon e reto, era seguida por grave quadro de colite, histologicamente muito semelhante a RCUI e a CE (BILOTTA; WAYE, 1989, MARTINEZ, 2010).

Pravda, (2005) propôs a Teoria por Indução de Radicais *(Radical Induction Theory)* para explicar os fenômenos iniciais que precedem à infiltração inflamatória da mucosa intestinal em portadores de RCUI. Segundo o autor, a agressão inicial a mucosa desencadeadora da doença é atribuída à formação aumentada de RLO pela própria célula epitelial da mucosa com modificações em seu metabolismo energético. Essa produção aumentada de RLO, aliada as deficiências do sistema antioxidante intracelular, possibilita a formação exagerada do radical hidroxila $OH^{\cdot}$. O radical $OH^{\cdot}$ em excesso, difundindo-se pelo interior da célula, ocasiona lesão das diferentes linhas de defesa da barreira permitindo a migração de antígenos e bactérias

existentes no interior da luz intestinal para a intimidade da submucosa estéril. Na tentativa de combater esta infiltração bacteriana, leucócitos migram para o interior da parede intestinal, dando origem ao processo inflamatório encontrado na doença. Enzimas citolíticas liberadas pelo processo inflamatório, aliadas a maior produção de RLO decorrentes da resposta inflamatória, alterariam, ainda mais, a permeabilidade seletiva. Contudo, o próprio autor em seu artigo original, chamou a atenção para a dificuldade em demonstrar, com os modelos experimentais de colite habitualmente utilizados, as etapas iniciais de sua teoria. Nenhum modelo adotado para o estudo da RCUI era capaz de reproduzir, com fidelidade, as etapas iniciais da agressão à mucosa cólica. A maioria deles parte da agressão química ao epitélio mucoso, não levando em conta a possibilidade da formação aumentada de RLO, pela própria célula epitelial com metabolismo energético alterado, estar relacionada à agressão inicial. Nesses modelos a lesão da mucosa cólica é provocada pela infusão de substâncias químicas que agridem diretamente a mucosa destruindo a eficiente barreira epitelial. Desse modo, modelos que empregam a aplicação de clisteres contendo ácido acético, H_2O_2, sulfato de dextrana (DSS), ácido trinitrobenzeno sulfônico (TNBS) são limitados na sua capacidade de reproduzir a etapa inicial (produção aumentada de RLO pelas células epiteliais) que antecedem a infiltração leucocitária. Esses modelos não consideram evidências recentes de que a agressão inicial surge a partir de distúrbios metabólicos da própria célula epitelial da mucosa. Da mesma forma, por destruírem os mecanismos de adesão celular não permitem verificar se RLO formados pelas células do intestino excluso poderiam danificar as proteínas constituintes da barreira epitelial cólica. Foi por essa razão que neste estudo resolvemos utilizar o modelo de CE, pois teríamos a convicção que o dano às junções estaria acontecendo por RLO.

2.2 Colite de Exclusão e Estresse Oxidativo

A CE e a colite ulcerativa, apesar de serem entidades clínicas distintas quando se consideram a sintomatologia, achados endoscópicos, histopatológicos e as principais estratégias terapêuticas, apresentam várias semelhanças. Em ambas o doente queixa-se de sangramento retal, aumento da eliminação de muco e dor pélvica. Ao exame endoscópico a mucosa cólica é friável, edemaciada e apresenta formação ulcerações aftóides superficiais, com nítido apagamento dos vasos da submucosa e sangramento ao simples contato do colonoscópio (SOUSA et al., 2009). Ao exame histológico existe perda epitelial com formação de úlceras mucosas profundas, infiltrado inflamatório neutrofílico nas criptas e estroma circunvizinho e intensa congestão vascular na submucosa (SOUSA et al., 2009). Na CE é possível encontrar-se focos de hiperplasia linfóide que representa um dos achados mais característicos da doença (SOUSA et al., 2009; MARTINEZ, 2010). As semelhanças

entre a CE e a colite ulcerativa tornam-se ainda mais evidentes ao verificar-se que as estratégias terapêuticas propostas para ambas as doenças utilizam nutrientes ou drogas com atividade antioxidante. Essas evidências sugerem que a produção de RLO aumentada pela mucosa inflamada é um achado comum tanto na CE quanto na colite ulcerativa. Reforçam essas suspeita o fato de que a aplicação tópica de ácido 5-aminosalicílico (mesalazina) por meio de enemas, é estratégia eficaz para o tratamento das duas enfermidades (TRIANTAFILLIDIS et al., 1991; TRIPODI; GORCEY; BURAKOFF, 1992; MARSHALL et. al, 2012). As semelhanças ficam ainda mais claras com os resultados de estudos que mostraram que a aplicação de enemas contendo a associação de substâncias antioxidantes (como o ácido 5-aminosalicílico) e soluções ricas em AGCC (butirato) são mais eficazes na regressão da inflamação do epitélio cólico do que o uso isolado de uma ou outra substância (SONG; CHIA; LI, 2006).

Uma série de estudos confirmou que a CE é uma síndrome de deficiência nutricional, decorrente da interrupção crônica do fornecimento de AGCC, que compromete às células epiteliais da mucosa cólica (ROEDIGER, 1990; NEUT et al., 1995; MORTENSEN e CLAUSEN, 1996; SCHEPPACH et al., 1997; KIELY et al., 2001; NASRRI et al., 2008). Fortalecem essas suspeitas os resultados de estudos experimentais mostrando que o uso de inibidores da *β*-oxidação dos AGCC, interfere no metabolismo energético dos colonócitos e aumenta, consideravelmente, a produção tecidual de RLO, sugerindo que o estresse oxidativo resultante possa ser um dos eventos iniciais no aparecimento da CE (CHRISTL et al., 1996; LIU et al., 2001; HAMER et al., 2010; MARTINEZ et al., 2010, MARTINEZ et al., 2010a). Dessa maneira seria interessante utilizar um modelo de CE para confirmar essas suspeitas.

O modelo experimental de CE proposto para este trabalho é facilmente reprodutível em animais de pequeno porte tendo sido empregado anteriormente por outros autores (BIONDO-SIMÕES, 2000; MARGARIDO et al., 2003; NASRRI et al., 2008). Quando comparado aos modelos experimentais de colite induzida por drogas os modelos experimentais de CE, do ponto de vista metodológico, possuem maior paralelismo com o que acontece no homem. Neles, o processo inflamatório da mucosa e submucosa cólica surge espontaneamente a partir de deficiências no metabolismo celular, sem que haja a necessidade de danificá-lo, artificialmente, pela infusão de agentes químicos. Estudos anteriores validaram modelo de CE para avaliar as etapas iniciais da agressão à mucosa cólica (SOUSA et al., 2008; NONOSE et al., 2009; MARQUES et al., 2010; MARTINEZ et al., 2010a; MARTINEZ et al., 2010b; CALTABIANO et al., 2011; CUNHA et al., 2011; LAMEIRO et al., 2011; ALMEIDA et al., 2012; MARTINEZ et al., 2012; KADRI et al., 2013; CHAIM et al., 2014; BONASSA et al. 2015; MARTINEZ et al., 2015). Nos modelos de colite quimicamente induzida torna-se difícil avaliar as etapas iniciais da agressão epitelial, assim

com a quebra dos diferentes sistemas de defesa que formam a barreira epitelial, uma vez que o dano a esses mecanismos de defesa pode estar sendo ocasionado pela própria substância aplicada. Um estudo experimental comparando os modelos de CE e colite quimicamente induzida pela aplicação intrarretal de TNBS, mostrou que os níveis de estresse oxidativo nos segmentos sem trânsito fecal surgiam precocemente e estavam relacionados às alterações inflamatórias avaliadas pela análise histológica, assim como pela dosagem de níveis tecidual de COX-2, Inos e PGE-2. Portanto, no modelo de CE, o estresse oxidativo e a inflamação tecidual acontecem nas etapas iniciais do processo inflamatório possibilitando o estudo da relação entre o estresse oxidativo, a inflamação e a quebra dos mecanismos de defesa da barreira epitelial. Esses achados eram diferentes nos animais submetidos à colite induzida por TNBS, onde o aumento da produção de RLO ocorria apenas após quebra da barreira mecânica do epitélio cólico (LONGATTI et al., 2010). Portanto, nos modelos de colite quimicamente induzida não seria possível estudar a relação entre a integridade das diferentes linhas de defesa que compõem a barreira mecânica da mucosa cólica e as etapas iniciais da patogênese da colite.

A barreira de muco que recobre a mucosa do cólon representa a primeira linha de defesa mecânica da barreira epitelial do intestino grosso. As mucinas representam as principais proteínas constituintes do muco do trato gastrointestinal. Estudos mostraram que na mucosa exclusa de trânsito intestinal existe redução do conteúdo tecidual de mucinas neutras e ácidas que se agrava com o decorrer do tempo de exclusão (NONOSE et al., 2009). Essa depleção no conteúdo das mucinas mostrou-se diretamente relacionada aos níveis de RLO produzidos pelo epitélio cólico desprovido de trânsito fecal. De modo semelhante ao que ocorre na colite ulcerativa a redução se dá, principalmente, às custas das mucinas ácidas. Existem dois subtipos principais de mucinas ácidas na mucosa cólica: as sulfomucinas e sialomucinas (MARTINEZ et al., 2010a). Estudos mostraram que na mucosa cólica de doentes portadores de colite ulcerativa existe desaparecimento das sulfomucinas fazendo que as sialomucinas passem a predominar na população de células caliciformes presentes nas glândulas intestinais. De maneira oposta, estudos mostraram que nas glândulas da mucosa exclusa de trânsito ocorre predomínio das sulfomucinas, existindo desaparecimento das sialomucinas (MARTINEZ et al., 2010a). Apesar dessas diferenças, a redução de ambos os subtipos de mucinas ácidas encontra-se diretamente relacionada à maior produção de RLO pelo epitélio inflamado (MARTINEZ et al., 2010b).

As aplicações de substâncias com atividade antioxidante ou AGCC, mostraram-se estratégias eficazes para o tratamento da CE experimental. A aplicação de enemas contendo n-acetilcisteína e ácido 5-aminosalicílico, substâncias com reconhecida atividade antioxidante, em um modelo experimental de CE mostrou-se estratégia eficaz para a melhora do processo inflamatório da mucosa cólica, bem como para redução dos níveis de estresse oxidativo

tecidual (CALTABIANO et al., 2011; ALMEIDA et al., 2012; MARTINEZ et al., 2013). Até mesmo, extrato obtido de plantas com reconhecida atividade antioxidante, mostraram-se eficazes para redução dos níveis teciduais de estresse oxidativo e melhora da inflamação epitelial no modelo de CE proposto (CUNHA et al., 2011). De modo semelhante, a aplicação de clisteres com AGCC além de reduzir os níveis de RLO melhorava as alterações inflamatórias na mucosa cólica desprovida de trânsito fecal (LAMEIRO et al., 2011). De modo distinto a exposição da mucosa cólica exclusa de trânsito a substâncias com grande poder oxidante agrava significativamente a lesão epitelial (MARQUES et al., 2010). Em última análise, esses resultados sugerem que a intervenção da mucosa cólica com substâncias antioxidantes é capaz de reduzir a inflamação da mucosa cólica desprovida de trânsito fecal.

Recentemente, foram publicados resultados parciais do presente trabalho, onde verificou-se que a aplicação de enemas contendo SCF, além de reduzir os níveis teciduais de RLO, preserva o conteúdo de ambos os tipos de mucinas assim como dos dois subtipos de mucinas ácidas (PEREIRA et al., 2013; CHAIM et al., 2015; BONASSA et al., 2015; MARTINEZ et al., 2015b). Esses achados confirmaram que o SCF além de possuir propriedades cicatrizantes e protetoras do epitélio inflamado é um potente antioxidante capaz de sequestrar RLO produzidos pelas células da mucosa cólica desprovidas do suprimento de AGCC.

Ao avaliar o comportamento das proteínas que compõe os sistemas de junção intercelular no mesmo modelo de CE proposto, verificou-se que ocorria redução significativa das proteínas que formavam as junções de oclusão e aderentes quando comparávamos a mucosa cólica com e sem trânsito intestinal (MARTINEZ, 2010, MARTINEZ et al., 2010a; MARTINEZ et al., 2012; KADRI et al., 2013; MARTINEZ et al., 2015). Esses achados mostraram a importância do AGCC para manutenção da integridade das junções intercelulares. Os resultados desses estudos mostraram ainda, que a queda no conteúdo tecidual dessas proteínas estava relacionada à maior produção de RLO pelas células carentes do suprimento energético (MARTINEZ, 2010; MARTINEZ et al., 2010a). Restava agora verificar se a aplicação do SCF também poderia preservar o conteúdo e a integridade dessa outra linha de defesa da barreira epitelial representada pelas junções intercelulares.

2.3 Junções Intercelulares

As células epiteliais da mucosa cólica unem-se umas às outras por sistemas de adesões célula-célula, as quais suportam grande parte do estresse mecânico. Para manter esse mecanismo, filamentos proteicos de actina que formam o citoesqueleto celular, atravessam o citoplasma de cada célula epitelial e unem-se a junções especializadas localizadas na membrana plasmática. Esse mecanismo é composto por numerosas moléculas de adesão celular. As moléculas de adesão celular são proteínas que podem

situar-se entre duas células vizinhas ou entre uma célula e a lâmina basal. Na mucosa do cólon essas proteínas são dependentes de cálcio (Ca^{++}) e podem ser destruídas quando se expõe o epitélio a substâncias quelantes do íon Ca^{++}, como o etileno-diamino-tetra-acético (EDTA) ou a RLO (LI et al., 2015). As moléculas de adesão celular da família das integrinas são responsáveis por manter as células epiteliais firmemente aderidas à lâmina basal, enquanto as junções celulares e os desmossomos respondem pelo sistema de adesão intercelular (ALBERTS et al., 2004; LODISH et al., 2004).

As junções intercelulares especializadas localizam-se em pontos onde existe contato entre duas células ou entre uma célula e a matriz extracelular. Para que as células funcionem de maneira integrada em um arranjo compacto, são necessárias estruturas de adesão especializadas, formadas por moléculas de adesão celular agrupadas (ALBERTS et al., 2004; LODISH et al., 2004). Há três grupos funcionais de junções intercelulares: junções de oclusão, junções aderentes e junções comunicantes. Existem também as junções de adesão com a matriz extracelular ou lâmina basal representada pelos hemidesmossomos.

As junções de oclusão têm como principal função selar, hermeticamente, as membranas entre duas células com intuito de evitar que moléculas pequenas migrem de um lado para outro do espaço intercelular (ALBERTS et al., 2004). Ao mesmo tempo, permitem a passagem de nutrientes selecionados através do epitélio para o fluído extracelular (LODISH et al., 2004). As junções de oclusão são formadas, principalmente por proteínas da família das claudinas (1-24), ocludinas e tricelulinas (Figura 2)

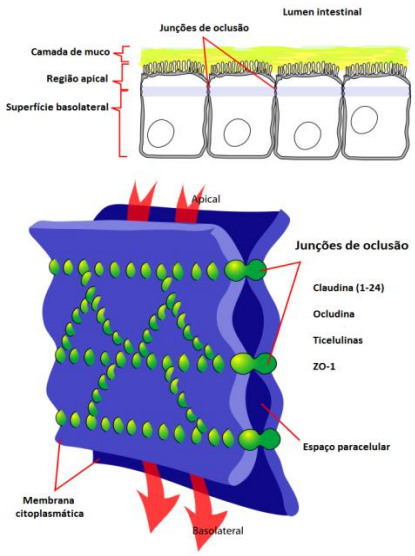

Figura 2 - Junções de oclusão. (Figura modificada de R.J. Medina). Fonte: http://gutcritters.com/part-three-dietary-fat-chylomicrons-and-endotoxemia/tight-junctions

A família das claudinas representa as principais proteínas constituintes das junções de oclusão, já se descrevendo 24 subtipos diferentes. A maioria dos subtipos de claudinas tem importância capital nos mecanismos de adesão celular, enquanto outros se relacionam

mais a regulação do trânsito de moléculas entre as células. Na mucosa do trato gastrointestinal as claudinas 1, 2, 3 e 23 são as mais importantes e, no cólon, as claudina-1 e 3, parecem ser primordiais nas junções de oclusão intercelulares. Estudos mostraram que o estresse oxidativo pode ocasionar a ruptura da claudina por um mecanismo tirosinoquinase dependente (HANBY et al., 1996). A função da ocludina ainda é pouco compreendida. Recentes estudos sugerem que a proteína isolada tenha pequena função nos mecanismos de junção intercelular, todavia quando associada às proteínas da família das claudinas apresenta papel nos mecanismos de adesão entre as células e na diferenciação celular (SAITOU et al., 2000). Já se demonstrou que a atividade inflamatória tecidual ocasionando aumento de citocinas e RLO é capaz de diminuir a expressão tecidual da ocludina interferindo na capacidade de barreira epitelial, sendo um dos mecanismos encontrados em portadores de DII (RAO et al., 1997). Estudos mostraram em portadores de DII que as proteínas constituintes das junções de oclusão encontram-se danificadas possibilitando a migração de bactérias e toxinas para o interior do meio interno (GASSLER et al., 2001; KUCHARZIK et al., 2001).

Plöger et al. (2012) demonstraram que os AGCC, em particular o butirato, são importantes para manutenção da integridade das junções de oclusão celular. Estudo em modelo experimental de CE mostrou pela primeira vez na literatura que existe redução significativa das proteínas claudina-3 e ocludina na mucosa cólica desprovida de trânsito fecal (MARTINEZ et al., 2015a). A redução no conteúdo tecidual de ambas as proteínas estavam diretamente relacionadas à piora da inflamação na mucosa cólica e aos maiores níveis de estresse oxidativo tecidual. Esses achados sugerem que substâncias que protejam a mucosa cólica e possuam atividade antioxidante possam ser úteis na manutenção da integridade das junções de oclusão.

As junções aderentes representam o segundo sistema de adesão intercelular. Localizam-se logo abaixo das junções de oclusão e conecta o citoesqueleto interno de uma célula a outra, através de um complexo proteico formado por proteínas da família das caderinas, cateninas, vinculinas e α-actina. As junções aderentes também se comunicam com proteínas das vias de sinalização intracelulares capacitando-as a participarem dos mecanismos de sinalização existentes no interior das células (ALBERTS et al., 2004; LODISH et al., 2004).

As caderinas são as principais proteínas existentes nas junções aderentes. São moléculas de adesão celular dependentes de Ca^{++} e desempenham papel fundamental na diferenciação tecidual, polarização e estratificação epitelial (DUBAND e THIERRY, 1990). Já se descreveram mais de 40 tipos de caderinas segundo o tecido onde são encontradas, porém as mais comuns são as caderinas presentes nos tecidos epiteliais (E-caderina). A E-caderina, é principal molécula de adesão celular encontrada no epitélio cólico (GUMBINER e

MCREA, 1993). É uma proteína transmembrana com peso molecular de 120 kDa, transcrita a partir do gene *cdh1*, localizado no cromossomo 16q22.1 que, caracteristicamente, possui duplo domínio funcional, um extracelular e outro citoplasmático. O domínio citoplasmático da E-caderina se une a uma ou mais proteínas de ancoramento intracelular (α-cateninas, β-cateninas, proteína p120), enquanto o domínio extracelular interage com o domínio extracelular de outra molécula homóloga, proveniente da célula vizinha, por um mecanismo dependente do Ca^{++}. Entre as moléculas de E-caderina, no espaço intercelular, íons Ca^{++} se posicionam entre cada molécula, mantendo as proteínas juntas com intuito de constituir um mecanismo de adesão mais rígido. A E-caderina atua como proteína de adesão transmembrana ligando, indiretamente, a actina do citoesqueleto de duas células vizinhas. No domínio citoplasmático a E-caderina interage com filamentos de actina de forma indireta, por meio de um grupo de proteínas de ancoramento denominadas cateninas, localizadas no citoplasma celular, paralelas ao folheto interno da membrana plasmática (ALBERTS et al., 2004; LODISH et al., 2004).

No epitélio cólico normal o maior contingente da proteína E-caderina se expressa nas junções de aderência situadas logo abaixo das junções ocludentes na superfície luminal das células. Estudos utilizando técnicas imunoistoquímicas demonstraram que a E-caderina é encontrada, principalmente, nas membranas das células epiteliais especializadas voltadas para a luz intestinal (JANKOWSKI; BEDFORD; KIM, 1997; JANKOWSKI et al., 1998). Durante a diferenciação celular e em algumas DII existe redução no conteúdo da E-caderina, alterando, sensivelmente, os mecanismos de adesão célula-célula.

Para que ocorra o ancoramento entre a E-caderina e a actina do citoesqueleto celular, proteínas da família das cateninas (α-catenina, β-catenina, γ-catenina e p120) são imprescindíveis. As cateninas comunicam o domínio citoplasmático da E-caderina com a actina celular por meio de interações entre as diferentes isoformas da proteína (DEMETTER et al., 2000). A β-catenina é uma proteína codificada pelo gene *ctnnb1* localizado no cromossomo 8q32 com peso molecular de 88 kDa. A isoforma β-catenina apresenta dupla função na biologia molecular das células, pois, além de contribuir para mecanismos de adesão intercelular tem importância capital no sistema de sinalização celular mediado pela via Wnt. Os níveis citoplasmáticos da β-catenina são regulados por um complexo protéico formado pelas proteínas APC, glicogênio-sintetase-quinase-3β e axina. Quando existe dano às pontes de E-caderina no espaço intercelular, ocorre acúmulo de β-catenina livre no citoplasma celular. O maior contingente citoplasmático faz com que a proteína migre para o interior do núcleo celular induzindo a transcrição de genes-alvo relacionados à divisão celular pela via Wnt (MORIN et al., 1997). Assim sendo, a β-catenina além ser parte fundamental das junções aderentes participa da via de sinalização Wnt, responsável pela indução da divisão celular. Provavelmente, essa dupla função tenha importância relevante

nos mecanismos de renovação das células do epitélio cólico, constantemente substituídas. É possível que na colite a β-catenina apresente maior expressão na zona proliferativa das criptas cólicas, onde se localizam as células tronco multipontentes, principais responsáveis pelo processo de renovação celular. A Figura 3 mostra as interações entre as proteínas constituintes das junções de adesão intercelular.

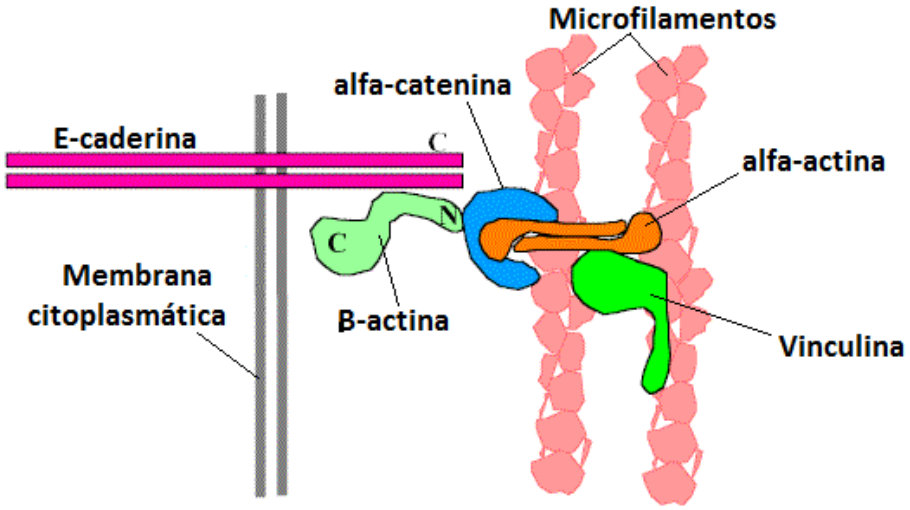

Figura 3 - Junções aderentes (Figura modificada de Universidade de Edimburgo). Fonte: http://www.bms.ed.ac.uk/research/others/smaciver/Cell%20biol.topics/Cell-cell%20adherens.htm

Diferentes fatores são capazes de destruir as junções aderentes no epitélio cólico e, dentre eles, o estresse oxidativo merece lugar de destaque (PARRISH et al., 1999). Estudos mostram que exposição da mucosa cólica a altas concentrações de RLO pode oxidar o Ca^{++} que mantém unidas as moléculas de E-caderina situadas entre duas células. Quando existe ruptura das moléculas de Ca^{++}, aumenta o conteúdo citoplasmático da β-catenina e, consequentemente, a divisão celular (KATSUBE; TSUJI; ONODA, 2007). Os RLO também podem dissociar as junções entre a E-caderina e a β-catenina no citosol celular, por um mecanismo tirosinoquinase dependente, o que também pode levar ao acúmulo citoplasmático da β-catenina induzindo a divisão célula. Porém, essa possibilidade é difícil de ser estudada nos modelos de colite quimicamente induzida, pois a ruptura das junções aderentes pode ocorrer pela ação dos agentes lesivos como o TNBS.

Estudos realizados no LIM-USF avaliando a expressão e o conteúdo tecidual das proteínas constituintes das junções aderentes mostrou que, na mucosa cólica sem trânsito intestinal, existe redução significativa do conteúdo de E-caderina e β-catenina na superfície apical das células epiteliais. A redução do conteúdo dessas proteínas estava relacionada à piora do processo inflamatório e maiores níveis de estresse oxidativo tecidual (MARTINEZ,

2010; MARTINEZ et al., 2012; KADRI et al., 2013). Outros estudos confirmaram a relação entre estresse oxidativo e ruptura das junções de oclusão e aderentes, bem como os benefícios da aplicação de substâncias com atividade antioxidante. Contudo, até a presente data não se avaliou o conteúdo das proteínas E-caderina e β-catenina em modelos experimentais de CE.

Finalmente, as junções comunicantes são formadas por uma proteína denominada conexina, capaz de controlar a passagem de moléculas, íons, proteínas, impulsos elétricos ou químicos de uma célula à outra, estabelecendo uma complexa rede de comunicação entre um grupo de células epiteliais justapostas (ALBERTS et al., 2004; LODISH et al., 2004).

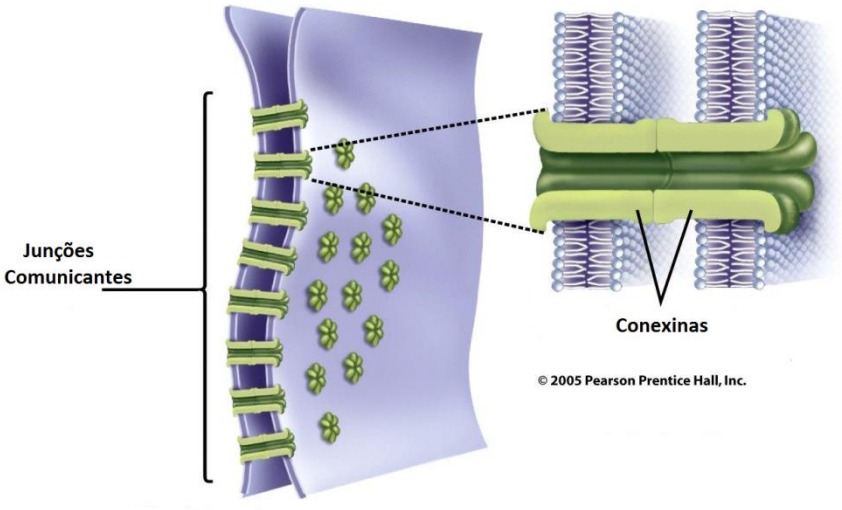

Figura 4 - Junções comunicantes. (Figura modificada de Pearson Pretntice Hall, Inc.). Fonte: https://www.studyblue.com/notes/note/n/bio-350-study-guide-2014-15-mcnichols/deck/13793549

2.4 O Sucralfato

Há mais de duas décadas a aplicação de enemas contendo SCF isolado ou associado aos corticóides ou ao 5-ASA, vem sendo utilizada como opção terapêutica medicamentosa no tratamento das DII (CARLING; KAGEVI; BORVALL, 1986, SINGAL; ANAND, 1998, PIENKOWSKI et al., 1989). Zahavi et al. (1989), foram os primeiros autores a avaliar os efeitos do SCF na colite induzida por ácido acético em ratos. Os autores chamaram a atenção para os efeitos positivos da aplicação de enemas com SCF na

redução dos níveis de prostaglandina E2 e na redução da atividade inflamatória tecidual, apesar de não descreverem os mecanismos pelos quais a droga agia. Outros estudos também mostraram que o SCF era capaz de reduzir o grau de inflamação no cólon de ratos com colite ulcerativa induzida por DSS (BJÖRCK et al, 1997). Estudo comparando o uso de enemas com SCF e corticóides em doentes com colite ulcerativa distal, mostrou que ambas as drogas mostraram benefícios terapêuticos, embora o SCF tenha melhorado o sangramento retal e a frequência das evacuações quando comparado ao uso do corticóide isolado (RILEY; GUPTA; MANI, 1989). Kochhar et al. (1990), utilizaram enemas contendo 2m/100mL de água de SCF, em portadores de retite pós-radioterapia e colite ulcerativa distal. Constaram melhora clínica e endoscópica em 86% e 82% dos doentes, respectivamente, sem efeitos colaterais, sugerindo a eficácia e a segurança do tratamento. O mesmo autor, posteriormente, num estudo prospectivo, duplo cego e randomizado comparando o uso de sulfasalazina associado à aplicação de corticóides por via retal a aplicação isolada pelo reto de enemas com SCF em portadores de retite actínica concluiu que, a aplicação do SCF estava associada a melhor resposta clínica, melhor tolerabilidade, menores custo e efeitos colaterais (KOCHHAR et al., 1991). Recentemente, o mesmo grupo mostrou que portadores de retite actínica que apresentavam sangramento retal grave ou moderado, tratados com enemas de SCF na concentração de 10% duas vezes ao dia apresentavam redução do sangramento com resposta terapêutica sustentada (KOCHHAR et al., 1999).

Os mecanismos de ação do SCF ainda não se encontram totalmente elucidados. Apesar do efeito tópico protetor à mucosa intestinal pela formação de uma barreira protetora estudos vêm mostrando que a substância aumenta a síntese de prostaglandina endógena (LIGUMSKY; KARMELI; RACHMILEWITZ, 1986). Estudo experimental, avaliando o efeito da aplicação de enemas com SCF na retite induzida por radiação constatou que a substância aumenta a produção de fator de crescimento tecidual, reduz a apoptose celular induzida pelas proteínas Tp53 e Bax/Bcl2, protegendo as células tronco intestinais existentes no interior das criptas cólicas, o local mais sensível ao dano do DNA (MATSUU-MATSUYAMA et al., 2006). Mais recentemente demonstrou-se que o a porção ativa da molécula do SCF, previne o retardo da cicatrização epitelial na colite induzida por H_2O_2, provavelmente pela indução da expressão de COX-2 e inativação dos mecanismos celulares de indução de apoptose. Esses efeitos provavelmente encontram-se relacionados à ativação da via do NF-kB (SHINDO et al., 2006).

Apesar de já ter sido demonstrado que o SCF apresenta efeitos antioxidantes nas lesões gástricas e cólicas quimicamente induzidas apenas três estudos relacionados às etapas iniciais desta Tese avaliaram os efeitos do SCF em modelo experimental de CE. No

primeiro deles verificamos que a aplicação de enemas diários com a substância melhorou o processo inflamatório na mucosa desprovida de trânsito fecal (PEREIRA et al., 2013). Num segundo estudo, com o objetivo de verificar se os efeitos do SCF estavam relacionados à sua ação antioxidante, verificamos que os animais submetidos à intervenção com o SCF apresentavam menores níveis de peroxidação lipídica e melhora do processo inflamatório da mucosa cólica. Esses achados parecem confirmar a ação antioxidante da substância (MARTINEZ et al., 2015b). Finalmente, com o objetivo de verificar se os efeitos tópicos do SCF na manutenção das mucinas que recobrem o epitélio cólico, foram realizados dois outros estudos (CHAIM et al., 2014; BONASSA et al., 2015). Os resultados desses estudos mostraram que a aplicação de clisteres com SCF preserva o conteúdo das mucinas neutras e ácidas, assim como dos dois subtipos de mucinas ácidas as sulfomucinas e as sialomucinas. Cabe destacar que os efeitos da substância foram dependentes da dose utilizada, bem como do período de tempo utilizado (CHAIM et al., 2014; BONASSA et al., 2015).

Entretanto, do melhor do nosso conhecimento, nenhum estudo avaliou os efeitos do SCF na manutenção da integridade das junções de oclusão e de aderência em modelos experimentais de CE. Desse modo, a proposta deste estudo é inédita.

2.5 Malondialdeído (MDA)

Durante o processo de respiração celular, quando existe redução do fornecimento de oxigênio tetravalente às células epiteliais fazendo com que elas aumentem a capacidade de formar RLO (FERREIRA e MATSUBARA, 1997). Na mucosa cólica normal essa situação é mantida em equilíbrio pelos sistemas antioxidantes intrínsecos das células epiteliais que neutralizando o excesso de RLO produzidos evitam o estresse oxidativo (ANTUNES et al., 2008). Nosso grupo demonstrou que a exclusão de trânsito intestinal modifica o processo de respiração celular ocasionando aumento na produção de RLO e, consequentemente, estresse oxidativo (MARTINEZ, 2010b). O excesso na produção de RLO é responsável por rupturas nos diferentes mecanismos de defesa da barreira epitelial determinando o aparecimento das alterações inflamatórias que caracterizam a CE.

Todas as estruturas que formam as células epiteliais e os mecanismos de junção intercelular são passíveis de sofrerem agressão pelos RLO. Todavia, as membranas celulares é uma das estruturas mais susceptíveis em decorrência da peroxidação de lipídios, principais constituintes da estrutura física das membranas (FERREIRA e MATSUBARA, 1997; ANTUNES et al., 2008). Quando o aumento da produção de RLO determina a oxidação dos fosfolípides existentes nas membranas celulares, os produtos dessa peroxidação lipídica, como o malondialdeído (MDA), podem ser quantificados por técnicas

bioquímicas e utilizados como marcadores da ação dos RLO no organismo (FERREIRA e MATSUBARA, 1997; NIELSEN et al., 1997). Em outras palavras, a dosagem do MDA, indiretamente, reflete o grau de estresse oxidativo que os tecidos estão sofrendo. Diante desses achados, a dosagem tecidual do MDA vem sendo rotineiramente como marcador dos níveis de estresse oxidativo em diferentes situações clínicas onde o estresse oxidativo é o denominador comum. Cabe destacar que o MDA é tóxico para as células provocando danos tanto às estruturas celulares citoplasmáticas, quanto às junções celulares e ao DNA.

A quantificação dos níveis de MDA nos tecidos, como destacado anteriormente, é um parâmetro importante para avaliação do estresse oxidativo em tecidos (ANTUNES et al., 2008). O MDA pode ser quantificado por diferentes tipos de ensaios laboratoriais destacando-se os ensaios bioquímicos avaliando a reatividade do MDA com o ácido tiobarbitúrico por espectrofotômetro e cromatografia líquida de alta eficiência. O princípio do método da avaliação da reatividade do MDA com o ácido tiobarbitúrico é que o produto formado é passível de ser detectado a partir de leitura espectrofotométrica na região onde apareça visível. Esse método ainda é o mais utilizado para a quantificação tecidual dos níveis de MDA, apesar de não ser totalmente específico para detecção dos produtos da lipoperoxidação, uma vez que quantifica a soma das diferentes substâncias reativas ao ácido tiobarbitúricos, denominadas como TBARS (STEGHENS et al., 2001). Todavia, a dosagem da peroxidação lipídica por esse ensaio bioquímico apresenta a vantagem de apresentar baixo custo, fácil exequibilidade e ter sido validada em vários estudos. A cromatografia líquida de alta eficiência, apesar de mais precisa requer a utilização de cromatógrafos de alto desempenho pouco disponíveis e com custo elevado para a sua execução. Foram esses fatores, aliados à experiência adquirida em estudos anteriores, que nos levaram a determinar os níveis de estresse oxidativo por meio da dosagem do MDA pelo ensaio bioquímico inicialmente descrito por Yagi (1976), com pequenas modificações (MARTINEZ et al., 2015b).

2.6 Mieloperoxidase

A mieloperoxidase (MPO) é uma proteína (enzima) catiônica, com peso molecular de 144 kD, formada por dois dímeros idênticos ligados entre si por pontes de bissulfeto sendo cada dímero composto de uma sub unidade de cadeia leve e uma pesada, com grupos heme funcionalmente idênticos. É a principal proteína constituinte dos grânulos azurófilos dos neutrófilos (85%-90) e o restante em monócitos. Após o recrutamento de neutrófilos para um foco de invasão bacteriana a MPO é prontamente liberada após a ativação por agonistas distintos da degranulação neutrofílica. A MPO, através de reações com o H_2O_2,

forma RLO e outras substâncias oxidantes difusíveis com atividade bactericida. A enzima possui papel fundamental na produção de RLO e existem evidências de que alguns metabólitos gerados a partir de reações químicas catalizadas pela MPO possam estar envolvidos em mecanismos de sinalização celular. Dentre os principais produtos formados a partir da reação da MPO encontra-se o RLO oxigênio singlete, um tipo de radical importante no combate a patógenos invasivos do meio interno. Por aumentar a produção de RLO nos tecidos, a enzima pode provocar estresse oxidativo tecidual aumentando a intensidade da lesão tecidual.

A dosagem tecidual da MPO é utilizada como um marcador específico para a avaliação da presença de infiltrado neutrofílico nos tecidos inflamados (MARTINEZ, 2015b). Seus valores traduzem indiretamente a atividade do processo inflamatório. A dosagem tecidual da MPO vem sendo utilizada em vários trabalhos clínicos e experimentais que estudam a intensidade da colite, bem como a ação de diferentes substâncias na melhora do índice de atividade inflamatórias e doentes e modelos experimentais de colite (LONGATTI et al., 2010). Existem vários métodos descritos para a avaliação dos níveis teciduais de MPO. Todavia, a maioria deles deriva do método descrito por (BRADLEY et al., 1982). Assim como foi feito para a avaliação dos níveis teciduais de MDA, utilizou-se o ensaio bioquímico proposto por Bradley, Christensen e Rothstein (1982), com pequenas adaptações pela familiaridade com o ensaio (MARTINEZ et al., 2015b).

3. OBJETIVOS

3.1 - Objetivo principal;

3.1.1 - Avaliar os efeitos da aplicação de enemas contendo SCF no conteúdo tecidual das proteínas claudina-3, ocludina, E-caderina, β-catenina, e atividade anti-inflamatória e antioxidante na mucosa cólica exclusa de trânsito fecal;

3.2 – Objetivos secundários;

3.2.1 - Avaliar os efeitos da aplicação de enemas com SCF na inflamação da mucosa cólica desprovida de trânsito fecal;

3.2.2 - Verificar se existe influência do tempo de intervenção com SCF no conteúdo tecidual das proteínas claudina-3, ocludina, E-caderina, β-catenina e mieloperoxidase na mucosa cólica exclusa de trânsito fecal;

3.2.3 - Avaliar os efeitos antioxidantes do SCF na mucosa cólica desprovida de trânsito fecal.

4. MATERIAL E MÉTODO

A realização deste estudo obedeceu à Lei Federal 11.794 de 08/10/2008 (Lei Sérgio Arouca) e às orientações do Colégio Brasileiro de Experimentação Animal (COBEA). Este trabalho foi aprovado pelo Comitê de Ética no Uso de Animal em Pesquisa (CEUA) da Universidade São Francisco, Bragança Paulista, São Paulo (Anexo I). O Projeto foi aprovado com o parecer Nº. 002.04.10 (ANEXO 1).

4.1 Animal de Experimentação

Utilizou-se 36 ratos machos (*Rattus norvegicus albinus)* da linhagem *Wistar*, provenientes do Biotério Central da Universidade São Francisco, Bragança Paulista, com peso variando entre 300 a 320g e média de idade de quatro meses.

4.2 Grupos Experimentais

Foram constituídos, aleatoriamente, três grupos experimentais com 12 animais cada, divididos segundo terem sido submetidos à aplicação diária de clisteres contendo, solução fisiológica, solução de SCF na concentração de 1,0 g/kg/dia (SCF-1) e solução de SCF na concentração de 2,0 g/kg/dia (SCF-2), respectivamente. Seis animais de cada um desses grupos experimentais foram sacrificados em duas semanas enquanto os 18 remanescentes, seis de cada grupo experimental, em quatro semanas (Fig.5).

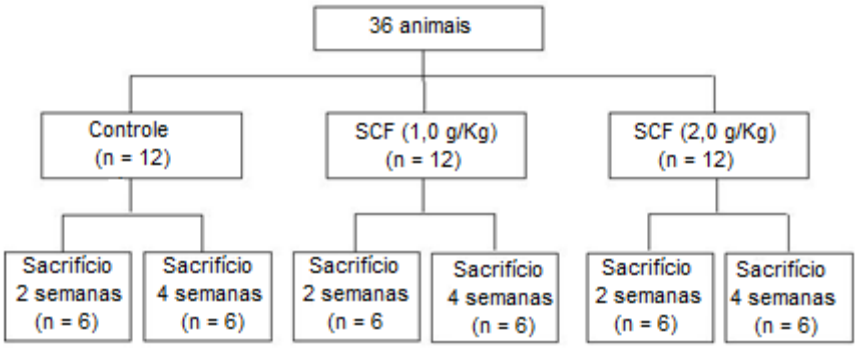

Figura 5- Algoritmo da divisão dos grupos experimentais.

4.3 Etapa Pré-operatória

Durante o período de vigilância epidemiológica (sete dias), os animais foram mantidos em gaiolas individuais, em ambiente climatizado, com controle de temperatura, luminosidade, umidade e ruídos. Todos permaneceram em jejum durante 24 horas, exceto para água, antes da intervenção cirúrgica. Identificou-se cada gaiola com o número do animal, o grupo e o subgrupo experimental a que pertencia. Esses mesmos dados foram tatuados com tinta da China (tinta nanquim) na cauda de cada animal.

4.4 Anestesia

No dia da intervenção, pesou-se os animais para cálculo da dose anestésica, utilizando-se, para tal, o cloridrato de xilazina 2% (Anasedan®) e o cloridrato de quetamina (Dopalen®) na dose de 0,1ml/100g, administradas por via intramuscular na pata traseira esquerda.

4.5 Técnica Operatória

Depois de anestesiados e fixos à mesa cirúrgica, em decúbito dorsal horizontal, foi realizada tricotomia da região abdominal, da pelve até as rebordas costais, com aparelho elétrico de depilação. Foi utilizada a polivinilpirolidona-iodo para antissepsia da área depilada, posteriormente, recoberta por campo cirúrgico fenestrado estéril. Abriu-se a cavidade abdominal por meio de incisão longitudinal mediana com três centímetros de extensão. Terminada essa etapa, o ceco foi identificado. Com o auxílio de um paquímetro se mediu no intestino grosso o local escolhido para a secção do cólon direito, situado a quatro centímetros após a papila ileocecal. Após ligadura dos vasos da arcada cólica marginal, seccionou-se o cólon no ponto pré-determinado e foi exteriorizado o segmento proximal, como colostomia terminal, através de incisão circular, com três milímetros de diâmetro, realizada na região da fossa ilíaca direita. A colostomia foi fixada à pele com pontos separados de fio absorvível monofilamentar 4-0 (Monocryl®) nos quatro pontos cardinais, e depois entre eles, amarrando com três nós.

Terminada a confecção da colostomia proximal, cateterizou-se o segmento caudal do intestino grosso seccionado com sonda de polivinil medindo 12 F de diâmetro interno, e o irrigamos com 80 ml de solução fisiológica aquecida a 37ºC, até que o efluente drenado pelo ânus não apresentasse a saída de síbalos fecais. Concluída a irrigação, o cateter foi

removido e o cólon distal exteriorizado como colostomia (fístula mucosa distal) na face lateral superior direita da parede abdominal. Fixou-se a colostomia distal com a mesma técnica utilizada na colostomia proximal. Foi realizada a síntese da parede abdominal em dois planos de sutura: peritônio e aponeurose com pontos contínuos de fio de ácido poliglicólico 4-0 (Vicryl®) e a pele com pontos separados de nylon 4-0 (Mononylon®).

4.6 Pós-operatório

Concluída a operação, os animais foram mantidos por 10 minutos sob aquecimento por meio de lâmpada incandescente e, após a recuperação anestésica, alojados nas gaiolas individuais previamente identificadas, liberada a ingestão de água e ração padronizada (Nuvilab CR1®), após terem recuperado completamente o estado de vigília.

Os animais permaneceram em gaiolas individuais até a data do sacrifício (duas ou quatro semanas), nas mesmas condições ambientais de umidade, luminosidade e temperatura do período de vigilância epidemiológica. Após a intervenção, não foi tomado qualquer cuidado adicional com relação à ferida operatória ou aos estomas, nem utilizados analgésicos ou antimicrobianos.

4.7 Intervenção com as Soluções Propostas

Os animais foram submetidos à aplicação de enemas diários com as soluções de intervenção padronizadas. A aplicação das substâncias foi realizada através de cateter de polietileno graduado em centímetros, com diâmetro interno de 14 F. O cateter foi introduzido, cuidadosamente, pelo ânus do animal numa profundidade padronizada em 3 cm a partir da margem anal. A seguir, aplicava-se o clister contendo a solução de intervenção correspondente para cada grupo experimental, à temperatura ambiente, até que a mesma saísse pela fístula mucosa distal (colostomia exclusa de trânsito fecal). As soluções de intervenção foram infundidas com auxílio de uma bomba de infusão numa velocidade padronizada em 20 mL/min.

4.7.1 Coleta do material

Na véspera do dia programado para a coleta do material, os animais foram novamente pesados e mantidos em jejum por 24 horas, exceto para água. Todos os animais receberam a aplicação do clister com a solução de intervenção, na manhã da data programada para a eutanásia, sempre realizada no período da tarde. Para a remoção dos

fragmentos cólicos a serem estudados, os animais foram anestesiados com a mesma técnica anteriormente descrita, realizando-se, posteriormente, incisão xifopúbica. Após liberação das aderências, caso presentes, removeu-se todo o cólon, incluindo a colostomia, desprovida de trânsito fecal, incluindo o ânus. Os animais anestesiados foram sacrificados com dose inalatória letal de éter.

Depois de removidos, abriu-se longitudinalmente os segmentos do cólon, pela borda anti-mesocólica, e lavou-se, com soro fisiológico a 0,9 % aquecido a 37 ºC, para remoção de resíduos fecais, muco ou tampão de sucralfato eventualmente formado. Foram retirados três fragmentos, medindo cada um deles 30 mm de comprimento, interessando toda a parede intestinal. Todos os fragmentos foram removidos do cólon desprovido de trânsito intestinal, submetido às soluções de intervenção. Desprezou-se um segmento de 10 mm do cólon a partir da fixação do estoma ao peritônio parietal, bem como um segmento de 4 cm acima do ânus, que incluía o canal anal. Dois dos três fragmentos colhidos foram encaminhados para estudo histológico através da técnica de coloração hematoxilina-eosina (HE) e imunoistoquímico e o terceiro foi enviado ao laboratório para mensuração dos níveis de malondialdeído (MDA). Para a dosagem do MDA, foram isolados fragmentos contendo exclusivamente a mucosa, obtidos por microdissecação realizada com o auxílio de lupa entomológica e, acondicionados em frasco com solução tampão, após identificação foram congelados a -22 ºC.

4.8 Técnica Histológica

4.8.1 Fixação e processamento

Para a realização do estudo histopatológico, dispôs-se e fixou-se com alfinetes os fragmentos com 30 mm de extensão, retirados dos cólons submetidos à intervenção com as soluções propostas, em superfície plana de cortiça com a face mucosa voltada para cima. Após identificação, o material foi acondicionado em frascos contendo solução de formaldeído tamponado a 10%, com a superfície mucosa voltada para baixo. Os fragmentos de cólon permaneceram imersos totalmente em formaldeído por um período de 72 horas. Em seguida os espécimes foram acondicionados e identificados individualmente em recipientes adequados e foram processados na seguinte ordem: lavados em água corrente e água destilada para, em seguida, serem desidratados em sucessivas concentrações crescentes de álcool, diafanizados em xilol e impregnados em banhos de parafina líquida a uma temperatura prévia e constante de 60ºC. Após, o material foi incluído em blocos parafina. Cada bloco foi submetido à microtomia onde foram realizados seis cortes longitudinais, com 5µm de espessura, para confecção das lâminas destinadas ao estudo

histológico e imunoistoquímico.

Para a avaliação histológica da presença de colite, as lâminas foram coradas com hematoxilina-eosina (HE) e montadas com lamínulas e resina.

4.8.2 Técnica de coloração hematoxilina-eosina

Para a realização do estudo de atividade inflamatória utilizou-se a técnica de coloração Hematoxilina-Eosina (HE). Os espécimes depois de coletados, processados, cortados e identificados foram colocados em lâminas de vidro específicas e expostos à temperatura de 60ºC por doze horas. Concluído este período para desparafinização foram submersos em dois banhos de xilol, ali permanecendo por dez minutos em cada banho. Em seguida as lâminas foram submetidas a três banhos de álcool absoluto por passagem e, hidratadas em água corrente por cinco minutos. Após a hidratação, todas as lâminas foram submersas em corante Hematoxilina de Harris por um minuto, realizando-se em seguida a lavagem das lâminas com água corrente para a remoção do excesso do corante. Terminada esta etapa, as lâminas foram expostas ao corante Eosina por dois minutos, novamente lavadas com água corrente e, logo a seguir, desidratadas através de três banhos de álcool absoluto, um banho com xilol/álcool e três banhos de xilol. Em seguida foram montadas utilizando-se lamínulas e resina para a avaliação do escore inflamatório.

4.9 Avaliação Histológica da Presença de Colite

A análise das lâminas foi feita com microscópio óptico comum, Nikon Eclipse DS-50, com magnificação final de 200x, por patologista experiente em doenças colorretais que desconhecia a origem do material e os objetivos do estudo. As fotomicrografias foram feitas com câmera de vídeo-captura (DS-Fi-50), acoplada ao microscópio e, posteriormente, digitalizadas em computador.

Para confirmar o diagnóstico histopatológico de colite, consideraram-se os seguintes parâmetros histológicos: presença de ulcerações no epitélio e escore inflamatório, segundo escala anteriormente proposta (GUPTA et al., 2007), modificada (QUADRO 1).

Quadro 1 – Escala de graduação do escore inflamatório		
Grau de inflamação	**Escore**	**Características histopatológicas**
Ausente	0	Sem infiltração neutrofílica tecidual
Leve	1	Infiltração neutrofílica (<50% das criptas) ou Infiltração neutrofílica (<50% dos campos) + Ausência de perda epitelial
Moderado	2	Infiltração neutrofílica (≥ 51% das criptas) ou Infiltração neutrofílica (≥ 51% dos campos) + Ausência de perda epitelial
Intenso	3	Perda epitelial

Baseada em Gupta et al. (2007) - Mount Sinai Hospital – Modificada

4.10 Técnica Imunoistoquímica

4.10.1 Proteínas e-caderina, β-catenina, claudina-3 e ocludina

Para realização da técnica de imunoistoquímica para pesquisa das proteínas E-caderina, β-catenina, caludina-3, ocludina e da enzima MPO nos blocos previamente confeccionados, foram realizados cortes histológicos, com 5 µ de espessura em todas as amostras colhidas dos animais dos diferentes grupos experimentais (segmentos submetidos às soluções de intervenção) nos dois períodos propostos. Os cortes foram dispostos em lâminas de vidro com extremidades foscas previamente silanizadas e identificadas com o grupo experimental, subgrupo, número do animal e local de onde o fragmento tinha sido removido. Após esses procedimentos foram submersas em solução de Trilogy em uma diluição de 1:100 (Trilogy, Marca Cell Marque). A exposição das lâminas ao Trilogy teve como objetivo promover a desparafinização, hidratação e recuperação antigênica. Essa exposição foi realizada em uma temperatura de 95 ºC em banho-maria por 45 minutos. Em seguida as lâminas foram transferidas para uma segunda cuba também contendo a solução de Trilogy, previamente aquecida à mesma temperatura, permanecendo em incubação por 10 minutos. Posteriormente, retirou-se a cuba com a solução de Trilogy com as lâminas do banho-maria mantendo a solução em temperatura ambiente por 30 minutos. Em seguida as lâminas foram lavadas com dois banhos de água destilada por dois minutos cada e

submetidos posteriormente em dois banhos solução com tampão de PBS com duração de dois minutos cada. O bloqueio das peroxidases endógenas foi realizado através da incubação das lâminas em uma solução de água oxigenada 10 volumes (V) à 3% por dez minutos em temperatura ambiente. Terminada esta fase as lâminas foram novamente lavadas com dois banhos de água destilada por dois minutos e dois banhos com tampão PBS, também por dois minutos cada. Para pesquisa da proteína E-caderina utilizou-se o anticorpo primário Anti-E-Caderina, (Dako do Brasil, São Paulo, Brasil; Clone: NCH-38) na diluição de 1:100. Foi realizada pela adição de 100 µL do anticorpo primário sobre os cortes em uma câmara úmida onde ficaram expostos por uma hora em temperatura ambiente. Para pesquisa da proteína β-Catenina utilizou-se anticorpo anti-β-Catenina (Dako do Brasil, São Paulo; Clone: β-Catenin-1) na diluição de 1:200. Na pesquisa da proteína claudina-3 utilizou-se anticorpo primário anti-claudina-3, (Spring, Bioscience) na diluição de 1:300. Para identificar a imunoexpressão tecidual da proteína ocludina adotou-se o anticorpo primário anti-Ocludina (Spring, Bioscience) na diluição de 1:100. Depois de realizada a diluição recomendada para cada anticorpo primário, 100 µL deste foi adicionado sobre os cortes em uma câmara úmida e, expostos por 1 hora à temperatura ambiente. Após a exposição ao anticorpo primário, as lâminas foram lavadas com água destilada, dois banhos com duração de dois minutos cada banho e dois banhos com solução tampão de PBS também com duração de dois minutos cada. Posteriormente realizou-se a incubação com sistema de avidina-biotina (anticorpos secundários) do Kit LSAB+System-HRP (Dako do Brasil, São Paulo, Brasil) com um tempo de exposição das lâminas por um período de 35 minutos em cada reagente. Terminado este tempo, os cortes foram lavados com dois banhos em solução de tampão de PBS, e revelados através da utilização do Kit Líquid DAB + Substrate (Dako do Brasil, São Paulo, Brasil) na diluição de 1 gota de cromógeno em 1 mL de solução tampão, sendo adicionado 100 µL do cromógeno sobre os cortes, por um período de incubação entre três a cinco minutos a temperatura ambiente. Após a revelação, os cortes foram lavados em água corrente e realizada a contra coloração com hematoxilina de Harris por 30 segundos. Depois, as lâminas foram lavadas novamente com água corrente até a remoção total do excesso de hematoxilina. Por fim, foram desidratadas em três banhos de álcool absoluto, um banho de Xilol/álcool e dois banhos de xilol, para serem montadas com lamínulas e resina.

4.10.2 Proteína mieloperoxidase (MPO)

Para realização da técnica de imunoistoquímica com a finalidade de pesquisa da enzima MPO, nos blocos previamente confeccionados, foram realizados cortes histológicos

com 5 µ de espessura em todas as amostras colhidas dos animais dos diferentes grupos experimentais (segmentos submetidos às soluções de intervenção) nos dois períodos propostos. Os cortes foram dispostos em lâminas de vidro com extremidades foscas previamente silanizadas e identificadas como: grupo experimental, subgrupo, número do animal e de onde o fragmento tinha sido removido. Após esses procedimentos as lâminas foram submersas em solução de Trilogy em uma diluição de 1:100, Trilogy (Cell Marque), sendo que, a exposição das lâminas à solução tinha como finalidade promover a desparafinização, hidratação e a recuperação antigênica. A exposição ao Trilogy foi realizada em uma temperatura de 95 ºC em banho-maria por um período de 45 minutos. Posteriormente, as lâminas foram transferidas para uma segunda cuba com solução de Trilogy previamente aquecida à mesma temperatura onde permaneceram por um período de incubação de 10 minutos, quando foram retiradas do banho-maria e mantidas em temperatura ambiente por 30 minutos. Em seguida, as lâminas foram lavadas em dois banhos de água destilada (dois minutos cada banho) e dois banhos com PBS (dois minutos cada banho). O bloqueio das peroxidases endógenas foi realizado através da incubação das lâminas com água oxigenada 10 V à 3%, por um período de dez minutos em temperatura ambiente. Em seguida as lâminas foram lavadas novamente com dois banhos de água destilada por dois minutos e dois banhos com PBS, pelo mesmo período de tempo. A incubação com o anticorpo primário anti-mieloperoxidase (MPO) (Cell Marque), na diluição de 1:100, foi realizada através da adição de 100 µL do anticorpo primário sobre os cortes em uma câmara úmida e expostos por 1 hora à temperatura ambiente. Após a exposição ao anticorpo primário, as lâminas foram lavadas com água destilada, em dois banhos de dois minutos e dois banhos com solução tampão de PBS pelo mesmo período. A seguir, as lâminas foram incubadas com amplificador por 10 minutos, e novamente lavadas com dois banhos de PBS e incubadas com o polímero de detecção por 10 minutos. O amplificador e o polímero de detecção compõem o kit Sistema de detecção de alta sensibilidade com polímeros HiDef (Cell Marque). No final do processo os cortes foram lavados com dois banhos com PBS. A revelação dos cortes ocorreu através da utilização do DAB Kit 12ml DAB e 200ml Bufer. (Cell Marque) na diluição de 1 gota de cromógeno em 1 mL de solução tampão, onde foi adicionado 100 µL do cromógeno sobre os cortes, por 5 minutos à temperatura ambiente. Após a revelação os cortes foram lavados com água corrente e contracorados com Hematoxilina de Harris por 30 segundos, e lavadas novamente com água corrente até a remoção total do excesso do corante, após esta remoção, foram desidratadas em três banhos de álcool absoluto, um banho de Xilol/álcool e dois banhos de xilol, e por fim montadas com lamínulas e resina.

A imunocoloração para a enzima MPO foi considerada positiva quando se detectava coloração acastanhada intensa que marcavam os neutrófilos presentes nas camadas

mucosa e submucosa, como recomenda o fabricante.

4.11 Medida do Conteúdo Tecidual de E-caderina, β-catenina, Claudina-3, Ocludina e MPO

A expressão das proteínas foi avaliada segundo o local e o conteúdo da imunocoloração. O conteúdo tecidual total de E-caderina, β-catenina, Claudina-3, Ocludina e MPO foi mensurado por análise de imagem assistida por computador (morfometria computadorizada), quantificando-se o conteúdo de ambas em três criptas cólicas contíguas, em três campos aleatórios. A imagem selecionada, após adequadamente focada, foi capturada por videocâmera acoplada ao microscópico óptico. A seguir a imagem captada foi processada e analisada pelo programa NIS-Elements instalado em um computador com boa capacidade de processamento de imagens.

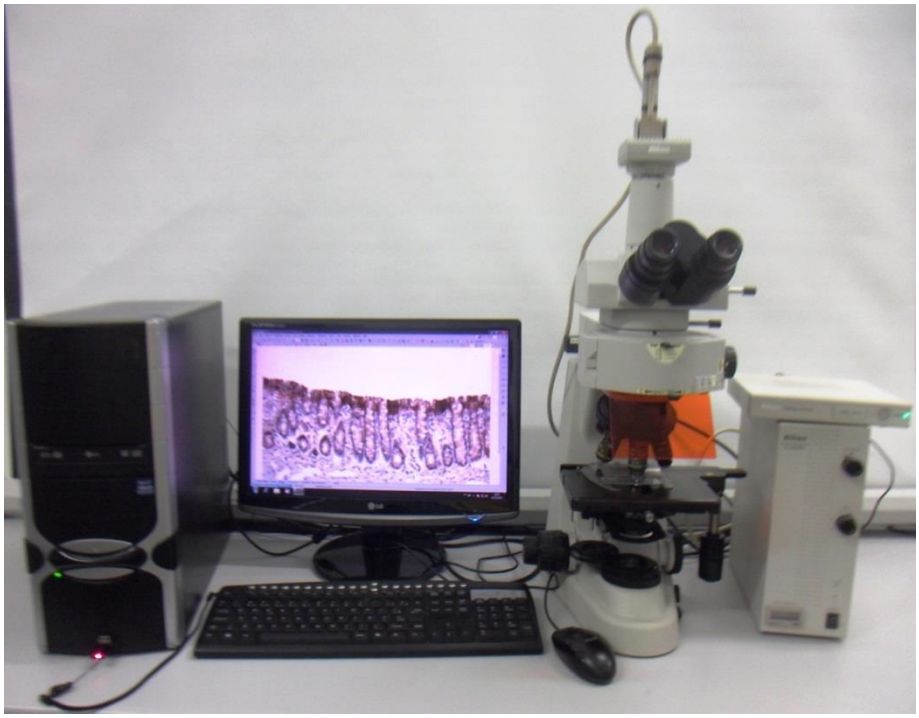

Figura 6 – Sistema de análise de imagens assistida por computador, utilizado para mensuração do conteúdo tecidual das proteínas E-caderina, β-catenina, claudina-3, ocludina e MPO.

A quantificação das proteínas foi sempre realizada após a calibração do programa para o aumento selecionado. A calibração era refeita após a leitura de cada lâmina. Para a quantificação da densidade de cor encontrada em cada campo selecionado, utilizou-se o filtro RGB adotando-se todo comprimento de onda que continha a cor parda-acastanhada (cor que identificava a imunoexpressão tecidual de ambas as proteínas). Com o programa, transformou-se a coloração parda-acastanhada, onde havia a imunoexpressão na cor branca e o restante do campo de visão capturado, sem imunocoloração, em preto. Os valores encontrados para o conteúdo tecidual das proteínas estudadas foram sempre expressos em porcentagem das proteínas por campo analisado (%/campo). O valor final adotado para os animais dos subgrupos controle e experimento (segmentos providos e desprovidos de trânsito intestinal) foi sempre representado pelo valor médio, com o respectivo desvio padrão. Todas as imagens selecionadas foram arquivadas para posterior documentação fotográfica.

A Figura 7A mostra a expressão da de β-catenina na camada mucosa do cólon em segmento provido de trânsito fecal após 18 semanas de derivação intestinal. A figura 7B mostra a quantificação da β-catenina no mesmo campo mostrado pela figura 7A utilizando o método de análise de imagem assistida por computador.

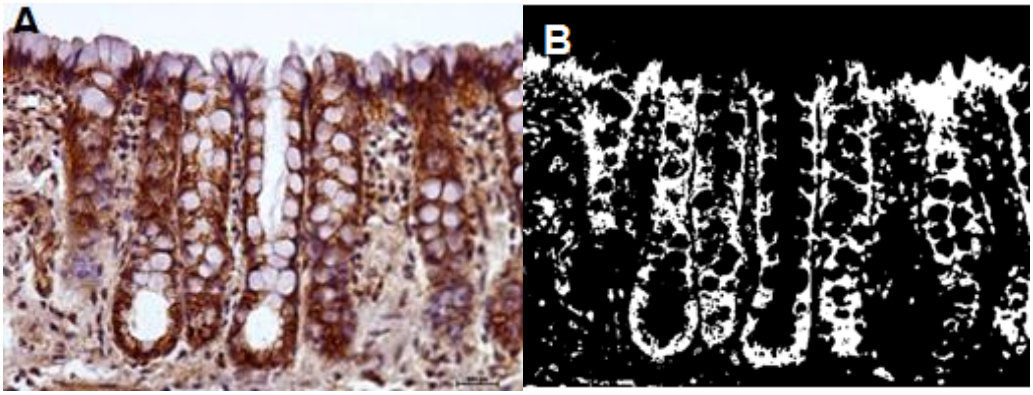

Figura 7A – Expressão da proteína β-catenina em segmento cólico desprovido de trânsito de animal submetido à exclusão fecal por 18 semanas. Imunoistoquímica para β-catenina. 200x. **Figura 7-B** – Quantificação da expressão tecidual da proteína β-catenina (coloração branca) por análise de imagem assistida por computador no mesmo campo descrito na figura 7-A. (200x)
Fonte: Figura utilizada com autorização de MARTINEZ, 2010, p. 59

4.12 Dosagem Tecidual de Malondialdeído (MDA)

Os níveis de peroxidação de lípidos foram avaliados através da medida dos níveis de substâncias que reagem ao ácido tiobarbitúrico (TBARS), tal como ao MDA, com metodologia previamente descrita (BRADLEY et al., 1982). O MDA é um produto secundário

da oxidação lipídica e é considerado um potencial candidato como um biomarcador gerando estresse oxidativo. Para a quantificação dos níveis teciduais da MDA, 1g de cada fragmento foi colocado em 5 mL de tampão fosfato e homogeneizado num vórtice e em ultra-sonicador durante 30 segundos, alternadamente, repetindo o processo três vezes. Em seguida, 250 uL do sobrenadante obtido a partir do processo de homogeneização foi transferida para um tubo de plástico que continha 25 mL de 4% BHT metanólico e novamente homogeneizada em vórtice. A amostra foi então misturada com 1 mL de ácido tricloroacético 12%, 1 mL de ácido tiobarbitúrico de 0,73% e 750 uL de tampão Tris/HCl e incubados em banho-maria a 100 °C durante 60 minutos. Após este passo, os tubos foram imediatamente colocados num recipiente com gelo para bloquear a reação, sendo adicionado 1,5 mL de n-butanol e, em seguida, misturados por vortex novamente por 30 segundos. As amostras foram separadas por centrifugação durante 10 minutos a 5.000 rpm. Finalmente, o sobrenadante foi removido, e a absorvância a 532nm da fase orgânica foi analisada utilizando um espectro fotómetro UV/VIS,6105 (Jenway, BibbyScientificLimited,Staffordshire, RU).

4.13 Método Estatístico

Os resultados foram sempre descritos pela mediana com respectivo erro padrão. Adotou-se sempre nível de significância de 5% ($p<0,05$). Foi utilizado o teste de Mann-Whitney para analisar o escore de graduação inflamatória e o conteúdo tecidual das proteínas E-caderina, β-catenina, claudina-3, ocludina, MPO e MDA, comparando os animais do grupo controle e experimental (sendo os segmentos irrigados com solução fisiológica e sucralfato nas concentrações de 1,0 g/Kg/dia e 2,0 g/Kg/dia). Empregou-se o teste de Kruskal-Wallis para análise de variância do conteúdo tecidual das proteínas E-caderina, β-catenina claudina, ocludina, MPO e MDA com relação ao tempo de intervenção. Para o estudo estatístico utilizou-se o programa SPSS (versão 13.0).

5. RESULTADOS

5.1 Avaliação Histológica da Presença CE

As Figuras 8A e 8B mostram a parede cólica em segmentos desprovidos de trânsito fecal em animais submetidos à intervenção com SF e SCF 2,0 g/kg/dia por 2 e 4 semanas.

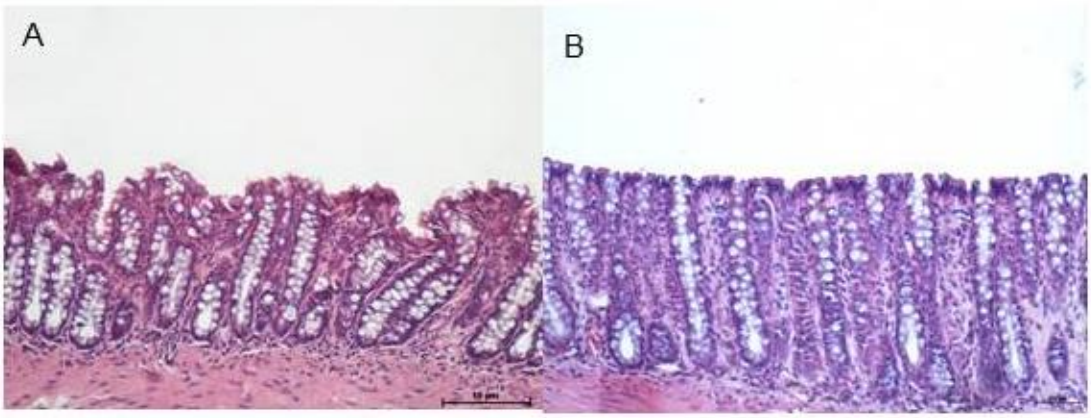

Figura 8A – Epitélio cólico sem trânsito fecal de animal submetido à intervenção com SF por 2 semanas, com perda da arquitetura linear da superfície epitelial e formação de micro-úlceras (HE 200 x). **Figura 8B** – Epitélio cólico sem trânsito fecal após intervenção com SCF 2,0 g/kg/dia por 4 semanas onde observa-se a preservação epitelial e a manutenção da altura das glândulas cólicas (HE 200x)

As Figuras 9A e 9B mostram a parede cólica em segmentos desprovidos de trânsito fecal em animais submetidos à intervenção com SCF 1.0 g/kg/dia por duas semanas e com SCF 2,0 g/kg/dia por quatro semanas.

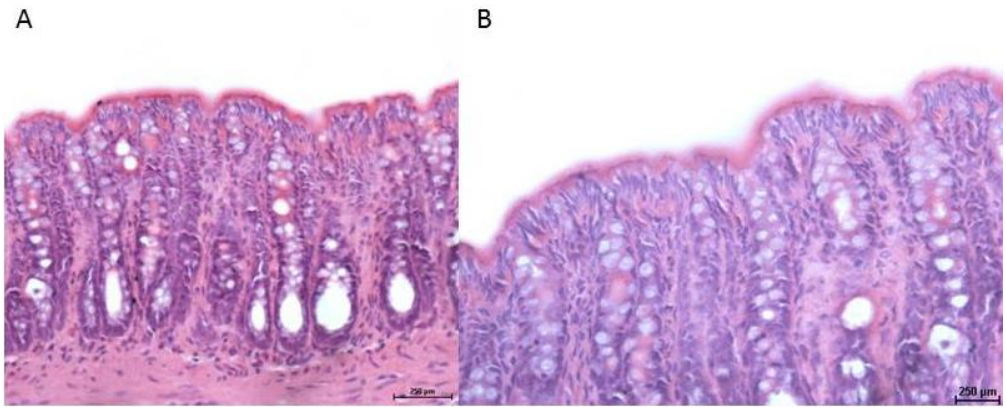

Figura 9A – Epitélio cólico sem trânsito fecal de animal submetido à intervenção com SCF 1,0 g/kg/dia por 2 semanas, com formação de uma película protetora de sucralfato sobre sua superfície com preservação epitelial (HE 200 x). **Figura 9B** – Epitélio cólico sem trânsito fecal após intervenção com SCF 2,0 g/kg/dia por 2 semanas, com formação de uma película de sucralfato sobre sua superfície com preservação epitelial (HE 400x)

5.2 Úlceras Epiteliais

A Figura 10 mostra a perda epitelial nos animais submetidos à intervenção com SF, SCF 1,0 g/kg/dia e 2,0 g/Kg/dia por 2 e quatro semanas. Ao analisar os níveis de perda epitelial verificou-se que nos animais irrigados com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia por duas semanas, em média com respectivo erro padrão, os valores foram de 2,2 ± 0,7, 2,2 ± 0,7 e 1,6 ± 0,6, respectivamente. Ao analisar os níveis de perda epitelial verificou-se que nos animais irrigados com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia por quatro semanas, em mediana com respectivo erro padrão, os valores foram de 2,6 ± 0,5, 2,5 ± 0,7 e 1,0 ± 0,1, respectivamente. Ao comparar os resultados foi constatado que a irrigação com SCF 2,0 g/kg/dia por quatro semanas reduz significativamente a perda epitelial ($p<0,05$) em relação aos animais do grupo controle e àqueles irrigados com SCF 1,0 g/kg/dia também por quatro semanas.

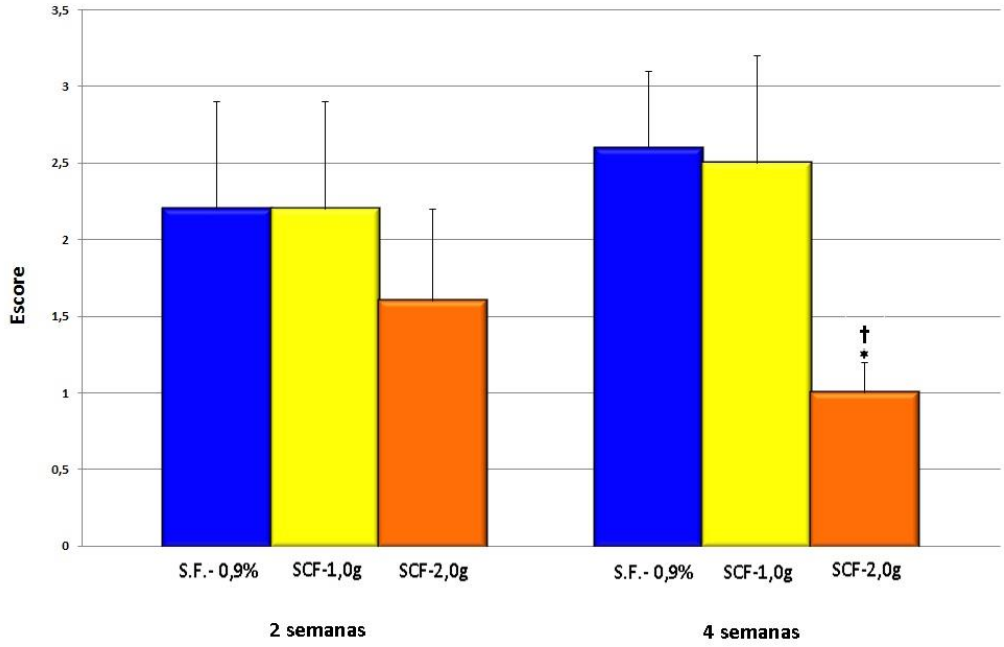

Figura 10 – Valores médios da intensidade (escore) da perda epitelial nos animais submetidos à intervenção com SF, SCF 1,0 g/kg/dia e 2,0 g/Kg/dia, por 2 e 4 semanas. * = significante (SCF 2,0 g/Kg/dia × SF); † = significante (SCF 2,0 g/kg/dia × SCF 1,0 g/kg/dia). Teste de Mann-Whitney

A Figura 11 mostra os escores encontrados quando se analisou o infiltrado inflamatório nas camadas mucosa e submucosa, dos segmentos cólicos irrigados com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por duas e quatro semanas. Os resultados mostraram que a intervenção do cólon excluso de trânsito intestinal com SCF na concentração de 2,0 g/kg/dia

foi capaz de diminuir o escore apenas após quatro semanas de irrigação quando comparado aos animais irrigados com SF (p = 0,003) e SCF 1,0 g/kg/dia (p=0,004).

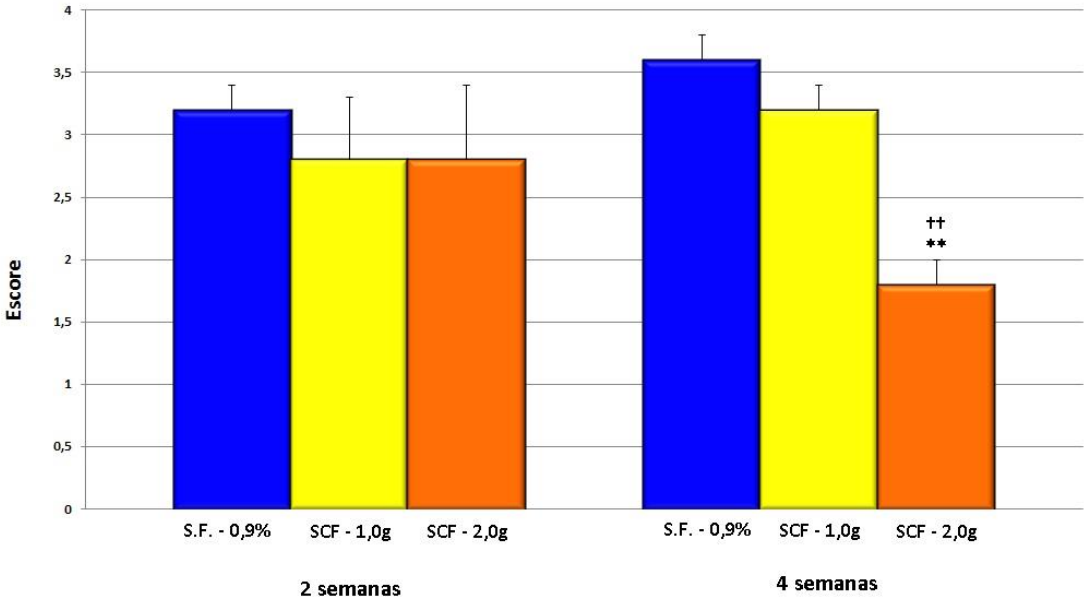

Figura 11– Valores médios do escore inflamatório encontrado nos animais submetidos à intervenção com SF, SCF 1,0 g/Kg/dia e 2,0 g/Kg/dia por 2 e 4 semanas. ** = significante (SCF 2,0 kg/dia × SF) (p<0,01); †† = significante (SCF 2,0 g/kg/dia × SCF 1,0g/Kg/dia). Teste de Mann-Withney.

5.3 Expressão Tecidual da Proteína E-caderina

A Figura 12A mostra a expressão da proteína E-caderina nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF por 4 semanas. A figura 12B mostra a expressão da proteína E-caderina nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SCF 2,0 g/kg/dia por 4 semanas.

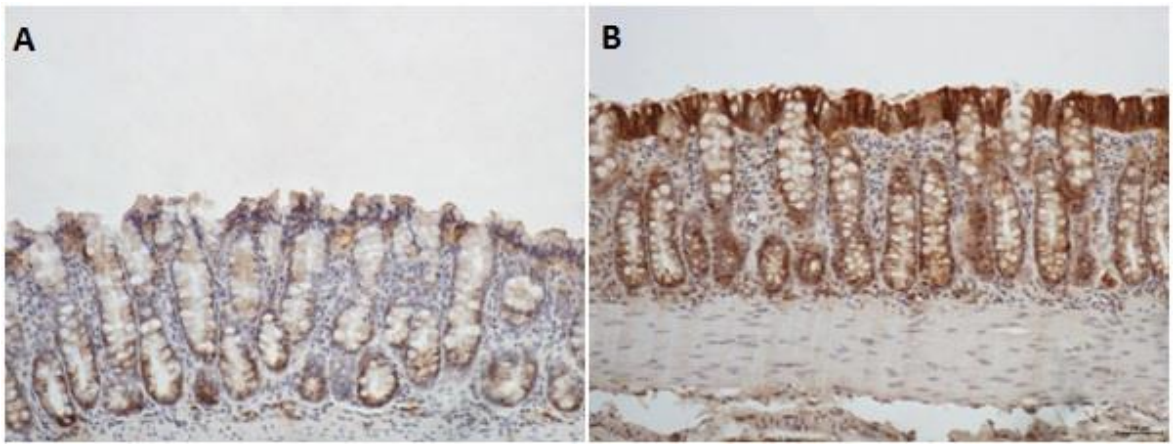

Figura 12A – Epitélio cólico sem trânsito fecal de animal submetido à intervenção com SF por 4 semanas, com perda da expressão de E-caderina na superfície epitelial e formação de úlceras (200 x). **Figura 12B** – Epitélio cólico sem trânsito fecal após intervenção com SCF 2,0 g/kg/dia por 4 semanas com expressão da proteína E-caderina na porção apical das criptas cólicas (200x)

5.4 Conteúdo Tecidual da Proteína E-caderina

A figura 13 mostra os valores encontrados do conteúdo tecidual da proteína E-caderina nos animais submetidos à intervenção diária com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia por duas e quatro semanas. Ao mensurar o conteúdo da proteína E-caderina, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por duas semanas, encontrou-se, em média com respectivo erro padrão, o conteúdo em porcentagem/campo histológico de 2.8 ± 0.43, 5.68 ± 1.2 e 7.62 ± 1,16, respectivamente. Esses resultados mostraram que a intervenção do cólon excluso de trânsito intestinal com SCF na concentração de 1,0 g/kg/dia por duas semanas aumenta, significativamente, o conteúdo da proteína E-caderina ($p=0,01$). Esse aumento é ainda maior nos animais irrigados com 2,0 g/kg/dia ($p=0,001$). Quando medimos o conteúdo da proteína E-caderina, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por quatro semanas, em média com respectivo erro padrão, o conteúdo em porcentagem/campo histológico foi de 3.5 ± 0.20, 6.38 ± 0.64 e 6.12 ± 0.80, respectivamente. Esses resultados mostram que independente da concentração utilizada houve aumento significativo ($p=0,001$).

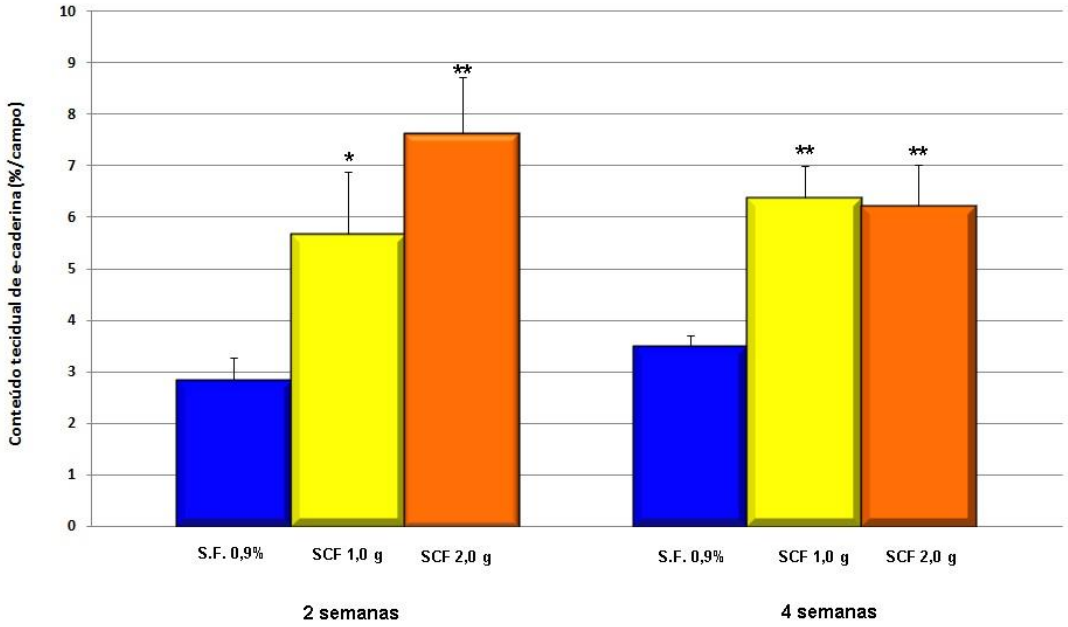

Figura 13 – Valores médios do conteúdo da proteína E-caderina encontrado nos animais submetidos à intervenção com SF, SCF 1,0 g/Kg/dia e 2,0 g/Kg/dia por 2 e 4 semanas. * = significante (p<0,05) ** = significante (p<0,01). Teste de Mann-Withney

5.5 Expressão da Proteína β-catenina

A Figura 14A mostra a expressão da proteína β-catenina nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF por 4 semanas. A figura 14B mostra a expressão da proteína β-catenina nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SCF 2,0 g/kg/dia por 4 semanas.

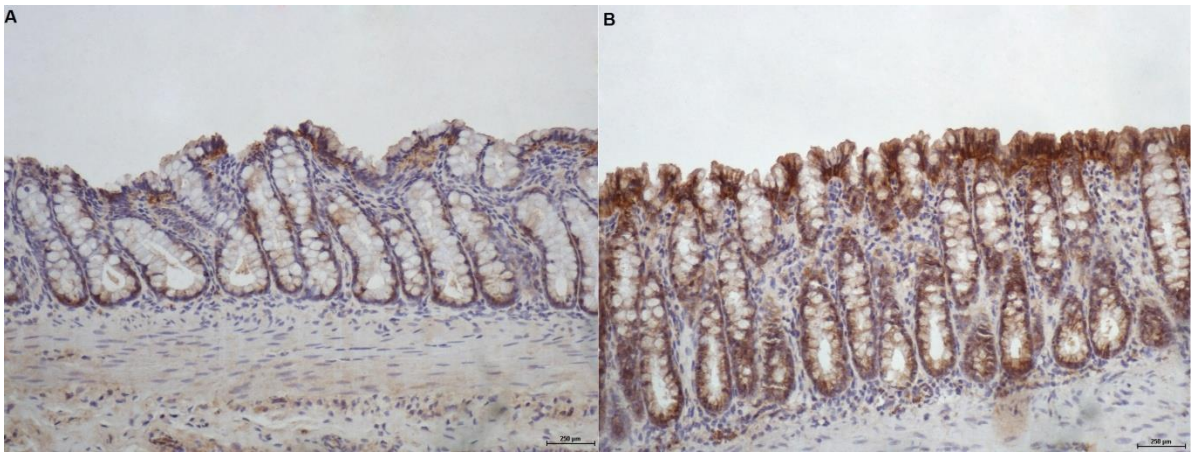

Figura 14A – Epitélio cólico sem trânsito fecal de animal submetido à intervenção com SF por 4 semanas, com perda da expressão de β-catenina na superfície epitelial com a formação de úlceras (200 x). **Figura 14B** – Epitélio cólico sem trânsito fecal após intervenção com SCF 2,0 g/kg/dia por 4 semanas com expressão da proteína β -catenina na porção apical das glândulas cólicas (200x).

5.6 Conteúdo Tecidual da Proteína β-catenina

A Figura 15 mostra os valores encontrados do conteúdo tecidual da proteína β-catenina nos animais submetidos à intervenção diária com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia por duas e quatro semanas. Ao mensurar o conteúdo da proteína β-catenina, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por duas semanas foi encontrado, em média com respectivo erro padrão, o conteúdo, em porcentagem/campo histológico, de 2.89 ± 0.38, 5.28 ± 0.5 e 6.41 ± 0,44, respectivamente. Esses resultados mostraram que a intervenção do cólon excluso de trânsito intestinal com SCF na concentração de 1,0 g/kg/dia por duas semanas aumenta, significativamente, o conteúdo da proteína β-catenina (p=0,004). Esse aumento é ainda maior nos animais irrigados com 2,0 g/kg/dia (p=0,0007). Quando se mediu o conteúdo da proteína β-catenina, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por quatro semanas, em média com respectivo erro padrão, o conteúdo em porcentagem/campo histológico foi de 3.51 ± 0.22, 5.34 ± 0.64 e 7.49 ± 0.75, respectivamente. Esses resultados mostram que houve aumento significativo no conteúdo de β-catenina nos animais irrigados com SCF 1,0 g/kg/dia (p=0,04), e maior ainda com concentração de 2,0 g/kg/dia(p=0,003). Não houve variação no conteúdo de β-catenina com o decorrer do tempo de intervenção, independente da concentração de SCF utilizada (p>0,05).

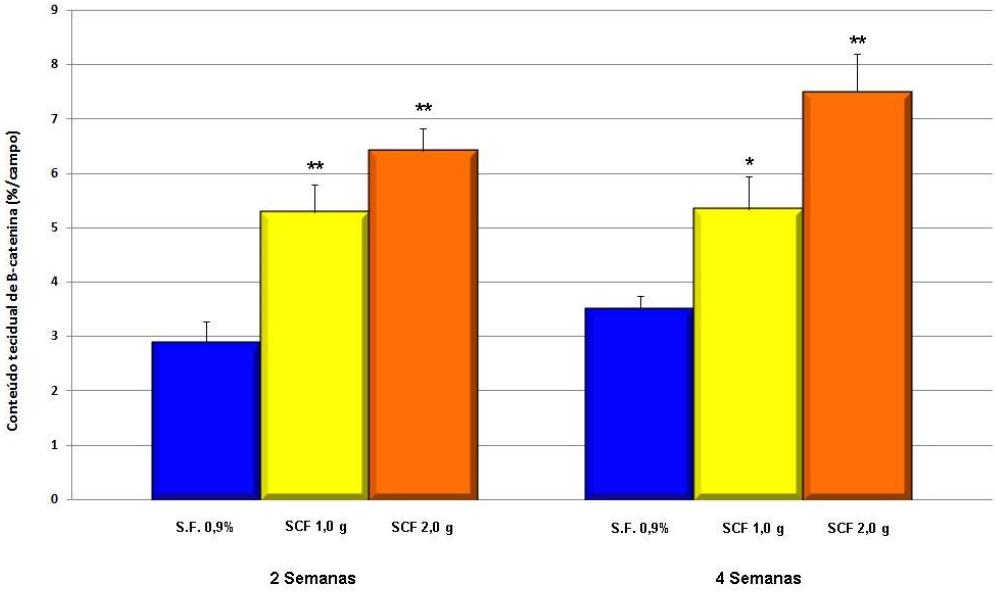

Figura 15 – Valores médios do conteúdo tecidual de β-catenina encontrado nos animais submetidos à intervenção com SF, SCF 1,0 g/Kg/dia e 2,0 g/Kg/dia por 2 e 4 semanas. * = significante (p<0,05); **= significante (p<0,001). Teste de Mann-Withney

5.7 Expressão da Proteína Claudina-3

A Figura 16A mostra a expressão da proteína claudina-3 nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF por 4 semanas. A figura 16B mostra a expressão da proteína claudina-3 nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SCF 2,0g/kg/dia por 4 semanas.

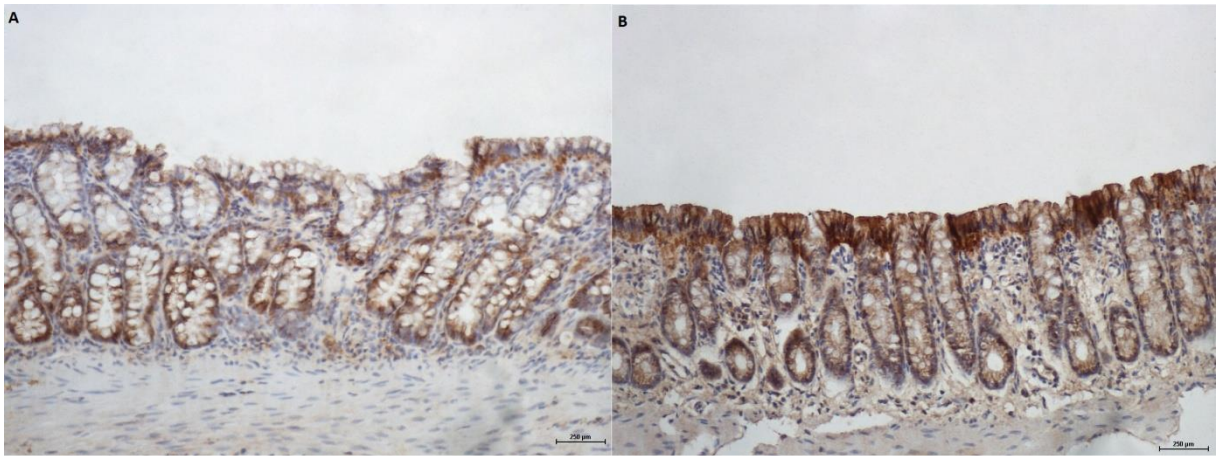

Figura 16A – Epitélio cólico sem trânsito fecal de animal submetido à intervenção com SF por 4 semanas, com perda da expressão de claudina-3 na superfície epitelial (200 x). **Figura 16B** – Epitélio cólico sem trânsito fecal após intervenção com SCF 2,0 g/kg/dia por 4 semanas com expressão da proteína claudina-3 na porção apical das glândulas cólicas (200x).

5.8 Conteúdo Tecidual da Proteína Claudina-3

A Figura 17 mostra os valores encontrados para o conteúdo tecidual da proteína claudina-3 nos segmentos cólicos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF, SCF 1.0 g/kg/dia e SCF 2,0 g/kg/dia por 2 e 4 semanas. Ao mensurar o conteúdo da claudina-3, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por duas semanas foi encontrado, em média com respectivo erro padrão, o conteúdo, em porcentagem/campo histológico, de 8.39 ± 0.87, 14.35 ± 0.89 e 19.69 ± 0,83, respectivamente. Esses resultados mostraram que a intervenção do cólon excluso de trânsito intestinal com SCF por duas semanas, independente da concentração utilizada aumenta, significativamente, o conteúdo da proteína claudina-3 ($p<0,0001$). Esse aumento após duas semanas de intervenção é maior nos animais irrigados com 2,0 g/kg/dia quando comparado aos irrigados com 1,0 g/kg/dia ($p=0,0003$). Quando se mensurou o conteúdo tecidual de claudina-3, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia

e 2,0 g/kg/dia por quatro semanas, em média com respectivo erro padrão, o conteúdo foi de 11.1 ± 0.45, 16.36 ± 1.52 e 17.94 ± 0.59, respectivamente. Esses resultados mostram que houve aumento significativo no conteúdo de claudina-3 nos animais irrigados com SCF 1,0 g/kg/dia (p=0,0001), e 2,0 g/kg/dia (p=0,0001). Esse aumento após duas semanas de intervenção é maior nos animais irrigados com 2,0 g/kg/dia quando comparado aos irrigados com 1,0 g/kg/dia (p=0,0001). Não houve variação no conteúdo tecidual de claudina-3 com o decorrer do tempo de intervenção, independente da concentração de SCF utilizada (p>0,05).

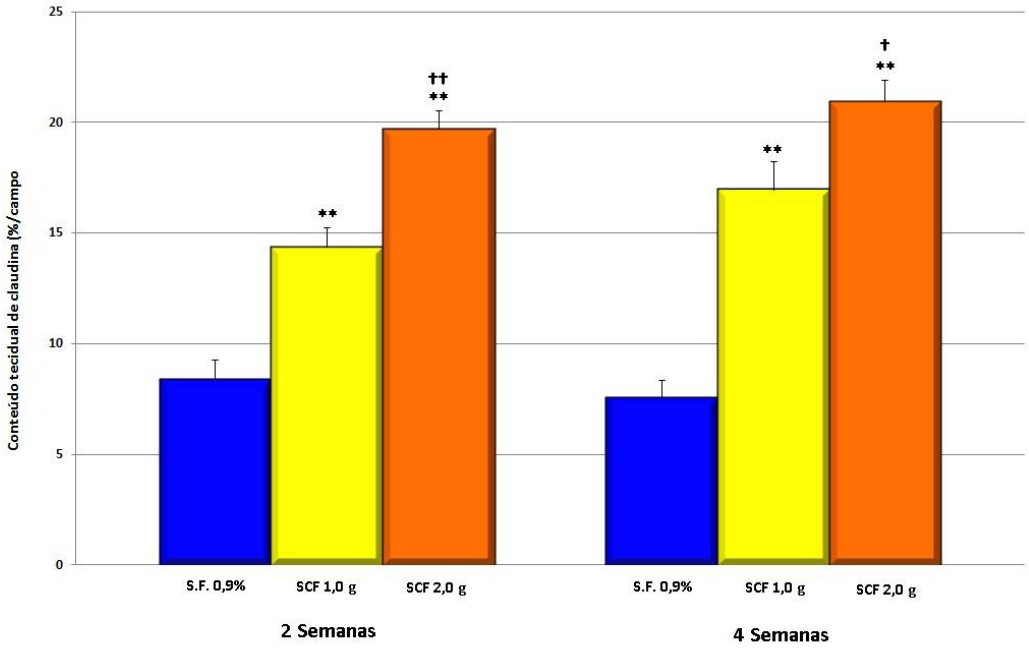

Figura 17 – Valores médios do conteúdo tecidual de claudina-3 encontrado nos animais submetidos à intervenção com SF, SCF 1,0 g/Kg/dia e 2,0 g/Kg/dia por 2 e 4 semanas. **= significante: SCF 1,0 g × SF e SCF 2,0 g × SF (p<0,0001); †= significante; SCF 2,0 g × SCF 1,0 g (p=0,01); †† = significante: SCF 2,0g × SCF 1,0 g (p=0,0003). Teste de Mann-Withney

5.9 Expressão da Proteína Ocludina

A Figura 18A mostra a expressão da proteína claudina-3 nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF por 4 semanas. A figura 18B mostra a expressão da proteína claudina-3 nas glândulas da mucosa cólica em segmentos desprovidos de trânsito fecal nos animais submetidos à intervenção com SCF 2,0 g/kg/dia por 4 semanas.

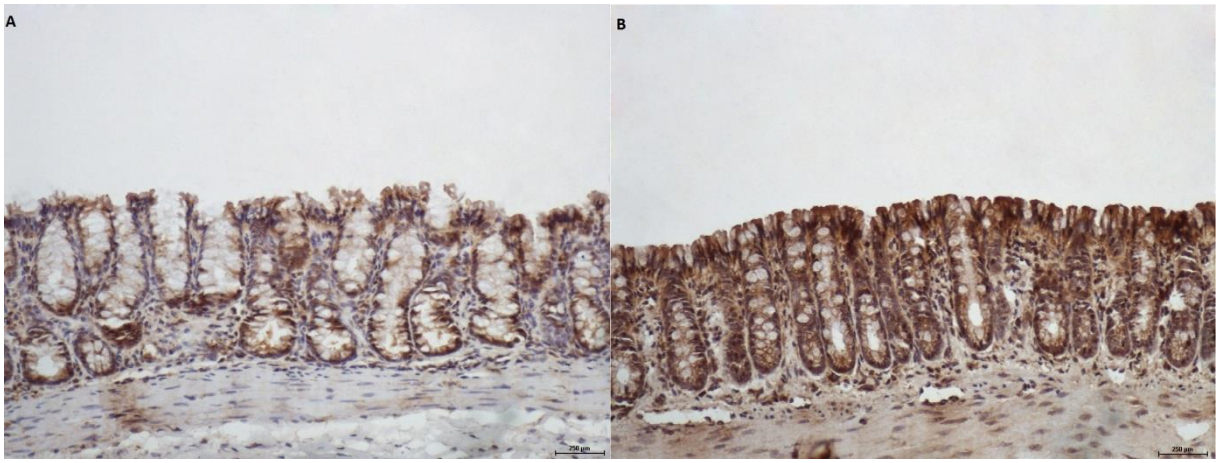

Figura 18A – Epitélio cólico sem trânsito fecal de animal submetido à intervenção com SF por 4 semanas, com perda da expressão de ocludina na superfície epitelial com a formação de úlceras (200 x). **Figura 18B** – Epitélio cólico sem trânsito fecal após intervenção com SCF 2,0 g/kg/dia por 4 semanas com expressão da proteína ocludina na porção apical das glândulas cólicas (200x).

5.10 Conteúdo Tecidual da Proteína Ocludina

A Figura 19 mostra os valores encontrados para o conteúdo tecidual da proteína ocludina nos segmentos cólicos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF, SCF 1.0 g/kg/dia e SCF 2,0 g/kg/dia por 2 e 4 semanas. Ao mensurar o conteúdo da ocludina, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por duas semanas encontrou-se, em média com respectivo erro padrão, o conteúdo, em porcentagem/campo histológico, de 9.73 ± 1.02, 13.21 ± 0.85 e 17.95 ± 0,60, respectivamente. Esses resultados mostraram que a intervenção do cólon excluso de trânsito intestinal com SCF por duas semanas, independente da concentração utilizada aumenta, significativamente, o conteúdo da proteína ocludina ($p<0,0001$). Esse aumento após duas semanas de intervenção é maior nos animais irrigados com 2,0 g/kg/dia quando comparado aos irrigados com 1,0 g/kg/dia ($p=0,0003$). Quando se mensurou o conteúdo tecidual de ocludina, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia por quatro semanas, em média com respectivo erro padrão, o conteúdo foi de 11.1 ± 0.45, 16.36 ± 1.52 e 17.94 ± 0.59, respectivamente. Esses resultados mostram que houve aumento significativo no conteúdo de ocludina nos animais irrigados com SCF 1,0 g/kg/dia ($p=0,0001$), e 2,0 g/kg/dia ($p=0,0001$). Não houve variação no conteúdo tecidual de ocludina com o decorrer do tempo de intervenção, independente da concentração de SCF utilizada ($p>0,05$).

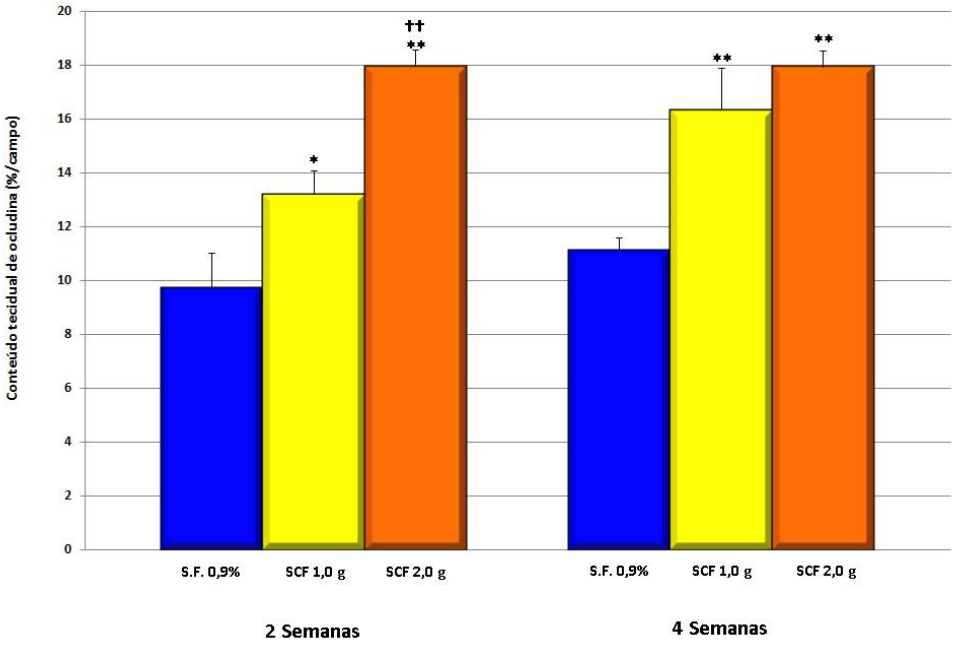

Figura 19 – Valores médios do conteúdo tecidual de ocludina encontrado nos animais submetidos à intervenção com SF, SCF 1,0 g/Kg/dia e 2,0 g/Kg/dia por 2 e 4 semanas. **= significante: SCF 1,0 g × SF e SCF 2,0 g × SF (p<0,0001); †† = significante: SCF 2,0g × SCF 1,0 g (p=0,0003). Teste de Mann-Withney

5.11 Expressão Tecidual da Enzima MPO

A Figura 20A mostra a expressão da enzima MPO em segmentos cólicos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF por 4 semanas. A figura 20B mostra a expressão da enzima MPO em segmentos cólicos desprovidos de trânsito fecal nos animais submetidos à intervenção com SCF 2,0 g/kg/dia por 4 semanas.

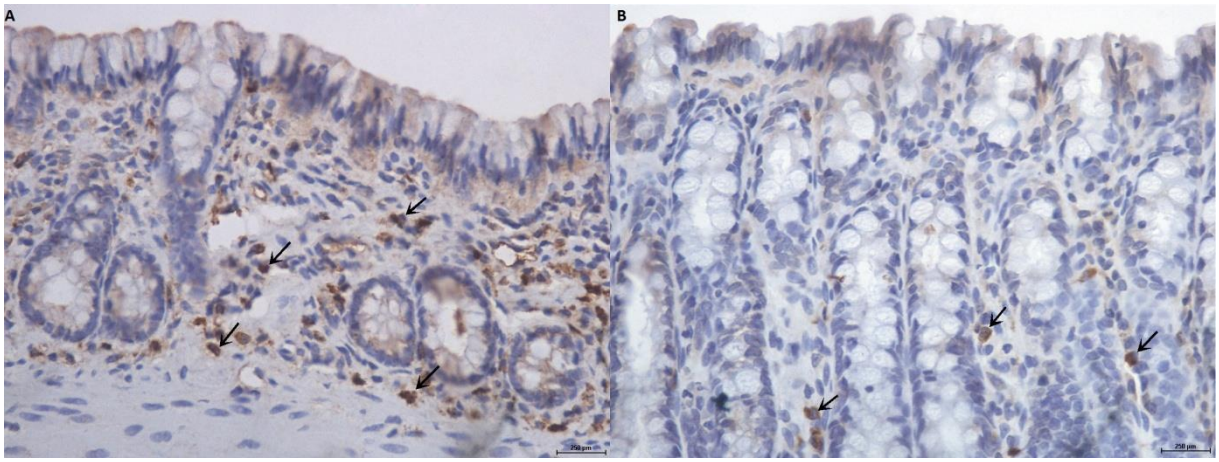

Figura 20A – Expressão de MPO em animal submetido à intervenção com SF por 4 semanas com um infiltrado de leucócitos do tipo neutrófilos marcados com setas (400 x). **Figura 20B** – Expressão de MPO após intervenção com SCF 2,0 g/kg/dia por 4 semanas. Com um menor número de leucócitos neutrófilos nas setas (400x).

5.12 Conteúdo Tecidual da Enzima MPO

A Figura 21 mostra os valores encontrados para o conteúdo tecidual da enzima MPO nos segmentos cólicos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF, SCF 1,0 g/kg/dia e SCF 2,0 g/kg/dia por 2 e 4 semanas. Ao mensurar o conteúdo de MPO, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por duas semanas encontrou-se, em média com respectivo erro padrão, o conteúdo, em porcentagem/campo histológico, de 13,77 ± 0,62, 10,32 ± 0,48 e 10,36 ± 0,54, respectivamente. Esses resultados mostraram que a intervenção do cólon excluso de trânsito intestinal com SCF por duas semanas, independente da concentração utilizada reduziu, significativamente, o conteúdo da enzima MPO ($p<0,0001$) mostrando diminuição do infiltrado neutrofílico. Essa redução após duas semanas de intervenção não dependia da concentração utilizada. Quando se mensurou o conteúdo tecidual de MPO, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia por quatro semanas, em média com respectivo erro padrão, o conteúdo foi de 14,70 ± 0,40, 9,82 ± 1,12 e 10,08 ± 0,46, respectivamente. Esses resultados mostram que, da mesma forma que ocorria após duas semanas de intervenção, houve redução do infiltrado neutrofílico nos animais irrigados com SCF 1,0 g/kg/dia e 2,0 g/kg/dia ($p=0,0001$). A diminuição do infiltrado neutrofílico após quatro semanas também não estava relacionado à concentração utilizada. Não houve variação no conteúdo tecidual de MPO com o decorrer do tempo de intervenção, independente da concentração de SCF utilizada ($p>0,05$).

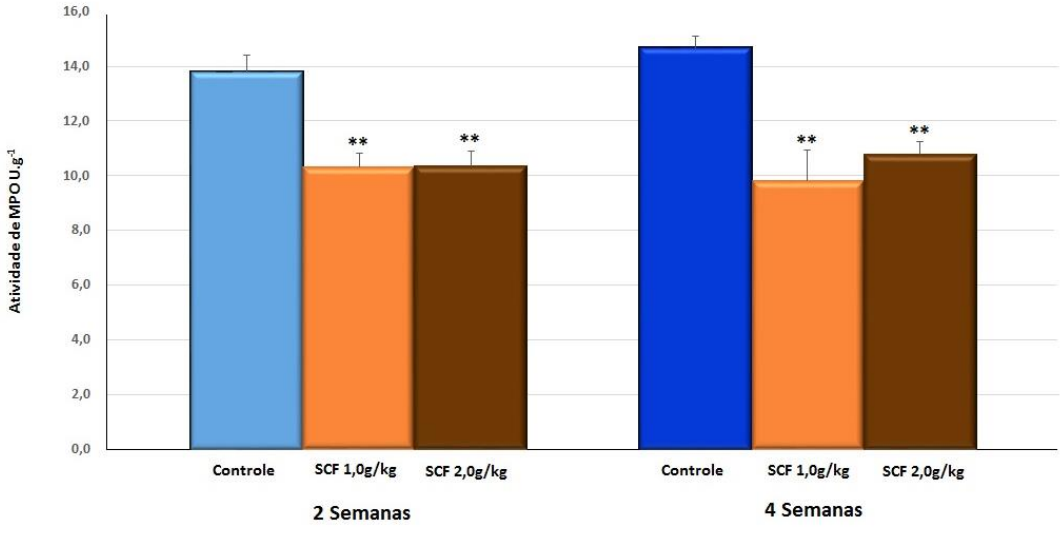

Figura 21– Valores médios do conteúdo tecidual de MPO encontrado nos animais submetidos à intervenção com SF, SCF 1,0 g/Kg/dia e 2,0 g/Kg/dia por 2 e 4 semanas. **= significante: SF × SCF 1,0 g e SCF 2,0 g ($p<0,0001$). Teste de Mann-Withney

5.13 Conteúdo Tecidual de MDA

A Figura 22 mostra os valores encontrados para o conteúdo tecidual de MDA nos segmentos cólicos desprovidos de trânsito fecal nos animais submetidos à intervenção com SF, SCF 1,0g/kg/dia e SCF 2,0 g/kg/dia por 2 e 4 semanas. Ao mensurar o conteúdo de MDA, no cólon excluso de trânsito fecal irrigado com SF 0,9%, SCF 1,0 g/kg/dia e 2,0 g/kg/dia, por duas semanas encontrou-se, em média com respectivo erro padrão, o conteúdo, em porcentagem/campo histológico, de 0,045200±0,003, 0,0074623±0,002 e 0,0106303±0,004, respectivamente. Esses resultados mostraram que a intervenção do cólon excluso de trânsito intestinal com SCF por duas semanas, independente da concentração utilizada reduziu, significativamente, o conteúdo da enzima MDA ($p<0,0001$) mostrando diminuição dos níveis de peroxidação lipídica. Essa redução após duas semanas de intervenção não dependia da concentração utilizada. Quando se mensurou o conteúdo tecidual de MDA, no cólon excluso de trânsito fecal irrigado com SF, SCF 1,0 g/kg/dia e 2,0 g/kg/dia por quatro semanas, em média com respectivo erro padrão, o conteúdo foi de 0,0610614±0,006, 0,023973±0,01 e 0,0114457±0,002, respectivamente. Esses resultados mostram que, da mesma forma que ocorria após duas semanas de intervenção, houve redução da peroxidação lipídica nos animais irrigados com SCF 1,0 g/kg/dia e 2,0 g/kg/dia ($p=0,0001$). A diminuição da peroxidação lipidica após quatro semanas também não estava relacionado à concentração utilizada. Não houve variação no conteúdo tecidual de MDA com o decorrer do tempo de intervenção, independente da concentração de SCF utilizada ($p>0,05$).

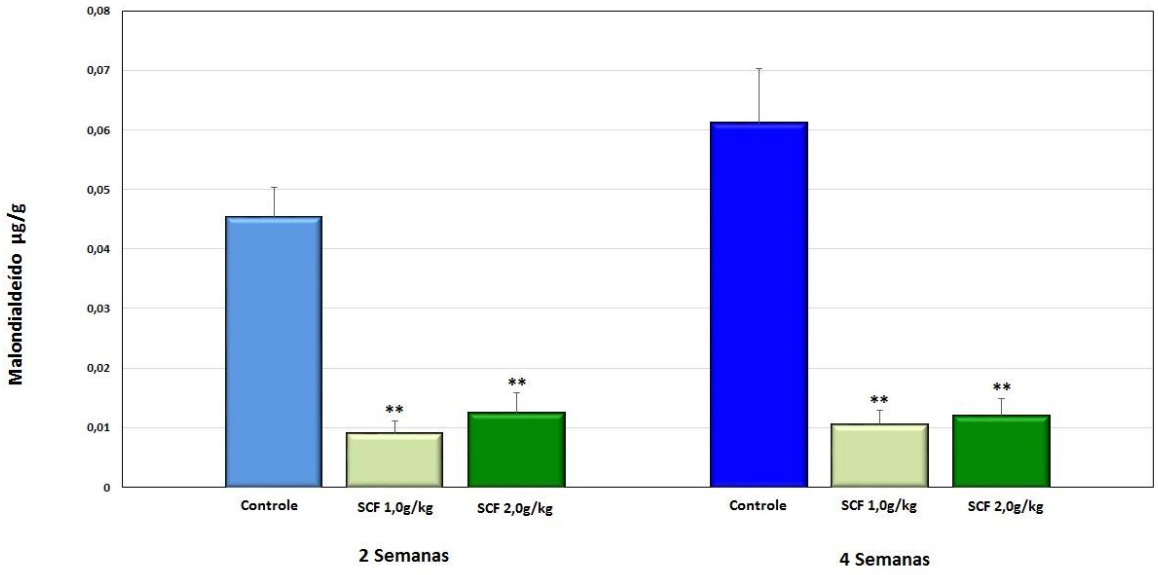

Figura 22 – Valores médios do conteúdo tecidual de MDA encontrado nos animais **ina, C** submetidos à intervenção com SF, SCF 1,0 g/Kg/dia e 2,0 g/Kg/dia por 2 e 4 semanas. **= **SCF 1** significante: SF × SCF 1,0 g e SCF 2,0 g ($p<0,0001$). Teste de Mann-Withney

A Tabela 1 mostra em mediana com o respectivo erro padrão, a variação para os valores encontrados para o conteúdo tecidual das proteínas E-caderina, β-catenina, claudina, ocludina, MPOe MDA nos segmentos cólicos desprovidos de trânsito fecal, nos animais submetidos à intervenção com SF 0,9%, SCF 1.0g/kg/dia e SCF 2,0 g/kg/dia após 2 e 4 semanas.

Tabela 1 – Variação no conteúdo tecidual das proteínas E-caderina, β-catenina, claudina, ocludina, MPO e MDA nos segmentos cólicos submetidos à intervenção com SF, SCF 1.0g/kg/dia e SCF 2,0 g/kg/dia em relação ao tempo de intervenção.

	2 semanas (M ± EP)			**4 semanas (M ± EP)**		
	SF-0,9%	**SCF-1**	**SCF-2**	**SF-0,9%**	**SCF-1**	**SCF-2**
E-caderina	2,81±0,38	4,35±1,20	6,18±1,16	3,50±0,22	6,38±0,64	6,35±0,87
β-catenina	2,68±0,38	4,95±0,50	6,03±0,44	3,36±0,22	5,31±0,64	7,99±0,75
Claudina	8,39±0,87	14,35±0,89	19,69±0,83	7,55±0,79	16,95±1,26	20,94±0,94
Ocludina	9,73±1,02	13,21±0,85	17,95±0,60	11,14±0,45	16,36±1,52	17,94±0,59
MPO	13,77±0,62	10,32±0,48	10,36±0,54	14,70±0,40	9,82±1,12	10,08±0,46
MDA	0,045±0,003	0,0074±0,002	0,010±0,004	0,0610±0,006	0,0092±0,003	0,0114±0,002

Teste de Kruskal-Wallis; M = Mediana; EP= Erro padrão.

6. DISCUSSÃO

6.1 Considerações Gerais

O SCF é o sal formado pelo dissacarídeo octosufalto de sacarose associado ao hidróxido de polialumínio (VOLKIN et al., 1993). A substância é considerada um complexo citoprotetor, sendo inicialmente utilizada para prevenir ou tratar doenças do trato digestivo superior, principalmente representadas pela doença ulcerosa péptica, úlceras de estresse e lesões agudas da mucosa gástrica (SZABO e HOLLANDER, 1989; SZABO, 1991). Posteriormente, em virtude da destacada capacidade de adesão sobre superfícies cruentas, a substância também se mostrou eficaz para prevenção e tratamento de úlceras varicosas crônicas, mucosites pós-radioterapia cervical, queimaduras e cicatrizes com aquelas decorrentes das hemorroidectomias (BANATI; CHOWDHURY e MAZUMDER, 2001; GUPTA et al., 2008; TUMINO et al., 2008; ALA et al., 2013; QUTOB et al., 2013).

A primeira referência ao uso do SCF em portadores de DII foi publicada em meados da década de 1980 (CARLING; KAGEVI; BORVALL, 1986). A partir de então uma série de autores publicaram os resultados do uso do SCF para o tratamento de diferentes enfermidades cólicas que evoluem com inflamação, entre elas a retocolite ulcerativa e a retite actínica (KOCHHAR et al., 1988; LADAS e RAPTIS, 1989; KOCHHAR et al., 1990; HIDALGO VERA et al., 1990; KOCHHAR et al., 1991; VALLS et al., 1991; HENRIKSSON; FRANZÉN; LITTBRAND, 1992; ARDIZZONE et al., 1996; STOCKDALE; BISWAS, 1997; O'BRIEN et al., 1997; SASAI et al., 1998; ZIMMERMANN; FELDMANN, 1998; KOCHHAR et al., 1999; MELKO et al., 1999; KNEEBONE et al., 2001; DENTON et al., 2002; O'BRIEN et al., 2002; GUL et al., 2002; SANGUINETI et al., 2003; MANOJLOVIC e BABIC, 2004; HOVDENAK; SØRBYE; DAHL, 2005; SEO, 2006; LEIPER; MORRIS, 2007, DE PARADES et al., 2008; HENSON, 2010; RUSTAGI e MASHIMO, 2011; NELAMANGALA RAMAKRISHNAIAH et al., 2012; HAWKINS; BILLINGHAM e BASTAWROUS, 2012; HANSON; MACDONALD; SHAUKAT, 2012; CHRUSCIELEWSKA-KILISZEK et al., 2013; SARIN; SAFAR, 2013; STACEY; GREEN, 2013; MCELVANNA; WILSON; IRWIN, 2014; MENDENHALL et al., 2014). Os resultados desses estudos são conflitantes, pois apesar da maioria mostrar que a aplicação de enemas com SCF melhora a sintomatologia e os achados endoscópicos em portadores de retite actínica, outros não foram capazes de mostrar benefícios significantes. Ao revisar a literatura somente quatro estudos, todos realizados com o Projeto Fomentado pela FAPESP relacionado a esta tese, avaliaram a eficácia do SCF num modelo experimental de CE (PEREIRA et al., 2013; CHAIM et al., 2014; BONASSA et al., 2015; MARTINEZ et al., 2015b). Cabe destacar que dois desses

estudos, já mostram parte dos resultados encontrados durante a elaboração desta Tese. No primeiro artigo verificou-se que a aplicação de enemas com SCF foi capaz de reduzir a perda epitelial e a formação de úlceras epiteliais. O SCF reduzia significativamente o grau de inflamação no cólon excluso de trânsito preservando a arquitetura normal do epitélio cólico (PEREIRA et al., 2013). O segundo artigo mostrou que a aplicação diária de SCF reduzia significativamente o infiltrado inflamatório avaliado pelos níveis teciduais de MPO, bem como os níveis de peroxidação lipídica, confirmando descrições anteriores que o SCF apresenta atividade antioxidante (MARTINEZ, 2015b).

Os efeitos terapêuticos do SCF nas lesões cutâneo mucosas estão relacionados à propriedade que a substância tem em aderir-se, firmemente, à superfície cruenta de lesões epiteliais, tornando difícil a remoção da camada gelatinosa que se forma (WADA et al., 1997). No presente estudo foram confirmados esses achados, pois em todos os animais que sofreram a intervenção com SCF, quando da abertura do cólon após a eutanásia, apresentavam um cilindro de coloração esbranquiçada e aspecto mucoso, firmemente aderido à mucosa intestinal. Quando se estudou microscopicamente esses animais, confirmou-se a formação de uma película protetora sobre a mucosa cólica, melhor observada nas figuras 9A e 9B.

A capacidade adesiva parece ser o principal mecanismo de ação da droga, todavia, recentemente, demonstrou-se que a molécula do SCF possui ainda, outras propriedades funcionais (ROBERT et al., 1979; REES, 1992). Demonstrou-se, que, o SCF aumenta a produção de muco pelas células caliciformes gastrointestinais por inibir a atividade da enzima acetiltransferase, e aumenta a produção de prostaglandinas E2(PGE2) (SLOMIANY et al., 1989). Como se sabe a camada de muco que recobre o epitélio intestinal além de servir como proteção mecânica, cria um meio essencial para que ocorra a restituição e reparação do epitélio lesado (SCHEIMAN et al., 1992). Estudo experimental que avaliou as características físicas e químicas do muco que recobre o epitélio gástrico mostrou que após a administração do SCF ocorria aumento de 8% na espessura da camada de muco (SLOMIANY et al., 1989). O estudo mostrou que o SCF aumentava em 63% o conteúdo de sulfomucinas e em 81% o de sialomucinas, aumentando a proteção epitelial conferida por esses subtipos de mucinas ácidas (SLOMIANY et al., 1989). Essas alterações foram acompanhadas de um aumento de quase duas vezes na viscosidade do muco e de 60% na hidrofobicidade (SLOMIANY et al., 1989). Essas modificações aumentam a capacidade adesiva do muco sobre a superfície epitelial conferindo proteção maior e por tempo mais prolongado contra agressões oriundas do meio externo. De modo semelhante, recentemente foi demonstrado que essas alterações, também ocorrem na mucosa do intestino grosso exclusa de trânsito fecal que desenvolve CE. Constatou-se, que após a aplicação de clistéres diários com SCF no cólon excluso, que existia significativo aumento

da produção de mucinas neutras e, principalmente ácidas (CHAIM et al., 2014). Num segundo estudo avaliando os efeitos do SCF sobre as subtipos de mucinas ácida, verificou-se que o aumento no conteúdo de mucinas ácidas se dava principalmente às custas de sialomucinas, justamente o subtipo que sofre a maior redução no cólon excluso como foi demonstrado em estudo anterior (MARTINEZ et al., 2010; BONASSA et al., 2015). Esses achados sugerem que o SCF apresenta papel de destaque aumentando a proteção fornecida pela camada de muco que recobre o epitélio cólico e que representa a primeira linha de defesa da barreira epitelial do intestino grosso.

Estudos demonstraram que o SCF é capaz de estimular a produção de fator de crescimento epitelial (EGF) e fator de crescimento derivado de plaquetas (PGDF) favorecendo a cicatrização de lesões epiteliais (SLOMIANY et al., 1992; MASUELLI et al., 2010). Com isso o SCF acelera a reparação dos tecidos por estimular a migração de fibroblastos para o local onde houve a lesão tecidual (ROBERT et al., 1979). Cabe destacar que esses dois efeitos se encontram relacionados ao estimulo da produção de mucinas, PGE2 e EGF. As prostaglandinas, particularmente a PGE2, representam os principais produtos do metabolismo do ácido araquidônico, desempenhando um papel crítico na manutenção da integridade do epitélio gastrointestinal (SLOMIANY et al., 1994). O SCF aumenta a produção de prostaglandina pelo epitélio gastrointestinal por estimular a produção e secreção da fosfolipase A2 (SLOMIANY et al., 1994). O aumento na produção de PGE2, principal metabólito da COX-1 e COX-2, é capaz de induzir a angiogênese local, a motilidade e a sobrevivência de células epiteliais e endoteliais. Cabe lembrar que o maior aporte sanguíneo é uma condição importante em todo processo de cicatrização tecidual. Dentre os vários mecanismos de ação que estimulam a cicatrização, estudos mostraram ainda na década de 1990, que o SCF aumenta significativamente a produção de TGF-α e que a associação de SCF e TGF-α estimula a proliferação de células da mucosa e aumenta ainda mais o suprimento sanguíneo local, favorecendo todo processo de reparação (LOUW et al., 1998). Apesar de não ter sido mensurado até a presente data os níveis de EGF, PGE-2, COX-2 e TGF-α, os resultados encontrados no presente estudo parecem corroborar esses achados. Os animais submetidos à intervenção com SCF, independente do tempo de aplicação e da concentração utilizada, apresentam melhora da integridade epitelial sugerindo que as duas concentrações, de alguma forma, melhorou a reparação tecidual. Os animais do grupo controle apresentavam maior quantidade de perda epitelial associada a maiores valores de escore inflamatório.

A aplicação tópica do SCF possui ainda, atividade antioxidante sendo capaz de reduzir a formação de RLO produzidos por neutrófilos presentes no tecido inflamado (KESHAVARZIAN et al., 1990; WADA et al., 1997; PAVLICK et al., 2002; MARQUES et al., 2010). Essa ação antioxidante protege contra a peroxidação de lipídios componentes das

membranas celulares, segunda linha de defesa epitelial, protegendo a mucosa gastrointestinal contra o estresse oxidativo tecidual (WADA; et al., 1997). Estudos experimentais em ratos mostraram que ao se expor a mucosa gastrointestinal ao H_2O_2, potente formador de RLO ocorre a formação de inflamação da mucosa cólica e formação de úlceras epiteliais semelhantes àquelas encontradas na colite ulcerativa (WADA et al., 1997; MARQUES et al., 2010). O SCF possui notável ação antioxidante, protegendo a mucosa não só pela presença do sulfato de alumínio na sua composição, como pelo aumento da síntese de mucinase PGs endógenas (LAUDANNO et al., 1990; LAUDANO et al., 1991). O SCF também possui destacada atividade contra o estresse oxidativo (KONTUREK; BRZOZOWSKI; PYTKO-POLONCZYK,1995). Recentemente, com intuito de confirmar essa ação antioxidante avaliou-se a eficácia antioxidante do SCF num modelo experimental de CE (MARTINEZ et al., 2015). Os resultados encontrados mostraram que os animais tratados com enemas de SCF apresentaram redução significativa dos níveis de lipoperoxidação de membranas, avaliado pela dosagem tecidual de malondialdeído, assim como do infiltrado neutrofílico, avaliado pela dosagem tecidual dos níveis de MPO. Esses achados não se mostraram relacionados à dose ou ao tempo de intervenção, mas diretamente relacionados à melhora do processo inflamatório (MARTINEZ et al., 2015). Esses resultados sugerem que a aplicação de enemas com SCF no cólon excluso melhora a o processo inflamatório tecidual, provavelmente, pela sua ação antioxidante.

6.2 Estresse Oxidativo e Lesão da Barreira Epitelial na CE

Em 2010, nosso grupo, pela primeira vez na literatura, foi demonstrado que o estresse oxidativo decorrente da maior produção de RLO pelas células do epitélio cólico desprovido de suprimento de AGCC, poderia ser um dos mecanismos etiopatogênicos envolvidos da CE (MARTINEZ et al., 2010b). A partir desse estudo pioneiro, dedicou-se especial atenção aos mecanismos pelos quais a produção aumentada de RLO poderia ocasionar quebra dos diferentes sistemas de defesa do epitélio intestinal. Inicialmente verificou-se que a exclusão do trânsito intestinal ocasionava alterações morfológicas estruturais em diferentes camadas da parede cólica (SOUSA et al., 2008). Foi constatado que no cólon excluso havia importante redução na altura das glândulas cólicas com formação de úlceras superficiais na mucosa, configurando que existia importante quebra da barreira epitelial cólica (SOUSA et al., 2008). Paralelamente, encontrou-se maior infiltrado inflamatório e maiores níveis de estresse oxidativo tecidual na mucosa exclusa de trânsito quando comparada à mucosa com trânsito preservado.

A partir da constatação de que a exclusão intestinal modificava, reduzia a espessura da parede cólica e provocava quebra da barreira epitelial, iniciou-se a avaliação das

diferentes linhas de defesa, componentes do epitélio cólico. Como a camada de muco representa a primeira linha e defesa da mucosa cólica e, recentemente, vem sendo considerada um dos mais importantes mecanismos de defesa contra a agressão bacteriana julgou-se interessante avaliar sua integridade mecânica e funcional no modelo de CE proposto. Verificou-se que a exclusão intestinal modificava consideravelmente aspectos morfofuncionais relacionados à proteção conferida pela camada de muco que recobre o epitélio intestinal. Foi constatada redução na população de células caliciformes produtoras de muco existentes nas glândulas intestinais (MELLO et al., 2012). Essas células no cólon excluso apresentavam depleção acentuada de mucinas no citoplasma celular estando a maior quantidade de muco concentrada na luz das glândulas cólicas (MELLO et al., 2012). Ao analisar a camada de muco do ponto de vista bioquímico, foi verificado que nos segmentos sem trânsito fecal ocorria redução no conteúdo dos diferentes subtipos de mucinas pela mucosa cólica e que esta redução se dava principalmente à custa das mucinas ácidas. Ao avaliar os subtipos de mucinas ácidas constatou-se que a depleção se dava principalmente das sialomucinas, importante fator de proteção da mucosa intestinal (NONOSE et al., 2009; MARTINEZ et al., 2010a; MELLO et al., 2012). Ao medir os níveis de estresse oxidativo tecidual verificou-se que existia correlação inversamente proporcional ao conteúdo de mucinas (MARTINEZ et al., 2010a). Dessa forma, ficou demonstrado que, o estresse oxidativo era capaz de alterar de modo significativo a proteção conferida pela camada de muco possibilitando a invasão bacteriana da parede cólica.

A segunda linha de defesa da barreira mucosa cólica é representada pelo complexo sistema de adesão intercelular, formados pelas junções de oclusão, adesão e comunicantes. Estudos em modelos de colite experimental e em portadores de DII mostraram que a quebra das junções intercelulares é um evento precoce na etiopatogênese das DII (SCHMITZ et al., 2000; GASSLER et al., 2001; KUCHARZIK et al., 2001; OSHIMA; MIWA e JOH, 2008; SCHULZKE et al., 2009; SU et al., 2009; JOHN; FROMM; SCHULZKE, 2011; HERING; FROMM; SCHULZKE, 2012; IWAYA et al., 2012). Demonstrou-se que o estresse oxidativo é um dos principais mecanismos envolvidos na quebra desses sistemas de junção intercelular (JOHN; FROMM; SCHULZKE, 2011, HAIDARI; ZHANG; WAKAME, 2013). As junções de oclusão, situadas mais próximas da região apical das células do epitélio cólico selam hermeticamente o espaço intercelular impedindo a passagem de antígenos e bactérias para o meio interno. As junções de adesão, situadas logo abaixo das junções de oclusão unem o esqueleto intercelular de uma célula epitelial a sua vizinha aumentando a resistência epitelial e também contribuindo para a impermeabilidade do espaço intercelular. As junções comunicantes, situadas na região basocelular permitem a passagem de íons e pequenas moléculas de uma célula a outra. Estudos mostraram que essas junções se encontram comprometidas em diferentes formas de colite e que a deficiência de AGCC pode ocasionar

quebra das junções intercelulares (VIVINUS-NÉBOT et al., 2014). A integridade das junções intercelulares, também foi estudada pelo nosso grupo no modelo experimental de CE proposto (MARTINEZ et al., 2012; KADRI et al., 2013; MARTINEZ et al., 2015). Nesses estudos, mensurou-se o conteúdo tecidual das principais proteínas componentes das junções de oclusão (claudina-3 e ocludina) e de adesão intercelular (E-caderina e β-catenina) comparando os cólons providos e desprovidos de trânsito fecal após seis, doze e dezoito semanas de derivação. Ao mensurar o conteúdo da proteína E-caderina verificou-se que existia acentuada redução nas células do epitélio excluso de trânsito fecal e que essa redução se relacionava ao aumento dos níveis de estresse oxidativo tecidual avaliado pelo ensaio em cometa (KADRI; et al., 2013). No mesmo grupo de animais, foi avaliado o conteúdo da proteína β-catenina comparando segmentos providos e desprovidos de trânsito fecal. Como ocorria com a E-caderina, encontrou-se redução do conteúdo da proteína nas porções apicais das células epiteliais, porém aumento do conteúdo nas células localizadas nas regiões proliferativas das criptas cólicas (MARTINEZ et al. 2012). Esses achados sugeriam que havia quebra das junções de adesão com migração do conteúdo de β-catenina do citoplasma para o núcleo celular, justamente onde estava ocorrendo maior proliferação celular (MARTINEZ et al., 2012). Como se sabe a proteína β-catenina faz parte da via de sinalização Wnt e é um importante sinalizador de genes envolvidos na divisão celular. A redução do conteúdo da proteína β-catenina nas junções de adesão intercelular também estava inversamente relacionada aos maiores níveis de estresse oxidativo.

Com o objetivo de verificar se o mesmo fenômeno ocorria com as junções de oclusão intercelular, recentemente foram publicados os resultados da avaliação do conteúdo das proteínas claudina-3 e ocludina, principais constituintes das junções de oclusão, nos mesmos animais (MARTINEZ et al., 2015). De modo semelhante aos resultados antes descritos para as proteínas componentes das junções de adesão, verificou-se que também ocorria expressiva redução do conteúdo de ambas as proteínas nas células superficiais do epitélio desprovido de trânsito intestinal (MARTINEZ et al., 2015). Essa redução era mais evidente no conteúdo de claudina-3 principal proteína constituinte das junções de oclusão da mucosa cólica. Assim como ocorria com as junções aderentes, a redução da expressão tecidual de ambas as proteínas das junções de oclusão estava inversamente relacionada aos níveis de estresse oxidativo e a piora da inflamação tecidual (MARTINEZ et al., 2015).

Os resultados encontrados nos estudos acima descritos sugeriam que o emprego de substâncias com atividade antioxidante talvez pudesse ser eficaz na preservação do epitélio intestinal desprovido de trânsito fecal. Com o objetivo de estudar esse postulado, avaliaram-se os efeitos da aplicação de clisteres contendo ácido 5-aminosalicilico (5-ASA) e n-acetilcisteína (NAC), substâncias com reconhecida atividade antioxidante, no modelo experimental de CE. Nos dois estudos verificou-se que ambas as substâncias além de

reduzirem os níveis de estresse oxidativo tecidual, diminuíam a inflamação tecidual avaliada tanto do ponto de vista histológico, utilizando escala de graduação inflamatória previamente validada, quanto bioquímico pela avaliação do grau de infiltração neutrofílica estimado pelos níveis teciduais de MPO (CALTABIANO et al., 2011; ALMEIDA et al, 2012; MARTINEZ et al., 2013). A importância dos RLO na etiopatogenia da CE ficou ainda mais evidente quando foi demonstrado que mesmo a aplicação de extratos obtidos de plantas que possuem menor atividade antioxidante era capaz de melhorar a inflamação tecidual no modelo experimental de CE (CUNHA et al., 2011). De modo contrário, a exposição da mucosa do cólon excluso de trânsito a substâncias capazes de aumentar a formação de RLO piorava sensivelmente o processo inflamatório tecidual (MARQUES et al., 2010). O simples restabelecimento do suprimento de butirato ao colón excluso ou soluções nutricionais ricas em AGCC era capaz de reverter o processo inflamatório tecidual (NASRRI; et al., 2008; LAMEIRO et al., 2011).

Entretanto, todas essas substâncias quando aplicadas no cólon excluso, permaneciam pouco tempo em contato com a mucosa cólica por não apresentarem boa adesividade. A substância ideal para o tratamento da CE, além de possuir propriedades antioxidantes e anti-inflamatórias deveria apresentar boa adesão com a superfície epitelial inflamada. Revisando a literatura foi verificado que o SCF apresentava essas propriedades, sendo utilizados em diferentes enfermidades do trato gastrointestinal que cursavam com a formação de úlceras epiteliais. Constatou-se que o SCF já vinha sendo utilizado desde a década de 1980 para o tratamento das ulcerações epiteliais existentes na colite ulcerativa e na colite pós-radioterapia (CARLING et al, 1986; KOCHHAR et al., 1988; LADAS e RAPITIS, 1989; HIDALGO VERA et al., 1990; KOCHHAR et al, 1990; KOCHHAR et al., 1991; ARDIZZONE et al., 1996; DELANEY et al., 1997; O'BRIEN et al., 1997; STOCKDALE; BISWAS,1997; DE PARADES et al., 1998; SASAI et al., 1998; KOCHHAR et al., 1999; MELKO et al., 1999; DENTON et al., 2002; GUL et al., 2002; SANGUINETI et al., 2003; MANOJLOVIC e BABIC, 2004; HOVDENAK et al., 2005; SEO, 2006; HENSON, 2010; HANSON et al., 2012; STACEY; GREEN, 2013). Recentemente com o objetivo de aumentar a adesividade do SCF no interior do reto assim como prolongar o tempo de contato da substância com a parede cólica, formulações utilizando o SCF em forma de pasta vem se demonstrando mais eficazes quando comparada ao uso da emulsão atualmente disponível no comércio (MCELVANNA et al., 2014). Entretanto, a utilização do SCF para o tratamento da CE clínica ou experimental nunca tinha sido avaliada anteriormente. Nosso grupo foi pioneiro ao testar a eficácia da aplicação do SCF num modelo experimental de CE (PEREIRA et al., 2013; CHAIM et al., 2014; BONASSA et al., 2015).

Inicialmente, num primeiro estudo, foi verificada a eficácia da aplicação de enemas diários com SCF em duas concentrações diferentes na mucosa do cólon de ratos submetidas à exclusão por duas e quatro semanas (PEREIRA et al., 2013). Os resultados

encontrados nesse primeiro estudo mostraram que a aplicação diária de enemas com SCF diminuía a perda epitelial, a formação de abscessos nas criptas cólicas, o infiltrado inflamatório e a presença de fibrose tecidual não relacionada ao tempo de intervenção. A intervenção com SCF preservava a população de células caliciformes sugerindo que existia a manutenção da produção de mucinas pelas células da mucosa cólica submetida à intervenção. Os efeitos da substância estavam relacionados à concentração utilizada e ao tempo de intervenção (PEREIRA et al., 2013). Esses resultados levaram à avaliação dos efeitos do SCF na produção de mucinas pelo epitélio cólico desprovido de trânsito (CHAIM et al., 2014). Verificou-se que a aplicação de SCF no cólon excluso além de diminuir o processo inflamatório mucoso, aumentava o conteúdo de mucinas ácidas e neutras no interior das células caliciformes sugerindo que a substância é capaz de estimular a produção de mucinas e aumentar a proteção sobre a mucosa cólica (CHAIM et al., 2014).Todavia, era importante verificar se a substância era capaz de aumentar a produção de sialomucinas, o subtipo de mucinas ácidas que sofre a maior depleção no cólon excluso (MARTINEZ et al., 2010). Foi constatado que a aplicação do SCF aumentava a produção de ambos os subtipos de mucinas ácidas (sulfomucinas e sialomucinas), porém o aumento do conteúdo de sialomucinas além de ser maior estava relacionado à concentração utilizada e ao tempo de intervenção (BONASSA et al., 2015). Todos esses resultados mostram que o SCF além de melhorar o processo inflamatório do ponto de vista histológico aumenta a proteção conferida pela primeira linha de defesa do epitélio intestinal, representada pela camada de muco.

Restava verificar se a substância era eficaz na manutenção da integridade das junções intercelulares, estruturas importantes para isolar o meio interno do lúmen intestinal evitando a infiltração de antígenos e bactérias para o meio interno. Assim sendo, no presente estudo foram avaliados os efeitos do SCF na expressão e conteúdo tecidual das proteínas das junções de oclusão (claudina-3 e ocludina) e de adesão (E-caderina e β-catenina), apenas nos segmentos exclusos de trânsito. A expressão dessas proteínas no cólon com trânsito fecal já tinha sido objeto de estudos anteriores (MARTINEZ et al., 2012; KADRI et al., 2013; MARTINEZ et al., 2015). Os resultados encontrados no presente estudo parecem confirmar os efeitos benéficos do SCF na preservação da integridade epitelial e das junções ocludentes e aderentes intercelulares. Ao sacrificar os animais submetidos à intervenção com SCF, independente da concentração ou tempo de intervenção considerado, havia a formação de uma camada esbranquiçada sobre a superfície epitelial. Em alguns animais era possível identificar a formação de uma rolha de muco no interior do lume intestinal, o que não acontecia nos animais submetidos à intervenção com SF. Ao analisar as lâminas histológicas dos animais submetidos à intervenção com SCF, era facilmente observada a formação de uma fina película sobre a superfície epitelial, como se houvesse a

formação de um filme protetor sobre a mucosa intestinal. Esses achados sugerem que o SCF é capaz de se aderir firmemente sobre a mucosa intestinal.

Ao estudar a ação do SCF no grau de inflamação no epitélio intestinal avaliado por escala de graduação previamente validada, constatou-se que os animais submetidos à intervenção com maiores concentrações e por período maior de tempo apresentavam menor perda epitelial e redução no escore de graduação inflamatória. Ao avaliar o processo inflamatório por meio da pesquisa de infiltrado neutrofílico pesquisada pela identificação imunoistoquímica da enzima MPO, constatou-se que nos animais submetidos à intervenção com SCF, ocorria redução significativa da população de neutrófilos nas camadas mucosa e submucosa, sugerindo que o SCF possua atividade anti-inflamatória. A redução do infiltrado neutrofílico não estava relacionada à concentração utilizada nem ao tempo de intervenção mostrando que mesmo com concentrações menores e utilizadas por um curto período de tempo o SCF era eficaz na redução da resposta inflamatória aguda. Seria interessante avaliar o conteúdo tecidual de citocinas, TNF-α, COX-2, bem como da via do NF-κB. Da mesma forma, seria interessante avaliar a expressão dos genes envolvidos na transcrição dessas proteínas. Entretanto, não se dispunha de recursos financeiros que possibilitasse aprofundar mais essa avaliação. Quem sabe em outra oportunidade seja possível dar continuidade a essa pesquisa.

Em estudos anteriores utilizando modelo experimental de CE foi demonstrado que a exclusão do trânsito fecal reduzia significativamente o conteúdo de E-caderina e β-catenina nas glândulas do epitélio intestinal, principalmente na superfície luminal das células (MARTINEZ et al., 2012; KADRI et al., 2013). No presente estudo, ao avaliar o conteúdo tecidual de E-caderina e β-catenina, na mucosa dos segmentos desprovidos de trânsito verificou-se que a intervenção diária com SCF preservava o conteúdo tecidual de ambas as proteínas nas glândulas cólicas, independente da concentração utilizada. Foi constatado que a aplicação de enemas diários com SCF na concentração de 1,0 g/kg/dia por duas semanas mantinha o conteúdo de E-caderina significativamente mais elevados que os animais do grupo controle. Utilizando concentrações mais elevadas, o conteúdo era ainda maior. Esses dados sugerem que os efeitos da substância após duas semanas de aplicação estão relacionados à concentração utilizada. De modo distinto, ao realizar a intervenção por maior período de tempo (quatro semanas), apesar do conteúdo tecidual de E-caderina manter-se significativamente mais elevados, esses valores não estavam relacionados à concentração utilizada. O SCF possui uma molécula de glicose na sua composição química. A glicose é um substrato energético também utilizado pelas células epiteliais da mucosa cólica para obtenção de energia e, consequentemente, estimular a síntese de proteínas. Apesar disso, a aplicação isolada de SCF por maior período de tempo (4 semanas) não foi suficiente para manter o conteúdo de SCF estável. Esses achados sugerem que a menor

oferta de AGCC ao colón excluso pode influenciar na capacidade de síntese da proteína pela célula epitelial. Essa possibilidade em breve poderá ser confirmada após a análise dos resultados obtidos com um novo experimento, onde, está sendo avaliado o conteúdo das proteínas componentes das junções intercelulares no cólon excluso submetido à intervenção com SCF associada ou não aos AGCC.

Em estudos anteriores mostrou-se que a derivação do trânsito intestinal, reduzia de modo significativo o conteúdo tecidual das duas principais proteínas constituintes das junções de oclusão tecidual (claudina-3 e ocludina) (MARTINEZ et al., 2015). Relembrando, as junções de oclusão intercelular são as principais responsáveis pela preservação da permeabilidade altamente seletiva do espaço intercelular. Quando se constata que na CE existe quebra das proteínas constituintes desse sistema de junção intercelular, é lícito supor que exista maior possibilidade de migração de antígenos e bactérias para o meio interno, agravando e perpetuando o processo inflamatório local. Assim, qualquer substância que aplicada no cólon excluso, possa preservar o conteúdo das proteínas formadoras dessas junções, seria interessante no tratamento da CE. Ao analisar o efeito da aplicação de enemas contendo SCF no conteúdo de claudina-3, verificou-se que ocorria aumento no conteúdo tecidual da proteína quando comparado aos animais do grupo controle. Esse efeito era dose-dependente mostrando que a aplicação de concentrações mais elevadas de SCF estavam relacionadas ao maior conteúdo tecidual de claudina-3, independente do tempo de aplicação considerado. Esses achados estavam relacionados à melhora do processo inflamatório tecidual avaliado tanto pela intensidade de infiltração neutrofílica (níveis teciduais de MPO), quanto pela simples análise histológica do cólon irrigado.

De modo semelhante, ao avaliar os efeitos do SCF na segunda proteína mais importante do sistema de junção de oclusão intercelular, a ocludina, verificou-se que havia aumento no conteúdo tecidual nos animais tratados com SCF. O aumento no conteúdo de ocludina já ocorria após duas semanas de intervenção e, da mesma forma que acontecia com a proteína claudina-3, era relacionado à concentração utilizada, particularmente após duas semanas de intervenção.

Todos esses resultados sugerem que a aplicação de enemas com SCF é capaz de preservar a integridade dos sistemas de junção de oclusão e de adesão celular, importantes constituintes da barreira mecânica da mucosa cólica. É possível que a película de SCF que se forma recobrindo o epitélio intestinal possa dificultar a lesão dessas importantes estruturas de defesa. Quando se sabe que o SCF possui destacada ação antioxidante, e que o estresse oxidativo é um dos mecanismos etiopatogênicos responsáveis pelo desenvolvimento da CE, é coerente suspeitar que a ação antioxidante do SCF possa estar contribuindo para a preservação das proteínas das junções intercelulares de oclusão e adesão por diminuir o estresse oxidativo local. Esses resultados tornam-se mais evidentes

quando se encontra menor infiltração neutrofílica tecidual nos animais tratados com SCF. O menor infiltrado neutrofílico nos animais tratados com SCF se traduz numa menor produção de RLO e, consequentemente, menor dano epitelial provocado por RLO produzidos por essas células. Dessa forma, o SCF reduzindo a produção de RLO, indiretamente está protegendo os sistemas de junção intercelular do dano ocasionado pelos RLO.

Os achados do presente estudo mostram que o SCF é eficaz na manutenção da integridade das junções de oclusão e adesão celular. Sua capacidade de adesão epitelial e efeitos anti-inflamatórios sugerem que a droga possa ser utilizada no tratamento da CE.

7. CONCLUSÕES

Considerando a metodologia empregada os resultados obtidos no presente estudo é possível concluir que:

O conteúdo das proteínas claudina-3, ocludina, E-caderina e β-catenina nos animais submetidos à intervenção com SCF é maior quando comparado aos submetidos a intervenção com SF 0,9%;

A aplicação tópica de SCF reduz o processo inflamatório da mucosa do cólon excluso de trânsito intestinal, independente da concentração utilizada e do tempo de intervenção;

Não houve variação no conteúdo das proteínas claudina-3, ocludina, E-caderina e β-catenina segundo o tempo de intervenção com o SCF;

A aplicação tópica de SCF reduz os níveis de estresse oxidativo tecidual independente da concentração utilizada e do tempo de intervenção adotado.

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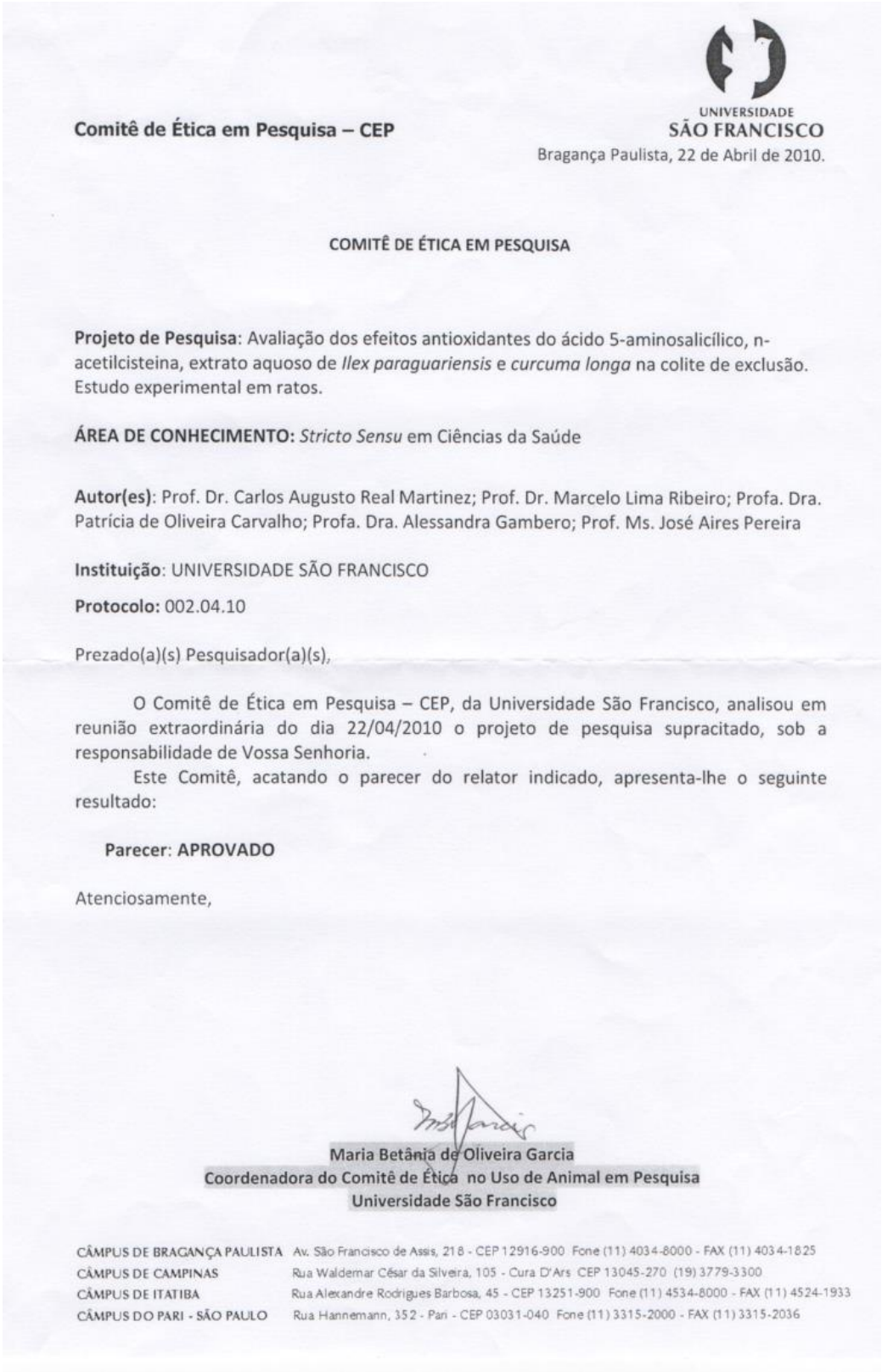
9. ANEXOS

9.1 Carta de aprovação do comitê de ética



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SÃO FRANCISCO

Comitê de Ética em Pesquisa – CEP

Bragança Paulista, 22 de Abril de 2010.

COMITÊ DE ÉTICA EM PESQUISA

Projeto de Pesquisa: Avaliação dos efeitos antioxidantes do ácido 5-aminosalicílico, n-acetilcisteina, extrato aquoso de *Ilex paraguariensis* e *curcuma longa* na colite de exclusão. Estudo experimental em ratos.

ÁREA DE CONHECIMENTO: *Stricto Sensu* em Ciências da Saúde

Autor(es): Prof. Dr. Carlos Augusto Real Martinez; Prof. Dr. Marcelo Lima Ribeiro; Profa. Dra. Patrícia de Oliveira Carvalho; Profa. Dra. Alessandra Gambero; Prof. Ms. José Aires Pereira

Instituição: UNIVERSIDADE SÃO FRANCISCO

Protocolo: 002.04.10

Prezado(a)(s) Pesquisador(a)(s),

O Comitê de Ética em Pesquisa – CEP, da Universidade São Francisco, analisou em reunião extraordinária do dia 22/04/2010 o projeto de pesquisa supracitado, sob a responsabilidade de Vossa Senhoria.

Este Comitê, acatando o parecer do relator indicado, apresenta-lhe o seguinte resultado:

Parecer: APROVADO

Atenciosamente,

Maria Betânia de Oliveira Garcia
Coordenadora do Comitê de Ética no Uso de Animal em Pesquisa
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9.2 Artigos publicados relacionados a essa tese

9.2.1 Claudin-3 and occludin tissue content in the glands of colonic mucosa with and without a fecal stream

J Mol Hist
DOI 10.1007/s10735-015-9610-y

ORIGINAL PAPER

Claudin-3 and occludin tissue content in the glands of colonic mucosa with and without a fecal stream

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Received: 3 November 2014 / Accepted: 28 January 2015

Abstract The synthesis of the proteins of the apical tight junctions (TJs) depends on a continuous supply of short-chain fatty acids (SCFAs) in colonic epithelium. No studies have evaluated the tissue contents of the TJs proteins in colon segments devoid of a fecal stream. To evaluate the contents of claudin-3 and occludin in the glands of colonic mucosa devoid of a fecal stream. Forty-five rats underwent a diversion of the fecal stream via a left side colostomy and distal mucous fistula. Three groups of 15 animals each were sacrificed at 6, 12 or 18 weeks after surgery. The presence and severity of colitis were defined by histology and inflammation grading scales, respectively. The expression of claudin-3 and occludin were evaluated by immunohistochemistry, and their contents were evaluated by computer-assisted image analysis. Mann–Whitney and Kruskal–Wallis tests were used to evaluate the results at a significance level of 5 % ($p < 0.05$). The colonic epithelium without a fecal stream had a higher degree of inflammation. Colonic glands without a fecal stream showed a reduction in claudin-3 content independent of the time and reduction in occludin content after 12 weeks of intestinal exclusion. The content of claudin-3 and occludin were mainly reduced at the apical surfaces of the colon glands, whereas segments retaining the fecal stream were maintained. The content of claudin-3 was not reduced with time, although the levels of occludin were reduced after 6 weeks and did not vary thereafter. Deficiencies in SCFAs decreased the content of claudin-3 and occludin in colonic glands with the areas of worst inflammation, confirming the importance of an adequate supply of SCFAs in maintaining the integrity of TJ proteins.

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Published online: 04 February 2015

Keywords Colitis · Fatty acids · Volatile · Cell adhesion molecules · Tight junctions · Claudin-3 · Occludin

Introduction

The colonic epithelium is one of the most effective morphofunctional barriers in living organisms (Pravda 2005). In addition to allowing the absorption of the water, nutrients and molecules required for cellular energy metabolism, the colonic mucosa can also prevent bacteria and antigen invasion from the intestinal lumen (Laukoetter et al. 2008). Therefore, the establishment and preservation of two distinct and separate compartments depend on the integrity of the colonic epithelial barrier (Clayburgh et al. 2004; Watson et al. 2005). The epithelial cells of the colonic glands adhere to one another and to the extracellular matrix through proteins known as cell adhesion molecules (Ding et al. 2013). Cell adhesion molecules form a complex system of intercellular adhesion that is responsible for maintaining the selective permeability of the intestinal epithelium (Laukoetter et al. 2008). These complex systems, known as intercellular junctions (IJs), are mainly composed of tight junctions (TJs), adherent junctions (AJs) and gap junctions (Clayburgh et al. 2004; Laukoetter et al. 2008). IJs are formed by proteins that cross the cellular membrane of the colonic epithelial cells and attach to proteins in the cytoskeleton of neighboring cells or structures (Laukoetter et al. 2008; Kadri et al. 2013). In addition to maintaining adherence between epithelial cells, these specialized junctions also enable functional communication between the cells (Ozawa et al. 1990; Schmitz et al. 1999; Usami et al. 2006, Turner 2009). Changes in the contents and chemical structures of AJ proteins have been demonstrated in patients with infectious colitis, inflammatory bowel diseases (IBD), colorectal cancer (CRC) and experimental models of TNBS-induced colitis (Kucharzik et al. 2001; Laukoetter et al. 2008; Abdallah and Ismael 2011). Recently, two articles showed in an experimental model of diversion colitis (DC) that deficiencies in the normal supply of short-chain fatty acids (SCFAs) to the cells of the colonic epithelium can reduce the contents of E-cadherin and β-catenin, the main protein constituents of the AJs of the colonic epithelial glands (Martinez et al. 2012; Kadri et al. 2013). The authors suggest that the steady supply of SCFAs play an important role in maintaining the integrity of the AJs, consequently worsening the mucosal inflammatory process found in the DC (Martinez et al. 2012; Kadri et al. 2013). In these studies, the authors draw attention to the possibility that the tissue content of the protein constituents of TJs can also be altered due to deficiencies in the supply of SCFAs; however, they did not further investigate this possibility.

TJs include the cell adhesion molecules that are often found in the epithelium, joining specific regions of the internal and external surfaces of adjacent cells. TJs act as a primary barrier to the diffusion of solutes through the intercellular space and create a boundary between the apical and basolateral domains of the plasma membrane (Schneeberger and Lynch 1984), and their major members include the claudin family of proteins and occludin (Lu et al. 2013; Martinez et al. 2013). Claudins are the principal constituent of TJs and form the paracellular barrier that controls the flux of ions and small molecules in the intercellular space (Lu et al. 2013; Wongdee et al. 2013). Claudins are connected to the actin of the cellular cytoskeletons by occludin and cytosolic proteins with lower functional importance, namely, ZO-1, ZO-2 and ZO-3 (Lal-Nag and Morin 2009). Claudin-3 is one of the members of the claudin family present in the glands of the colon and rectum (Lu et al. 2013). Despite their variable contents in TJs, claudins are considered reliable immunohistochemical markers of TJs in the colonic epithelium. The presence of colitis has differential effects on the claudins in colonic epithelial cells (Prasad et al. 2005). Occludin has not only a structural role but also an important functional role in TJs and has been closely linked to the formation of the paracellular permeability barrier (Schneeberger and Lynch 1984; Furuse et al. 1998). Alterations in the tissue contents and patterns of occluding expression in the colonic epithelial glands have been associated with changes in the physiological function of TJs and their pathological conditions (Balda et al. 1996; Hirase et al. 1997).

Reductions in the tissue expression of TJ proteins have been reported in several diseases, such as UC, Crohn's disease (CD) and CRC (Schmitz et al. 1999; Clayburgh et al. 2004; Laukoetter et al. 2008; Ding et al. 2013). Most authors consider the reduction in claudin-3 content and occludin in the colonic epithelial glands a consequence of the inflammatory process. To the best of our knowledge, alterations in the tissue contents of claudin-3 and occludin in the crypts of the colonic mucosa devoid of the fecal stream have not been evaluated, either clinically or experimentally. Thus, the purpose of this study was to confirm the effect of a diminished supply of SCFAs on the integrity of the TJs. Thus, the aim of this study was to evaluate the tissue contents of claudin-3 and occludin in the glands of the colonic mucosa diverted from the fecal stream.

Methods

Animals

This study was performed in accordance with the Federal Law No. 11,794 and the guidelines of the Brazilian College

Table 1 Grading scale for inflammation score

Degree of inflammation	Score	Histopathological characteristics
Absent	0	Without neutrophil infiltration into tissue
Mild	1–3	Neutrophil infiltration <50 % of crypts or neutrophil infiltration <50 % of fields and absence of erosion or ulcers
Moderate	4–6	Neutrophil infiltration ≥50 % of crypts or neutrophil infiltration ≥50 % of fields and absence of erosion or ulcers
Severe	7–9	Neutrophil infiltration ≥50 % of crypts or neutrophil infiltration ≥50 % of fields and presence of erosion or ulcers

Based on Gupta et al. (2007)—Mount Sinai Hospital (modified)

for Animal Experimentation (COBEA) and was approved by the Research Ethics Committee of São Francisco University in Bragança Paulista, São Paulo, Brazil (Process No. 2211/07).

Forty-five SPF male Wistar rats (*Rattus norvegicus Brakenhoff*) weighing 300–350 g were randomly assigned to three groups of 15 rats each and were sacrificed at 6, 12 or 18 weeks after the operative procedure described below.

Surgical technique: diversion of the fecal stream

All of the animal surgeries were performed by the same surgical team. General anesthesia was induced by the intraperitoneal administration of 0.1 mL/100 g of a 1:1 (v/v) ketamine (50 mg/mL) and xylazine (20 mg/mL) solution. After superficial shaving, the abdominal cavity was accessed through a 5-cm midline incision. Fecal stream diversion was established by means of a left side colostomy and a distal mucous fistula, as previously described (Martinez et al. 2012). Then, the distal segment of the sectioned colon was catheterized with a 12F polyvinyl catheter and irrigated with 0.9 % physiological solution until the effluent collected from the anus was free of fecal material. The abdominal incision was closed in two layers. During the postoperative period, the animals were maintained in individual cages with no antibiotics. Analgesia was improved by diluting dipyrone (15 mg/kg) into the water offered daily.

Sample collection

On the day scheduled for euthanasia (6, 12 or 18 weeks after the surgical procedure), all animals were anesthetized as described above, and colon segments were removed from the excluded and non-excluded colon segments. Two-colon specimens measuring 30 mm (with and without a fecal stream) were opened longitudinally through the anti-mesenteric border. These segments were carefully washed with 0.9 % saline at 37 °C to remove either fecal waste or mucus.

The segments were then laid out on a flat cork surface, secured with the mucosal surface facing up and fixed in 10 % formaldehyde for 3 days at room temperature. After fixation, the tissues were dehydrated using increasing concentrations of alcohol and clarified in xylene. The segments were embedded in paraffin and cut into 5-μm longitudinal sections. The slides were stained with hematoxylin and eosin (HE) and subjected to histological analysis to confirm the presence of colitis. The presence of colitis in the colon segments devoid of the fecal stream was confirmed by the presence of two independent histological parameters: mucosal–submucosal neutrophil infiltration and epithelial erosion or ulceration (− to 9+). The severity of the colitis inflammation in the excluded tissues was established in accordance with a previously validated scale (Table 1; Gupta et al. 2007).

Immunohistochemical staining

For the immunohistochemical analysis, 5-μm thick sections of the colon segments (with and without a fecal stream) were cut and placed on previously silanized surfaces. The sections were mounted on slides with frosted edges that had been previously labeled with the animal number and the experimental colonic site from which the segment had been taken. The sections were dewaxed, rehydrated in alcohol (100, 95, 80 and 50 %) and washed in distilled water. Next, the sections were submerged in PBS (0.05 M, pH 7.2) for 10 min and dried with filter paper. Endogenous peroxidase was blocked with 3 % H_2O_2 in a moist chamber for 10 min at room temperature, followed by another 10-min wash in PBS. Antigen retrieval was performed by incubation in sodium citrate (10 mM, pH 6.0) in a 95 °C water bath for 45 min. Then, the slides were left at room temperature for 20 min, followed by another 5-min wash with PBS. An anti-claudin-3 monoclonal antibody (Ref. E-3834, Lot. 110520, Spring Bioscience, Pleasanton, CA, USA) was mixed 1:50 in bovine serum albumin (1 %), or a monoclonal mouse anti-human anti-occludin (Ref. E-17464, Lot. 111207S, Spring Bioscience, Pleasanton,

CA, USA) was mixed 1:100 in bovine serum albumin (1 %). All of the slides were covered with approximately 100 µL of this solution and incubated at 4 °C for 24 h. Next, they were washed once more with PBS, incubated with a secondary antibody (Lot: H1011 Histofine Code: 414191N, Spring Bioscience, Pleasanton, CA, USA), and then incubated for 45 min with the streptavidin–biotin-peroxidase complex (ABC Staining System, Dako A/S, Glostrup, Denmark), which was freshly prepared at a dilution of 1:100 in PBS. The chromogenic reaction was developed with a freshly prepared solution of DAB (diaminobenzidine tetrahydrochloride, 10 mg in 10 mL of PBS), which was added to 3 mL of hydrogen peroxide 5 min before the end of the ABC incubation. This solution was applied to the slides for 3 min, and then the slides were washed and counterstained with methyl green for 1 min before another wash in distilled water. Next, the slides were dehydrated by immersion in 50, 80, 95 and 100 % ethanol, followed by xylene. Finally, the slides were mounted, labeled and kept in a horizontal position for 24 h.

Immunostaining was considered to be positive when a diffuse brownish color with spots of varying intensity and a homogeneous distribution in the apical or basolateral cellular membrane was observed. As recommended by the manufacturer, a negative control was prepared without the addition of the primary antibody, and a positive control was prepared using human CRC tissue, which is known to be positive for both proteins.

The slides were analyzed under an optical microscope (Nikon Inc., Tokyo, Japan) at a magnification of 200×. The slides prepared for both histology and immunohistochemistry (anti-claudin-3 and anti-occludin) were reviewed by a pathologist who was unaware of the origin of the material or the objectives of the study. The tissue contents of claudin-3 and occludin immunostaining were evaluated by three independent observers. The observers examined three different histological fields that had at least three contiguous and intact colonic glands and classified the intensity of the immunostaining in each of the locations in crosses (0 to + + + +). The staining intensity score was recorded as follows: absent as 0, marginal as +1, light intensity as +2, moderate intensity as +3, and strong intensity as +4. The final content of tissue expression adopted for each slide was the median found after reading the three different fields. Conflicting results were subsequently analyzed together. None of the observers knew the location in the colon from which the material had been removed or the objectives of the study.

Statistical analysis

Statistical analysis was performed using a 5 % significance level ($p < 0.05$). The data obtained from the colon segments with and without fecal streams are expressed as the mean values and respective standard errors using SPSS for Windows statistical software (version 13.0). The Mann–Whitney test was used to compare the inflammation scores and tissue contents of claudin-3 and occludin, comparing the segments with and without a fecal stream. The Kruskal–Wallis test was used to analyze the variance in inflammatory score, claudin-3 and occludin tissue levels at three different time points.

Results

Histopathological findings

Figure 1a, b shows the colonic glands of the colon mucosa in segments, with and without a fecal stream, after 18 weeks. In the segments with a fecal stream, we can see that the epithelial surface is full and not recovery by mucus. The colonic glands are aligned and juxtaposed, and the goblet cells are filled with mucus. A small inflammatory infiltrate can be observed at the base of the crypts. In the colon segments without a fecal stream, we can see that the epithelial surface resembles a "brush border", and the ostium of the colonic glands is covered with a mucus layer. The goblet cells were filled with high amounts of mucus, and the colonic glands were dilated with large amounts of mucus in the crypts, extruding to the colon lumen. An increased number of inflammatory cells can be identified at the base of the crypts.

Inflammatory score

Figure 2 compares the inflammation grading scores for colon segments with and without a fecal stream in the different experimental groups. We also verify that the inflammatory grades in segments with a fecal stream at 6, 12 and 18 weeks had mean values of 2.0 ± 0.48, 3.0 ± 0.28 and 3.0 ± 0.28, whereas those segments without a fecal stream had values of 3.0 ± 0.40, 8 ± 0.37 and 8.0 ± 0.37, respectively. We could verify a significant increase in the inflammatory grade of segments without a fecal stream after 12 and 18 weeks compared to the segments with a fecal stream or from the control group ($p = 0.0001$). The inflammatory grading score was unchanged in the colon segments with a fecal stream during the scope of the experiment.

Tissue expression of claudin-3 in the colon glands

Figure 3a shows the tissue expression of claudin-3 in the colon glands with a fecal stream in animals subjected to fecal exclusion for 18 weeks, and Fig. 3b shows the expression of claudin-3 in the colon glands without a fecal stream. It is easy to verify that, in the epithelial surface of

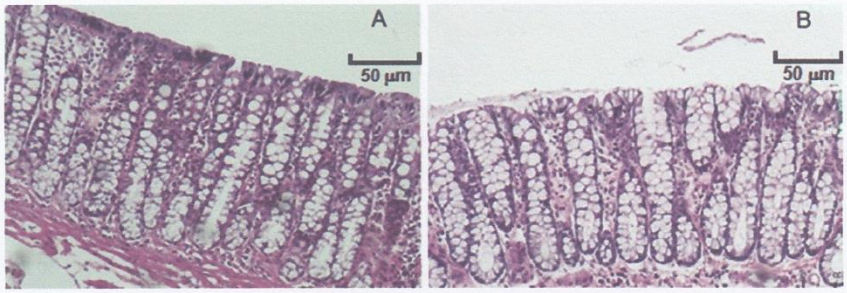

Fig. 1 **a** Colonic segments with a preserved fecal stream. **b** Colonic segments after 18 weeks of deviation of the fecal stream

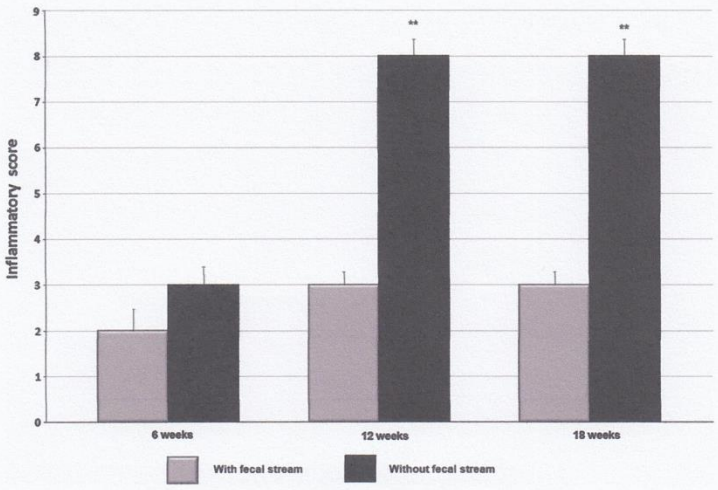

Fig. 2 Inflammation grading score comparing colon segments with and without a fecal stream after 6, 12 and 18 weeks. **Significant ($p < 0.01$). Mann–Whitney test

the animals with a fecal stream, the main tissue expression of claudin-3 is located in the apical portion of the colonic glands with the largest number of specialized cells and in the region in direct contact with the intestinal lumen with higher bacterial concentrations. When we compare these values with those of animals submitting to 18 weeks of fecal stream diversion, we observed a significant reduction in the claudin-3 content in the apical surface of the colon glands, with increasing goblet cell substitution for other specialized cells.

Tissue content of claudin-3 in the colon glands, with and without a fecal stream

Figure 4 compares the tissue contents of claudin-3 in colon segments with and without a fecal stream after 6, 12 and 18 weeks of fecal stream diversion. In the colon glands with a fecal stream, the content of claudin-3 were 2.55 ± 0.12, 3.55 ± 0.17 and 3.52 ± 0.16 (%/fields), whereas in colon glands without a fecal stream, the levels of claudin-3 were 1.9 ± 0.17, 1.83 ± 0.16 and 1.82 ± 0.17 (%/fields), respectively. We show that, in colons glands without a fecal stream, the tissue contents of claudin-3 were significantly lower than the values obtained from the segments with a fecal stream, independent of the time of the exclusion considered ($p = 0.0001$). The tissue content of claudin-3 in the colon glands with a fecal stream increase between six and 12 weeks stabilized thereafter, although the segments without fecal streams showed no variation with time.

Tissue expression of occludin in the colon glands

Figure 5a shows the tissue expression of occludin in the colon glands with the fecal stream after 18 weeks, whereas

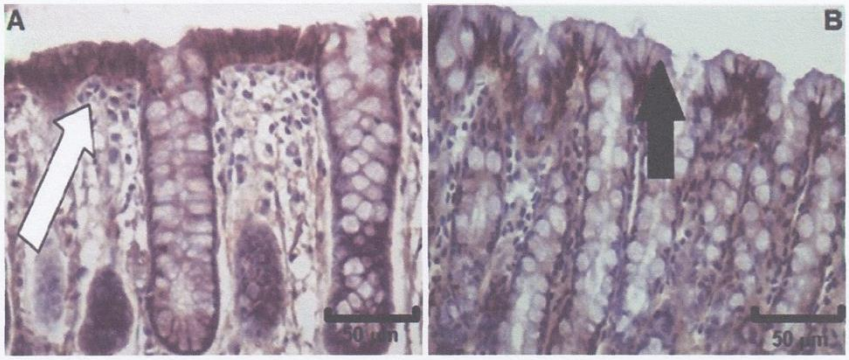

Fig. 3 **a** Strong expression of the claudin-3 protein at the apical surface of the colonic glands (*white arrow*) in the colon segments with a fecal stream after 18 weeks. **b** Reduction of the tissue contents of the claudin-3 protein in the apical region of the colonic glands (*black arrow*) after 18 weeks

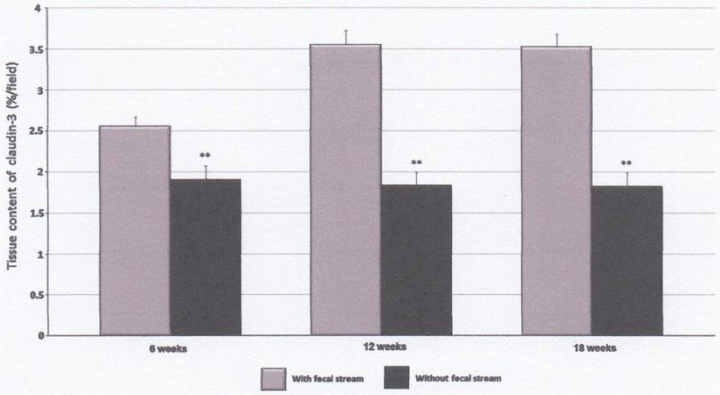

Fig. 4 Tissue content of claudin-3 in the colon glands with and without a fecal stream after 6, 12 and 18 weeks. **Significant ($p < 0.01$). Mann–Whitney test

Fig. 5b shows the pattern of tissue expression of occludin in the colon glands without the fecal stream at the same time point.

Tissue contents of occludin in the colon glands, with and without a fecal stream

Figure 6 compares the tissue contents of occludin in the colon glands, with and without a fecal stream, after 6, 12 and 18 weeks of fecal stream diversion. In the colon glands with a fecal stream, the contents of occludin were 2.70 ± 0.20, 2.77 ± 0.30 and 2.88 ± 0.28 (%/fields), whereas in the colon segments without a fecal stream the levels of occludin were 2.48 ± 0.21, 1.51 ± 0.19 and 1.48 ± 0.17 (%/fields), respectively. We show that, in the colon glands without a fecal stream, the tissue contents of occludin after 12 weeks were significantly lower than the values obtained from glands with a fecal stream ($p = 0.0001$). The tissue content of occludin in the colon glands with a fecal stream did not change with time, whereas segments without the fecal stream showed reduced contents that stabilized 6 weeks thereafter.

Discussion

Intestinal epithelial integrity is vital for nutrition absorption and host defense against pathogens. The intestinal epithelium limits the free passage of toxic molecules and infectious agents from the intestinal lumen while allowing selective paracellular absorption (Edelblum and Turner 2009). Compromised epithelial barrier function and TJ

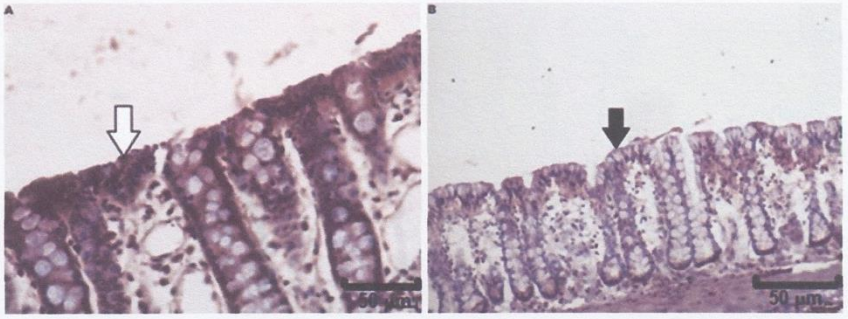

Fig. 5 **a** Strong expression of occludin in the apical surface of the colon glands with a fecal stream after 18 weeks (*white arrow*). **b** Reduction of the tissue expression of occludin proteins at the apical surface of the colonic glands without a fecal stream after 18 weeks (*black arrow*)

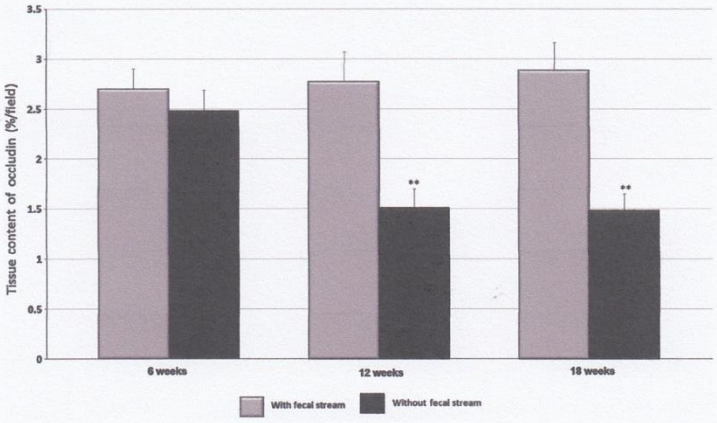

Fig. 6 Tissue contents of occludin in the colon glands, with and without a fecal stream, after 6, 12 and 18 weeks. **Significant ($p < 0.01$). Mann–Whitney test

alterations are hallmarks of a number of GI disorders, such as UC, CD, UC-associated colorectal cancer and CRC (Schmitz et al. 1999; Clayburgh et al. 2004; Usami et al. 2006; Laukoetter et al. 2008; Weber et al. 2008; Mees et al. 2009; Su et al. 2009). Luminal antigen uptake occurs via TJ discontinuities and epithelial gross lesions, which are likely to induce many other changes to the epithelium besides simply altering the TJ barriers (Hollander et al. 1986; Su et al. 2009; Lu et al. 2013). TJs are the most apical component of the IJs complex and provide the most efficient form of cell-to-cell adhesion in the colonic epithelium (Mitic and Anderson 1998; Ding et al. 2013), and claudins appear to be the most important TJs protein (Mitic and Anderson 1998; Van Itallie et al. 2009; Lu et al. 2013; Van Itallie and Anderson 2014). Currently, 24 claudin members have been described in mammals. Claudins-1, -3, -4, -7 and -8 represent the most important members presents in the TJs of the colonic epithelium, whereas claudins-2, -7, -8 and -9 are mainly found in the upper digestive tract (Prasad et al. 2005; Mees et al. 2009; Lu et al. 2013). No studies have evaluated the expression of any member of the claudin family in the colonic epithelium without a fecal stream. There are few studies evaluating the tissue expression of claudin-3 in the colonic epithelium, and most have studied the expression of proteins in human tissues from patients with IBD or CCR (Resnick et al. 2005; Weber et al. 2008; Mees et al. 2009). This context represents one of the reasons motivating us to evaluate the tissue expression of claudin-3 in the colon lacking a fecal stream. Another reason is that claudin-3 is present throughout the normal colonic epithelium but is reduced or redistributed in the surface of inflamed epithelium, similar to IBD findings

(Van Itallie et al. 2000). Thus, we analyzed the expression of claudin-3 in both normal colonic glands and in glands subjected to a restricted supply of SCFAs.

Occludin was the first transmembrane TJ protein to be identified (Furuse et al. 1993). Studies have shown that occludin is present in TJ strands and plays a functional role in the connection between the intracellular filaments that form claudin and the cytoskeleton (Saitou et al. 2000). Studies have evaluated the tissue expression of the occludin protein in both normal and inflamed mucosa from patients with IBD (Kucharzik et al. 2001). The colonic mucosa from patients with UC revealed a dramatic, global down-regulation of occludin in regions of active trans-epithelial neutrophil migration, as well as in quiescent areas (Kucharzik et al. 2001). Significant decreases in occludin tissue expression were observed at the protein and mRNA levels. In contrast, the expressions of other TJ and AJ proteins, such as claudins, ZO-1, β-catenin, and E-cadherin, were down-regulated only in epithelial cells immediately adjacent to transepithelially migrating neutrophils (Kucharzik et al. 2001). These results suggest that the tissue expression of occludin is diminished in the inflamed colon by mechanisms that are distinct from those regulating the expression of other intercellular junction proteins; indeed, these findings were the major reason behind our evaluation of occludin contents in this study. It has been speculated that the down-regulation of epithelial occludin might play a role in the enhanced paracellular permeability and transepithelial migration of neutrophils that is observed in active IBD. Studies have demonstrated that the intestinal tissue expression of occludin is markedly decreased in patients with intestinal permeability disorders, including UC, CD, and celiac disease, as well as in animal models of IBD (Fries et al. 1999; Gassler et al. 2001; Ciccocioppo et al. 2006; Zeissig et al. 2007; Ding et al. 2013; Wu et al. 2014). It has been proposed that decreased intestinal occludin expression may represent an important mechanism leading to a greater permeability of intestinal epithelial TJs. Experimental studies have shown that, under normal healthy conditions, the intestinal epithelial barrier is effective in preventing macromolecular flux but readily allows the flux of ions and small molecules through the claudin-regulated pore pathway (Van Itallie et al. 2009). However, in pathological conditions, occludin depletion leads to the opening of the nonrestrictive pathway, permitting the paracellular flux of macromolecules, including bacterial antigens capable of inducing an inflammatory response (Nazli et al. 2006; Ding et al. 2013). Similar to existing data on claudin-3, no studies have evaluated the default tissue expression of occludin in colon mucosa glands devoid of a SCFA supply.

Glotzer et al. (1981) became the first to describe the development of an inflammatory process in the mucosa of the colon without a fecal stream; this new form of inflammatory colorectal disease was called DC. Various theories regarding the pathogenesis of DC have been proposed, but most authors believe that DC arises due to the absence of SCFAs in the intestinal lumen due to the diversion of the fecal stream (Pacheco et al. 2012; Vieira et al. 2012). SCFAs represent the most important fuel for colonic epithelial cells. Butyrate production in the large intestine depends on bacterial butyryl-CoA/acetate-CoA transferase activity and is highest when fermentable fiber and nonstructural carbohydrates are balanced (Plöger et al. 2012). Gastrointestinal epithelia seem to use butyrate and butyrate-induced endocrine signals to adapt proliferation, apoptosis, and differentiation to the growth of the bacterial community (Nazli et al. 2006). Butyrate has a potential clinical application for the treatment of various inflammatory diseases that damage the colonic mucosa (Plöger et al. 2012; Vieira et al. 2012). Experimental studies have shown that a deficiency in the SCFAs supplied to the colonic epithelial cells resulting from fecal stream deviation cause the atrophy and ulceration of the colonic mucosa, increased neutrophil infiltration, and reduced expression of the genes related to the transcription of several proteins crucial for proper cellular metabolism (Gaudier et al. 2004; Sauer et al. 2007; Sousa et al. 2008; Gaudier et al. 2009; Peng et al. 2009). Experimental studies have demonstrated that SCFA deficiencies modify the cellular respiratory metabolism, leading to the production of large amounts of ROS (Martinez et al. 2010a). The oxidative stress that then arises may disrupt the different lines of defense in the colonic mucosa (Martinez et al. 2010b). Conversely, the delivery of SCFAs to the epithelium deprived of the colonic fecal stream reduces the levels of oxidative tissue damage resulting from the increased production of ROS (Lameiro et al. 2011). The deviation of the fecal stream, generating oxidative stress, reduces the mechanical and bactericidal protection offered by the mucus layer overlying the colonic epithelium by decreasing mucin production by goblet cells, thereby modifying the type of mucin produced (Nonose et al. 2009; Martinez et al. 2010b; John et al. 2011). In an experimental model of DC, it was recently demonstrated that supplementing SCFA deficiencies may significantly reduce the tissue contents of two main AJs protein constituents, leading to the inflammatory changes that characterize DC (Martinez et al. 2012; Kadri et al. 2013). The importance of adequate SCFA delivery for the maintenance of colonic mucosa integrity has been reinforced by studies showing that the restoration of fecal transit or SCFA supplementation, particularly butyrate, may reverse the characteristic inflammatory process (Nassri et al. 2008; Pacheco et al. 2012; Vieira et al. 2012). Likewise, the use of enemas with substances that have antioxidant activity or SCFAs may

reverse inflammation by restoring epithelial integrity (Nassri et al. 2008; Cunha et al. 2011; Caltabiano et al. 2011; Pacheco et al. 2012; Vieira et al. 2012; Pereira et al. 2013). All of these studies show that deficiencies in the SCFA supply can precipitate the rupture of the colonic epithelial barrier. However, despite these findings, to the best of our knowledge, no studies have evaluated the importance of SCFA deficiencies within the expression of the main constituent proteins of TJs.

Some authors suggest that SCFA, especially butyrate and n-3 polyunsaturated fatty acids, may contribute to the restoration of the TJ barrier in IBD by affecting the expression of claudins, occludin, cingulin, and zonula occludens proteins (ZO-1, ZO-2) via the inhibition of the release of TNF-α and interleukin 13 and of the activity of histone deacetylase (Zeissig et al. 2007; Li et al. 2008; Plöger et al. 2012). A study using cell cultures treated with 4 mM sodium butyrate showed that butyrate might play an important role in recovering the intestinal TJs, exerting a positive effect on the maintenance of the gut integrity (Ma et al. 2012). The effects of depriving the AGCC supply on the tissue contents and patterns of expression in the colonic glands of the major proteins of the TJs have never been evaluated. These points motivated us to evaluate the claudin-3 protein and occludin in an experimental model of CD we have studied for almost two decades.

The results of this study show that this deficiency in the SCFA supply modified the colonic mucosa and, more significantly, the tissue contents of the two major protein constituents of the TJs. We observed a reduction in the tissue contents of claudin-3, mainly in the apical surface of the colonic epithelium without a fecal stream, when compared with the segments within the intestinal transit. Deviated colonic segments revealed 25.51, 48.86 and 48.30 % content reductions after six, 12 and 18 weeks, respectively. The most significant reduction in the claudin-3 contents occurred after 6 weeks and stabilized thereafter. The reduction of the claudin-3 tissue content was inversely related to the increase in the tissue inflammatory score. Interestingly, epithelial inflammation also became more intense after the sixth week of the diversion of the fecal stream. However, in the glands of the colonic segments with a fecal stream, the tissue contents of claudin-3 increased significantly after 6 weeks and remained stable thereafter. These findings provide strong evidence supporting the importance of maintaining a SCFA supply for the contents of both proteins and, indirectly, for the integrity of TJs.

Similarly, we found reductions in the tissue contents of occludin in the colon segments without a fecal stream of 18.05, 54.51 and 48.62 % at 6, 12 and 18 weeks, respectively. Likewise, the reduction in the tissue contents of occludin were found to be inversely related to the inflammatory score and directly related to the reduction of the tissue contents of claudin-3. It should be noted that, in the glands of the colon with intestinal transit tissues, the contents of occludins remained constant throughout the 18 weeks of intestinal transit exclusion. Similarly, the maintenance of the occludin content seems to be directly related to the ongoing maintenance of the SCFAs supply formed from the fermentation of fecal waste in colons with a preserved fecal stream.

All of these results suggest that in colonic mucosa without a supply of SCFAs, the degree of inflammation tends to intensify, whereas the contents of claudin-3 and occludin tend to reduce. This finding suggests that there is increased breakage of TJs and thus a greater propensity for the migration of antigens and bacteria through the intercellular space, increasing the inflammatory process in the colonic mucosa, reinforcing the importance of SCFAs in the maintenance of TJ integrity in patients with IBD.

The absence of quantifying techniques, such as Western-blotting, immunoassays or real-time polymerase chain reactions, represents a weakness of our study. However, using immunohistochemistry as a sole approach has been shown to be valid and reliable in many settings, including studies of TJ protein expressions (Resnick et al. 2005; Weber et al. 2008; Mees et al. 2009; Kadri et al. 2013). Different studies have shown that the results obtained by immunohistochemical techniques are comparable to the results generated by quantification techniques, such as Western-blotting or microarrays (Dhawan et al. 2005; Gröne et al. 2007; Mees et al. 2009). It should be remembered also that the aim of our study was to evaluate the contents of these proteins in TJ exclusively on the colonic glands. The use of biochemical techniques, such as Western-blotting, requires a large amount of tissue obtained from all segments of the colon wall. Thus, we were able to add TJ proteins from other segments of the intestinal wall. Using biochemical techniques, we cannot be certain that the results found in the quantification of these proteins were related only to their presence in the glands of the colon mucosa. Furthermore, using immunohistochemical techniques, we were able to illustrate more clearly the pattern of expression of the proteins in the glands, with and without a fecal stream. The same arguments can be used to support our choice of measuring the tissue contents of claudin-3 and occludin by computerized morphometry. There are several published studies that have confirmed the validity and accuracy of this methodology (Nonose et al. 2009; Martinez et al. 2010b, 2012; Kadri et al. 2013; Kozlowski et al. 2013; Kobayashi et al. 2014). A recent study showed that computer-assisted image analysis shows a 94.5 % specificity and 95.6 % sensitivity rate (Malekian et al. 2013). Computer-assisted image analysis has the additional advantage of further enabling the measurement of the protein contents

only in the colonic glands, excluding the expression of these proteins in the other layers of the intestinal wall. Using other biochemical methods, such an analysis would be very difficult to perform.

Different reasons could explain the reduction in the contents of claudin-3 proteins and occludin in the glands of the colon mucosa devoid of a fecal stream. In a previous study using the same tissue specimens as in this study, we verified that, as increasingly long diversions of the fecal stream were applied, the level of oxidative stress in the cells of the colonic glands increased significantly, as evaluated by a comet-assay, and was related to a worsening inflammatory score. Subsequently, two other studies in our group analyzing the protein components of AJs (e-cadherin and β-catenin) showed that the levels of AJs proteins decreased as the time of fecal diversion increased, in correlation with higher levels of oxidative stress in the colonic glands (Martinez et al. 2012; Kadri et al. 2013). These results raise the possibility that oxidative stress resulting from diversion of the fecal stream can damage TJs by reducing the tissue contents of claudin-3 and occludin. The lack of available SCFA, the major nutrient of the specialized cells of the colon mucosa, may also have contributed to the decreased claudin-3 and occludin tissue contents in the colon glands. The lack of this important nutrient, as previously demonstrated, can reduce the gene expression and protein synthesis of the components that form the colonic epithelial barrier (Gaudier et al. 2004, 2009). We are currently studying the tissue contents of TJ proteins and the levels of oxidative stress in colonic glands without the fecal stream that were subjected to enemas containing SCFAs. If the results show that the application of enemas containing SCFAs preserves the contents of TJs proteins and reduces the level of tissue oxidative stress, we will have further evidence regarding the importance of ROS in the mechanism of injury to IJs in the epithelial barrier. Based on the evidence described above, we believe that tissue oxidative stress and nutritional deficiencies among the cells of the colonic glands are the main players behind the reduced tissue contents of the protein components of the TJs in DC.

Conclusion

Based on the results of this study, it can be concluded that there is reduction in the tissue contents of claudin-3 proteins and occludin in the glands of the colonic mucosa devoid of a fecal stream, reinforcing the importance of SCFAs in maintaining the integrity of the colonic epithelium.

Acknowledgments Fundação de Amparo a Pesquisa do Estado de São Paulo—FAPESP: Project No. 2010/12.492-7.

Conflict of interest The authors declare that they have no conflict of interest.

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9.2.2 Tissue content of sulfomucins and sialomucins in the colonic mucosa, without fecal stream, undergoing daily intervention with sucralfate

4 - ORIGINAL ARTICLE
MODELS, BIOLOGICAL

Tissue content of sulfomucins and sialomucins in the colonic mucosa, without fecal stream, undergoing daily intervention with sucralfate[1]

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DOI: http://dx.doi.org/10.1590/S0102-865020150050000004

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ABSTRACT

PURPOSE: To measure the content of acidic mucin, sialomucin, and sulfomucins in the colonic mucosa without fecal stream submit to intervention with sucralfate (SCF).

METHODS: Thirty-six rats were submitted to a right colostomy and a distal mucous fistula and divided into two groups according to sacrifice to be performed two or four weeks. Each group was divided into three subgroups according daily application of enemas containing saline, SCF at 1.0 g/kg/day or 2.0 g/kg/day. Colitis was diagnosed by histological analysis. Acid mucins were determined with the Alcian-Blue and sulfomucin and sialomucin by high iron diamine-alcian blue (HID-AB) techniques. The mucins were quantified by computer-assisted image analysis. Mann-Whitney and ANOVA tests were used to analyze the results establishing the level of significance of 5% for both ($p<0.05$).

RESULTS: SCF enemas decreased the inflammation score and was related to the concentration used and time of the intervention. SCF at both concentrations increased the content of acid mucin, which was related to the concentration used and to the improvement in the inflammatory score. There was an increase in the content of sulfomucins and sialomucins in SCF groups. SCF increased sulfomucins from 2 weeks of intervention, which was not related to the dose or time of application. The increase in sialomucin content was related to the time and dose used in the intervention.

CONCLUSION: Sucralfate increased the content of acidic mucins, primarily at the expense of sialomucin, which was affected by the dose and time of intervention.

Key words: Colon. Colitis. Mucins. Sialomucins. Sucralfate. Image Processing, Computer-Assisted. Rats.

Introduction

The colon mucosa is considered the most perfect human barrier: it protects humans against invading bacteria, antigens and existing toxins from the intestinal lumen[1]. A thin layer of mucus overlying the colonic epithelium constitutes the first line of defense, and most of its protective effect is due to the presence of mucins, which predominantly consist of glycoproteins[2,3]. Epithelial cells produce two types of mucins, neutral and acid[4,5]. Acidic mucin, in turn, consist of two subtypes, according to the predominance of sulfate radicals (sulfomucins) or sialic acid (sialomucin)[2,6]. In the normal colonic mucosa, the ratio of acidic to neutral mucins remains constant throughout the digestive tract, but it may be modified in various inflammatory bowel diseases (IBDs)[2,7].

Previous studies have shown that the capacity of the epithelium of the large intestine to produce mucins depends on an adequate supply of short-chain fatty acids (SCFAs) formed by the fermentation of the fiber ingested in the diet[4-6]. SCFAs, particularly butyric acid, are essential for mucous cells to express genes related to the transcription of the protein fraction of mucin[3,8]. The diversion of the fecal stream, which prevents the supply of ACGG to the excluded colon, reduces the ability of the goblet cells to produce mucins and tends to worsen with time exclusion[5,6]. Experimental studies using an experimental model of diversion colitis (DC) have shown that in colonic mucosa without fecal stream, there is a reduction in the level of total acidic mucins, primarily at the expense of sialomucins[5,6]. The reduction in the tissue content of acidic mucins was related to the inflammatory process in the mucosa of the colon devoid of the supply of SCFAs[5,6]. These studies suggest that in colon segments devoid of SCFAs, there are changes in the energy metabolism that lead to ~~the~~ increased production of reactive oxygen species (ROS). The resulting oxidative stress damages the layer of mucus that covers the colonic epithelium, modifying different types of mucins[5,6,9].

The application of enemas containing natural or synthetic substances, with antioxidant effects or rich SCFA solutions, improves tissue inflammatory changes detected in the DC model[10-14]. Conversely, the exposure of the colonic mucosa to substances with great oxidant power, such as hydrogen peroxide (H_2O_2), worsens the epithelial damage[15]. This evidence suggests that it would be interesting to evaluate the effect of substances with antioxidant effects in protecting the mucin layer to promote the recovery of the colonic epithelium.

Sucralfate (SCF) is the salt formed by disaccharide sucrose octosufalto associated with polyaluminum hydroxide[16]. The substance is considered a cytoprotective complex that was initially used to prevent or treat diseases of the upper digestive tract[17]. The therapeutic effects of SCF mucosal skin lesions are related to the property that the formed complex must cling firmly to the bloody surface of epithelial lesions. However, it has recently been shown that the SCF has other functional properties, such as stimulating mucus production and increasing the production of prostaglandins (PGE2) and epidermal growth factor (EGF)[18]. A study also showed that the administration of SCF can stimulate the formation of acid in the upper digestive tract mucins[19]. The topical application of SCF also showed antioxidant activity in reducing the formation of ROS produced by neutrophils present in inflamed tissue[20]. Kochhar *et al.*[21], were the first authors to demonstrate the effectiveness of the application of enemas containing SCF in controlling rectal bleeding that is caused by radiation proctitis (RP). Since then, a number of studies have confirmed the effects of SCF on clinical, endoscopic and histological improvement in patients with RP, and it is now used to treat other IBDs that evolve with the formation of epithelial ulcers[22-27]. Despite the DC course with reduced mucin content in the glands of the colonic epithelium and the formation of epithelial ulceration related to tissue oxidative stress, to the best of our knowledge, the effects of SCF in the production of acid mucins and its subtypes, sulfomucins and sialomucins, have not yet been evaluated in patients or experimental models of DC[5,6,9]. Therefore, the objective of this study is to determine, in an experimental model of DC, the effects of daily enemas with SCF on the levels of acidic mucin, sialomucin and sulfomucins in the colonic mucosa in the absence of a fecal stream.

Methods

The experiments were performed in accordance with the principles outlined by Federal Law nº 11.794 (10/08/2008)), and approved by the Ethics Committee in Animal Research of São Francisco University (Nº 2211/2007).

Thirty-six male, SPF, Wistar rats (300–350g) were obtained from the São Francisco University School of Medicine barrier facility and maintained on light/dark cycles of 12 hours; they were fed a standard rodent chow diet. They were deprived of food, but not water, for 24h prior to the surgical procedure.

Surgical technique

The diversion of the fecal stream was performed in all animals under general anesthesia by the intramuscular administration of 0.1 ml/100 g of a 1:1 (v/v) solution of ketamine (50 mg/ml) and xylazine (20 mg/ml). The abdomen

was shaved, and a 3-cm-long midline incision was made. The left colon was exteriorized and sectioned in its mid-portion, corresponding to the descending colon, approximately 3 cm above the Peyer's lymphoid patch, which is located in the rectal-sigmoid transition. Two circular skin pellets, 3 mm in diameter and 3 cm apart, were made in the left side of the abdominal wall at the same vertical level. The proximal end of the colon was exteriorized through the cranial cutaneous orifice, and the distal stoma was exteriorized through the caudal skin opening after splitting the abdominal wall muscles. The proximal end and distal stoma were fixed to the skin with full-thickness sutures. Before the fixation of the distal stoma to the skin, the distal colon was cleaned with the infusion of a physiologic solution until the fecal contents were completely removed. The abdominal incision was closed in two stages (aponeurosis and the skin). In this way, two colostomies were performed; one proximal colostomy was performed as a terminal colostomy with intestinal transit, and the second colostomy was performed as a distal stoma devoid of the fecal stream. Rats were maintained in individual cages, and no particular care was taken with respect to the stomas and abdominal incisions.

Experimental groups

The animals were divided into two experimental groups with 18 animals each, according to whether they were euthanized after two or four weeks. Each experimental group was divided into six subgroups (n=6) according to the intervention solution employed and the time of intervention. In the first and second subgroups, 12 animals received daily rectal enemas consisting of 40 ml of 0.9% saline solution (control subgroup) at 37°C for two weeks (n=6) and four weeks (n=6). In the second subgroup, 12 animals received daily rectal enemas consisting of 40 ml of SCF (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 1.0 g/kg for two weeks (n=6) and four weeks (n=6). Finally, 12 animals of the third subgroup received daily enemas consisting of 40 ml of SCF at a concentration of 2.0 g/kg for two weeks (n=6) and four weeks (n=6). To standardize the speed and time of application, the enemas were administered in all animals with an infusion pump, whose speed was standardized at 2 ml/min.

Sample collection

Upon completion of the pre-determined irrigation period, the animals were anesthetized as described above, and the midline incision was opened again. In both groups, specimens were collected from the intra-abdominal part of the excluded region (colon without fecal stream) subjected to irrigation with the proposed substances. The removed specimen, measuring approximately 4.0 cm, was longitudinally opened through the anti-mesenteric border fixed in a piece of cork and subjected to histological and histochemical analysis

Histological and histochemical analysis

Fragments prepared for histological analysis were immersed in 10% neutral formalin buffer (Sigma-Aldrich, St. Louis, MO, USA) for 24h, dehydrated by exposure to increasing ethanol concentrations, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 µm on a rotary microtome (Leica Biosystems, Nussloch, Germany), mounted on a glass slide, cleared, hydrated and stained with hematoxylin-eosin (HE) to evaluate them for the presence of colitis. Slide analysis was performed using an optical microscope (Eclipse DS-50, Nikon Inc., Osaka, Japan) with a final magnification of 200×. Photomicrographs were collected with a digital video-capture camera (DS-Fi-50; Nikon Inc., Osaka, Japan) coupled to the microscope body and digitized by a computer system of image analysis (NIS-Elements; Nikon Inc., Osaka, Japan). The diagnosis of colitis was made based on the presence of the following three independent histological parameters: reduction of the crypt length, neutrophil infiltration of the mucosa and epithelial loss. These variables were stratified as crosses, according to the degree of each, as follows: a) + absent or no alterations; b) ++ when intensity was mild d) +++ moderate and e) ++++ intense. For all analyzed variables, the final value considered for each animal was the mean value after quantification of three distinct histological fields.

The tissue expression of the acid mucins was determined individually by means of the Alcian Blue (AB) histochemical technique. The total acid mucin content stained blue[6]. To identify the subtypes of acid mucins (sulfomucins and sialomucins), the slides were stained using the high iron diamine-alcian blue (HID-AB) histochemical technique in accordance with the standardized methodology[6]. In summary, the slides were deparaffinized in two successive xylol baths for 10 minutes, treated with an alcohol rinse in three successive baths of pure alcohol and then hydrated in running water for five minutes. After this step, they were washed in three successive baths of distilled water and then exposed to high-iron diamine (HID) for six hours. The HID solution consisted of 120 mg de NN-dimethyl-meta-phenylenediamine-dihydrochloride, 20 mg de NN-dimethyl-paraphenylenediamine-dihydrochloride, 50 ml

of distilled water and 1.4 ml of a recently prepared solution of 60% ferric chloride. At the end of the HD staining, the sections were washed in running water for five minutes and then in three successive baths of distilled water. Subsequently, they were exposed to the staining agent alcian blue (AB) for 10 minutes and then washed in running water for five minutes followed by three successive baths of distilled water. The 1% AB staining medium was prepared in acetic acid (pH 2.5). At the end of this step, the slides were counterstained with neutral red for eight minutes and washed again in running water. They were then dehydrated in three successive baths of pure alcohol and diaphanized in three baths of xylol, and cover slips were mounted using resin. Through the HID-AB technique, sulfomucins stained brown, and sialomucins stained blue. A pathologist with expertise in diseases of the digestive tract, who was unaware of the origin of the material and objectives of the study, evaluated the presence of colitis and the pattern of tissue expression.

Image processing, computer-assisted

The tissue content of acid mucins, sulfomucins and sialomucins was quantified by means of computer-assisted image processing and was always performed in a focal field in which there were at least three complete and contiguous colonic crypts, with a magnification of x200. The selected images were captured on a video camera that had been coupled to an optical microscope. These images were processed and analyzed using the NIS-Elements 3.1 software (Nikon Inc., Osaka, Japan), installed in a microcomputer. By means of colored histograms in the RGB system, the software determined the color intensity in the number of pixels in each selected field, and the final data were transformed into percentage expressions by analyzed fields (%/fields). The final value measured for each slice was the mean of the values found from evaluating three different fields. The presence of sulfomucins and sialomucins was quantified in the same glands.

Statistical analysis

The statistical analysis on the results obtained with a significance level of 5% ($p \leq 0.05$). The data from each colon segment analyzed in each experimental group were expressed as the mean value with the respective standard error and were analyzed using the Biostat for Windows statistical software (version 5.0). To compare the mucin content in the different groups, the Mann-Whitney test was used. To analyze the variance in the mucin expression between the different experimental groups, ANOVA was used with the Newman-Keuls post-test.

Results

Figure 1A shows that the obtained colon segment was irrigated with 0.9% saline solution for 4 weeks, and Figure 1B shows a colon irrigated with SCF at a concentration of 2.0 g/kg/day for the same period of time. The animals in the control group had obvious epithelial loss, formation of epithelial ulceration and breakdown in the alignment of colic glands, while in animals treated with 2.0 g/kg/day SCF, the epithelial surfaces in preserved intestinal crypts are shown aligned, with a standard normal distribution and preservation of the population of goblet cells. It is possible to observe a thin layer of SCF coating the epithelial surface of the colon mucosa as an increase in the intensity of staining on the luminal portion of the epithelial cells.

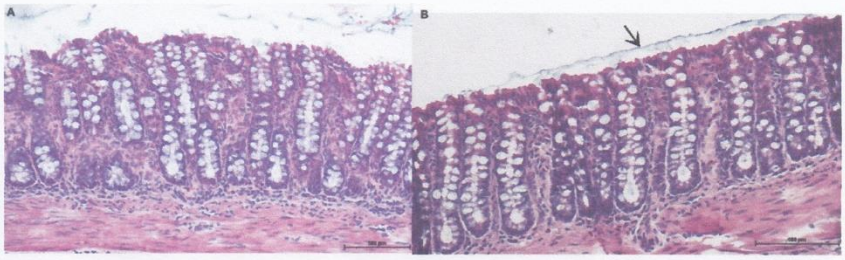

FIGURE 1 - A: colonic mucosa devoid of fecal stream after irrigation with 0.9% saline for four weeks (HE x100). **B**: colonic mucosa without fecal stream after irrigation with SCF at a concentration of 2.0 g/kg/day for four weeks. SCF layer covering the mucosal surface (black arrow) (HE x200).

Figure 2 A shows that the colon segment irrigated with 0.9% saline solution for four weeks, and Figure 2B shows a colon irrigated with SCF at a concentration of 2.0 g/kg/day for the same period of time. The animals submitted to intervention with saline (Figure 2A) have predominance of tissue expression of sulphomucins and absence of sialomucins. Differently, the animals underwent intervention with sucralfate have tissue expression of sialomucins mainly in the lower portions of the colon crypts (Figure 2B).

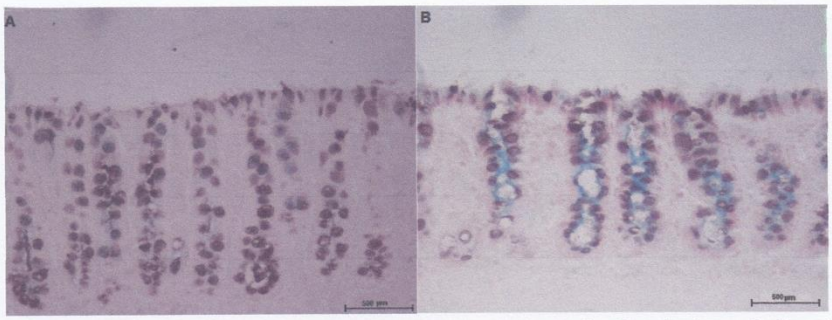

FIGURE 2 - A: colonic mucosa devoid of fecal stream after irrigation with 0.9% saline for four weeks with predominance of sulfomucins (HE x100). **B**: colonic mucosa without fecal stream after irrigation with SCF at a concentration of 2.0 g/kg/day for four weeks with presence of sialomucins (HE x200).

Figure 3 shows the inflammatory scores found in colonic segments without a fecal stream two or four weeks after intervention with saline and 1.0 g/kg/day and 2.0 g/kg/day SCF. The results show that intervention with SCF at a concentration of 2.0 g/kg/day in colon segments devoid of the fecal stream reduces the colonic inflammation scores after four weeks of irrigation compared with the control group ($p = 0.03$).

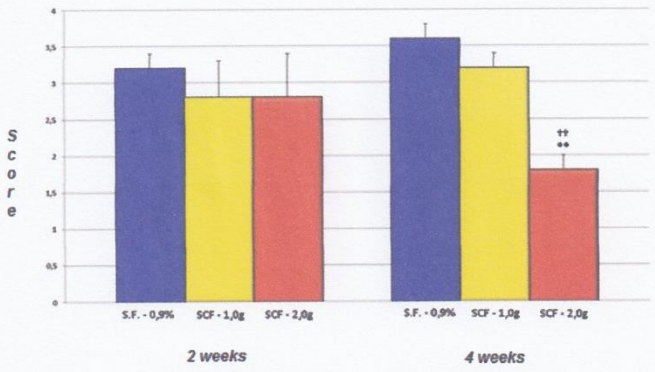

FIGURE 3 - Inflammatory score in the colon without fecal stream for the animals from the control (saline), SCF1 (SCF 1.0 g/kg/day) and SCF2 (2.0 g/kg/day) groups at two to four weeks. * = Significant (SCF2 × Control - four weeks). Mann-Whitney test.

Figure 4 shows the content of the total acid mucins found on the segments without fecal stream after intervention with saline and 1.0 g/kg/day and 2.0 g/kg/day SCF at two to four weeks. The results show that intervention with SCF at a concentration of 1.0 g/kg/day or 2.0 g/kg/day in the colon segments without a fecal stream significantly increased the content of total acidic mucins after two and four weeks of irrigation compared with the control group animals ($p<0.01$).

Tissue content of sulfomucins and sialomucins in the colonic mucosa, without fecal stream, undergoing daily intervention with sucralfate

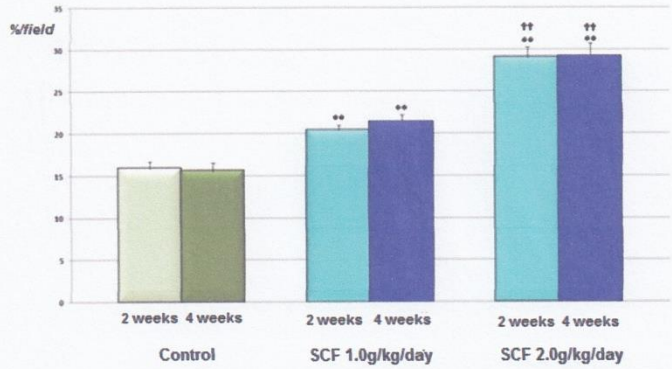

FIGURE 4 - Tissue content of acidic mucins in the control group animals SCF1 (SCF 1.0 g/kg/day) and SCF2 (2.0 g/kg/day) groups, which were subjected to daily intervention with SCF for 2 and 4 weeks. ** = p <0.01 (SCF1 × Control; SCF2 × Control); †† = p <0.01 (SCF1 × SCF2). Mann-Whitney test.

Figure 5 shows the contents of the sulfomucins found in colon segments without fecal stream after intervention with saline and 1.0 g/kg/day and 2.0 g/kg/day SCF for two to four weeks. The results show that intervention with SCF at a concentration of 1.0 g/kg/day or 2.0 g/kg/day in a colon devoid of the fecal stream significantly increased the content of sulfomucins two and four weeks after irrigation compared with animals in the control group ($p < 0.01$).

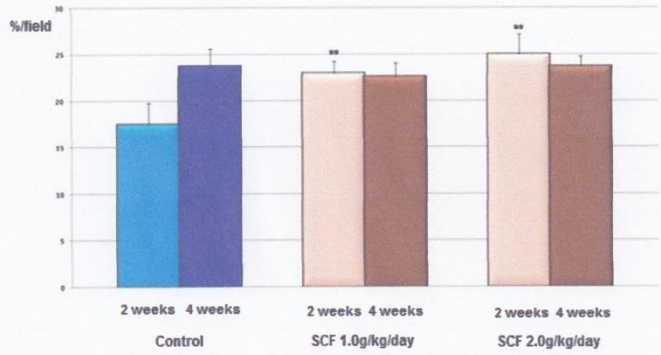

FIGURE 5 – Tissue content of sulfomucins in the control group and SCF1 (SCF 1.0 g/kg/day) and SCF2 (2.0 g/kg/day) groups, which were subjected to daily intervention with SCF for 2 and 4 weeks. ** = p <0.01 (SCF1 × Control; SCF2 × Control). Mann-Whitney test.

Figure 6 shows the tissue content of sialomucins found in the segments without fecal stream after intervention with saline and 1.0 g/ kg/day and 2.0 g/kg/day SCF in two to four weeks. The results show that intervention with SCF at a concentration of 1.0 g/kg/day or 2.0 g/kg/day in the colon without fecal stream significantly increased the content of sialomucins at two and four weeks after irrigation compared with animals in the control group. There is a relationship to the concentration used after two ($p<0.01$) and four weeks ($p < 0.05$).

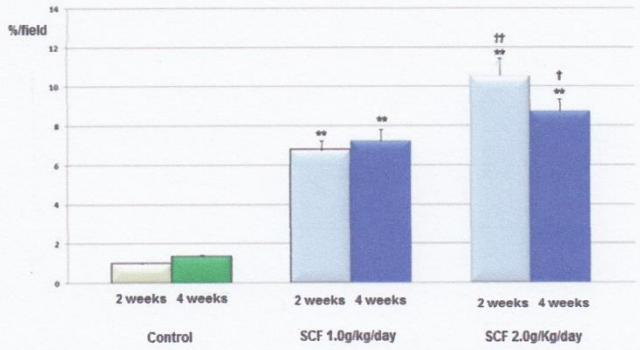

FIGURE 6 – Tissue content of sialomucins in the control group and SCF1 (SCF 1.0 g/kg/day) and SCF2 (2.0 g/kg/day) groups, which were subjected to daily intervention with SCF for two and four weeks. ** = p <0.01 (SCF1 × Control; SCF2 × Control); †† = p <0.01 (SCF2 × SCF1) after two weeks; † = p <0.05 (SCF2 × SCF1) after four weeks. Mann-Whitney test.

Table 1 shows the mean values with the corresponding standard error of the variation in the tissue content of total acidic mucins, sulfomucins, and sialomucin in animals subjected to intervention with saline, SCF 1.0 g/kg/day and 2.0 g/kg/day according to the intervention time (two or four weeks).

TABLE 1 - Variation of the tissue content of acidic mucin, sulfomucin and sialomucin in animals subjected to intervention with saline, SCF 1.0 g/kg/day and SCF 2.0 g/kg/day and after two and four weeks.

	Mean ± Standard error		
***Total* acidic mucins**	**2 weeks**	**4 weeks**	**p**
Saline	15.91±0.86	15.53±1.00	NS
SCF1	20.36±0.63	21.33±0.91	NS
SCF2	28.99±1.30	29.06±1.65	NS
Sulfomucins			
Saline	17.46±2.30	23.68±1.90	<0.05*
SCF1	22.93±1.93	22.57±1.70	NS
SCF2	24.95±2.20	23.65±1.00	NS
Sialomucins			
Saline	0.97±0.04	1.32±0.07	NS
SCF1	6.74±0.65	7.19±0.63	NS
SCF2	10.53±0.81	8.72±0.70	<0.05*

NS = not significant; * = significant (p ≤0.05) ANOVA.

Discussion

SCF is a cytoprotective drug that has been used for decades to treat inflammation of the esophagus, stomach and duodenum, and its cytoprotective action in the gastrointestinal epithelium occurs by different mechanisms[17,28,29]. SCF increases the thickness of the layer of mucus that coats the gastrointestinal mucosa, thereby increasing blood flow to the mucosa, preserving cell viability, and stimulating the production of prostaglandins and endogenous growth factors related to tissue repair[28]. Therefore, it is likely that the SCF has a dual mechanism of action, which is related to the direct interaction of the substance or its components with the surface of injured tissues and, in part, by stimulating the effects of tissue repair[28].

The increased production of mucins from goblet cells induced by SCF assists in the protection afforded by the mucus layer that covers the intestinal mucosa against aggression by different biological, physical or chemical agents in the intestinal lumen. In contact with epithelial ulceration of the gastrointestinal mucosa, SCF clings tightly to albumin and fibrinogen in the deep ulcer surface, thus forming a stable and insoluble complex that covers and protects the ulceration and functioning as a true mechanical barrier. Moreover, SCF accelerates tissue repair by stimulating the migration of fibroblasts to the site where there is erosion of the gastrointestinal mucosa[28,29]. These two effects are related to the ability of the substance to stimulate mucin production, PGE2 and EGF[30]. The increase in PGE2 production, the principal metabolite of the COX-1 and COX-2, can regulate the local angiogenesis, survival and motility of epithelial cells, and this effect is dose-dependent[30]. A published study showed that SCF significantly increases the production of TGF-α and that the association of SCF with TGF-α can stimulate cell proliferation, promoting mucosal healing[31].

Slomiany *et al.*[19], evaluated the influence of the administration of SCF on the content, composition and physical properties of mucus overlying the gastric mucosa. SCF could increase the thickness of the mucus layer by 8% and increase its viscosity 1.9-fold. When analyzing the production of acidic mucins, there was a significant increase in the production, with an increase of 63% and 81% in the sulfomucins and sialomucins, respectively[19]. These results suggest that the therapeutic effects of SCF are also related to their ability to increase the content of acidic mucin present in the mucus layer that covers the gastrointestinal tract[19,33]. Among the different mechanisms of action, studies have shown that SCF has remarkable antioxidant action by inhibiting the production of superoxide radicals[33,34]. Laudano *et al.*[34], exposed the gastrointestinal mucosa of rats to H_2O_2, which potently induces the formation of ROS, and found that SCF has antioxidant effects, protecting the mucosa not only by the presence of aluminum sulfate but also by leading to an increased synthesis of mucins and endogenous prostaglandins.

Most authors believe that the pathogenesis of DC is related to a deficiency in the supply of SCFAs to the mucosa of the colon or rectal segments that lack a fecal stream[35,36]. This possibility is supported by the previous studies reporting that when the supply of SCFAs is deficient, the cells of the colon mucosa lack intestinal transit, which is related to the development of DC, whereas the restoration of the fecal stream or irrigation of the excluded segments with AGCC improves the symptoms and decreases the endoscopic and histological findings in the disease[12]. Despite the important role of maintaining the supply of SCFAs to prevent the development of DC, the molecular mechanisms that cause epithelial lesions have only recently begun to be understood[9]. Experimental studies have shown that the epithelial lesions found in the models of DC are related to the tissue oxidative stress that results from the increased production of ROS by colonic mucosa that is devoid of a fecal stream, a location that is known to be deficient in antioxidant enzyme systems[37,38]. ROS are produced in excess by colonic mucosa without intestinal transit. The increased production of superoxide radicals, hydroxyl and hypochlorous acid, which cause oxidative stress, can damage the various defense systems of the colonic mucosa[39]. The damage to these defense systems enables the migration of antigens and bacteria in the intestinal lumen to the intima of sterile layers of the colon wall, thus triggering the inflammatory process found in DC[5,6,9]. An experimental study showed that it is possible to trigger the onset of epithelial lesions such as those found in the DC by applying enemas with hydrogen peroxide, a potent donor of ROS[15]. The possibility that ROS may trigger the onset of DC gained more support after studies demonstrated that the application of enemas with antioxidants, such as 5-ASA and N-acetylcysteine, to the excluded colon is effective in controlling the disease[11,14,36].

The tissue expression and content of the mucins in the excluded colon in humans or experimental models with DC has rarely been studied[1,5,6]. The results from these studies demonstrate that transit modifies the tissue content of neutral and acidic mucin present on the colonic mucosa glands. It is possible that the oxidative stress resulting from intestinal exclusion is the molecular mechanism responsible for the damage to the mucus layer. Nonose *et al.*[5], measured the content of neutral and acidic mucins in the colonic glands in an experimental model of DC and found a reduction in the tissue content of neutral and acidic mucins in the segments without fecal stream. Subsequently, the same group, measuring only acidic mucin subtypes (sulfomucins and sialomucins) found a reduction in the mucins, mainly at the expense of sialomucin[6]. The authors drew attention to the possibility that the reduction in the tissue content of acidic mucins could be related to production of higher levels of ROS in the excluded colon[5,6]. Corroborating this evidence, experimental studies have shown that the preventive application of natural substances, such as the aqueous extract of *Ilex paraguariensis* or synthetic as n-acetylcysteine and 5-ASA, which are potent antioxidants, reduced the levels of oxidative stress and protected the mucosa that lacked a fecal stream against the deleterious effects of ROS[10-14].

It seems obvious that a substance that stimulates the production of mucus by the colonic epithelium and possesses antioxidant activity may also be useful for treating DC. However, one study evaluated the effects of SCF application in an experimental model of DC. The results of this study showed that application of enemas with increasing concentrations of SCF in the excluded colon reduced the loss of epithelial colonic mucosa, decreased mucosal ulcers, impaired the formation of abscesses in the colon crypts and reduced mucosal inflammatory infiltrate[40]. Despite this evidence, no clinical or experimental studies have evaluated the effects of SCF on the synthesis of sulfomucins and sialomucins by cells of the colonic mucosa without fecal stream. Similar to that described above, the results of this study show that the daily use of daily enemas with SCF in the excluded colon transit reduces inflammation of the colon mucosa. This effect was directly related to the concentration and duration of the intervention. Animals subjected to the intervention with the highest concentration (SCF 2.0 g/kg/day) and for a longer period of time (4 weeks) showed significant reduction in the inflammatory score, confirming the results of a previous study[40]. We conducted an intervention for a maximum period of four weeks because this

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The tissue expression and content of the mucins in the excluded colon in humans or experimental models with DC has rarely been studied[1,5,6]. The results from these studies demonstrate that transit modifies the tissue content of neutral and acidic mucin present on the colonic mucosa glands. It is possible that the oxidative stress resulting from intestinal exclusion is the molecular mechanism responsible for the damage to the mucus layer. Nonose *et al.*[5], measured the content of neutral and acidic mucins in the colonic glands in an experimental model of DC and found a reduction in the tissue content of neutral and acidic mucins in the segments without fecal stream. Subsequently, the same group, measuring only acidic mucin subtypes (sulfomucins and sialomucins) found a reduction in the mucins, mainly at the expense of sialomucin[6]. The authors drew attention to the possibility that the reduction in the tissue content of acidic mucins could be related to production of higher levels of ROS in the excluded colon[5,6]. Corroborating this evidence, experimental studies have shown that the preventive application of natural substances, such as the aqueous extract of *Ilex paraguariensis* or synthetic as n-acetylcysteine and 5-ASA, which are potent antioxidants, reduced the levels of oxidative stress and protected the mucosa that lacked a fecal stream against the deleterious effects of ROS[10-14].

It seems obvious that a substance that stimulates the production of mucus by the colonic epithelium and possesses antioxidant activity may also be useful for treating DC. However, one study evaluated the effects of SCF application in an experimental model of DC. The results of this study showed that application of enemas with increasing concentrations of SCF in the excluded colon reduced the loss of epithelial colonic mucosa, decreased mucosal ulcers, impaired the formation of abscesses in the colon crypts and reduced mucosal inflammatory infiltrate[40]. Despite this evidence, no clinical or experimental studies have evaluated the effects of SCF on the synthesis of sulfomucins and sialomucins by cells of the colonic mucosa without fecal stream. Similar to that described above, the results of this study show that the daily use of daily enemas with SCF in the excluded colon transit reduces inflammation of the colon mucosa. This effect was directly related to the concentration and duration of the intervention. Animals subjected to the intervention with the highest concentration (SCF 2.0 g/kg/day) and for a longer period of time (4 weeks) showed significant reduction in the inflammatory score, confirming the results of a previous study[40]. We conducted an intervention for a maximum period of four weeks because this

time period was used in most previous studies[10,11,14,15,40]. However, the inflammatory process may worsen with time. Further studies evaluating the effects of SCF in improved tissue inflammation that also adopt a longer exclusion period are still needed to confirm whether the use of the drug for longer periods maintains a sustained clinical response. Authors who used SCF in patients with RP and DII showed that the effects were more evident with an increasing time of application[21-25].

The present study demonstrated that the application of enemas containing SCF, independent of the applied concentration, increased the content of acid mucin in the glands of the colon that lacked a fecal stream. Compared with the control group, the increase in the production of acid mucins was independent of the concentration. The increased content of acidic mucins was directly related to the dose used; the animals that received 2.0 g/kg/day had a tissue mucin acid content that was significantly higher than those irrigated with a lower concentration. There are several possible explanations for the action of SCF in the colonic mucosa that lacks a fecal stream. It is possible that the preventive application of the substance into a gelatinous layer on the colonic mucosa impairs the contact and penetration of antigens and bacteria in sterile and deeper layers of the intestinal wall, slowing the colonic lumen-derived aggression. However, it is worth remembering that although bacteria, antigens, and chemicals are still present in the excluded colon, it is likely that the levels of these aggressive agents are lower due to fecal exclusion. SCF might provide an antimicrobial effect in the colon, thus reducing the bacterial population and possibility of invasion from other layers of the colon wall and, therefore, reducing the inflammatory process. We are currently evaluating the bacterial flora in the excluded colon that underwent intervention with SCF to evaluate whether the substance has antimicrobial action. Because SCF stimulates the production of PGE2 and increases local blood flow, it is possible that the increased production of acid mucins may be related to the properties of the substance. It is worth remembering that although we found increased tissue levels of sulfomucins and sialomucins in the animals treated with SCF, this increase primarily occurred at the expense of sialomucins, which is the subtype of mucin that has the greatest reduction in the mucosa of the colon lacking a fecal stream[6]. It has been demonstrated that SCF primarily stimulates the production of mucins rich that are in sialic acid[19]. Because the acidic mucins are found in the germ portions of the cramping glands, it is possible that the SCF protection of the colonic mucosa to decrease the need for cellular replication stabilizes the yield of sialomucin. Studies evaluating the content of PGE2, EGF and vascular endothelial growth factor in animals undergoing intestinal intervention with SCF derivation are still needed to confirm this possibility.

SCF has antioxidant activity, and the intestinal bypass significantly increases the production of ROS[9,33]. As demonstrated with other substances that possess antioxidant activity (5-ASA, n-acetylcysteine and aqueous extract of *Ilex paraguariensis*), it is also possible that the SCF has the same antioxidant effects on excluded sigmoid traffic mucosa, preventing epithelial assault, neutrophil infiltration and apoptosis of cells producing mucin[10,11,14]. In an unpublished study in which we measured the levels of oxidative tissue stress by measuring malondialdehyde, SCF intervention reduced the levels of lipid peroxidation (oxidative stress) and the presence of myeloperoxidase. It is noteworthy that these effects were related to the expression and content of different types and subtypes of acid mucins.

As noted, all of these questions merit further study. There are still questions about the mechanisms of action of SCF in the excluded colon. Nevertheless, we found that the application of the substance enhances the inflammatory process and increases the production of acid mucins in models of DC[5]. Ultimately, these results suggest that the application of enemas with SCF could become a valid strategy for treating DC in patients with a stoma.

Conclusion

The daily application of enemas containing sucralfate increases the tissue content of sulfomucins and sialomucins and reduces the inflammatory changes found in experimental diversion colitis.

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Received: Jan 14, 2015
Review: Mar 12, 2015
Accepted: Apr 15, 2015
Conflict of interest: none
Financial source: Sao Paulo Research Foundation (FAPESP). Project n°. 2010/12492-7.

[1]Research performed at Laboratory of Medical Investigation, Medical School, Sao Francisco University (USF), Bragança Paulista-SP, Brazil. Part of Master degree thesis, Postgraduate Program in Health Sciences, USF. Tutor: Carlos Augusto Real Martinez.

9.2.3 Evaluation of the application of enemas containing sucralfate in tissue contente of neutral and acid mucins in experimental modelo of diversion colitis

1 - ORIGINAL ARTICLE
MODELS, BIOLOGICAL

Evaluation of the application of enemas containing sucralfate in tissue content of neutral and acid mucins in experimental model of diversion colitis[1]

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DOI: http://dx.doi.org/10.1590/S0102-86502014001500001

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ABSTRACT

PURPOSE: To evaluate the effects of sucralfate on tissue content of neutral and acids mucins in rats with diversion colitis.

METHODS: Thirty-six rats were submitted to a proximal right colostomy and a distal mucous fistula. They were divided into two groups according to sacrifice to be performed two or four weeks after intervention. Each group was divided into three subgroups according daily application of enemas containing saline, sucralfate at 1.0 g/kg/day or 2.0 g/kg/day. Colitis was diagnosed by histological analysis and neutral and acid mucins by Periodic Acid Schiff and Alcian Blue techniques, respectively. The contents of mucins were quantified by computer-assisted image analysis. Student's t paired and ANOVA test were used to compare the contents of both types of mucins among groups, and to verify the variance with time, establishing level of signification of 5% for both ($p<0.05$).

RESULTS: Enemas containing sucralfate improves the inflammation and increases the tissue contents of neutral and acid mucins. The content of neutral mucins does not change with the time or concentration of sucralfate used, while acid mucins increases with concentration and time of intervention.

CONCLUSIONS: Sucralfate enemas improve the inflammatory process and increase the tissue content of neutral and acid mucins in colon without fecal stream.

Key words: Colitis. Colostomy. Fatty Acids, Volatile. Mucins. Sucralfate. Image Processing, Computer-Assisted. Rats.

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Introduction

Glotzer *et al.*[1] were the first to describe the development of an inflammatory process in the mucosa of colon segments without fecal stream. They called this new form of inflammatory bowel disease (IBD) diversion colitis (DC). Subsequently, it has been demonstrated that the etiopathogenesis of DC is related to intraluminal deficiency of short-chain fatty acids (SCFAs), the main energy substrate for the cells of the colon epithelium[2]. The most common histological finds in patients with DC include epithelial erosions or ulcerations, decreased length or modified architecture of the colonic glands, inflammatory infiltrate and, depletion of the number of goblet cells, with consequent modifications on tissue content of mucins[2-4]These histological alterations are similar to those found in other forms of IBD, particularly in the distal proctitis founded after pelvic radiotherapy and in rectal compromising of ulcerative colitis (UC)

The mucous layer covering the colon epithelium is the first line of defense against invasion of the intestinal wall by bacteria and antigen that are present in the intestinal lumen[4,5]. Most of the protective effect provided by the mucous layer relates to the presence of mucins, which are the predominant glycoproteins in the chemical composition of colonic mucous[6-8]. Mucins in addition to forming a mechanical barrier exhibit antimicrobial activity that protects the intestinal wall against existing pathogens in the intestinal lumen[9]. The histochemical expression of mucins in the colonic mucosa is well known and, selectively, the presence of neutral and acid mucins can be demonstrated[10]. The proportions between neutral and acid mucins may be modified in IBD and changes to the tissue expression of this glycoprotein in the colon mucosa of patients with UC and Crohn's disease have already been demonstrated[11,12]. The capacity of the colonic epithelium to produce mucins depends on adequate supply of SCFAs[7]. Recent studies have shown the importance of maintaining the supply of SCFAs, particularly butyrate, in order to have adequate gene expression relating to mucin formation and content of the glycoprotein[4,5,8,13].

Despite the histopathological similarities between UC and DC, few studies has evaluated the modifications in the tissue contents and pattern of expression of neutral and acid mucins on glands of the colonic mucosa without fecal stream[4,5,13]. The results of these studies showed that the deviation of the fecal stream reduce the content and modify the pattern of tissue expression of neutral and acid mucins. In other words, the deficiency in the supply of SCFAs can modify the mucus that overlying the colonic mucosa, reducing the effectiveness of this first line of defense against luminal aggression[5,13].

Sucralfate (SCF) is a basic aluminum salt of sucrose octasulphate which was employed for prevention and treatment of several gastrointestinal diseases[14]. SCF has used as a topical treatment to healing several types of epithelial wounds such as venous ulcers, mucositis and perianal wounds[15,16]. The molecule of SCCF has other properties such as antioxidant, stimulating the formation of gastrointestinal mucus, increase the production of prostaglandins and epidermal growth factor (EGF)[14]. Kochhar *et al.*[16] were the first author to demonstrate the efficacy of enemas containing SCF in control of rectal bleeding due to radiation proctitis (RP). Since then, a string of well-conducted studies have confirmed the beneficial therapeutic effects of the substance in clinical, endoscopic and histological improvement in patients with RP, as well as in other forms of IBD where occur the formation of ulcers in the colonic mucosa, such as UC and solitary rectal ulcer[17,18]. Despite the DC course with the formation of superficial epithelial ulcerations and aphthous ulcers in the colonic mucosa, to the best of our knowledge, the effects of SCF on tissue expression of mucins has not yet been evaluated in patients or experimental models of DC. Therefore, the objective of this study is to determine the effects of application of daily enemas with SCF on tissue content of mucins in colonic mucosa devoid of the fecal stream.

Methods

The experiments were performed in accordance with the principles outlined by Federal Law nº 11.794 (10/08/2008) and were approved by the Ethics Committee in Animal Research of Sao Francisco University (Nº 2211/2007).

Thirty-six male, SPF, Wistar rats (300–350g) were obtained from the Sao Francisco University, School of Medicine, barrier facility and maintained on light/dark cycles of 12 hours, and fed a standard rodent chow diet. They were deprived of food, but not water, for 24h prior to the surgical procedure.

Surgical technique

The diversion of the fecal stream was performed in all animals under general anesthesia by intramuscular administration of 0.1 ml/100 g of a 1:1 (v/v) solution of ketamine (50 mg/ml) and xylazine (20 mg/ml). The abdomen was shaved, and a 3 cm long midline incision was made. The left colon was exteriorized and sectioned in its mid-portion, corresponding to the descending colon, approximately 3 cm above the Peyer's lymphoid patch located in the rectal-sigmoid transition. Two circular skin pellets, 3 mm in diameter and 3 cm apart, were made in the left side

of the abdominal wall at the same vertical level. The proximal end of the colon was exteriorized through the cranial cutaneous orifice, and the distal stoma was exteriorized through the caudal skin opening after splitting the abdominal wall muscles. The proximal end and the distal stoma were fixed to the skin with full-thickness sutures. Before the fixation of distal stoma to the skin, the distal colon was cleaned by means of an infusion of a physiologic solution until the fecal contents were completely removed. The abdominal incision was closed in two stages (aponeurosis and the skin). In this way, two colostomies were performed: the proximal colostomy as a terminal colostomy with intestinal transit and the second colostomy, as a distal stoma devoid of the fecal stream. Rats were maintained in individual cages, without particular care being taken with regard to the stomas and abdominal incisions.

Experimental groups

The animals were divided into two experimental groups with 18 animals each according the euthanasia had done after two or four weeks. Each experimental group was divided into six subgroups (n=6) according to the intervention solution employed and time of intervention. In the first and second subgroups, 12 animals received daily rectal enemas containing 40 ml of 0.9% saline solution (control subgroup) at 37ºC for two weeks (n=6) and four weeks (n=6). In the second subgroup, 12 animals received daily rectal enemas containing 40 ml of SCF (Sigma-Aldrich, St Louis, MO, USA) at a concentration of 1.0 g/kg for two weeks (n=6) and four weeks (n=6). Finally, 12 animals of the third subgroup received daily enemas containing 40 ml of SCF at a concentration of 2.0 g/kg for two weeks (n=6) and four weeks (n=6). In order to standardize the speed and time of application, the enemas were administered in all animals with an infusion pump whose speed was standardized at 2/ml/min.

Sample collection

Upon completion of the pre-determined irrigation period, the animals were anesthetized as described above, and the midline incision was opened again. In both groups, specimens were taken from the intra-abdominal part of the excluded (colon without fecal stream) subjected to irrigation with the proposed substances. The removed specimen, measuring approximately 4.0 cm, was longitudinally opened through the anti-mesenteric border fixed in a piece of cork and referred to histological and histochemical analysis.

Histological and histochemical analysis

Fragments prepared for histological analysis were immersed in 10% neutral formalin buffer (Sigma-Aldrich, St. Louis, MO, USA) for 24 h, dehydrated by exposure to increasing ethanol concentrations, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 µm on a rotary microtome (Leica Biosystems, Nussloch, Germany), mounted on a glass slide, cleared, hydrated and stained with hematoxylin-eosin (HE) for evaluation of the presence of colitis. Slide analysis was performed with optical microscope (Eclipse DS-50, Nikon Inc., Osaka, Japan) with final magnification of x200. Photomicrographs were taken with a digital video-capture camera (DS-Fi-50; Nikon Inc., Osaka, Japan) coupled to the microscope body and digitized by a computer system of image analysis (NIS-Elements; Nikon Inc., Osaka, Japan). The diagnosis of colitis was made based on presence of three independent histological parameters: reduction of the crypt length, neutrophil infiltration of the mucosa and epithelial loss. These variables were stratified as crosses, according to the degree of each, as follows: a) + absent or no alterations; b) ++ when intensity was mild d) +++ moderate and e) ++++ intense. For all variables analyzed, the final value considered for each animal was the mean value after quantification of three distinct histological fields.

The tissue expression of the neutral mucins was determined individually by means of the histochemical technique of Periodic Acid Schiff (PAS), while the expression of acid mucins was determined using the Alcian Blue (AB) technique. The neutral mucins stained magenta, while the acid mucins stained blue[19]. The pattern of tissue expression was by a pathologist with experience of diseases of the digestive tract who was unaware of the origin of the material and the objectives of the study.

Image processing, computer-assisted

The tissue content of neutral and acid mucins was quantified by means of image processing computer-assisted and was always performed in a focal field in which there were at least three complete and contiguous colonic crypts, with a magnification of x200. The images selected were captured on a video camera that had been coupled to an optical microscope. These images were processes and analyzed using the NIS-Elements 3.1 software (Nikon Inc., Osaka, Japan), installed in a microcomputer. By means of colored histograms in RGB system the software determined the color intensity in number of pixels in each field selected, and transformed the final data into percentage expressions by analyzed fields (%/fields). The final value taken for each field measured in the segments with and without intestinal transit was the mean of the values found from evaluating three different fields.

Statistical analysis

The statistical analysis on the results obtained was performed by taking the significance level of 5% ($p < 0.05$). The data from each colon segment analyzed, in each experimental group, were expressed as the mean value with the respective standard error, and were analyzed using the Biostat for Windows statistical software (version 5.0). To compare the mucin content in the segments with and without transit, Student's t paired test was used. To analyze the variance in the mucin expression between the different experimental groups, ANOVA was used with the Newman-Keuls post-test.

Results

Figure 1A shows the colonic epithelium without fecal stream submitted to intervention with S.F. 0.9% for 4 weeks, while Figure 1B shows the colon segment without fecal stream irrigated with SCF at concentration of 2.0 g/kg/day.

Figure 2 shows the inflammatory score comparing animals submitted to intervention with saline, SCF 1.0 g/kg/day and SCF 2.0 g/kg/day for two and four weeks.

Figure 3A show the tissue expression of neutral mucins, evaluated in segments without fecal stream submitted to intervention with S.F. 0.9% for four weeks, while Figure 3B shows the colon segment without fecal stream irrigated with SCF at concentration of 2.0 g/kg/day.

Figure 4 shows the mean, with respective standard error, from quantification of neutral mucins comparing colon segments without intestinal transit, submit to daily intervention with saline and SCF at concentration of 1.0g/kg and 2.0g/kg. We found a significant increase in tissue content of neutral mucins in animals subjected to irrigation with SCF compared to animals irrigated with S.F. 0.9%, regardless of the concentration and duration of intervention. The tissue contents of neutral mucins did not vary with the time of intervention established, independent of concentration.

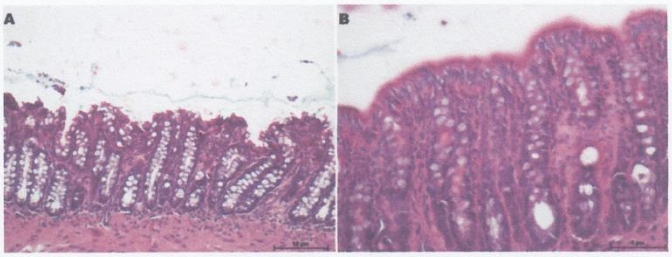

FIGURE 1 – A. Colonic mucosa devoid of the fecal stream submitted to intervention with saline for four weeks. Note the epithelial loss with formation of superficial ulcers and irregularity of the colonic glands (HE-x100). **B.** Colonic mucosa devoid of the fecal stream submitted to intervention with SCF (2.0 g/kg/day) for four weeks. It's possible verify the formation of a protective layer of SCF on the colonic mucosa, preservation of the epithelial colonic surface without the formation of ulcers.

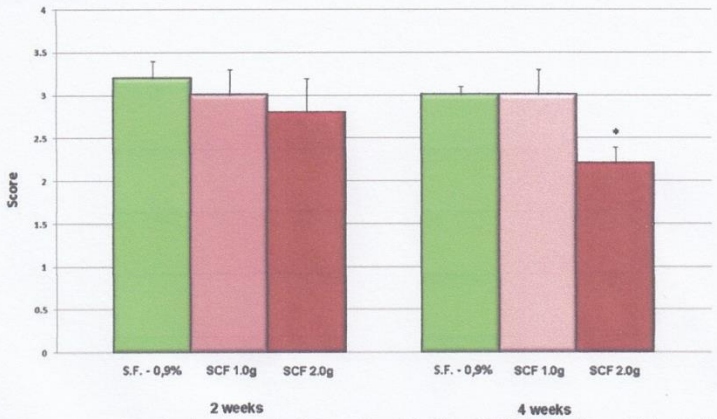

FIGURE 2 - Inflammatory score comparing animals submitted to intervention with saline, SCF 1.0 g/Kg/day and 2.0 g/Kg/day for two and for weeks. * = significant ($p<0.05$). Student t test.

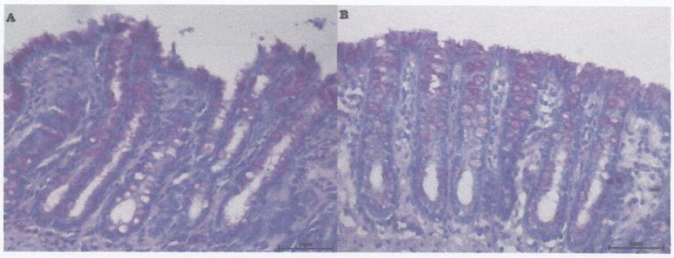

FIGURE 3 – A. Tissue content of neutral mucins in animals submitted to intervention with saline for four weeks. It's possible verify loss of epithelium surface, distortion of the colon crypts and reduction of tissue content of neutral mucins in colon glands (PAS-x200). **B.** Neutral mucins on glands of colonic mucosa devoid of the fecal stream submitted to intervention with SCF 2.0 g/Kg/day for four weeks. The epithelial surface is preserved, and the neutral mucin expression occurs uniformly along the colonic glands (PAS-x200).

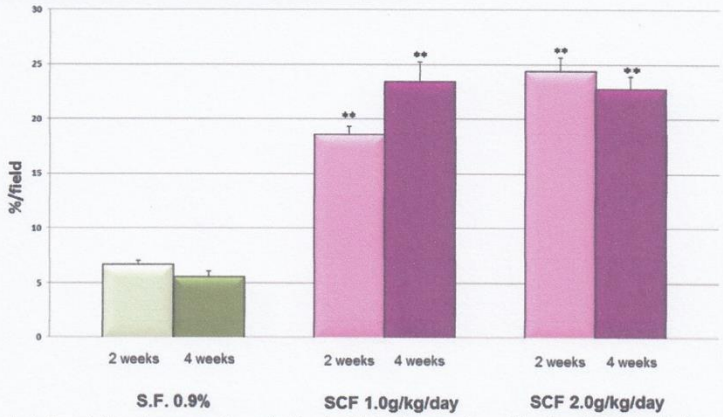

FIGURE 4 - Tissue content of neutral mucins comparing animals submitted to intervention with saline, SCF 1.0 g/Kg/day and 2.0 g/Kg/day for two and for weeks. ** = significant ($p<0.01$). Student t test.

Figure 5A shows the tissue expression of acid mucins, evaluated using the Alcian-Blue technique in colonic segments without fecal stream submitted to intervention with S.F. 0.9% for four weeks, while Figure 5B shows the colon segment without fecal stream irrigated with SCF at concentration of 2.0 g/kg/day.

Figure 6 shows the mean, with respective standard error, from quantification of acid mucins comparing colon segments without intestinal transit, submmit to daily intervention with S.F.0.9% and SCF at concentration of 1.0g/kg and 2.0g/kg. We found a significant increase in tissue content of acid mucins in animals subjected to irrigation with SCF compared to animals irrigated with S.F. 0.9%, regardless of the concentration and duration of intervention. The tissue contents of acid mucins increase with the concentration established, independent of the time.

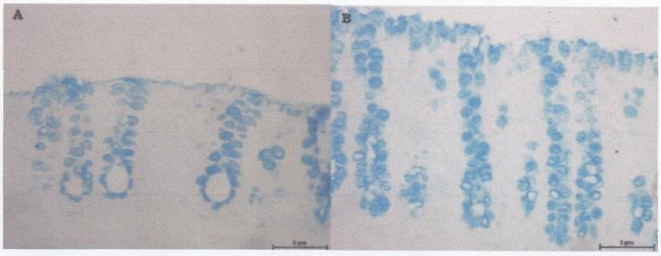

FIGURE 5 – A. Tissue content of acid mucins in animals submitted to intervention with saline for four weeks. Reduction of tissue content of acid mucins in colon glands (AB-x100). **B.** Acid mucins on glands of colonic mucosa devoid of the fecal stream submitted to intervention with SCF 2.0 g/Kg/day for four weeks. The expression of acids mucin occurs uniformly along the colonic glands (AB-x200).

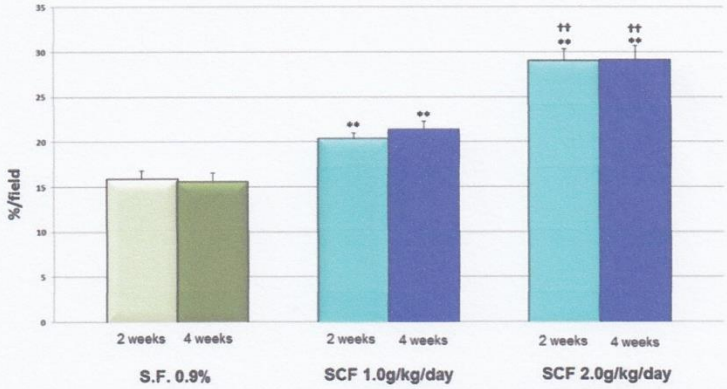

FIGURE 6 - Tissue content of acid mucins comparing animals submitted to intervention with saline, SCF 1.0 g/Kg/day and 2.0 g/Kg/day for two and for weeks. ** = significant ($p<0.01$) **SCF 1.0 g/kg/day and SCF 2.0 g/kg/day × Saline. †† SCF 1.0 g/kg/day × SCF 2.0 g/kg/day Student t test.

Discussion

DC is defined as an IBD that affects colorectal segments without fecal stream[2]. A variety of theories have been put forward to explain the etiopathogenesis of DC. Prominent among these is a theory that relates the appearance of this disease to decreased quantities of SCFAs in the intestinal lumen[2,3,20-22]. SCFAs are the main energy source for colonocytes and account for approximately 80% of the energy needs of the colon epithelium[20]. In colon segments without fecal stream, absence of the diet fibers impedes the formation of SCFAs and also their absorption and use by the epithelial cells. Recent studies have shown that an absence of SCFAs modifies energetic metabolism of the mitochondrial respiratory chain of the colonocytes leading an increase on production of reactive oxygen species (ROS)[23]. The increased production of ROS in a tissue recognized to be deficient in antioxidant systems can cause oxidative damage to the cells of the colonic epithelium[23]. It has been shown the deleterious effects of ROS against the mechanisms of defense of colonic epithelial barrier, in particular the mucus layer overlying the intestinal mucosa and the cellular adhesion moleculles[5,13,24-25]. These studies showed a relationship between increased of ROS production and reduction in tissue content of neutral and acid mucins in colon segments without fecal steam[5-13]. Reinforcing the importance of oxidative stress in the intestinal epithelium aggression in colonic segments devoid of fecal stream had been demonstrated that the application of enemas containing substances with antioxidant activity, such as 5-aminosalicylic acid and n-acetylcysteine, improves tissue damage by reducing levels of ROS by cells of the colonic mucosa[26-27].

The main function of mucus is to serve as protection for the epithelial cells of the intestinal mucosa, against aggression caused by enzymes, antigens and bacteria that are present in the intestinal lumen[6,9]. The expression of mucins in the colon epithelium is well known and can be categorized by histochemical techniques as neutral or acid[4,13]. The proportion among all types of mucins is usually constant in the mucosa of normal colon tissue, but may undergo changes in various diseases[5]. The capacity for mucin synthesis shown by the goblet cells of the colon mucosa depends on an adequate supply of energy substrate[7,8]. Reductions in the supply of SCFAs, as occurs during intestinal diversion operations, are accompanied by morphological and functional changes, thereby causing reductions in protein synthesis capacity[5,8]. The mechanisms through which shortages of SCFAs lead to lower capacity for mucins production are still not completely clear. Studies have shown that intestinal diversion reduces the quantities of dietary fiber and modifies the bacterial populations in the segment excluded and it decreases the production of SCFAs, thereby causing a state of nutritional deficiency for the cells of the colon mucosa[5,20,21,28]. This nutritional deficiency reduces the mucin synthesis capacity of these cells[7]. Reinforcing this evidence, studies have shown that administration of SCFA enemas increases the mucin synthesis capacity of the colon mucosa cells, while administration of enemas containing sodium bromo-octanoate (a powerful inhibitor of SCFA metabolization) completely blocks mucin synthesis and causes the appearance of colitis[8,28]. SCFAs also induce the expression of various genes that transcribe mucins, thereby increasing the expression of *MUC-2* around twentyfold[7,8]. SCFAs increase cell metabolism by more than 80% and the production and secretion of mucins by the colon epithelium more

than twentyfold[8,29]. Recent research has also demonstrated that shortages of SCFAs greatly reduces the expression of the *MUC-2* gene, which is the main agent responsible for transcription of the protein fraction of colon mucins[8,30]. Two studies adopting an experimental model of DC showed that the deviation of the fecal stream reduce the tissue content of neutral and acid mucins, particularly scialomucins quantified by computer assisted image analysis, confirming the importance of correct supply of SCFAs in maintaining the synthesis of the glycoprotein[5,13]. All these points reinforce the important role played by SCFAs in relation to the capacity of the colon epithelium to produce mucins and consequently to protect the mucosa against aggression coming from the intestinal lumen[5,7,13,31].

These features suggest that the ideal substance for the treatment of DC should have as principal precepts: enhance protection of the intestinal epithelium that lost the protection afforded by the mucus layer, stimulating the production of mucins by intestinal epithelium and improve the tissue repair of the damaged epithelium. If the substance still presents an antioxidant activity, could reduce the oxidative tissue stress and consequently the epithelial damage caused by overproduction of ROS[23].

SCF is a cytoprotective agent that has been used for more than three decades in the treatment of several disorders such gastritis, duodenal, stress ulcers, burns, skin lesions, and DII[14-18,32]. SCF does not have any adverse effects and it's use is known to have multiple beneficial effects on mucocutaneous wound healing, because the drug can induce the proliferation of dermal fibroblasts, and limiting the inflammation might decrease fibrosis formation and EGF expression as well as the expression of other factors involved in tissue repair processes[32,33]. Stimulating effects on vascular factors, such as angiogenesis, which play important roles in tissue repair, have been demonstrated by SCF[14,35]. The substance is a sucrose and sulfate-aluminum complex which, when in contact with gastrointestinal secretions, forms a viscous gel that adheres to the mucosa creating a physical barrier that protects the mucosa[15-18]. The complex formed, in addition to preventing the degradation of the mucus that covers the gastrointestinal epithelium has antimicrobial properties[31,36]. Studies have shown that SCF also has a bactericidal effect, effective against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*[37]. SCF adheres tightly to proteins on the surface of ulcerations, mainly albumin and fibrinogen, thus forming a stable and insoluble complex, creating a protective layer that covers and protects the ulcerated epithelium. Recent studies have shown that SCF stimulates the production of prostaglandin E2 (PGE2), EGF and gastrointestinal mucus[38]. It has been shown that the use of SCF increase in 8% the dimension of the mucus gel layer, and increase the contents of acid mucins, sulfomucins and sialomucin, 63 and 81%, respectively. Furthermore, SCF showed a marked increase in the proportion of the high-molecular-weight form of mucin[38]. The results indicate that the protective qualities of SCF lie mainly in its ability to enhance the mucus gel viscosity, content of mucin and its proportion in the high-molecular-weight form. Recently it was shown that the use of high concentrations of SCF decreases the production of ROS by leukocytes or by the xanthine-xanthine oxidase system after cell injury induced by H_2O_2, showing that the substance has antioxidant activity[39]. SCF prevented the delay of wound repair in cells damage induced by H_2O_2 probably through induction of COX-2 and an anti-apoptotic mechanism. These effects of SCF might be given through the activation of the NF-kappaB pathway.

When we consider the issues involved in the pathogenesis of DC all of these pharmacological properties suggest that the use of SCF may be an interesting therapeutic strategy for treating DC. Although the application of enemas containing SCF have been used successfully for the treatment of RP, UC and other diseases associate with a formation of ulcers and erosions on colonic epithelium, to the best of our knowledge, only a single experimental study of our group and recently published evaluated the effects of this strategy in an experimental model of DC[14,15,29]. In this study we verified that daily use of enemas with SCF decreased epithelial loss, abscess formation in the colon crypts, inflammatory infiltrate and was able to preserve the population of goblet cells[40]. However, although in this study we showed that the application of enemas containing SCF was able to increase the population of goblet cells, we did not studied modifications on tissue content of neutral and acidic mucins in colonic epithelial glands.

The results of the present study confirm the beneficial effects of the use of enemas with SCF in the prevention of the most frequently found histological alterations in DC. We found that the topical application of SCF in the proposed model reduced of the crypt atrophy, neutrophil infiltration of the mucosa, and epithelial loss mainly when the substance is applied at a higher concentration and for a longer period of time. However, the improvement of the inflammatory score can be obtained when using a higher concentration of the substance and a longer intervention time. It is possible that the results could be even better if we had used a higher concentration of the substance, as well as more frequent interventions (e.g. every 12 h). Despite these aspects our findings were similar to those found by other authors who evaluated the effects of SCF on chemically-induced colitis model[41]. In the animals from the group where intervention was performed with saline, the epithelial surface was irregular, with a

"brush border" aspect, and in some areas we observed the formation of small or larger ulcers (Fig.1A). In all animals treated with SCF, regardless of the concentration used, there was the formation of a gelatinous layer on the colonic mucosa that preserved the epithelial surface, without epithelial ulcerations (Fig. 1B).

Previous studies have shown that there is significant reduction in the content of neutral and acidic mucins in the mucosa devoid of the fecal stream[13]. The authors found that although there is a reduction in both types of mucins the reduction of the tissue content of acidic mucins was more important[13]. Subsequently, the same group studying the tissue content of both subtypes of mucins in an experimental model of DC suggested that the reduction was given, mainly at the expense of the mucins rich in sialic acid (sialomucins), usually found in the deeper regions of colonic[5]. The results of both studies clearly show that the derivation of fecal stream interferes with the synthesis of mucus layer that cover the intestinal mucosa. The absence of this morphofunctional barrier expose the colonic epithelial surface biological and antigens of the in the intestinal lumen, determining the onset of colitis. In this study when we evaluated the content of neutral mucins within the goblet cells throughout the length colonic glands, we verified that intervention with SCF increased the content of neutral mucin when compared to animals treated with saline intervention regardless of concentration used. The contents of neutral mucins not vary with time independent of the concentration used intervention. Similarly, with occurs with tissue content of neutral mucins we found that daily intervention with SCF can increase the tissue content of acid mucins. Other than that was verified with the content of neutral mucins, the increase in tissue content of acidic mucins was directly related to the concentration used. However, the tissue content did not change with the time of intervention. These results suggest that the use of enemas SCF is able to maintain the release of acidic mucin content in goblet cells even after 4 weeks of derivation of de fecal stream. These results showed that there application of SCF enemas maintains production of mucins from goblet cells of colon crypts. It is possible that this effect may be related protective properties of the substance to the colonic epithelium, stimulation of mucin by the cells to increase production of EGF, antibacterial action, and antioxidant effects that the substance presents, reducing the aggression by ROS.

Studies correlating tissue mucins with tissue levels of PGE-2, EGF and ROS are needed to verify if the effects of SCF are related only to increased production of mucus or even the substance can reduce levels of oxidative stress found in transit devoid of intestinal mucosa. We are currently studying in the same group of animals the tissue levels of oxidative stress, PGE-2, EGF, NK-kappaB and COX-2 with immunohistochemical and biochemical techniques to assess this possibility.

Conclusion

The daily application of enemas containing sucralfate, in an experimental model of diversion colitisC, decrease the inflammatory alterations and increases tissue content of neutral and acidic mucins on colonic mucosa. The increase on tissue content of acidic mucin is related to concentration used of sucralfate.

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Received: Apr 15, 2014
Review: Jun 17, 2014
Accepted: July 14, 2014
Conflict of interest: none
Financial source: Sao Paulo Research Foundation (FAPESP). Project N°. 2010/12492-7.

[1]Research performed at Laboratory of Medical Investigation, Medical School, Sao Francisco University (USF), Bragança Paulista-SP, Brazil. Part of Master degree thesis, Postgraduate Program in Health Sciences, USF. Tutor: Carlos Augusto Real Martinez.

9.2.4 Evaluation of sucralfate enema in experimental diversion colitis

J COLOPROCTOL. 2013;33(4):182-190

Journal of Coloproctology

www.jcol.org.br

Original article

Evaluation of sucralfate enema in experimental diversion colitis*

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ARTICLE INFO

Article history:
Received 20 July 2013
Accepted 23 August 2013

Keywords:
Colon
Experimental colitis
Short chain fatty acids
Sucralfate
Rats

ABSTRACT

Diversion colitis (DC) is an inflammatory disease that develops in segments with fecal diversion. Sucralfate (SCF) complex, which consists of sucrose octasulfate and polyaluminum hydroxide, has been demonstrated to be effective in the treatment of different forms of colitis. However, until now, the effects of SCF have not been evaluated in DC.

Objective: to evaluate whether the use of enemas containing SFC improves histological findings in experimental DC.

Methods: Thirty-six rats underwent right colon bypass procedure through the creation of a proximal colostomy and a distal mucous fistula. The animals were divided into two groups according to the euthanization procedure to be performed two to four weeks after surgery. Each experimental group was divided into three subgroups of six animals, which were submitted to daily application of enemas containing saline solution 0.9% or SCF at concentrations of 1.0 g/kg/day or 2.0 g/kg/day, respectively. The diagnosis of DC in segments with fecal diversion was established by histopathological study considering the following variables: epithelial loss, formation of crypt abscesses, the population of goblet cells, inflammatory infiltrate and presence of fibrosis. For statistical analysis, the nonparametric Mann-Whitney and Kruskal-Wallis tests were used, with a significance level of 5% ($p < 0.05$).

Results: It was observed that the daily application of SCF enemas decreased epithelial loss, formation of colon crypt abscesses, inflammatory infiltrate and tissue fibrosis ($p < 0.05$), unrelated to time of intervention. The intervention with SCF preserves the goblet cell population. The effects of the substance on the preservation of colonic epithelium; the decrease in the inflammatory process and subsequent abscess formation in the colon crypts are associated with the concentration used, whereas tissue fibrosis decrease is associated with the concentration and time of intervention.

Conclusion: Preventive application of SCF enemas reduces the inflammatory process in the colon with fecal diversion

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2237-9363/$ - see front matter.
http://dx.doi.org/10.1016/j.jcol.2013.08.005

Avaliação dos efeitos da aplicação de enemas com sucralfato em modelo experimental de colite de exclusão

Palavras-chave:
Cólon
Colite experimental
Ácidos graxos de cadeia curta
Sucralfato
Ratos

RESUMO

A colite de exclusão (CE) é uma doença inflamatória que se desenvolve em segmentos desprovidos de trânsito fecal. O sucralfato (SCF) complexo formado pelo octossulfato de sacarose e hidróxido de polialumínio vem se demonstrando eficaz para o tratamento de diferentes formas de colite, porém, até a presente data, os efeitos do SCF ainda não foram avaliados na CE.

Objetivo: avaliar se a aplicação de clisteres contendo SFC melhora as alterações histológicas encontradas em modelo experimental de CE.

Métodos: trinta e seis ratos foram submetidos à derivação do trânsito no cólon direito pela confecção de colostomia proximal e fístula mucosa distal. Os animais foram divididos em dois grupos experimentais de acordo com o sacrifício ser realizado após duas ou quatro semanas do procedimento cirúrgico. Cada grupo experimental foi dividido em três subgrupos de seis animais segundo terem sidos submetidos à aplicação diária com enemas contendo solução fisiológica a 0,9% ou SCF nas concentrações de 1,0g/kg/dia ou 2,0 g/kg/dia. O diagnóstico de CE nos segmentos sem trânsito foi estabelecido por estudo histopatológico considerando-se as seguintes variáveis: perda epitelial, formação de abscessos nas criptas, população de células caliciformes, infiltrado inflamatório e a presença de fibrose. Para análise estatística adotou-se os testes não paramétricos de Mann-Withney e Kruskal-Wallis estabelecendo-se para ambos, nível de significância de 5% ($p < 0,05$).

Resultados: verificou-se que a aplicação diária de enemas com SCF diminui a perda epitelial, a formação de abscessos nas criptas cólicas, o infiltrado inflamatório e a presença de fibrose tecidual ($p < 0,05$), não relacionada ao tempo de intervenção. A intervenção com SCF preserva a população de células caliciformes. Os efeitos da substância na preservação do epitélio cólico, na redução do processo inflamatório e consequente formação de abscessos nas criptas cólicas encontram-se relacionado à concentração utilizada, enquanto a redução da fibrose tecidual a concentração e ao tempo de intervenção.

Conclusão: a aplicação preventiva de enemas com SCF reduz o processo inflamatório em segmentos cólicos desprovidos de transito intestinal.

Introduction

Sucralfate (SCF) is the salt formed by the association of disaccharide sucrose octasulfate and poly-aluminum hydroxide.[1] This substance is a cytoprotective complex, which was initially used to prevent or treat several diseases of the upper digestive tract, mainly represented by gastroesophageal reflux disease (GERD), gastritis, peptic ulcers, stress ulcers and acute gastric mucosal lesions.[2] Subsequent studies demonstrated that SCF had beneficial effects on skin and mucosal wound healing, and being successfully used for the treatment of lesions found in varicose ulcers, aphthous stomatitis, oral and genital ulcers in Behcet's disease, peristomal dermatitis, burns and post-hemorrhoidectomy or fistulectomy surgical wounds.[3-9]

The therapeutic effects of the topical use of SCF on skin and mucosal lesions are related to the property that the formed complex has to adhere strongly to the raw surface of epithelial ulcers, making it difficult to remove the gelatinous layer that forms on the lesion. The adhesive capacity over the raw surface of the wounds seems to be the main mechanism of action of the drug. However, more recently it was shown that the molecule possesses other properties, such as stimulating the formation of mucus and increasing the production of prostaglandins and epithelial growth factor (EGF).[10] The topical use of SCF has also antioxidant activity and is capable of reducing the formation of oxygen-free radicals (reactive oxygen species or ROS) produced by leukocytes present in the inflamed tissue. This antioxidant action prevents the peroxidation of cell membrane lipids and, therefore, prevents tissue damage.[11]

Kochhar et al. were the first to demonstrate the short-term effectiveness of the application of enemas containing SCF for the control of rectal bleeding due to radiation proctitis.[12] The authors showed that the use of enemas containing a 10% SCF suspension inhibited rectal bleeding in most of the patients with radiation proctitis of moderate or severe intensity. From then on, a sequence of well-conducted studies confirmed the beneficial therapeutic effects of the drug in clinical, endoscopic and histological improvement in patients with radiation proctitis, as well as in other forms of inflammatory bowel disease (IBD) accompanied by the formation of ulcers in the colonic mucosa, such as ulcerative colitis (UC) and solitary rectal ulcer.[12-18]

Glotzer et al. in 1981, described for the first time the development of aphthous ulcers, similar to those found in the UC, in segments of the colonic mucosa with fecal diversion in patients who had no history of IBD.[19] The authors called this new form of IBD "diversion colitis" (DC). It has been shown that the DC appears as a result of the deficiency in the sup-

ply of intraluminal short-chain fatty acids (SCFA) to epithelial cells of the colonic mucosa, caused by bowel bypass. Recent studies suggest that epithelial injury in DC is caused by the increased production of ROS by the epithelial cells of the colonic mucosa itself, along with changes in its metabolism as a result of a deficiency in the supply of its main energy substrate represented by the SCFA.[20-23]

Different therapeutic strategies have been used in the treatment of DC. The restoration of SCFA supply either by reconstitution of the fecal stream or by applying nutritional solutions rich in SCFA is able to ameliorate epithelial lesions. Recent studies have shown that the use of enemas containing substances with antioxidant activity, such as 5-ASA and N-acetylcysteine, as they decrease the levels of oxidative stress in the tissue, they may also improve the histological findings in experimental DC models.[21,24] Although DC is accompanied by the formation of ulcers in the colonic mucosal epithelium , to the best of our knowledge, the effects of SCF have not been evaluated in patients or experimental models of DC. Therefore, the objective of this study is to determine whether the use of SCF enemas is effective in reducing histological alterations found in the colonic mucosa of an experimental model of DC.

Method

This study followed the recommendations of the Federal Law N. 11.794 and the guidelines of the Brazilian College of Animal Experimentation (COBEA). The research project was approved by the Ethics Committee on the Use of Animals in Research of Universidade São Francisco.

Experimental animals

A total 36 male Wistar rats, weighing 300 to 350 g, obtained from the Central Animal Facility of Universidade São Francisco were used in this study. The animals were kept in individual cages in air-conditioned environment with controlled temperature, light, humidity and noise level. On the eve of the surgical intervention, they fasted for 12 hours, except for water which was offered ad libitum. The cages were identified with the number, experimental group and subgroup to which they belonged and that data was tattooed with India ink on the tail of each animal. The rats were always fed the same chow, suitable for rodents, and weighed weekly.

Surgical technique

The fecal diversion in all animals was performed under general anesthesia by intramuscular administration of 0.1 mL/100 g of 1:1 (v / v) solution of ketamine (50 mg/mL) and xylazine (20 mg/mL) in the left hind leg. After being anesthetized and fixed to the operating table, the abdominal cavity was opened by making a median incision, 3 cm long. After Peyer's patch identification, the distance between the patch and the location chosen for the left colon section was measured with a caliper, 4 cm above the upper edge of the patch.
After ligation of the marginal arcade vessels, the colon was sectioned at the chosen point, externalizing the proximal segment, as the end colostomy in the left hypochondrium, fixing the colostomy to the skin with interrupted sutures using absorbable 4-0 monofilament thread in the four cardinal points, and between them. After the fixation of the proximal colostomy, the caudal segment of the left colon was catheterized and irrigated with 40 mL of 0.9% saline solution (SS) at 37°C until the effluent drained through the rectum showed no fecal residues.

After the irrigation, the catheter was removed and the distal colon was exteriorized as colostomy (distal mucous fistula) on the lower left lateral face of the abdominal wall. The distal stoma was fixed with the same technique used in the proximal one. The closure of the abdominal wall was performed in two planes of sutures: peritoneum and aponeurosis with running sutures using 4-0 polyglycolic acid thread and the skin with 4-0 nylon thread.

Experimental groups

Fig. 1 shows the algorithm for the experimental group formation. The 36 animals were randomly divided into three groups with 12 rats in each. The first group received daily enemas containing 0.9% saline solution (control group). The second and third groups (experimental groups) received daily enemas containing SCF (EMS do Brasil Ltda., São Paulo, Brazil) at two different concentrations (1.0 g/kg and 2.0 mg/kg, respectively). In each group, six animals were sacrificed two weeks and the other six four weeks after the intervention.

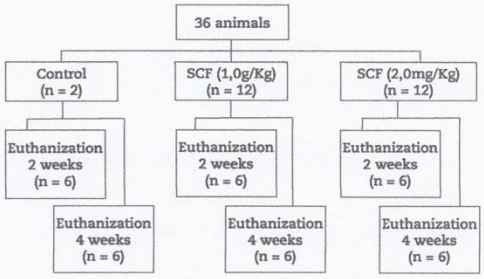

Fig. 1 - Algorithm of the experimental groups formation.

Sample collection

Two to four weeks after the intervention with the proposed substances, the animals were anesthetized using the same technique described before. The abdominal cavity was opened again, removing two 4-cm fragments from the colon submitted or not to irrigation with the solutions at the proposed concentrations. The removed segments were opened longitudinally from the antimesenteric border, washed with saline solution and divided into two 2-cm long fragments, used for the histological analysis.

Histological analysis

The fragments removed for histological analysis were submerged in a 10% formaldehyde buffered solution (Sigma, St. Louis, MO, USA) for 24 hours, dehydrated by exposure to increasing concentrations of ethanol and embedded in paraffin. From each paraffin block, two 5-mm thick histological sections were obtained for slide mounting. Once assembled, they were cleared, hydrated and stained by hematoxylin-eosin (HE) for the diagnosis of colitis and by trichrome Masson (TM) for the assessment of total collagen content (fibrosis).

Slide analysis was performed under a light microscope (Eclipse DS-50, Nikon Inc., Osaka, Japan) by an experienced pathologist in IBD, blinded to the source of the material and the purpose of the study. Histological photographs were taken using a digital videosystem (DS-Fi-50, Nikon Inc., Osaka, Japan) previously attached to the microscope. The specimen analyses were always performed with a 200x final magnification. The analysis of slides in the irrigated segments was performed in sites where there were at least three contiguous intact colon crypts.

The following histological parameters were considered for the diagnosis of DC: loss of epithelial surface (epithelial ulcerations), colon crypt abscesses, inflammatory infiltrate intensity and presence of epithelial fibrosis. The variables epithelial loss, presence of colon crypt abscesses and inflammatory infiltrate were stratified as crosses, according to the degree of each, as follows: a) absent when there were no alterations; b) + when intensity was mild; c) + + moderate d) + + + intense. The intensity of tissue fibrosis was evaluated by the total collagen content, quantified by computerized morphometry and stratified according to the percentage found per histological field studied, considering: 0, no fibrosis was identified; 1 when the content was ≥ 1% and ≤ 5% ; 2 when the content was > 5% and ≤ 10 % and finally 3 when the content was > 10%. For all variables analyzed, the final value considered for each animal was the mean value after quantification of three distinct histological fields.

The morphometric analysis of collagen content was performed using the imaging analysis program NIS-Elements® (Nikon Inc., Japan), release 3.0. The program used RGB (red, green, blue) system color histograms and determined the intensity of the chosen color (in this case blue, the color in which collagen is expressed when the slides are stained with TM) in number of pixels per selected field, transforming the final collagen content into percentage per field (%/field). The final value considered for each animal was the mean value obtained after reading three histological fields at the established magnification (200x).

Statistical analysis

The data was described according to the median with its respective standard error. The comparison between groups was assessed by the median test and analysis of variance with Kruskal-Wallis test. A significance level of 5% ($p < 0.05$) was used for the statistical analysis of the results, using the computer program Biostat® release 5.0.

Results

Fig. 2A shows the segment obtained from colon irrigated with 0.9% saline for four weeks, while Fig. 2B shows the colon irrigated with SCF at a concentration of 2.0 g/kg/day at the same period of time. It can be observed that in animals submitted to intervention with 0.9% SS there is clear epithelial loss, increased goblet cell population, disarray of the architecture and alignment of colic glands. In animals submitted to intervention with SCF 2.0 g/kg/day, the epithelial surface is preserved and the intestinal crypts are aligned, with normal distribution pattern and preservation of the goblet cell population

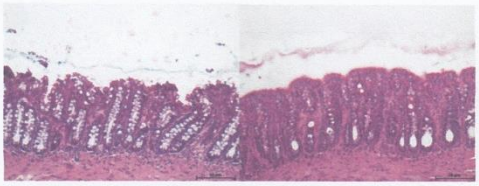

Fig. 2 – A, Colon segment with fecal diversion submitted to intervention with 0.9% saline solution for four weeks, where we observe the formation of epithelial ulceration, increase in the population of goblet cells and disarray in the architecture of colic glands (HE × 200). B, Colon segment with fecal diversion submitted to therapy with SCF (2.0 g / kg / day) for four weeks, where we can observe the integrity of the colonic mucosal epithelium, maintenance of the goblet cell population and the cytoarchitecture of colic glands (HE 200 ×).

Fig. 3A shows the segment obtained from the colon irrigated with 0.9% saline solution for four weeks, while Fig. 3B shows the colon irrigated with SCF at a concentration of 2.0 g/kg/day. In animals submitted to intervention with 0.9% saline solution, there is mucus accumulation inside the goblet cells, which have replaced part of the absorptive cells of the epithelial surface. One can identify the superficial epithelial ulcers in animals that underwent the intervention with SS. One can verify, through the tissue content of total collagen identified by TM, that animals submitted to intervention with 0.9% SS have more fibrosis in the mucosal and submucosal layers when compared to animals submitted to intervention with SCF.

Fig. 4 shows the values of scores found when analyzing the epithelial loss of colonic mucosa in the segments with fecal diversion of animals irrigated with 0.9% SS, SCF 1.0 g/kg/day and 2.0 g/kg/day. It was observed that irrigation of the colon with fecal diversion with SCF at a concentration of 2.0 g/kg/day preserved the mucosal epithelium, preventing the formation of epithelial ulcerations when compared

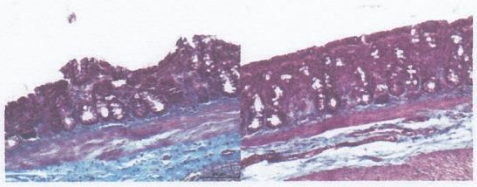

Fig. 3 – A, Colon segment with fecal diversion submitted to therapy with 0.9% saline solution for four weeks, where we observe the intense deposition of collagen in the mucosal and submucosal layers of the colon wall (TM 200 ×). B, Colon segment with fecal diversion submitted to therapy with SCF (2.0 g / kg / day) for four weeks, which shows that there was less collagen deposition when compared to animals irrigated with 0.9% saline solution (TM 200×).

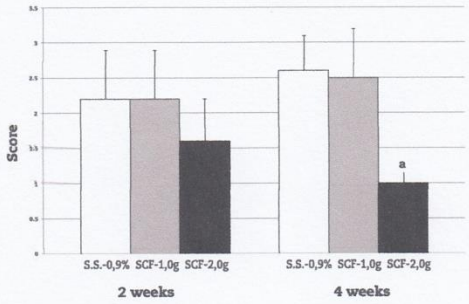

Fig. 4 – Epithelial loss comparing animals submitted to daily irrigation with 0.9% saline solution (SS), sucralfate 1.0 g / kg and 2.0 g / kg after two and four weeks of intervention. Mann-Whitney test. [a] = $p < 0.05$ (SCF 2.0 g / kg vs. 0.9% saline solution).

to animals irrigated with 0.9% SS (p = 0.03). The mucosal epithelium of the animals irrigated with SCF was regular and had a smaller population of goblet cells. In animals irrigated with SCF it was observed an increased amount of mucus covering the epithelial surface, when compared to control animals. When assessing the level of epithelial loss with increasing duration of intervention, although it is related to the concentration used, it was observed that it did not worsen with increasing duration of intervention time, regardless of the concentration of SCF used.

Fig. 5 shows the scores obtained by analyzing the presence of crypt abscesses in the segments irrigated with 0.9% SS, SCF 1.0 g/kg/day and 2.0 g/kg/day. The results show that the intervention in the colon with fecal diversion using a SCF concentration of 2.0 g/kg/day reduced the formation of abscesses after four weeks of irrigation, when compared to animals irrigated with 0.9% SS (p = 0.02) and those irrigated with SCF at a concentration of 1.0 g/kg/day (p = 0.02). After four weeks of intervention, the reduction in abscess formation in the intestinal crypts was related to the SCF concentration used. Similarly to what occurred in relation to epithelial loss, the formation of colon crypt abscesses, even though related to the SCF concentration used, did not change with increasing time of intervention regardless of the SCF concentration used.

Fig. 6 shows the scores obtained when analyzing the inflammatory infiltrate in the mucosal and submucosal layers of the colon irrigated with 0.9% SS, SCF 1.0 g/kg/day and 2.0 g/kg/day for two and four weeks. The results showed that the intervention on the colon with fecal diversion with SCF at a concentration of 2.0 g/kg/day was able to decrease the inflammatory infiltrate only after four weeks of irrigation, when compared to animals irrigated with 0.9% SS (p = 0.03). There was no worsening in the inflammatory infiltrate in the course of the intervention time, regardless of the SCF concentration used (p = 0.18).

Fig. 7 shows the contents of tissue collagen found in the mucosal and submucosal layers of the colon wall in segments irrigated with 0.9% SS, SCF 1.0 g/kg/day and 2.0 g/kg/day for two and four weeks of intervention. It was observed that irrigation of the colon with fecal diversion with SCF at a concentration of 2.0 g / kg / day for four weeks was able to reduce the total tissue collagen content when compared to animals treated with daily irrigation with SS 0.9% and SCF at a concentration of 1.0 g/kg (p = 0.009). The presence of fibrosis, albeit reduced with higher concentrations of the drug, did not change with increasing duration of intervention time, regardless of the SCF concentration used.

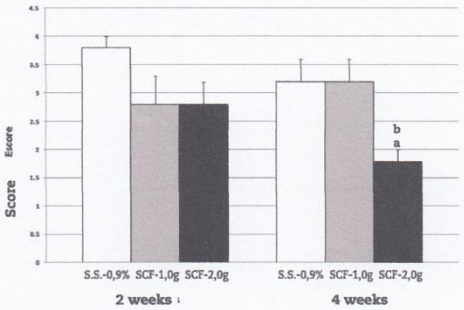

Fig. 5 – Presence of abscesses in crypts comparing with animals treated with daily irrigation with 0.9% saline solution (SS), 1.0 g SCF / kg and 2.0 g / kg after two and four weeks of intervention. Mann-Whitney Test. [a] e [b] = $p < 0,05$ ([a] = SCF 2.0 g/kg vs. SS 0.95; [b] = SCF 2.0 g/kg vs. SCF 1.0 g/kg).

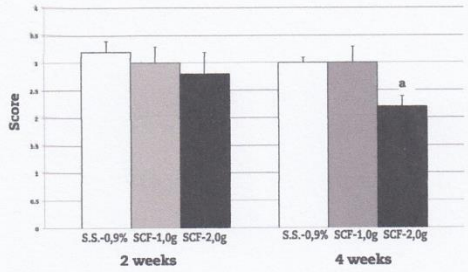

Fig. 6 – Inflammatory infiltrate in animals subjected to daily irrigation with 0.9% SS, SCF 1.0 g/kg and 2.0 g/kg after two and four weeks of intervention. Mann-Whitney Test. [a] = $p < 0.05$ ([a] = SCF 2.0 g/kg vs. SS 0.95).

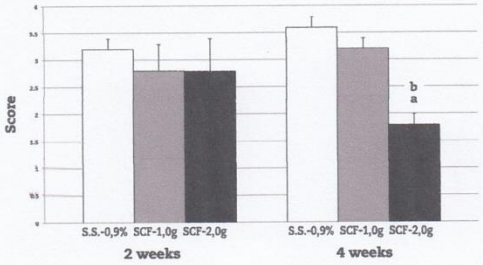

Fig. 7 – Content of tissue collagen in animals submitted to daily irrigation with 0.9% SS, SCF 1.0 g / kg and 2.0 g / kg after two and four weeks of intervention. Mann-Whitney Test. [a] e [b] = $p < 0.01$ ([a] = SCF 2.0 g/kg vs. SS 0.95; [b] = SCF 2.0 g/kg vs. SCF 1.0 g/kg).

Discussion

DC is an inflammatory disease that affects the large intestine segments with fecal diversion.[25] Endoscopic examination of the colon or rectum with fecal diversion shows endoscopic alterations that are characteristic of the disease in all patients after a period of time ranging from three to 36 months the diversion.[25] The endoscopic appearance of the intestinal mucosa may vary depending on the intensity and duration of the disease and common findings are: the absence of the vascular pattern of the submucosal layer due to the inflammatory infiltrate, frailty in the excluded mucosa caused by greater vascular congestion, formation of superficial ulcerations that determine spontaneous bleeding or at minimal local trauma and stiffness of the intestinal wall by greater deposition of collagen, leading to fibrosis of the colon wall.[26]

In more severe cases there may be formation of larger aphthous ulcers, making it difficult to make the differential diagnosis with other forms of IBD, particularly URC.[27] The presence of ulcerations on the mucosal surface and the intense inflammatory process are the most significant alterations, as they are directly related to the main symptoms of the disease - presence of blood and mucus in stools - reported by most patients. Thus, when establishing therapeutic strategies for the treatment of DC, these aspects should be considered.

The physiopathological bases for the development of DC are not yet fully understood.[28] However, most authors believe that the etiopathogenesis of DC is related to a deficiency in the supply of SCFA to the colonic mucosa.[29] This possibility is supported by the results of studies showing that the deficiency in the supply of SCFA to the cells of the colonic mucosa is related to the development of DC, while the restoration of fecal stream or irrigation of the excluded segments with SCFA improves symptoms and reverts endoscopic and histological changes found in the disease.[30]

Despite the important role played by the maintenance of SCFA supply to prevent the development of DC, the molecular mechanisms that cause epithelial lesions have been understood only recently.[20] Experimental studies have shown that epithelial lesion found in DC models are related to tissue oxidative stress due to an increased production of ROS by the colonic mucosa, a site known to be deficient in antioxidant enzyme systems.[31,32] It is well established that ROS such as superoxide ($O_2^{\cdot}$), hydroxyl (OH), hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO) are produced in excess by the colonic mucosa devoid of fecal stream and are harmful to it.[33] ROS are capable of damaging the different defense systems of the colonic mucosa that prevent the migration of antigens and bacteria present in the intestinal lumen to the proximity of the sterile layers of the colon wall.[20,22,23,25] The possibility that deficiencies in the supply of SCFA may trigger the onset of DC gained more support after studies demonstrated that substances that inhibit β-oxidation of SCFA in the intestinal lumen are able to trigger the onset of DC, whereas the use of enemas in the colon without fecal stream with antioxidants, such as 5-ASA and n-acetylcysteine have been successfully used for the treatment of the disease.[21,24,34]

SCF is a cytoprotective agent that has been used for more than three decades in the treatment of duodenal peptic ulcers, stress ulcers and GERD. The substance is a sucrose and sulfate-aluminum complex which, when in contact with hydrochloric acid in the stomach, forms a viscous gel that adheres to the gastric mucosa creating a physical barrier that protects the mucosa and prevents the diffusion of hydrochloric acid into the gastric wall. The complex formed, in addition to preventing the degradation of the mucus that covers the gastrointestinal epithelium, stimulates the production of bicarbonate, acting as a buffer with cytoprotective properties. When in contact with the raw surface of epithelial lesions of the digestive tract mucosa, SCF adheres tightly to proteins on the surface of ulcerations, mainly albumin and fibrinogen, thus forming a stable and insoluble complex, creating a protective layer that covers and protects the ulceration. Recent studies have shown that SCF stimulates the production of prostaglandin E2 (PGE2), epithelial growth factor (EGF) and gastric mucus.[10,35]

It has been previously shown that the PGE-2 is the primary product of arachidonic acid metabolism, playing a critical role in maintaining the integrity of the gastrointestinal epitheli-

um.[36] The increase in PGE-2 production, the main metabolite of COX-1 and COX-2, can regulate the angiogenesis, motility and survival of epithelial and endothelial cells. A number of studies have shown that SCF increases the production of PGE-2 and prostaglandin-F1 by cells and that this effect is dose-dependent.[37]

Louw et al. have shown that SCF also significantly increases the production of TGF-α and that the combination of SCF and TGF-α is able to induce the proliferation of mucosal cells and increase local blood supply, favoring the healing process.[38]

The inflammatory aggression that develops after tissue damage is often involved in the induction of cell apoptosis, which is considered the main reason for decreased cellularity during the different stages of wound healing. SCF can inhibit cell apoptosis after tissue damage. Matsuu-Matsuyama et al. demonstrated that SCF protects the distal colonic epithelium of rats submitted to radiotherapy, by reducing the level of cell apoptosis by inhibiting the activation of caspase-3 and that this phenomenon may be dependent on p53 protein pathway due to its capacity to decrease, along with p21 protein expression, the Bax/Bcl2 ratio in the colon cells.[14]

With better knowledge of SCF mechanisms of action, it was observed that in addition to working as a mechanical barrier, it is also able to preserve vascular integrity, increase mucus secretion and EGF production, responsible for stimulating epithelial regeneration, angiogenesis and epithelialization, considered the main phase of cutaneous-mucous wound healing. The formation of a stable complex, firmly adhered to ulcers of the digestive tract, maintains EGF production for a long period of time, by exerting a continuous trophic stimulation on the gastrointestinal mucosa. This property protects the lining of the digestive tract from new aggressions, as well as stimulates the migration and proliferation of cells from germinal regions of the intestinal crypts.[39]

Studies have shown that SCF also has a bactericidal effect, effective against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It has been shown that reducing the bacterial population surgical wounds located in the intestinal lumen is an important aspect of the healing process.[40] Recently it was shown that the use of high concentrations of SCF decreases the production of ROS by leukocytes or by the xanthine-xanthine oxidase system after cell injury induced by H_2O_2, showing that the substance has antioxidant activity.[10]

Fig. 8 shows a summarized version of the known mechanisms of action of how the SCF molecule acts at different stages of the healing process in surgical wounds.

All these SCF properties combined with the etiopathogenic mechanisms of DC make interesting the assessment of the topical effects of the substance in experimental models of DC. However, as far as we know, this possibility has not yet been studied. The results of the present study confirm the beneficial effects of the use of enemas with SCF in the prevention of the most frequently found histological alterations in DC. We found that the topical application of SCF in the proposed model reduced epithelial loss, mainly when the substance is applied at a higher concentration and for a longer period of time.

In the animals from the group where intervention was performed with 0.9% SS, the epithelial surface was irregular, with a "brush border" aspect, and in some areas we observed

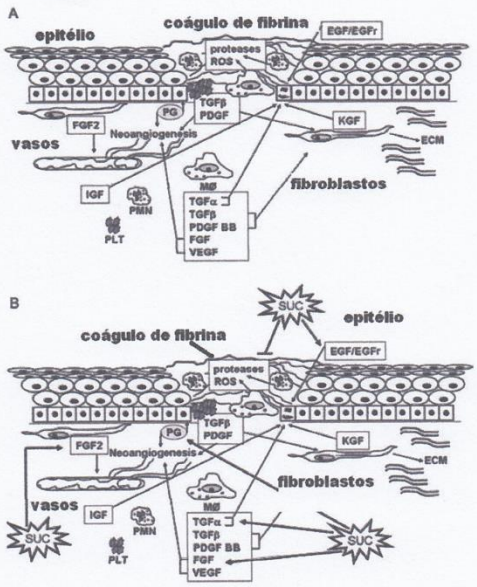

Fig. 8 – Molecular mechanisms of action of SCF on epithelial healing. A, Release of growth factors involved in epithelial wound healing. B, SCF increases the bioavailability of growth factors and prostaglandins and decreases the production of ROS, enhancing angiogenesis, granulation and tissue re-epithelialization. EGF, epidermal growth factor; EGFr epidermal growth factor receptor; ROS, reactive oxygen species; ECM , extracellular matrix; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β; KGF, keratinocyte growth factor ; FGF2 , fibroblast growth factor 2 ; TGF-α , transforming growth factor α ; PDGF BB , platelet-derived growth factor , FGF , fibroblast growth factor; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; PG , prostaglandin ; PLT , platelets, PMN, polymorphonuclear leukocytes; Mø, macrophages, SUC: sucralfate. (Modified from Masuelli et al. , 2010[10].)

the formation of small (Fig. 2A) or larger ulcers (Fig. 3A). In all animals treated with SCF, regardless of the concentration used, there was the formation of a gelatinous layer on the colonic mucosa that preserved the epithelial surface, without epithelial ulcerations (Figs. 2B and 3B).

These findings seem to confirm the substance's cytoprotective and regenerative properties of the mucosal epithelium. Likewise, it was observed that in animals submitted to intervention with SCF, there was a greater amount of mucus covering the epithelial surface and that the goblet cells did not have their cytoplasm filled with mucus as happened in animals submitted to irrigation with 0.9% SS (Fig. 2A, B).

The epithelial surface integrity in animals submitted to intervention with SCF may also be related to the capacity of the substance to increase the local levels of PGE-2, TGF-α, EGF and FGF, all of which, besides exerting a trophic effect on the intestinal epithelium, accelerate the healing process by stimulating cell proliferation and migration.[11,36] The improvement in epithelial regeneration in the present study showed to be dose-dependent. One possible explanation for this fact is the greater capacity of mechanical protection when using a higher concentration as well as the increased production of PGE-2, TGF-α, EGF and FGF, which is also dose-dependent.[36]

The animals submitted to irrigation with higher SCF concentrations and for a longer period of time had lower rates of abscess formation in crypts and less inflammatory infiltrate (Fig. 4). It is possible that this finding may be related to agreater protection of the epithelial surface provided by the gelatinous layer that forms on the intestinal mucosa, as well as the increased amount of mucus on the site.

The formation of this additional protective layer can hinder the migration of antigens and bacteria from the intestinal lumen into the sterile layers of the colon wall, decreasing the local inflammatory response. The lower inflammatory infiltrate, as well as the lower formation of cryptic abscesses may also be related to the bactericidal activities of the substance. It must be also recalled that SCF has an antioxidant activity, neutralizing ROS formed by epithelial cells with energy metabolism altered by a deficiency in the supply of SCFA due to the absence of fecal stream. The neutralization of these ROS can decrease levels of oxidative stress on site, making it difficult for these radicals to attack the epithelial surface cells. At the moment we are measuring in these same animals, the tissue levels of ROS by assessing lipid peroxidation of membranes (tissue levels of malondialdehyde) and of oxidative stress to cell DNA (8-OHdG by immunohistochemistry), in order to confirm the importance of the antioxidant activity of SCF in the prevention of oxidative tissue damage found in DC .

When analyzing the presence of tissue fibrosis comparing animals preventively irrigated with SCF or 0.9% SS, we found that the use of enemas with SCF at higher concentrations significantly reduced the content of collagen tissue, suggesting the presence of less local fibrosis. It is possible that this effect is related to a lower local inflammatory process resulting from mechanical protection given by the substance by reducing bacterial infiltration and inflammation resulting from antioxidant and antibacterial activities. By modulating the production and release of pro-inflammatory cytokines, stimulating the production of PGE-2 and EGF, it is possible that collagen deposition, as well as epithelial replacement, are carried out in a more harmonic way when compared to the animals irrigated with 0.9% SS.

As described by other authors, the protective layer on the epithelium formed by the SCF causes the release of EGF and FGF on site for a longer period of time which improves the healing of epithelial injury.[2,11,41] The increased activity of FGF seems to be a major mechanism of action of SCF.[11,42] FGFs are a class of heparin-binding proteins, mainly represented by basic FGF (bFGF) and acidic FGF (FGF), which stimulate mitogenic, chemotactic and angiogenesis activity in many cell types, including mesenchymal and neural epithelial cells.[11] Because of their myogenic activity on endothelial cells, chondrocytes and fibroblasts, FGFs play a key role in all stages of wound healing. It is possible that greater stimulation to EGF and FGF production can modulate tissue epithelialization, decreasing collagen deposition as it occurs in animals irrigated with 0.9% SS

The results of this study suggest that the properties of the SCF molecule of improving the mechanical barrier function and accelerating wound healing by stimulating the production of growth factors, especially FGF, can improve the healing of the colonic mucosa without fecal stream. Moreover, the induction of prostaglandin production, as well as protection against apoptosis, promoting tissue re-epithelialization, can improve the healing process of the colonic mucosa. These biological properties have encouraged the clinical use of the substance as a topical agent for the treatment of different types of colitis caused by inflammation, infection and physical damage, such as in URC and actinic rectitis. According to the clinical evidence reported in the literature, it seems that SCF promotes healing of the intestinal mucosal epithelium in these patients.

The results of this study suggest that for all its properties, topical application of SCF can also be a valid strategy for the prevention and treatment of DC. However, clinical studies in humans, with a significant number of cases are still necessary to validate the experimental results found in this study.

Conclusion

Considering the conditions of this study, topical application of enemas containing SCF improves epithelial changes found in experimental DC.

Funding

Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Process N. 2010/12492-7.

Conflicts of interest

The authors declare no conflicts of interest.

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9.2.5 Evaluation of the anti-inflammatory and antioxidante effects of the sucralfate in diversion colitis

J COLOPROCTOL (RIO J). 2015;35(2):90–99

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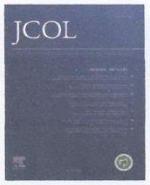

Original Article

Evaluation of the anti-inflammatory and antioxidant effects of the sucralfate in diversion colitis☆

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ARTICLE INFO

Article history:
Received 8 December 2014
Accepted 20 February 2015
Available online 29 April 2015

Keywords:
Sucralfate
Myeloperoxidase
Malondialdehyde
Lipid peroxidation
Oxidative stress
Short-chain fatty acids
Rats

ABSTRACT

Sucralfate enemas present good results in the treatment of colitis, however the mechanism of action of the drug is not yet fully clarified.

Objective: To evaluate the anti-inflammatory and antioxidant effects of sucralfate enemas in diversion colitis model.

Method: Thirty-six Wistar rats underwent intestinal bypass by end colostomy in the descending colon and distal mucous fistula. The animals were divided into 3 experimental groups according to the daily dose of enemas received containing 0.9% SF, sucralfate enemas or sucralfate enemas 1 g/kg/day or 2 g/kg/day. Each group was divided into two subgroups according to euthanasia to be performed 2–4 weeks after derivation. The tissue grade of inflammation was assessed histologically, and neutrophil infiltration by the tissue expression of myeloperoxidase (MPO) identified by immunohistochemistry and quantified by computerized morphometry. Oxidative stress was measured by tissue levels of malondialdehyde (MDA). To compare the results the Student's *t* test variance was used, and also the variance by ANOVA test, establishing a level of significance of 5% ($p < 0.05$) for both.

Results: The intervention with sucralfate enemas showed improvement in the intensity of tissue inflammation related to the concentration used and the duration of the intervention. Intervention with sucralfate enemas reduced the tissue levels of MPO, independent of concentration or time of intervention ($p < 0.01$). There was a reduction of MDA levels in animals irrigated with sucralfate enemas, independent of concentration or duration of the intervention ($p < 0.01$).

☆ Study conducted at the Medical Research Laboratory, Graduate Program in Health Sciences, Universidade São Francisco, Bragança Paulista, SP, Brazil. Study awarded with the prize Journal of Coloproctology during the 63rd. Brazilian Congress of Coloproctology Congress (Brasilia (DF), 2014).

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http://dx.doi.org/10.1016/j.jcol.2015.02.007

Conclusion: Enemas with sucralfate enemas reduce inflammation, neutrophil infiltration and oxidative stress in the excluded colon suggesting topical application of the substance to be a valid therapeutic option for the treatment of diversion colitis.

Avaliação dos efeitos anti-inflamatório e antioxidante do sucralfato na colite de exclusão

Palavras-chave:
Sucralfato
Mieloperoxidase
Malondialdeído
Peroxidação dos lípidos
Estresse Oxidativo
Ácidos graxos de cadeia curta (AGCC)
Ratos

RESUMEN

A aplicação de clisteres com sucralfato (SCF) apresenta bons resultados no tratamento de colites, entretanto seu mecanismo de ação ainda não encontra-se esclarecido.

Objetivo: Avaliar os efeitos anti-inflamatórios e antioxidantes do SCF em modelo de colite de exclusão.

Método: Trinta e seis ratos, foram submetidos a derivação intestinal por colostomia terminal no cólon descendente e fistula mucosa distal. Os animais foram divididos em 3 grupos experimentais segundo receberem clisteres diários com SF 0,9%, SCF 1 g/kg/dia ou SCF 2 g/kg/dia. Cada grupo foi dividido em dois subgrupos segundo a eutanásia ser realizada após 2 ou 4 semanas da derivação. O grau de inflamação tecidual foi avaliado por estudo histológico e a infiltração neutrofílica pela expressão tecidual de mieloperoxidase (MPO) identificada por imunoistoquímica e quantificada por morfometria computadorizada. O estresse oxidativo foi mensurado pelo conteúdo de malondialdeído (MDA). Para análise dos resultados utilizou-se os teste t de Student, e ANOVA, estabelecendo-se para todos os testes nível de significância de 5% ($p < 0,05$).

Resultados: A intervenção com SCF melhorou o grau de inflamação tecidual relacionando-se a concentração utilizada e ao tempo de intervenção. A intervenção com SCF reduziu os níveis teciduais de MPO, independente da concentração ou do tempo de intervenção ($p < 0,01$). Houve redução dos níveis de MDA nos animais irrigados com SCF, independente da concentração ou tempo de intervenção ($p < 0,01$).

Conclusão: Enemas com SCF reduzem o processo inflamatório, infiltrado neutrofílico e estresse oxidativo no cólon excluso sugerindo que a substância possa se tornar uma opção terapêutica válida para o tratamento da colite de exclusão.

Introduction

Sucralfate (SCF) is formed by the association between sucrose octosulphate and polyaluminum hydroxide.[1] For more than three decades SCF has been used as a cytoprotective agent for treatment of gastrointestinal ulcer diseases.[2] Studies have shown that the therapeutic effects of SCF appear to be related to its ability to adhere to erosions and ulcerations in gastrointestinal mucosa, forming a difficult-to-remove mechanical barrier.[2] However, it was demonstrated later that SCF also presents other mechanisms of action.[3,4] This drug stimulates the secretion of prostaglandin E2 (PGE2), thus increasing the production and secretion of mucus by goblet cells of the gastrointestinal epithelium.[2] SCF enhances the production of epidermal growth factor (EGF), which induces cell division and promotes tissue reepithelialization.[3] SCF has antimicrobial activity, acting against the pathogenic bacterial flora present in the colonic lumen.[3] It has been shown also that the SCF molecule has remarkable antioxidant activity, reducing the production and removing oxygen free radicals (OFR) present in inflamed tissues.[4] This antioxidant action protects the epithelial cells of gastrointestinal mucosa against peroxidation of phospholipids, the main constituents of cytoplasmic membranes, thus reducing apoptosis.[3–5] All of these properties have led several authors to use SCF for treatment of colorectal inflammatory diseases.[2,6–11] The results of these studies confirm that the use of SCF enemas was effective in healing ulcers of rectal mucosa, as those found, for example, in actinic rectitis, ulcerative colitis, solitary ulcer of the rectum and, more recently, diversion colitis (DC).[2,6–11]

DC is an inflammatory bowel disease (IBD) that has its onset in colon segments excluded from intestinal transit.[12] It has been shown that epithelial cells of transit-excluded segments, devoid of their primary energy supply, represented by short-chain fatty acids (SCFA), undergo changes in their respiratory metabolism – increasing, as a result, the formation of OFR.[13,14] The resulting oxidative stress causes breakdown of those various defense systems that form the epithelial protective barrier.[15–17] The rupture of these defense mechanisms enables the invasion of sterile layers of the intestinal wall by bacteria from bowel lumen, triggering the inflammatory

response.[13,14] In this process, the intense neutrophil migration further increases OFR production, perpetuating the epithelial aggression that characterizes DC.[13]

When one considers that the oxidative stress resulting from an increased tissue production of OFR by mucous cells devoid of fecal transit, as well as neutrophil infiltration, are mechanisms involved in DC etiopathogenesis, it will be of interest to evaluate the antioxidant activity of SCF in an experimental model of DC. If SCF is able to enhance the inflammatory process at mucosal level, reduce neutrophil infiltration and diminish OFR production, this drug could become a valuable alternative for treatment of DC. The aim of this study was to evaluate antioxidant and anti-inflammatory effects of a topical application of SCF in an experimental model of DC.

Method

This study followed the recommendations of the Federal Law 11,794 and of the Brazilian College of Animal Experimentation (COBEA). The research project was approved by the Ethics Committee on Animal Use in Research (CEUA), Universidade de São Francisco (Opinion N° 22-11/2007).

Experimental animals

For the present study, 36 male Wistar rats weighing 300–350 g, provided by the Central Animal Facility, Universidade de São Francisco, were used. The animals were kept in individual cages under controlled conditions of temperature, humidity, light and noise. Prior to the surgical procedure, all animals were fasted, but with water, for 12 h. The cages were identified with the number of the rat and the experimental group to which it belonged. These same data were tattooed on the tail of each rat.

Surgical technique

The derivation of the intestinal transit was performed in all animals under general anesthesia. On the day of surgery, the animals were weighed to calculate the anesthetic dose. A 1:1 solution containing xylazine 2% (Anasedan, Agribrands do Brasil Ltda., São Paulo, Brazil) and ketamine hydrochloride (Dopalen, Agribrands do Brasil Ltda., São Paulo, Brazil.) in a dose of 0.1 mL/100 g intramuscularly in the left hind paw was used as anesthetic vehicle. Once anesthetized, the animals were fixed on the operating table and the entire anterior abdomen was shaved. Then antisepsis with PVP (povidone iodine, 10% topical solution) was carried out. The abdominal cavity was accessed via a 3-cm length midline incision. After examination of the cavity, the Peyer's patch, a lymphoid structure located on the anterior face of the colon, in the transition between the animal's rectum and sigmoid colon, was identified. With the aid of a caliper, the left colon was sectioned at a standard distance 4 cm above the upper limit of the Peyer's patch. The colon's proximal segment was externalized in the form of a terminal colostomy on the left flank, and fixed to the skin with separate points of 4.0 monofilament absorbable suture applied in four cardinal points and between these points. After proximal stoma maturation, the caudal segment

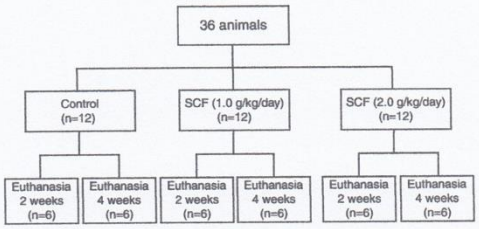

Fig. 1 – Distribution of experimental groups.

of the sectioned colon was catheterized with a 12F polyethylene tube and subjected to irrigation with 40 mL of 0.9% saline solution at 37 °C until the whole effluent drained by the animal's anus (previously dilated) no longer displayed any fecal waste output. After this mechanical cleaning, the catheter was removed and the distal colon exteriorized through the abdominal wall as a mucous fistula in the lower left face of the abdominal wall. The caudal colostomy was fixed to the skin with the same technique described for the cranial stoma. After stoma fixation, the abdominal wall was closed in two surgical plans: peritoneum and aponeurosis with 3–0 braided polyglycolic acid suture, and the skin with separate points of 4–0 monofilament nylon. Postoperatively, the animals did not receive antibiotics, and no further care regarding surgical incision and the stomata was taken.

Experimental groups

Fig. 1 shows the distribution of our experimental groups. Our 36 animals were divided randomly into three groups of 12 rats each. In the first group, the animals were subjected to an application of enemas with 0.9% saline warmed to room temperature (control group). The second and the third groups of animals (experimental groups) received daily applications of enemas containing SCF (EMS do Brasil Ltda., São Paulo, Brazil) in two different concentrations (1.0 g/kg/day and 2.0 g/kg/day, respectively). In all animals the application of intervention solutions was carried out with the aid of an infusion pump (KD Scientific Inc., Holliston, MA, USA) at a controlled infusion rate of 20 mL/min. In each of the three experimental groups, six animals were sacrificed after two weeks, and the other six after four weeks.

Sample collection

In the scheduled dates for euthanasia, animals were anesthetized with the same technique described above. The abdomen was reopened through a midline incision with greater length. The excluded colon (subjected to the intervention solutions), including the anus of all animals, was carefully removed. The removed colon segments were opened through their anti-mesenteric border and rinsed with PBS solution heated at 37 °C for 2 min. A fragment with 3 cm in length (excluding the anus and the region closest to the stoma) of each animal was fixed with the mucosa facing up on a piece of cork. The fragments fastened on cork were stretched with

the aid of pins. After this fastening procedure, the material was placed into vials containing 10% buffered formaldehyde (Sigma, St. Louis, MO, USA). During removal of the excluded colon, a second fragment measuring 1 cm was also removed, washed with PBS solution at 37 °C, packaged in cryoflasks and stored under ultra-cooling conditions, for further biochemical analysis of tissue levels of malondialdehyde (MDA).

Histological analysis

The fragments designated for histological study were kept in 10% formaldehyde for 48 h at room temperature to ensure proper specimen fixation. Then, the specimens were dehydrated by exposure to increasing concentrations of ethanol and embedded in paraffin. From each block, two 5 μm-thick fragments were cut with the aid of a manual microtome (Leica RM 2235, Leica do Brasil Importação e Comércio Ltda., São Paulo, Brazil), for slide mounting. One slide was stained by hematoxylin–eosin (HE) technique and sent for histopathological evaluation for the presence of colitis, as well as for the degree of tissue inflammation. The second slide was intended for immunohistochemistry, to detect the tissue expression of myeloperoxidase (MPO). All slides were analyzed with an ordinary optical microscope (Eclipse DS-50, Nikon Inc., Osaka, Japan) by a pathologist specialized in IBD diagnosis and blinded for the origin of the material and the study objectives. Histological photographs were taken using a digital video camera (DS-Fi-50, Nikon Inc., Osaka, Japan) previously attached to the microscope body. All specimens analyzed were photographed with a final magnification of 100×. The reading of each slide was always done in a histological field showing at least three intact and contiguous colonic glands. For each slide, three distinct histological fields were evaluated. The diagnosis of colitis and the degree of tissue inflammation were determined by histological (modified) criteria previously described by Akgun et al.[18] (Table 1). The following stratification for histological degree of tissue inflammation was adopted: 0–3, mild; 4–6, moderate; and 7–9, severe.

Table 1 – Variables used for stratification of the histological degree of tissue inflammation.

Findings	E score	Criterion
Epithelial loss	0	No epithelial loss in mucosa
	1	Loss of <5% of epithelial surface
	2	Loss between 5 and 10% of epithelial surface
	3	Loss >10% of epithelial surface
Crypt integrity	0	Intact crypts
	1	Loss <10% of crypts
	2	Loss between 10 and 20% of crypts
	3	Loss >20% of crypts
Inflammatory infiltrate	0	Absent
	1	Mild
	2	Moderate
	3	Severe

Modified from Akgun et al.[18]

Immunohistochemistry for study of tissue levels of MPO

For the immunohistochemical study, all blocks were sectioned in 5 μm-thick sections obtained from colon segments treated with the intervention solutions. These cuts were deposited in previously silanized slides and identified with the number of the rat and the group to which he belonged. Slides were diaphanized and rehydrated, and antigen retrieval was performed using the Trilogy solution (Cell Mark Inc., Rocklin, CA, USA). Next, the slides were rinsed with distilled water and subsequently immersed in PBS solution for 10 min and dried with filter paper. Endogenous peroxidases were blocked using 3% hydrogen peroxide (H_2O_2) in a humid chamber at room temperature for 10 min. Then, further washing was performed with PBS for 10 min. After this process, the slides were left resting at room temperature for 10 min and then washed with PBS for 5 min. The primary polyclonal anti-MPO antibody (Dako do Brazil Ltda., São Paulo, Brazil) with cross-reactivity to rats was diluted in saline containing bovine serum albumin (1%) diluted 1:100. All slides were coated with 100 μL of this solution and left resting at room temperature for a period of 2 h. Following exposure to primary antibody, the slides were rinsed with distilled water (2 baths) and PBS buffer (two baths of 2 min). Then, the slides were incubated with an avidin–biotin system (secondary antibody) comprising the LSAB + kit System-HRP (Dako do Brasil Ltda., Sao Paulo, Brazil) for a 35-min period of exposure for each reagent, and then washed with two baths of PBS. The section processing occurred by using the Liquid DAB + Substrate Kit (Dako do Brasil Ltda., São Paulo, Brazil) in a dilution of 1 drop of chromogenous solution in 1 μL of buffer solution. 100 μL of the chromogen was added over the sections for a period of 5 min at room temperature. After this processing, the sections were washed in running water and counterstained with Harris hematoxylin for 30 s. After this process, the slides were again washed in running water, until removal of excess dye. Finally, the slides were dehydrated in three baths with increasing concentrations of alcohol and two baths of xylene. The slides were then mounted with coverslips and resin.

Computerized morphometry

The immunostaining was considered positive when a diffusely brownish color was present, with variable intensity points and a homogeneous distribution in neutrophils. As recommended by the manufacturer, the negative control for the immunostaining was performed without the addition of primary antibody; and the positive control was made in human vermiform appendix suffering from acute appendicitis. The presence of a brownish color makes it possible to quantify its tissue content by computerized morphometry (computer-assisted image processing). The MPO content was then measured with the aid of NIS-Elements 4.0 software (Nikon Inc., Osaka, Japan). Like the histological analysis, the tissue content of MPO in each sample was always determined in a site where there were at least three contiguous crypts. The image analysis program using color histograms in RGB

(Red, Green, Blue) system is able to determine the amount of the selected color (in this case the brown color, that in which MPO is expressed) present in the tissue, converting color intensity into number of pixels in each selected field. Thus, the final content of MPO was determined in percentage by field (%/field). The final amount considered for each rat represented the average value obtained after reading three histological fields in the established magnification (100×).

Determination of malondialdehyde (MDA) levels

The levels of lipid peroxidation were evaluated by measuring the levels of thiobarbituric acid reactive substances (TBARS), as with MDA, with a previously described methodology.[19] MDA is a secondary product of lipid oxidation and is considered a potential candidate for being a general biomarker of oxidative stress. As to the quantification of tissue levels of MDA, 1 g of each fragment was placed in 5 mL of phosphate buffer and homogenized by vortex and ultrasound sonication for 30 s, alternately, repeating the process for three times. Then, 250 μL of the supernatant obtained from the homogenization process was transferred to a plastic test tube containing 25 mL of 4% methanolic BHT, with a new vortex homogenization. The sample was then mixed with 1 mL of 12% trichloroacetic acid, 1 mL of 0.73% thiobarbituric acid and 750 μL of Tris/HCl buffer, and then incubated in a water bath at 100 °C for 60 min. After this step, the tubes were immediately placed in a container with ice to block the reaction, with addition of 1.5 mL of n-butanol. Then, the mixture was again vortexed for 30 s. The samples were separated by centrifugation for 10 min at 5000 rpm. Finally, the supernatant was removed, and the absorbance at 532 nm of the organic phase was analyzed, using a UV/vis 6105 (Jenway, Bibby Scientific Limited, Cheshire, UK) spectrophotometer.

Statistical analysis

The results for the degree of inflammation were described according to the median of the values obtained. As to tissue levels of MPO and MDA, the results were described according to their media ± standard error. The comparison of results found among experimental groups was analyzed by Student's t test. ANOVA test was used to study the variation in results according to the intervention time in each experimental group. It was established for all tests the level of significance of 5% ($p < 0.05$), and we used one asterisk (*) to identify values of $p < 0.05$ and two asterisks (**) for values of $p < 0.01$.

Results

Fig. 2A shows colonic epithelium excluded from bowel transit, submitted to intervention with 0.9% saline for 4 weeks, while Fig. 2B shows the colon without transit submitted to irrigation with SCF at a concentration of 2.0 g/kg/day.

Fig. 3 shows the degree of tissue inflammation, compared with animals subjected to intervention with 0.9% saline, SCF 1.0 g/kg/day and SCF 2.0 g/kg/day for 2 and 4 weeks.

Fig. 4A shows the tissue expression of MPO in the segments without transit and subjected to intervention with 0.9% saline for 4 weeks, while Fig. 3B shows the colon without transit subjected to irrigation with SCF at a concentration of 2.0 g/kg/day for 4 weeks.

Fig. 5 shows MPO tissue content by comparing animals subjected to intervention with 0.9% saline, SCF 1.0/kg/day and SCF 2.0/kg/day for 2–4 weeks.

Fig. 6 shows MDA tissue content by comparing animals subjected to intervention with 0.9% saline, SCF 1.0 g/kg/day and SCF 2.0 g/kg/day for 2 and 4 weeks.

Discussion

The colonic epithelium is considered the most perfect functional barrier of the human body.[13] Formed by a single layer of cells, it separates the colonic lumen, an antigen and bacteria-rich environment, from the sterile internal environment.[16,17] This property becomes important when we consider that in the early stages of IBD, especially ulcerative colitis, the colonic mucosa is consistently impaired, suggesting that the

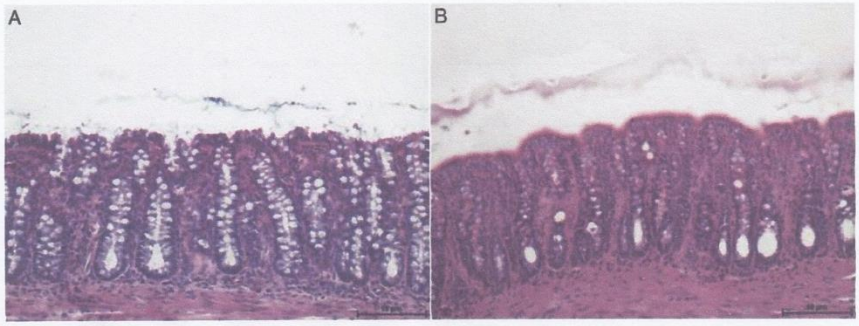

Fig. 2 – (A) Mucosa without traffic subjected to intervention with 0.9% saline for 4 weeks. Apparent irregularity of the mucosal surface with formation of erosion, tortuosity of colonic glands (HE – 100×). (B) Colonic mucosa without intestinal transit subjected to intervention with SCF (2.0 g/kg/day) for 4 weeks. Formation of a protective layer of SCF on the epithelial surface, that is preserved, without erosions and with colonic gland alignment (HE – 100×).

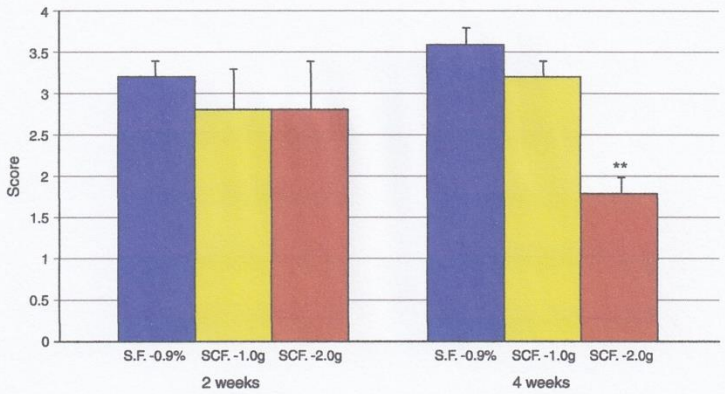

Fig. 3 – Degree of inflammation comparing animals subjected to intervention with 0.9% saline, SCF 1.0 g/kg/day and 2.0 g/kg/day for 2 and 4 weeks. **Significant: (SCF 2.0 g/kg/day x 0.9% saline and SCF 1.0 g/kg/day) ($p < 0.01$). Student's t test.

disruption of the epithelial barrier is an early event related to the pathogenesis of the disease.[14] The importance of epithelial barrier integrity is enhanced when one considers that the major experimental models proposed for colitis induction use chemicals such as trinitrobenzene sulphonic acid (TNBS), H_2O_2, acetic acid and dextran sulfate in order to disrupt the epithelial barrier, thus allowing the bacterial invasion and consequently an acute inflammatory response that characterizes the disease.[20]

However, the early molecular mechanisms that trigger the injury of those different defense systems that form the colonic barrier in patients with IBD have not been fully clarified.[14] One of the possible pathogenic mechanisms involved in the initial injury of the colonic mucosal barrier was suggested by Pravda in 2005, that proposed the theory of colitis induction by OFR.[14] According to Pravda, the pathogenesis of colitis had two distinct stages. In the first stage, called by the author as "initiation phase", the initial insult to the intestinal mucosa was a result of the increased production of OFR by the epithelial cells of the colon mucosa themselves, with changes in their energy metabolism. The overproduction of OFR by these cells would cause breakage of those different defense lines that make up the mucosal barrier, allowing migration of bacteria and antigens present in the intestinal lumen into the sterile intimacy of the submucosa.[14] In the second stage, called "spreading phase", neutrophils would migrate into the intestinal wall in an attempt to combat the bacterial infiltration, disseminating the inflammatory process.[14,21,22] The possibility that the increased production of OFR may cause injury to the colonic mucosal epithelium has been known for several years, when it was shown that H_2O_2 (a potent OFR generator) instillation inside the colon was followed by a severe picture of colitis, sometimes with a fatal outcome.[23–25] It should be noted that H_2O_2-induced colitis presents clinical, macroscopic and microscopic features very similar to those found in ulcerative colitis.[26]

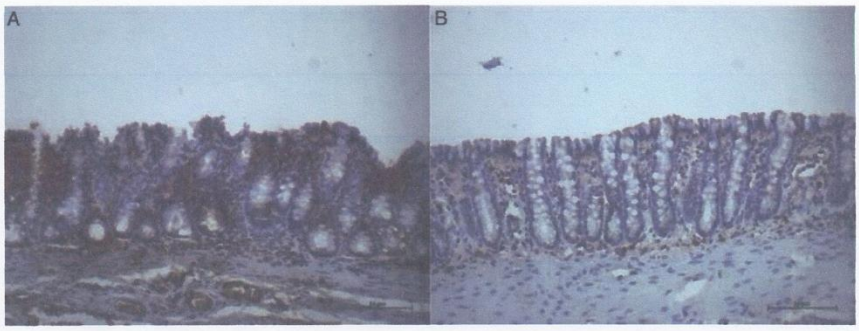

Fig. 4 – (A) Intense mucosal MPO expression, among colonic glands and inside submucosal blood vessels in an animal subjected to intervention with 0.9% saline solution for 4 weeks (HE – 100×). (B) Minor tissue expression in colonic mucosa of an animal submitted to intervention with SCF 2.0 g/kg/day for 4 weeks (HE – 100×).

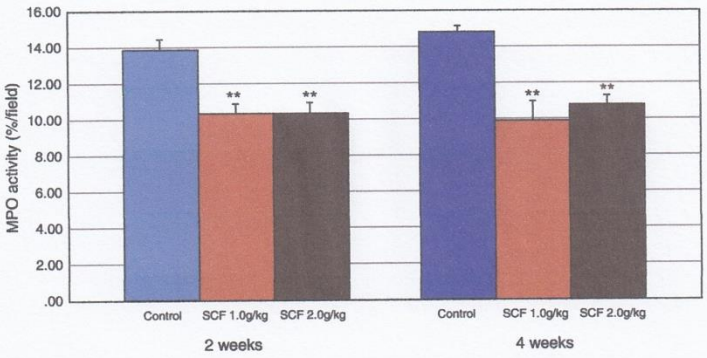

Fig. 5 – MPO tissue content, comparing animals subjected to intervention with 0.9% saline, SCF 1.0 g/kg/day and SCF 2.0 g/kg/day for two and four weeks. **Significant: SCF 2.0 g/kg/day and SCF 1.0 g/kg/day × 0.9% saline ($p < 0.01$). Student's t test.

However, in order to experimentally verify if the colon mucosa cells with changes in their energy metabolism would be able to produce OFR in sufficient quantity to damage the intestinal epithelium, it would be necessary to establish an experimental model of colitis where the initial damage to the mucosa was not caused by the exposure of the intestinal epithelium to chemicals, but by conditions that only would alter the cellular metabolism.[13] SCFA, represented by butyrate, propionate and acetate, account for 90% of all substrate used by colonic mucosa cells to obtain energy, and the simple deprivation of these substances to the mucosa alters the energy metabolism of colonocytes, leading to the appearance of DC.[2,13,16,17] In the face of this evidence, an experimental model of DC would assess the ability of epithelial cells excluded from bowel transit in producing larger amounts of OFR, and also would verify if the resulting oxidative stress could damage the different lines of epithelial defense. A number of studies using an experimental model of DC showed that the colonic mucosa cells excluded from fecal transit are subjected to tissue oxidative stress through an increase in the production of OFR.[13] It has also been demonstrated that an overproduction of OFR causes harm to the colonic mucosa, decreases the population of goblet cells, reduces the content and modifies the expression of mucins, causes disruption of protein constituents of intercellular junctions, and also causes oxidative damage to cellular DNA.[13,15–17,27–29] All these findings confirmed the higher capacity of OFR production by colonic cells with metabolic changes, as shown by the relationship between an increased production of OFR and the disruption of different defense systems of epithelial barrier.[13]

Based on these findings, several studies began to test the effectiveness of substances with antioxidant activity for the treatment of DC.[19,30–32] The results of these studies confirmed that natural or synthetic substances with antioxidant activity were able to reduce the production of OFR and to improve the histological changes that characterize DC.[19,29–32]

Riley et al. in 1989[33] were the first to demonstrate the benefits of using enemas with SCF in the acute phase of ulcerative

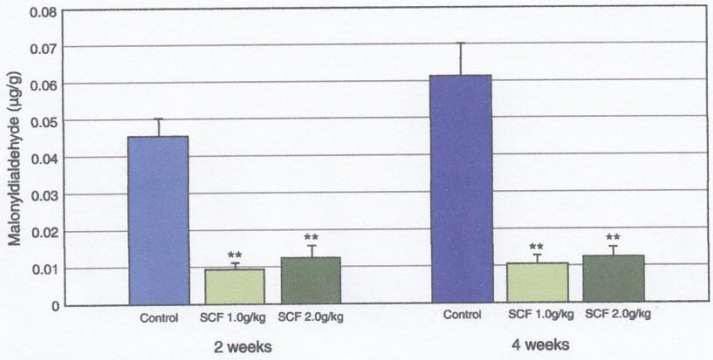

Fig. 6 – MDA tissue content, comparing animals subjected to intervention with 0.9% saline, 1.0 SCF/kg/day and SCF 2.0 g/kg/day for 2 and 4 weeks. **Significant: SCF 2.0 g/kg/day and SCF 1.0 g/kg/day × 0.9% saline ($p < 0.01$). Student's t test.

rectitis. Subsequently, other studies have confirmed the efficacy of the drug in other forms of colitis.[5,6,8–10] Most of these authors attributed the action of the drug to its adhesive capacity on the inflamed epithelium. Riley et al. in 1989[33] were the first to demonstrate the benefits of using enemas with SCF in the acute phase of ulcerative proctitis. Subsequently, other studies have confirmed the efficacy of the drug in other forms of colitis.[5,6,8–10] Most of these authors attributed the action of the drug to its adhesive propriety on the inflamed epithelium, regardless of its important antioxidant action, already known for decades.[7,34] The antioxidant activity of SCF was the result of the drug's ability to remove the OFR formed in the tissues under different experimental conditions.[35–37] Only one study evaluated the effects of the application of enemas with SCF in an experimental model of DC.[2] The authors found that this substance reduced significantly the epithelial loss, decreased the abscess formation within intestinal crypts, and also reduced the inflammatory infiltrate and local collagen deposition,[2] in line with other studies that attributed the improvement of the mucous inflammatory process to the SCF ability to form a protective layer on the inflamed mucosa.[2] While drawing attention to other mechanisms of action of this drug, these studies did not assess the possibility that the beneficial effects of the substance could be related to a possible antioxidant action.[2]

In this study, we aimed to verify if the therapeutic action of SCF could be related to its antioxidant properties. We used the DC model, because oxidative stress is now considered one of the molecular mechanisms related to the etiopathogenesis of this disease.[13–17] With the DC model, we could confirm the results previously described, as we showed that the use of enemas with SCF reduced the degree of tissue inflammation.[2] Using a previously validated inflammatory scale, we found that the degree of tissue inflammation decreased significantly in animals in which we applied enemas with higher SCF concentrations for a longer period. These findings showed that the topical effect of SCF depends on the concentration used and the intervention time. In all animals which underwent intervention with SCF, we could detect, through the histological study, the formation of a thin protective film covering the colon mucosa in most animals. This finding, coupled with the improvement of the degree of tissue inflammation, confirms the ability of this drug, in acting as a mechanical barrier hindering the contact between the epithelium with the existing flora in the intestinal lumen. However, if the action of this drug was purely mechanical, by force the tissue inflammation score should be lower from the first weeks of intervention, which suggests that other mechanisms of action are involved.

The colonic mucosa infiltration by inflammatory cells is another common finding in DC. In more severe cases, in the acute phase the infiltrate is composed predominantly of neutrophils, whereas in the chronic phase, the lymphocytes become the main cells, although still with neutrophils present in tissues.[15] MPO is an enzyme found primarily in the azurophilic granules of neutrophils.[13] While MPO may be present in other inflammatory cells, it is estimated that 95% of all its content comes from the neutrophils; this finding makes this substance an efficient marker for the presence of an acute inflammatory infiltrate. Previous studies used tissue dosage of MPO content in order to confirm the presence of a neutrophil infiltrate in DC.[13,38] In this study, when we analyze the infiltration of neutrophils for assessing the tissue content of MPO, we found that animals subjected to SCF intervention had a lower content of MPO compared to animals receiving intervention with 0.9% saline. The reduction in neutrophilic infiltrate was not related to the concentration of SCF used or to the intervention time. There are several possible explanations for this finding. Perhaps the formation of a mechanical barrier over the epithelial surface made it difficult for the occurrence of bowel wall invasion by bacteria from the colonic lumen, decreasing the neutrophil inflammatory response. Possibly the antimicrobial properties of SCF could decrease the number of bacteria in bowel lumen, thus decreasing the neutrophil infiltration. Likewise, if SCF can reduce OFR production, the epithelial injury will be smaller, which would decrease the bacterial infiltration. In turn, the penetration of a lower number would diminish the infiltrate and, thus, the production of OFR from neutrophils, which would confirm the antioxidant activity of SCF.

To evaluate the antioxidant action of SCF, we used MDA tissue content dosage. The products of lipid peroxidation of phospholipids present in cell membranes, such as MDA, can be used as indicators of OFR action in the body.[39] By far, MDA determination is the most popular indicator of oxidative damage to cells and tissues.[39] We noted that in the first two weeks of intervention with SCF, a significant reduction in MDA tissue content already had taken place, even when we applied a lower concentration of the substance. MDA tissue content remained low after four weeks of intervention, regardless of SCF concentration used. This showed that the substance maintained its antioxidant activity. On the other hand, in the control group animals, MDA levels progressively increased with the passage of time, showing that the longer the epithelial cells were deprived of their SCFA supply, the higher the level of tissue oxidative stress. These findings confirm the antioxidant power of SCF, since the action of this drug is not dependent on the concentration used or on the intervention time. The decrease in MDA levels was directly related to the improvement of the neutrophilic infiltrate within the first weeks of intervention, remaining that way throughout the intervention period. The lower MDA content was also directly related to the improvement of the degree of inflammation after four weeks of intervention. This finding shows that SCF reduces oxidative stress in tissue and preserves the epithelial barrier, resulting in a smaller acute inflammatory response.

The results of this study confirm that SCF has antioxidant action, as previously shown.[5] When one considers that DC pathogenesis is related to oxidative stress, the results suggest that the application of enemas with SCF is a new and effective therapeutic approach for the treatment of this disease. Its low cost, easy availability and the lack of serious side effects are additional advantages to be considered, to minimize the suffering of patients with DC already living with the limitations imposed by the presence of a stoma.

Conclusion

Under the conditions of this experimental study, we conclude that the application of enemas containing SCF in a colon with transit exclusion reduces the levels of tissue oxidative stress,

decreases neutrophil infiltration and improves tissue inflammation, confirming the antioxidant action of this substance.

Funding

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). Process N° 2010/12492-7.

Conflict of interests

The authors declare no conflicts of interest.

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