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AVALIAÇÃO DA ATIVIDADE ANTI-INFLAMATÓRIA DO ALCALÓIDE ISATINA EM RATOS SUBMETIDOS AO MODELO DE COLITE ULCERATIVA INDUZIDA POR ÁCIDO TRINITROBENZENOSSULFÔNICO

EVALUATION OF THE ANTI-INFLAMMATORY ACTIVITY OF THE ALKALOID ISATIN IN RATS SUBMITTED TO ULCERATIVE COLITIS MODEL INDUCED BY TRINITROBENZENE SULFONIC ACID

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Resumo

Retocolite ulcerativa inespecífica (RCUI) e doença de Crohn (DC) são as principais doenças inflamatórias intestinais (DII). São caracterizadas por inflamação crônica da mucosa do trato gastrintestinal, resultando em diarreia, fezes sanguinolentas, dores abdominais, anemia, febre, fadiga e perda de peso, tanto em homens quanto em mulheres. Acredita-se que tais manifestações sejam resultado de uma interação entre indivíduos geneticamente susceptíveis, fatores ambientais específicos, desbalanço na microflora intestinal e uma resposta imunológica exacerbada. Atualmente, as terapias vigentes visam atingir um estado remissivo, reduzindo a intensidade do processo inflamatório, bem como seus sintomas. Contudo, estas terapias apresentam custo elevado e uma série de efeitos adversos importantes. Neste contexto, pesquisas com produtos naturais mostram-se como alternativas promissoras no tratamento de DII. A isatina é um alcaloide indólico com atividades biológicas e farmacológicas já testadas que incluem atividades antimicrobiana, antitumoral, antioxidante e anti-inflamatória. Em vista disso, este trabalho avaliou os efeitos de isatina em dois protocolos de modelo de colite aguda induzida pelo ácido 2,4,6-trinitrobenzeno sulfônico (TNBS). No primeiro protocolo, os animais foram divididos nos seguintes grupos: não-colítico, colítico não-tratado e colíticos tratados com isatina (3, 6, 12.5, 18.75 e 25 mg/Kg) 72, 48, 24 e 2h antes do desafio pelo TNBS. A eutanásia dos animais ocorreu 96h após a exposição com TNBS, seguido de remoção do cólon para realização das análises macroscópicas e bioquímicas. Os resultados demonstraram que as doses de 6 e 25 mg/Kg foram eficazes em proteger a mucosa cólica contra o aumento de TNF-α, IFN-γ, PGE_2 e COX-2, bem como contra a redução dos níveis de IL-10. Estas doses foram efetivas, ainda, em prevenir a queda significativa dos níveis de GSH, bem como contra o aumento da atividade da GSH-Px. A dose de 6 mg/Kg também foi efetiva ao evitar a redução da atividade da SOD e o aumento da atividade da GSH-Gr. Em vista destes resultados, a dose de 6 mg/Kg foi novamente testada em um segundo protocolo experimental.

Neste, os animais foram desafiados e em seguida tratados uma única vez com a dose de 6 mg/Kg. Os animais foram eutanaziados três e sete dias após o desafio. Os resultados macroscópicos revelaram que a dose única do alcaloide reduziu de forma significativa os parâmetros macroscópicos da lesão. As análises bioquímicas revelaram que o alcaloide foi eficaz na redução do processo inflamatório a partir do terceiro dia, sendo este efeito prolongado até o sétimo dia. A Isatina foi eficaz em aumentar os níveis de GSH e aumentar a atividade da GSH-Px, além de reduzir a atividade da MPO. Além disso, o alcaloide foi efetivo na redução dos níveis de TNF-α, IFN-γ, IL-1β, IL-12, PGE2 e aumento dos níveis de IL-10. Os resultados obtidos em ambos os protocolos experimentais revelaram que a isatina, alcaloide indólico encontrado em certas plantas e em tecidos de mamíferos, apresenta uma atividade protetora significativa contra danos na mucosa intestinal. Este efeito dá-se, possivelmente, devido à uma combinação entre suas propriedades antioxidantes e anti-inflamatórias. No entanto, ainda são necessárias maiores investigações no intuito de se estabelecer todos os mecanismos envolvendo a isatina e seus efeitos farmacológicos.

Palavras chave: Doenças inflamatórias intestinais, Retocolite ulcerativa, isatina, atividade anti-inflamatória e atividade antioxidante.

Abstract

Ulcerative colitis (UC) and Crohn´s disease (CD) are the major forms of inflammatory bowel diseases (IBD). They are characterized by chronic mucosal inflammation of gastrointestinal tract, which results in diarrhea, blood stools, abdominal pain, anemia, fever, fatigue and weight loss, both in man and in woman. It is believed that such manifestations are the result of an interaction between genetically susceptible individuals, specific environmental conditions, imbalance in the intestinal microflora and imbalance of the immune response. Currently, therapies aim to achieve a remissive state, reducing the intensity of the inflammatory process, as well as its symptoms. However, these therapies have high cost and present many significant adverse effects. In this context, research on natural products appear as promising alternative in the treatment of IBD. Isatin is an indole alkaloid with biological and pharmacological activities already tested, such as antimicrobial, anti-tumor, antioxidant and anti-inflammatory activity. In view of this, this study evaluated the effects of isatin in two acute colitis model protocols induced by 2,4,6-trinitrobenzene sulfonic acid. In the first protocol, the animals were divided into seven groups: non-colitic, untreated colitic and five treated colitic groups. Doses of 3, 6, 12.5, 18.75 and 25 mg/Kg of isatin were tested, and the animals were pre-treated on times 72, 48, 24 and 2h prior the challenge. The euthanasia occurred 96h after challenge with TNBS, followed by colon removal for macroscopic and biochemical analyses. The results showed that both 6 and 25 mg/Kg doses were effective in protecting the colonic mucosa against the increase of TNF-α, IFN-γ, PGE2 and COX-2, as well as over IL-10 content reduction. These doses were also effective in preventing a significant reduction of GSH levels and the increase of GSH-PX activity. The dose of 6 mg/Kg was effective also in prevent the reduction of SOD activity and the increase of GSH-Gr activity. In view of these results, 6 mg/Kg dose was again tested in a second experimental protocol. In this, the animals were challenged and then treated once with 6 mg/Kg dose. The animals were sacrificed three and seven days after

challenge. The macroscopic results shows that a single dose of de alkaloid significantly reduced the macroscopic lesion. Biochemical analyses revealed that the alkaloid was effective in reducing the inflammation from the third day after induction, which sustained until the seventh day. Isatin was effective by increasing the GSH content and the GSH-PX activity, besides reducing MPO activity. The alkaloid was effective also in reduce the levels of TNF-α, IFN-γ, IL-1β, IL-12, PGE2 and increase IL-10 content. The results obtained in both experimental protocols showed that isatin, an indole alkaloid found in some plants and mammalian tissues, presents a significant protective effect against damage to the intestinal mucosa. This effect is possible due to a combination of its antioxidant and anti-inflammatory properties. However, it is still needed further investigation in order to establish all mechanisms involving the isatin and its pharmacological effects.

Keywords: Inflammatory bowel diseases, ulcerative colitis, isatin, anti-inflammatory activity, antioxidant activity.

Sumário

1. Introdução ... 12

1.1. Epidemiologia ... **12**

1.2. Etiologia ... **13**

1.2.1. Fatores genéticos ... **13**

1.2.2. Fatores Ambientais ... **15**

1.3. Mediadores Inflamatórios ... **17**

1.4. Estresse Oxidativo ... **20**

1.5. Terapêutica ... **21**

1.5.1. Plantas Medicinais ... **22**

1.5.2. Alcaloides ... **22**

2. Artigo: "Inhibition of tumor factor alpha and cyclooxigenase-2 by Isatin: A molecular mechanism of protection against TNBS-induced colitis in rats." ... 25

2.1. Objetivos ... **25**

2.2. Periódico publicado ... **25**

2.3. Correspondência de aceite ... **26**

2.4. Artigo ... **27**

2.5. Conclusões ... **35**

2.6. Considerações ... **36**

3. Artigo: "Anti-inflammatory properties of isatin: An inhibitory mechanism of tumor necrosis factor alpha and interleukin-1 beta". ... 37

3.1. Objetivos ... **37**

3.2. Periódico submetido ... **37**

3.3. Correspondência da revista ... **38**

3.4. Artigo ... **39**

3.5. Conclusões ... **65**

3.6. Considerações ... **66**

4. Conclusão geral do trabalho ... 67

Referências bibliográficas ... 70

1. Introdução

As doenças inflamatórias intestinais (DII), termo que compreende a Doença de Crohn (DC) e retocolite ulcerativa (RCU), são manifestações inflamatórias crônicas, reincidentes e remitentes que afetam o trato gastrintestinal. São doenças caracterizadas por episódios de quiescência e remissão, sendo o período ativo classificado de acordo com a intensidade do processo inflamatório (Gracie & Ford, 2015). Sua sintomatologia principal envolve inflamação crônica da mucosa gastrointestinal, resultando em diarreia, fezes sanguinolentas, dores abdominais, febre, anemia, fadiga e, consequentemente, perda de peso (Head & Jurenka, 2003). Em paciente com DC, o processo inflamatório é de caráter transmural, podendo levar a fibroses e obstruções clinicas, ocorrendo descontinuamente ao longo do trato gastrintestinal. Já na RCU, o processo inflamatório fica restrito a mucosa e a submucosa, envolvendo de forma consistente e contínua o cólon (Rungoe, Nyboe Andersen, & Jess, 2015).

As DII apresentam caráter multifatorial, desencadeadas por uma resposta imune impropria contra a microflora em indivíduos geneticamente susceptíveis (Nielsen, 2014). Como resultado, observa-se um aumento significativo no número de células imunes no sítio inflamado, conferindo maior expressão na produção de mediadores inflamatórios como citocinas, eicosanoides e espécies reativas de oxigênio (EROs) (Luchini et al., 2008).

1.1. Epidemiologia

Nas últimas décadas observou-se aumento nas taxas de incidência das DII, mais significativamente nas áreas industrializadas. No caso da RCU, 1,5 milhões de norte-americanos, 2,2 milhões de europeus e aproximadamente 30 mil brasileiros são acometidos pela doença (Ananthakrishnan, 2015b; B. C. da Silva, Lyra, Rocha, & Santana, 2014). Tais dados epidemiológicos sugerem que o estilo de vida próximo às áreas mais industrializadas seja um fator de risco para as DII. Tais levantamentos sugerem ainda que a RCU seja mais comum que a DC, tanto nos países do continente europeu quanto na américa do norte (Vatn, 2009).

Sua distribuição não apresenta diferenças estatísticas entre gêneros, sendo a população caucasiana mais susceptível (Head & Jurenka, 2003). Nas últimas décadas, apesar do aumento na incidência em diferentes grupos etários, notou-se um maior número de casos em indivíduos entre 30 e 40 anos, sendo este considerado o primeiro e mais significativo pico destas doenças. O segundo pico inflamatório se dá entre 60 e 80 anos (Kaplan, 2010; Sonnenberg, 2010).

1.2. Etiologia

Devido sua sua etiologia multifatorial, inúmeros estudos têm sido realizados objetivando melhorar o entendimento da fisiopatologia da doença bem como a elaboração de terapias mais eficazes, conforme revisado por Mayer (2010). Para tanto, são empregados modelos animais, estudos em tecidos humanos e a identificação de genes susceptíveis, além do reconhecimento mais amplo dos fatores ambientais envolvidos na patogênese das DII (Mayer, 2010). Estes fatores são de suma importância na etiologia das DII, sendo apresentados com maior detalhamento a seguir.

1.2.1. Fatores genéticos

Inicialmente, estudos de casos familiares e estudos com gêmeos acometidos por estes doenças direcionaram os interesses dos pesquisadores para os possíveis componentes genéticos envolvidos na patogênese das DII (Sarlos et al., 2014; Vatn, 2009). Na investigação gênica, foram identificados diversos *loci* em regiões cromossômicas distintas, como portadores de genes susceptíveis às DII (J. H. Cho & Abraham, 2007; Sarlos et al., 2014).Estes *loci* codificam sinais responsáveis pela manutenção da integridade da barreira epitelial, no reconhecimento de antígenos, na autofagia, na resposta imune inata, na coordenação da resposta imune adaptativa e no recrutamento de leucócitos (Sarlos et al., 2014).

A nível molecular, a maior diferença entre a DC e a RCU é encontrada em antígenos leucocitários humanos (HLA) classe II, em genes associados ao reconhecimento de padrões antigênicos, como nos domínios de oligomerização de ligação de nucleotídeos (NODs) e os receptores do tipo *Toll* (TLRs); na imunidade inata, no receptor para IL-23 (IL-23R), ou ainda em

vias autofágicas, como as vias ATG16L1 e IRGM (Ferguson, Shelling, Browning, Huebner, & Petermann, 2007; Sarlos et al., 2014; Stokkers et al., 1998).

O complexo antígeno leucocitário humano (HLA) é uma região densa, altamente polimórfica, presente no cromossomo 6p21.3. Este complexo compreende aproximadamente 130 genes expressos e dentre estes, genes altamente polimórficos de classe I e II, essenciais para a atividade normal de linfócitos (Beck et al., 1999; Shiina et al., 1999). Os genes clássicos HLA classe II codificam glicoproteínas de superfície celular expressas em células apresentadoras de antígenos (APCs), como é o caso das células dendríticas e dos macrófagos. Sua função é apresentar peptídeos para os receptores de células T e, como consequência, ativar tais células (Ahmad, Marshall, & Jewell, 2006).

Os NODs são uma família de receptores que funcionam como receptores de reconhecimento padrão (PRRs) intracelulares, reconhecendo e respondendo à produtos microbianos. O reconhecimento de tais produtos ativa a transcrição dos fatores pró-inflamatórios fator de transcrição nuclear κB (NF-κB) e da proteína quinase ativada por mitógenos (MAPKs) (Franchi, Wamer, Viani, & Nunez, 2009; Kanneganti, Lamkanfi, & Nunez, 2007; Siggers & Hackam, 2011). Um importante membro desta família é o domínio NOD2/CARD15, cuja importância nas doenças intestinais é suportada por inúmeros estudos que demonstram que variâncias neste gene estão associados à indivíduos predispostos à processos inflamatórios crônicos no intestino (Siggers & Hackam, 2011).

Já os receptores do tipo Toll (TLRs) são componentes essenciais da resposta imune inata. São receptores transmembranares que reconhecem componentes microbianos de diferentes bactérias, fungos ou vírus (Aderem & Ulevitch, 2000; Beutler, 2004; Kawai & Akira, 2006), sendo encontrados tanto na membrana celular (TLR1, 2, 4, 5 e 9) quanto em organelas intracelulares (TLR3, 7 e 8). Estes receptores são expressos por células epiteliais intestinais e células imunes em pacientes com DII. Sua sinalização no cólon pode inibir a resposta inflamatória e garantir a homeostase cólica (Sarlos et al., 2014).

1.2.2. Fatores Ambientais

Outra importante influência na patogênese das DII está relacionada às interações ambientais dos indivíduos e suas consequências contra a microbiota. Diversos estudos têm destacado um papel fundamental da microflora intestinal na patogênese destas condições inflamatórias (Ananthakrishnan, 2015a; Gevers et al., 2014; Morgan et al., 2012). Pacientes com DII exibem uma disbiose intestinal, resultando na redução da diversidade da microflora quando comparados à indivíduos saudáveis (Ananthakrishnan, 2015a). Esta disbiose se caracteriza pela redução de subpopulações de bactérias potencialmente protetoras, tais como *Firmicutes*, favorecendo o povoamento destes sítios por bactérias potencialmente patógenas, tais como *Escherichia coli* entero invasiva (Darfeuille-Michaud et al., 2004).

O ambiente externo tem grande influência sobre a microbiota e, consequentemente, modula a resposta imunológica do hospedeiro, bem como a integridade da barreira epitelial, sendo tal fato corroborado por estudos epidemiológicos que apontam a importância do ambiente no desenvolvimento das DII. Nas últimas décadas, evidenciou-se um aumento significativo no número de casos diagnosticados em diversos grupos étnicos e regiões geográficas distintas, sofrendo maior influência de estilos de vida industrializados. Tais estudos oferecem pistas intrigantes para a patogênese das DII, bem como sugerem que alterações em tais influências poderão desempenhar um papel importante tanto na prevenção destas doenças quanto na melhora da história natural em pacientes com a doença já estabelecida (Ananthakrishnan, 2015a; Sartor, 2006; Vatn, 2009). Dentre os fatores ambientais apontados como possíveis determinantes no desenvolvimento das DII, destacam-se o tabaco, a alimentação, fatores psicológicos e comportamentais, infecções bacterianas e virais e a utilização de certos fármacos (Head & Jurenka, 2003).

O tabagismo é o fator ambiental mais significativo e mais bem documentado para as inflamações intestinais, sendo estes dados reproduzidos recentemente em estudos de DII com gêmeos (O'Toole & Korzenik, 2014). A influência do tabaco em ambas doenças é marcante. Provas convincentes demonstram seu papel protetor na colite ulcerativa, levando a terapias de

reposição de nicotina em tratamentos da doença. No entanto, o tabagismo se mostra um fator de risco no desenvolvimento da DC, bem como efeitos adversos e complicações, o que inclui a doença perianal, recorrências pós-operatórias e a resistência à fármacos (Biedermann et al., 2013). Esta relação complexa entre o tabagismo e as DII destaca as diferenças na fisiopatologia das duas doenças (O'Toole & Korzenik, 2014).

Por ser uma enfermidade que acomete o trato gastrintestinal, muitos autores investigam as possíveis relações entre os hábitos alimentares e o desenvolvimento ou agravamento do processo inflamatório (Sicilia, Vicente, & Gomollon, 2009). Pacientes acometidos por tais enfermidades são obrigados a avaliar toda sua alimentação, afim de identificar possíveis componentes alimentares que possam desencadear o processo inflamatório. Dentre estes, destacam-se o açúcar refinado, carboidratos totais e dietas ricas em gordura e com baixa ingestão de fibras, denominada "dieta ocidental". Por outro lado, o consumo de café, de frutas, verduras e dietas ricas em fibras alimentares são apontadas como benéficas contra o desenvolvimento das DII (Collins, 2014; Sicilia et al., 2009).

Além dos fatores ambientais já acima citados, as DII são constantemente associadas a certas alterações comportamentais e psicológicas. Dentre tais alterações destacam-se o neuroticismo, a dependência e a ansiedade (Ananthakrishnan, 2015a). Estudos baseados em entrevistas com indivíduos acometidos por ambas doenças indicam a associação entre os principais agentes estressores, ansiedade ou depressão e o risco de incidência ou reincidência do processo inflamatório (Bitton et al., 2008; Camara, Schoepfer, Pittet, Begre, & von Kanel, 2011; Goodhand et al., 2012). Devido à esta possível relação, Li, Norgard, Precht, and Olsen (2004) atribuíram à estes agentes estressores a manutenção do processo inflamatório, em virtude das reações bioquímicas desencadeadas por tais agentes e de alteração significativa da microbiota.

Outros fatores ambientais são apontados por aumentar o risco de incidência das DII. A microbiota tem papel fundamental na manutenção da homeostase cólica e alterações em sua composição ou concentração podem influenciar a patogênese das DII. Neste contexto, a

exposição a certos fármacos, como antibióticos, especialmente na infância e adolescência, tem sido associada com a maior incidência destas doenças tanto na infância quanto na fase adulta (Shaw, Blanchard, & Bernstein, 2010; Singh, Graff, & Bernstein, 2009). Já as drogas anti-inflamatórias não-esteroidais (DAINES), contraceptivos orais e as terapias de reposição hormonal pós-menopausa parecem desempenhar um papel importante no desenvolvimento das DII através de seu efeito sobre o rompimento da barreira epitelial ou sobre a resposta imune dos indivíduos (Ananthakrishnan et al., 2012; Khalili et al., 2012; Khalili et al., 2013).

1.3. Mediadores Inflamatórios

O trato gastrintestinal é um componente importante do sistema imune, responsável por reconhecer e reagir contra estímulos ambientais. A interação entre o trato gastrintestinal e sua microbiota é altamente regulada, permitindo que estes organismos habitem o ambiente intestinal sem disparar um processo inflamatório na mucosa intestinal. A este mecanismo dá-se o nome de homeostase -intestinal (Pedersen, Coskun, Soendergaard, Salem, & Nielsen, 2014). Como dito anteriormente, as DII são doenças multifatoriais, que combinam fatores ambientais, genéticos e o microambiente intestinal do desenvolvimento de uma resposta imune indevida na mucosa intestinal. Como consequência, nota-se uma resposta inflamatória continua e impropria contra a microbiota, o que resulta em produção e liberação constante de mediadores pró-inflamatórios e consequente alteração da homeostase intestinal (Maloy & Powrie, 2011). Estas alterações são, em algum grau, determinadas geneticamente e podem conduzir à uma disfunção da integridade da barreira epitelial, a disfunção na detecção de microrganismos e na alteração da regulação da resposta imune adaptativa (Khor, Gardet, & Xavier, 2011).

No entanto, nas DII, a resposta imune não cessa e a produção de mediadores pró-inflamatórios é contínua. Este processo inflamatório resulta no recrutamento de células do sistema imune e consequente liberação de citocinas, como a interleucina 1 (IL-1), interleucina 6 (IL-6) e o fator de necrose tumoral alfa (TNF-α). Estas citocinas induzem a expressão de moléculas de adesão nas paredes das células endoteliais vasculares, às quais neutrófilos,

monócitos e linfócitos se ligarão, favorecendo o aumento da permeabilidade celular e induzindo a coagulação (Breese et al., 1994; Danese, 2012; Garrett, Gordon, & Glimcher, 2010; Murch, Lamkin, Savage, Walker-Smith, & MacDonald, 1991).

O epitélio intestinal se situa entre as células imunes da lâmina própria e a microbiota luminal, exercendo o papel de comunicador entre ambos. Esta interação é necessária para a manutenção da homeostase intestinal, bem como para evitar danos teciduais (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004).

Alterações na barreira intestinal podem conduzir a uma ativação imune constante, sendo este fato de grande valia nos processos inflamatórios do trato gastrintestinal (Sartor, 2006). Este processo resulta no recrutamento de células imunes para o sitio afetado e consequentemente, na liberação de citocinas, como a interleucina-1 (IL-1), interleucina-6 (IL-6) e TNF-α. Estas citocinas induzem a produção de moléculas de adesão nas paredes das células endoteliais vasculares, as quais neutrófilos, linfócitos e monócitos se ligarão, aumentando a permeabilidade e induzindo a coagulação. Outras citocinas que participam deste processo são a Interleucina-8 (IL-8) e o interferon gama (IFN-γ). Todos estes efeitos resultam em edemas e no acumulo de células leucocitárias nas áreas inflamadas (Benjamini, Coico, & Sunshine, 2000).

Uma das principais fontes de citocinas são os linfócitos T *helper* (T_H1, T_H2 e T_H17), diferenciadas a partir de células T CD4. Durante a resposta imune inicial, os antígenos têm papel fundamental na seleção da via de diferenciação das células T_H0 indiferenciadas. A quantidade e o tipo de antígenos apresentados à estas células durante a estimulação imune primária, também influencia o desenvolvimento dos subgrupos T_H1, T_H2 e T_H17 (Benjamini et al., 2000).

A diferenciação das células T_H0 em células T_H1 se dá na presença de IL-12, uma citocina do sistema imune inato altamente expressa por macrófagos e células dendríticas. Estas células por sua vez secretam altos níveis de IFN-γ, um agente importante na ativação de macrófagos

(Zenewicz, Antov, & Flavell, 2009). Sendo ainda responsáveis pela secreção de IL-1, IL-2, IL-6, IL-12, IL-18 e TNF-α (Benjamini et al., 2000; Neuman, 2004).

Na presença de interleucina-4 (IL-4), nota-se a diferenciação T_H2 seguido do aumento da produção de IL-4, células responsáveis por secretar uma gama de citocinas com funções distintas. Dentre elas, podemos destacar a IL-4, IL-5, IL-9, IL-10, IL-13 e IL-25 (Abbas, Lichtman, & Pillai, 2010; Benjamini et al., 2000; Zenewicz et al., 2009).

Na presença de antígenos potencialmente patógenos, o sistema imune é recrutado e tem por função reconhecer e combater tais patógenos. Para tanto, as células imunes se valem de uma importante família de receptores, os receptores do tipo *Toll* (TLRs). A função deste grupo de receptores é preparar o sistema imune inato, com considerável especificidade, contra uma vasta gama de agentes microbianos, fornecendo ao hospedeiro um mecanismo de resposta imediata contra tais agentes. Estes eventos são de grande importância para o desencadeamento da resposta imune adaptativa e consequente combate à estes agentes (Levin & Shibolet, 2008; Testro & Visvanathan, 2009). Esta família de receptores possui nove membros em células mamíferas, sendo TLR4, TLR5, TLR6, TRL1 e TLR2 localizados na membrana plasmática e TLR3, TLR7/TLR8 e TLR9, localizados em membranas intracelulares. Cada receptor reconhece diferentes componentes microbianos, como é o caso do TLR4, o qual é ativado na presença de lipopolissacarídeos (LPS) e tem por finalidade estimular a expressão de interferons tipo 1 e a produção de TNF-α, IL-1, IL-6, IL-8 e IL-12p40 através da expressão do NF-κB (Takeda & Akira, 2004; Testro & Visvanathan, 2009).

O receptor TLR2, por sua vez, associa-se a TLR1 ou TLR6, reconhecendo bactérias Gram-positivas. Assim como o TLR4, estes receptores, quando ativados, resultam na ativação e expressão de NF-κB e, consequentemente, na produção de TNF-α, IL-1, IL-6. A flagelina, proteína presente nos flagelos, é reconhecida pelo receptor TLR5. Quando ativado, este receptor culmina na degradação de IκB e ativação do NF-κB, o que resulta na expressão de IL-8 e de oxido nítrico sintase induzida (iNOS) (Cario, 2008; McNeilly et al., 2010).

TLR3 é ativado na presença de RNA dupla fita (dsRNA), resultando na sinalização majoritária de NF-κB, e levando à expressão de interferons tipo 1, à produção de IL-6, IL-12 e TNF-α e à maturação de células dendríticas (Botos, Liu, Wang, Segal, & Davies, 2009). Já TLR7 e TLR8 são responsivos ao RNA de fita simples (ssRNA) presente em vírus, assim como RNAs de algumas bactérias (como as do grupo B *Streptococcus*), o que resulta novamente na expressão de interferons tipo 1 e do NF-κB que, por sua vez, induz a produção de citocinas pró-inflamatórias (Takeuchi & Akira, 2010). Por fim, o reconhecimento de dinucleotídeos CpG não-metilados, presentes em DNA bacteriano e viral, se dá através do TLR9, sendo que a sinalização desta via culmina novamente na expressão de interferons tipo 1 (O'Neill, Bryant, & Doyle, 2009).

1.4. Estresse Oxidativo

Quando instaurado, o processo inflamatório tem como resposta inicial o recrutamento de células imunes ao sitio afetado bem como o acúmulo destas células no local. Além dos mediadores inflamatórios já mencionados, estas células também secretam outro grupo de substâncias, como é o caso das enzimas da via oxidativa e as espécies reativas de oxigênio (EROs) (Allgayer, 1991).

As EROs são formadas a partir da redução do oxigênio molecular (O_2) ou de derivados de produtos da redução do O_2. Dentre elas, encontram-se moléculas e fragmentos moleculares com um ou mais elétrons desemparelhados, o que lhes confere um considerável grau de reatividade, sendo denominados, então, radicais livres. A produção basal destas espécies é responsável pela regulação de diversos eventos fisiológicos, como o controle do tônus muscular, a regulação do crescimento celular, além da diferenciação e migração celular. No entanto, apesar destes compostos serem de suma importância na sinalização celular, sua produção exacerbada pode resultar em efeitos deletérios (Martinon, 2010; Valko et al., 2007).

De forma a evitar os possíveis efeitos deletérios das EROs, as células se valem de mecanismos antioxidantes enzimáticos e não-enzimáticos que reduzem o poder reativo destas espécies, convertendo-as em espécies menos agressivas. Dentre as defesas enzimáticas,

encontra-se as enzimas superóxido dismutase (SOD), glutationa peroxidase (GSH-Px) e catalase (CAT). Os mecanismos não enzimáticos, por outro lado, são representados pelo ácido ascórbico (vitamina C), α-tocoferol (vitamina E), glutationa (GSH), carotenóides, flavonóides, dentre outros antioxidantes (Valko et al., 2007). O desbalanço entre a formação destas EROs e a capacidade do organismo em eliminar tais radicais de forma a favorecer o acumulo destas EROs, instaura um estado celular conhecido com estresse oxidativo (Martinon, 2010).

Evidencias clinicas e experimentais sugerem um possível envolvimento das EROs na fisiopatologia das DII. No entanto, ainda não está claro se o aumento do estresse oxidativo no ambiente intestinal resulta de falhas metabólicas, especialmente nas mitocôndrias, ou é resultado da redução da capacidade de detoxificação. Deste modo, são necessárias mais análises de forma a acumular uma maior quantidade de dados sobre a participação das EROs na fisiopatologia das doenças inflamatórias intestinais (Piechota-Polanczyk & Fichna, 2014).

1.5. Terapêutica

De acordo com Mayer (2010), os fármacos desenvolvidos atualmente interagem com a microbiota ou com agentes mediadores da resposta imune, estimulando tanto a resposta inata quanto a resposta adaptativa. Desta forma, até os anos 90 os tratamentos vigentes para as DII eram focados na remissão dos sintomas, não atuando sobre a redução do processo inflamatório ou sobre a cicatrização da mucosa (Pineton de Chambrun, Peyrin-Biroulet, Lemann, & Colombel, 2010). Este cenário modificou-se após a década de 90, com a intensificação tanto dos estudos visando a redução do processo inflamatório e a cicatrização da mucosa. Desde então, a utilização de corticosteroides, aminossalicilatos e seus derivados, assim como a utilização de antibióticos, drogas imunomudolatórias e terapias biológicas foi intensificada. Como consequência, obteve-se uma redução significativa do processo inflamatório, bem como um aumento expressivo da cicatrização da mucosa. Entretanto, juntamente com estas metas alcançadas, estão atreladas uma série de efeitos adversos (Head & Jurenka, 2003; Piechota-Polanczyk & Fichna, 2014).

Com a intensificação dos estudos e, consequentemente, maior compreensão da fisiopatologia das DII, diversas abordagens terapêuticas tem surgindo, visando o entendimento dos mecanismos específicos de cada doença, com o intuito de proporcionar terapias mais eficientes e mais seguras (Nakamura, Nagahori, Kanai, & Watanabe, 2008). Dentre as novas abordagens terapêuticas, destacam-se as substâncias de origem natural, produzidas a partir do metabolismo secundário de plantas.

1.5.1. Plantas Medicinais

Plantas, bem como outros organismos unicelulares e multicelulares, contem moléculas orgânicas ubíquas (aminoácidos, carboidratos e ácidos graxos), denominadas metabólitos primários, essenciais para a estrutura básica e metabolismo da célula. Esses compostos também servem como substrato para a síntese de substâncias químicas denominadas metabólitos secundários, os quais se acumulam em menores concentrações e apresentam distribuições diferentes para cada espécie. Após a ingestão, alguns metabólitos secundários de plantas comestíveis interagem beneficamente, agindo como coativadores enzimáticos. A ingestão insuficiente desses metabolitos pode estar associada à patogênese de algumas doenças (Eastwood, 2001). No intuito de avaliar tais compostos secundários frente a modelos de inflamação, nosso grupo avaliou diversas classes de metabólito secundários em modelos de úlcera (Batista et al., 2014; de-Faria, Almeida, Luiz-Ferreira, Dunder, et al., 2012; de-Faria, Almeida, Luiz-Ferreira, Takayama, et al., 2012; de Almeida et al., 2012; Luiz-Ferreira et al., 2012; Luiz-Ferreira et al., 2011; Takayama et al., 2011), colite (M. S. da Silva et al., 2010; de Almeida et al., 2013; de Faria et al., 2012; Manzo et al., 2015; Socca et al., 2014)e modelos de dor e inflamação (Dunder et al., 2013), obtendo resultados promissores sobre tais processos.

1.5.2. Alcaloides

Dentre os compostos de origem natural encontram-se os alcalóides, compostos nitrogenados, biologicamente e farmacologicamente ativos, encontrados predominantemente em angiospermas. De acordo com Pelletier (1983), "*um alcalóide seria uma substancia orgânica de origem natural, cíclica, contendo um nitrogênio em um estado de oxidação*

negativo e cuja distribuição é limitada entre os organismos vivos." Devido à variedade estrutural dos alcaloides, as atividades biológicas reportadas a este grupo de moléculas abrangem um grande espectro, podendo-se citar sua atividade anticolinérgica, anti-hipertensiva, antitumoral, antioxidante e anti-inflamatória (Simões & Mariot, 2003).

Os alcaloides indólicos representam um grupo de alcaloides caracterizados pela presença de um núcleo indol ou derivados em sua estrutura. Atualmente, este grupo possui grande importância econômica em vista de suas atividades farmacológicas. Dentre os alcalóides indólicos, pode-se citar a vincristina e vimblastina, com atividade antineoplásica e a elipticina e a olivacina, com atividade antitumoral (J. F. M. da Silva, Garden, & Pinto, 2001; Pelletier & ScienceDirect (Online service), 2001; Popp, 1969).

A isatina (1H-indol-2,3-diona), composto heterocíclico encontrado em diversas espécies vegetais, como é o caso das plantas do gênero *indigofera,* apresenta-se como um alcaloide indólico com atividades farmacológicas promissoras (Chiang, Li, Leu, Fang, & Lin, 2013; Medvedev, Igosheva, Crumeyrolle-Arias, & Glover, 2005). Este alcaloide foi obtido primeiramente por Erdman e Laurent em 1841, como produto da reação de oxidação do corante índigo pelos ácidos nítrico e crômico (J. F. M. da Silva et al., 2001).

Desde sua descoberta, esta molécula tem sido amplamente estudada. Três importantes revisões foram publicadas focando sobre as propriedades químicas deste composto, publicadas por Sumpter (1944), Popp, Oneill, and Donigan (1978) e por J. F. M. da Silva et al. (2001). Estes trabalhos exploraram a composição química desta molécula, bem como sua utilização na síntese de derivados heterocíclicos indólicos e quinolínicos. Curiosamente, esta molécula também foi encontrada em tecidos de mamíferos (Medvedev et al., 2005). Este fato, atrelado a versatilidade desta molécula em produzir derivados heterocíclicos, despertou o interesse em suas possíveis atividades biológicas e farmacológicas (J. F. M. da Silva et al., 2001).

Este composto apresenta significante importância na química medicinal devido sua grande variedade de atividades biológicas e um amplo espectro de atividades terapêuticas

(Kandasamy et al., 2010; Pakravan, Kashanian, Khodaei, & Harding, 2013). Dentre as atividades farmacológicas apresentadas,destacam-se a atividade anti-inflamatória (Andreani & Maselli, 1977) e a atividade antineoplásica (Maysinger, Ozegovic, Bokulic, & Movrin, 1981), dadas devido a modulação de mediadores pró-inflamatórios, como a produção de citocinas, a redução da infiltração de células imunes, além da inibição da expressão de cicloxigenase 2 (COX-2) (Kandasamy et al., 2010).

Entretanto, os efeitos testados desse alcaloide indólico não foram ainda estudados em modelos de doenças inflamatórias intestinais. Portanto, neste trabalho, serão avaliados os efeitos do tratamento com o alcaloide isatina em modelo de colite ulcerativa induzida pelo ácido 2,4,6-trinitrobenzeno sulfônico (TNBS).

2. Artigo: “Inhibition of tumor factor alpha and cyclooxigenase-2 by Isatin: A molecular mechanism of protection against TNBS-induced colitis in rats.”

2.1. Objetivos

Avaliar macroscopicamente a formação do processo inflamatório e os possíveis efeitos de diferentes doses de isatina em modelo de colite aguda induzida pelo ácido 2,4,6-trinitrobenzeno sulfônico (TNBS).

Avaliar a produção de mediadores inflamatórios no processo inflamatório instaurado pelo TNBS, bem como verificar os possíveis efeitos preventivos de isatina sobre a produção destes mediadores em modelo agudo de colite ulcerativa induzida por TNBS.

Avaliar a produção de espécies reativas de oxigênio (EROs) no modelo inflamatório agudo induzido por TNBS, assim como verificar a os possíveis efeitos protetores desse alcaloide indólico sobre a produção destas EROs em modelo agudo de inflamação intestinal induzido por TNBS.

2.2. Periódico publicado

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2.3. Correspondência de aceite

Date: Nov 28, 2013
To: "Eduardo Augusto Rabelo Socca" eduardo_socca@yahoo.com.br,cinza03n@gmail.com
From: "Chemico-Biological Interactions" cbi@elsevier.com
Subject: Your Submission

Ms. Ref. No.: CHEMBIOINT-D-13-00359R1
Title: Inhibition of tumor necrosis factor and cyclooxigenase-2 by Isatin: A molecular mechanism of protection against TNBS-induced colitis in rats.
Chemico-Biological Interactions

Dear Dr. Rabelo Socca,

I am pleased to inform you that your manuscript entitled "Inhibition of tumor necrosis factor and cyclooxigenase-2 by Isatin: A molecular mechanism of protection against TNBS-induced colitis in rats." has been accepted for publication in a forthcoming issue of Chemico-Biological Interactions.

I have forwarded the manuscript to the publisher, from whom you will receive the galleys and a reprint order form. Any inquiries regarding the status of your paper should from then on be addressed to the publisher. Please ensure that the manuscript number appears on any correspondence.

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With kind regards,

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2.4. Artigo

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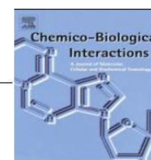

Inhibition of tumor necrosis factor-alpha and cyclooxigenase-2 by Isatin: A molecular mechanism of protection against TNBS-induced colitis in rats

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Isatin, an indole alkaloid has been shown to have anti-microbial, anti-tumor and anti-inflammatory effects. Due to its findings, we evaluated whether this alkaloid would have any effect on TNBS-induced colitis.

Animals (male Unib:WH rats, aged 8 weeks old) were induced colitis through a rectal administration of 2,4,6-trinitrobenzene sulphonic acid using a catheter inserted 8 cm into the rectum of the animals. The rats were divided into two major groups: non-colitic and colitic. The colitic group was sub-divided into 6 groups (10 animals per group): colitic non-treated, Isatin 3; 6; 12.5; 18.75 and 25 mg/kg.

Our main results showed that the oral treatment with Isatin 6 and 25 mg/kg were capable of avoiding the increase in TNF-a, COX-2 and PGE_2 levels when compared to the colitic non-treated group. Interestingly, the same doses (6 and 25 mg/kg) were also capable of preventing the decrease in IL-10 levels comparing with the colitic non-treated group. The levels of MPO, (an indirect indicator of neutrophil presence), were also maintained lower than those of the colitic non-treated group. Isatin also prevented the decrease of SOD activity and increase of GSH-Px and GSH-Rd activity as well as the depletion of GSH levels.

In conclusion, both pre-treatments (6 and 25 mg/kg) were capable of protecting the gut mucosa against the injury caused by TNBS, through the combination of antioxidant and anti-inflammatory properties, which, together, showed a protective activity of the indole alkaloid Isatin.

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1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel diseases (IBD), are characterized by damage to the intestinal epithelium and deeper layers, which is caused by an excessive immune response directed against normal constituents of the gut microflora [1]. In addition to this excessive immune response, environmental and genetic factors have also been considered as the major causes of IBD and, due to this diverse etiology, a safe therapy has not been developed so far [2]. The current treatment guidelines recommend a variety of therapeutic options depending on the disease activity, such treatments include glucocorticosteroids, aminosalicylates, immunosuppressors and biological therapies [3–5]. Although there is a large arsenal for the treatment of IBD, it usually results in recovery and sometimes mucosal healing, but there is no cure [6]. In addition, due to adverse effects, the treatments can be limited. In this context, there is a need to find new and safe compounds to contribute to the prevention or even to the treatment of IBD.

Plant-derived molecules are considered to possess anti-inflammatory properties and therefore were proposed as an alternative natural approach to prevent or treat chronic inflammatory diseases [7].

Isatin, 1H-indole-2,3-dione, is a heterocyclic compound found in various medicinal plant species [8]. This compound has significant importance in medicinal chemistry by its wide variety of biological activities and its relevance in a broad range of drug therapies [9,10]. Among these activities, the modulation of proinflammatory mediators such as cytokine production, reduction of leukocyte infiltration, inhibition, expression and activity of inducible isoforms cyclooxygenases [11,12] were studied. Based on these studies, we hypothesized that the inhibition of these mediators by Isatin might be a useful therapy target for IBD treatment. In the present study, we evaluated the ability of Isatin to inhibit intestinal inflammation in a TNBS-induced colitis model in rats.

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2. Materials and methods

2.1. Animals

Male Unib: WH rats (n = 7, 150–250 g) from Central Animal House of the Universidade Estadual de Campinas (CEMIB-UNICAMP; São Paulo, Brazil) were used. The animals were fed a certified Nuvilab (Nuvital) diet with free access to tap water under standard conditions of 12 h dark—12 h light, humidity (60 ± 1.0%) and temperature (21 ± 1 C). The experimental protocols were approved by the Institutional Animal Care and Use Committee (CEUA/IB/UNICAMP, No. 2773-1).

2.2. Induction of colitis and assessment of the inflammatory process

Colitis was induced using the method originally described by Morris [13]. Briefly, animals were fasted overnight and then anaesthetized with halothane. Under anesthesia, they were administered 10 mg of trinitrobenzenesulphonic acid (TNBS) dissolved in 0.25 ml 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm into the anus. During and after TNBS administration, the rats remained in a head-down position until they recovered from the anesthesia. Rats from the non-colitic (normal) group received 0.25 ml of saline instead of TNBS.

2.3. Acute colitis

Rats received doses of 3, 6, 12.5, 18.75 and 25 mg/kg of Isatin orally at 72, 48, 24 and 2 h before colitis induction. The drugs were administered by means of an oesophageal catheter (volume: 10 ml/kg). Two additional groups were included for reference: a non-colitic group that received saline intracolonically and the oral vehicle and a colitic group that received only the first dose of TNBS and the vehicle orally. The body weight of the animals, the occurrence of diarrhea (as detected by perianal fur soiling) were recorded daily. Animals from all groups (n = 8) were killed by CO_2 gas 72 h after colitis induction.

The colonic segments were obtained after laparotomy, and the occurrence of adhesions between the colon and adjacent organs was noted. The colonic segments were placed on an ice-cold plate, cleaned (fat removed) and then blotted on filter paper. The colon was weighed, and its length was measured under a constant load (2 g). The colon was opened longitudinally and scored for macroscopically visible damage on a 0–10 scale by two observers blinded to the treatments, according to the criteria described by Bell [14]. The colon was subsequently divided longitudinally into different pieces to be used for the following biochemical assays.

2.4. Superoxide dismutase activity (SOD)

SOD activity was analyzed by the reduction of nitroblue tetrazolium using a Xanthine–Xanthine oxidase system, that is, superoxide generation [15]. The results were expressed as SOD units per mg of protein.

2.5. Glutathione (GSH) content

Total GSH content was quantified with the recycling assay described by Anderson [16]. Samples were thawed, minced, diluted to a concentration of 1:20 (w/v) in ice-cold 5% (w/v) trichloroacetic acid and homogenized. The homogenates were centrifuged at 9000g for 15 min at 4 C, and the supernatants were used to quantify glutathione content. The results were expressed as nmol per mg of protein.

2.6. Glutathione peroxidase activity (GSH-Px)

GSH-Px activity was quantified by following the decrease in absorbance at 365 nm induced by 0.25 mM H_2O_2 in the presence of reduced glutathione (10 mM), NADPH, (4 mM), and 1 U enzymatic activity of GSH-Rd [17]. Results were expressed as nmol per min per mg of protein.

2.7. Glutathione reductase activity (GSH-Rd)

GSH-Rd activity was measured according to Carlberg and Mannervick [18], following the decrease in absorbance at 340 nm induced by oxidized glutathione in the presence of NADPH in phosphate buffer, pH 7.8. Absorbance changes were read between 1 and 10 min. Results were expressed as nmol per min per mg of protein.

2.8. Myeloperoxidase (MPO) activity

MPO activity was measured according to the technique described by Krawisz [19]. Samples were suspended in 1 ml of 50 mM phosphate buffer incorporating 0.5% hexadecyltrimethylammonium bromide (pH 6.0) and minced with scissors for 15 s on an ice-cold plate. The resultant suspension was subsequently diluted to a final 1:20 w/v ratio, homogenized for 1 min with an automatic Heidolph homogeniser, sonicated for 10 s and subjected to three freeze–thaw cycles. The homogenates were then centrifuged at 7000g and 4 C for 10 min, and the supernatants were assayed for MPO activity. The results were expressed as MPO units per g of protein.

2.9. IFN-c and IL-10 levels

Colonic samples for the determination of IFN-c and IL-10 levels were weighed, homogenized, minced on an ice-cold plate and suspended in a centrifugation tube with 10 mmol/L sodium phosphate buffer (pH 7.4) (1:5 w/v). The tubes were placed in a shaker submersed in a water bath (37 C) for 20 min and then centrifuged at 9000g for 30 s at 4 C; the supernatants were frozen at 80 C until assayed. IFN-c and IL-10 levels were quantified by ELISA MAX™ Delux Sets to measure natural and recombinant rat enzyme (Biolegend Inc., CA USA). The results were expressed as pg per mg of protein.

2.10. Isolation of cytoplasmic proteins and Western blotting analysis

Frozen colonic tissues were weighed and homogenized in icecold buffer [50 mM HEPES pH 7.9; 1 M KCl; 1 M $MgCl_2$; 0.1 M EDTA; 0.1 M NaF; 1% protease inhibitor cocktail (Sigma–Aldrich Co., MO, USA)]. Homogenates were incubated for 10 min in ice and centrifuged (12,000g, 45 min, 4 C). Cytoplasm proteins were collected from the supernatants. Protein concentration of the homogenate was determined following Bradford colorimetric method (Bradford, 1976). Aliquots of supernatants containing equal amounts of protein (100 lg) were separated on 10% acrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In the next step, the proteins were transferred onto a nitrocellulose membrane and incubated with specific primary antibodies for COX-1, COX-2 (Cayman Chemical, MI, USA) and TNF-a (R&D Systems, MN, USA) all diluted at 1:500. Each membrane was washed three times for 10 min and incubated with the secondary horseradish peroxidase-linked anti-rabbit for COX-1 and COX-2) (Santa Cruz Biotechnology, CA, USA) and anti-hamster for TNF-a (Invitrogen, NY, USA) all diluted at 1:5000. To prove equal loading, the blots were analyzed with standard protein dye ponceau [20]. Immunodetection was performed using enhanced chemiluminescence lightdetecting kit (SuperSignal West Femto Chemiluminescent Substrate, Pierce, IL, USA). Densitometric data were performed with G-BOX, Syngene following normalization to the control (ponceau) by GeneSys software.

2.11. PGE_2 levels

Colonic samples, for the determination of PGE_2, were weighed, homogenized, minced on an ice-cold plate and suspended in a centrifugation tube with 10 mmol/L sodium phosphate buffer (pH 7.4) (1:5 w/v). The tubes were placed in a shaker submersed in a water bath (37 C) for 20 min and then centrifuged at 9000g for 30 s at 4 C; the supernatants were frozen at 80 C until assayed. The prostaglandin E2 levels were quantified with an immune-enzymatic dosage kit from R&D Systems (USA) and the results expressed as pg per micro liter.

2.12. Statistical analysis

Results were expressed as mean ± standard error of means (SEM). The statistical significance of each test group in relation to the control was calculated using two-way ANOVA followed by Dunnet or Tukey's t-test.

3. **Results**

3.1. Protective effects of Isatin pre-treatment in TNBS-induced colitismodel

Rats challenged with TNBS revealed severe loss in body weight and chronic diarrhea. Macroscopic inspection of the cecum, colon and rectum showed that the colitic group presented severe colonic mucosal damage, with oedema, moderated adhesion to adjacent organs and deep ulcerations when compared with the animals that were not challenged with TNBS (Table 1). In addition, a significant increase in the colon weight/length ratio of the colon, an indicator of inflammation, was observed in colitic rats. On the other hand, rats pre-treated with Isatin revealed less body weight loss at doses of 6 mg/kg and 25 mg/kg with no diarrhea at the dose of 6 mg/kg.

3.2. Effect of Isatin pre-treatment on superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Rd) activity

Effect of Isatin pre-treatment on colon SOD, GSH-Px and GSHRd activities is shown in Table 2. The decrease of SOD activity in TNBS-induced colitis group was found to be significant (2.64 ± 0.17 o SOD per protein mg) when compared to non-colitic group (6.03 ± 1.95 SOD per mg of protein). The Isatin treatment (6 mg/kg B. wt.) prevented this decrease (4.62 ± 0.28), while the Isatin treatment (25 mg/kg B. wt.) did not prevent SOD activity decrease (2.51 ± 0.29). GSH-Px was found to be significantly increased ($p < 0.001$) in colitic control colon (12.33 ± 1.37 nmol/ min/protein mg), but this increase in enzyme activity was significantly ($p < 0.001$) prevented in Isatin pre-treatment (9.34 ± 0.27 and 7.58 ± 0.57 nmol/min/protein mg at 6 mg/kg and 25 mg/kg respectively). Similarly, GSH-Rd activity was found to be significantly increased ($p < 0.05$) after TNBS challenge (14.92 ± 1.32 nmol/min/protein mg). Isatin 6 mg/kg pre-treatment was found to significantly prevent ($p < 0.05$) the increase of enzyme activity (8.14 ± 1.87 nmol/min/protein mg) when compared to colitic group, however, Isatin 25 mg/kg pre-treatment did not prevent the increase in GSH-Rd activity (11.66 ± 5.43 nmol/min/ protein mg) when compared to colitic group. Non-colitic colon activities of SOD, GSH-Px and GSH-Rd were found to be 6.03 ± 1.95 SOD per mg of protein, 7.21 ± 0.87 nmol/min/protein mg and 9.25 ± 0.24 nmol/min/protein mg respectively (Table 2).

3.3. Determination of the glutathione (GSH) content in colon pretreated with Isatin

The first parameter of oxidative stress induced by TNBS in rat colon was measured by assessing glutathione (GSH) levels. As shown in Fig. 1, TNBS significantly reduced ($p < 0.01$) GSH levels in rat colon when compared with non-colitic group (0.78 ± 0.13 versus 2.97 ± 0.34 nmol per mg of protein), while Isatin pre-treatment 6 and 25 mg/kg was able to significantly prevent ($p < 0.05$) GSH reduction after TNBS challenge (2.10 ± 0.31 and 2.13 ± 0.44 nmol per mg of protein, respectively).

Table 1

Body weight changes and macroscopic results following experimental colitis and treatment with Isatin. Data expressed by mean ± SEM. Body weight changes and colon weight/ length ratio data were analyzed by ANOVA followed by Dunnett's t test. Adhesion, diarrhea and lesion score were analyzed by parametric Kruskal–Wallis test followed by Dunn's test. *p < 0.05; **p < 0.01; ***p < 0.001 significantly different from colitic group. Results were expressed as mean ± standard error of means (SEM).

Group	Body weight changes (g)	Adhesions score (0–2)	Diarrhea score (0–1)	Colon weight/length ratio (mg/cm)	Lesion score (0–10)
Non-colitic	1.80 ± 2.77***	0.0***	0.0**	0.10±0.01***	0.0***
Colitic	19.16 ± 4.26	1.0	1.0	0.16 ± 0.02	9.0
Isatin 3 mg/kg	11.33 ± 7.60	1.0	1.0	0.18 ± 0.02	8.0
Isatin 6 mg/kg	9.16 ± 1.33*	0.0***	0.0**	0.17 ± 0.03	5.5**
Isatin 12.5 mg/kg	13.5 ± 7.63	1.0	1.0	0.14 ± 0.02	7.5
Isatin 18.75 mg/kg	14.5 ± 3.39	1.0	1.0	0.15 ± 0.01	8.0
Isatin 25 mg/kg	7.57 ± 3.41**	1.0	1.0	0.15 ± 0.01	6.0*

Table 2

Effect of Isatin selected doses on colonic antioxidant enzymes in TNBS-induced colitis in rats. Data expressed as mean ± SEM ANOVA followed by Dunnetts t test. *p < 0.05; ***p < 0.001 significantly different from colitic group.

Group	SOD (SOD/protein mg)	GSH-Px (nmol/min/mg of protein)	GSH-Gr (nmol/min/mg of protein)
Non-colitic	6.03 ± 1.95***	7.21 ± 0.87***	9.25 ± 0.24*
Colitic	2.64 ± 0.17	12.33 ± 1.37	14.92 ± 1.32
Isatin 6 mg/kg	4.62 ± 0.28*	9.34 ± 0.27***	8.14 ± 1.87*
Isatin 25 mg/kg	2.51 ± 0.29	7.58 ± 0.57***	11.66 ± 5.43

Table 3

Effect of Isatin selected doses on colonic IFN-c, PGE2 and IL-10 in TNBS-induced colitis in rats. Data expressed as mean ± SEM ANOVA followed by Dunnetts test, *p < 0.05; **p < 0.01, ***p < 0.001; significantly different from colitic group.

Group	IFN-γ (pg/mg of protein)	PGE_2 (pg/mg of protein)	IL-10 (pg/mg of protein)
Non-colitic	2.27 ± 0.85***	620.20 ± 167.80**	22.00 ± 3.31***
Colitic	7.74 ± 0.69	1123.00 ± 74.57	9.65 ± 2.07
Isatin 6 mg/kg	5.23 ± 0.35***	733.70 ± 219.00*	23.15 ± 5.75***
Isatin 25 mg/kg	3.98 ± 0.63***	400.40 ± 114.20***	17.84 ± 2.10*

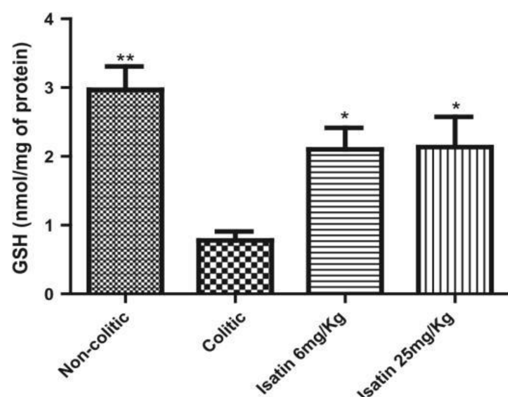

Fig. 1. Effect of Isatin on tissue glutathione levels in TNBS-induced colitis. Data expressed as mean ± SEM ANOVA followed by Dunnetts t test. ⁄p < 0.05; ⁄⁄p < 0.01 significantly different from colitic group.

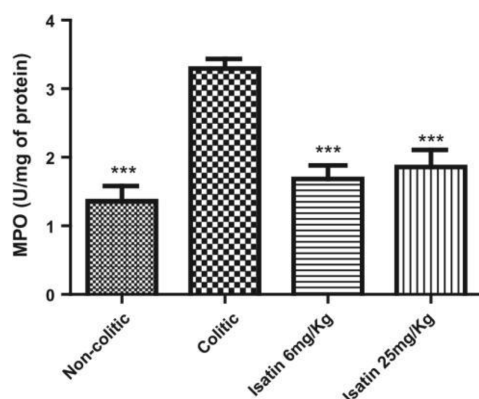

Fig. 2. Effect of Isatin pre-treatment in MPO activity in TNBS-induced colitis model. Data expressed as mean ± SEM ANOVA followed by Dunnetts t test. ⁄⁄⁄p < 0.001 significantly different from colitic group.

3.4. Effect of pre-treatment with Isatin on colon myeloperoxidase (MPO) activity during TNBS-induced colitis

The effect of pre-treatment with Isatin 6 and 25 mg/kg on colon myeloperoxidase (MPO) activity is shown in Fig. 2. The significant increase in MPO activity in colitic group (3.29 ± 0.14 U per mg of protein) was found to be significantly prevented by both pre-treatments with Isatin (1.686 ± 0.19 and 1.86 ± 0.25 U per mg of protein, $p < 0.001$). Non-colitic MPO activity was found to be 1.36 ± 0.22 U per mg of protein.

3.5. Assessment of colonic IFN-c, PGE_2 and IL-10 levels

Colonic levels of IFN-c, PGE_2 and IL-10 are shown in Table 3. In this acute TNBS challenge model, colitic samples showed a significant increase

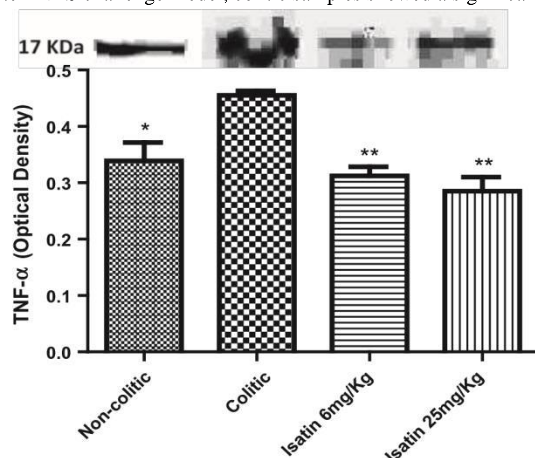

Fig. 3. Analysis of TNF-a protein expression by Western blotting. Densitometry data were analyzed following normalization with ponceau. Data expressed as mean ± SEM ANOVA followed by Dunnetts t test, ⁄p < 0.05; ⁄⁄p < 0.01 significantly different from colitic group.

in IFN-c levels (7.74 ± 0.69 pg per mg of protein). On the other hand, Isatin pre-treatment (6 and 25 mg/kg) was able to significantly prevent the increase in IFN-c levels (5.24 ± 0.35 and 3.98 ± 0.63 pg per mg of protein, respectively). In addition, we measured PGE_2 levels of colonic tissues, and TNBS challenge colon showed a significant increase in the prostanoid levels (1123 ± 74.57 pg per mg of protein). Both Isatin pre-treatments were able to prevent PGE_2 production (733.70 ± 219.00 and 400.40 ± 114.20 pg per mg of protein, respectively) with significant differences from colitic group. The colitic control group the TNBS control group samples showed a significant decrease ($p < 0.001$) in IL-10 levels (9.65 ± 2.07 pg per mg of protein). In this assay, Isatin pre-treatment showed to be able to significantly prevent ($p < 0.001$ and $p < 0.05$ respectively) IL-10 decrease levels (23.15 ± 5.75 and 17.84 ± 2.10 pg per mg of protein respectively) when compared to colitic group. Non-colitic levels of IFN-c, PGE_2 and IL-10 (Table 3) were 2.27 ± 0.85; 620.2 ± 167.80 and 22.00 ± 3.31 pg per mg of protein respectively.

3.6. *Analysis of TNF-a, COX-1 and COX-2 protein expression*

TNF-a, COX-1 and COX-2 expression were measured by Western blotting of cytosolic extract from colonic mucosa (Fig. 3 and Fig. 4). In this assay, TNF-a levels from colitic group showed a significantly increased ($p < 0.05$) after TNBS exposure (0.45 ± 0.01), while both Isatin pre-treatments showed to significantly prevent ($p < 0.01$) cytokine increased levels (0.31 ± 0.02 and 0.28 ± 0.02 respectively). COX-1 remained unchanged in all groups, indicating that COX-1 protein was constitutively expressed in colonic tissue and has no significant alterations after TNBS exposure or in presence of Isatin pre-treatment. Colitic tissues and pre-treated COX-1 expressions are 0.60 ± 0.04; 0.50 ± 0.03 and 0.53 ± 0.04 respectively.

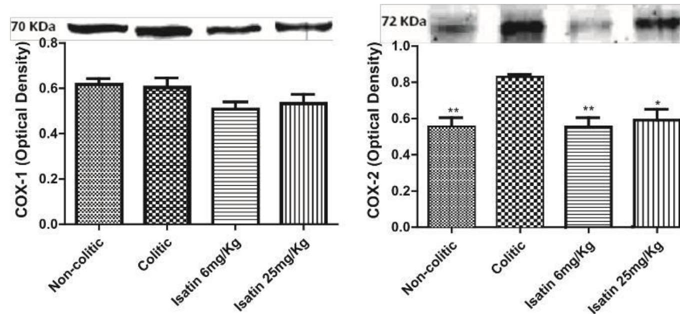

Fig. 4. Analysis of COX-1 (A) and COX-2 (B) protein expression by Western blotting. Densitometry data were analyzed following normalization with ponceau. Data expressed as mean ± SEM ANOVA followed by Dunnetts test, $*p < 0.05$; $**p < 0.01$ significantly different from colitic group

On the other hand, COX-2 protein expression was significantly increased ($p < 0.01$) after TNBS exposure (0.83 ± 0.01) and both Isatin pre-treatments were able to significantly prevent ($p < 0.01$ and $p < 0.05$ respectively) COX-2 expression (0.55 ± 0.05 and 0.59 ± 0.06 respectively). Non-colitic tissue levels of TNF-a, COX-1 and COX-2 were 0.34 ± 0.03; 0.62 ± 0.03 and 0.55 ± 0.05 respectively.

4. Discussion

The present study evaluated the effects of Isatin (1H-indole-2,3dione) on trinitrobenzene sulfonic acid (TNBS) model of acute colitis. This model is cited by many authors as an important experimental model of ulcerative colitis, which is characterized by an acute inflammatory response, followed by thickening of the colon wall, ulcerations and immune cell infiltration. Due to increased cell infiltration, there is a raise in the production of pro-inflammatory cytokines and reactive oxygen species (ROS), which surpasses the intestinal antioxidant defenses, resulting in inflammation and oxidative damage [11,21–25].

To evaluate the possible anti-inflammatory activity of Isatin, a few macroscopic parameters, such as weight evolution, diarrhea, adhesion and an inflammation score index [14] were evaluated. With these results we were capable of observing that 6 and 25 mg/kg doses were efficient in protecting the intestinal mucosal against the hapten (Table 1). These two doses were than selected to subsequent biochemical analysis.

Reactive oxygen species (ROS) are free radicals that contain an oxygen atom and include hydrogen peroxide (H_2O_2), superoxide anion (O_2) and hydroxyl radical (OH). These molecules are highly reactive due to the presence of unpaired electrons in the valence shell and are produced as a byproduct of oxygen metabolism in mitochondria [26]. Its production regulates several physiological responses, such as control of vascular tone, regulation of cell growth and angiogenesis. However, its sustained production may cause cellular damage [26].

As inflammation is intimately related to the formation of reactive species, oxidative stress has been proposed as a mechanism underlying the pathophysiology of the IBD [27]. The redox state of intestinal epithelium plays a fundamental role in maintaining cell integrity and function. The antioxidant system, which comprises non-enzymatic antioxidants (GSH) and enzymatic antioxidants (SOD, GSH-Px, GSH-Rd) is the first defense line of intestinal mucosa [28].

During oxygen metabolism, molecular oxygen receives an additional electron, forming superoxide anion (O_2). Superoxide is considered the ‘‘primary’’ ROS, and can further interact with other molecules to generate ‘‘secondary’’ ROS. Its production occurs mostly within the mitochondria of a cell via NADPH oxidase or Xanthine oxidase [29]. Superoxide dismutase (SOD) is the key enzyme which inactivates superoxide by converting it into H2O2, a more stable metabolite. During oxidative stress and inflammatory process, SOD activity tends to be decreased on inflamed tissues, when compared with non-inflamed tissues [30]. This lower SOD activity may permit the accumulation of superoxide, and subsequently its oxidative action on the tissue, adhesion of circulating leucocytes to intestinal epithelium, through the expression of adhesion molecules [30]. Isatin pre-treatment was able to prevent the decrease in SOD activity only at the dose of 6 mg/kg, maintaining its activity close to that found in non-inflamed tissues (Table 2).

The conversion of superoxide into H2O2 diminishes the reactivity within the tissue, but does not abrogate it. Hydrogen peroxide may cause cellular damage too, even being a less reactive molecule [31]. To protect the system from the possible damages caused by H2O2, the antioxidant system has others enzymatic and non-enzymatic mechanisms. In this case, reduced glutathione (GSH), a non-enzymatic endogenous tripeptide, is primarily used in a glutathione peroxidase (GSH-Px)-dependent fashion [32,33]. Its depletion, a crucial event in gut injury during the inflammation, has already been confirmed by other authors in human and experimental models [33–35]. During the inflammatory process, GSH consumption enhanced, and its levels become lower than normal at the inflamed site, letting gut mucosa susceptive to oxidative and, subsequently, inflammatory damage [18,36].

In our study, GSH levels showed to be depleted in TNBS group, supporting data found in the literature [37]. Our results with Isatin treatment (6 mg/kg and 25 mg/kg) show that the alkaloid was efficient at preventing GSH depletion, keeping its levels similar to those found in non-inflamed tissues. This data suggest that Isatin has the capacity of protecting intestinal mucosa from injuries, probably by protecting the depletion of this antioxidant barrier, and so, avoiding the action of reactive species (Fig. 1).

However, GSH itself does not reduce H_2O_2 levels. This reaction is mediated by GSH-Px, an antioxidant enzyme that helps scavenging and inactivating H_2O_2, thereby, protecting tissues from deleterious damage caused by the peroxide. The enzyme catalyses the reaction of H_2O_2 and a variety of organic peroxides to the corresponding stable alcohols and water using GSH as the reducing agent [30]. Reports show that the cytosolic GSH-Px activity in rat colon tissues is altered in response to oxidative stress [30,38]. In our study, GSH-Px (Table 2) activity was showed to be increased in colitic group when compared with non-colitic group whereas both Isatin (6 mg/kg and 25 mg/kg) treatments prevented the activation of this enzyme. This result supports those found in GSH, since Isatin prevented GSH decrease and GSH-Px was less active.

In an attempt to restore GSH levels after H2O2 scavenging, another reaction is activated, mediated by glutathione reductase (GSH-Rd), which is crucial to maintain an intracellular reducing environment, which is crucial to cell against the oxidative stress [36]. Its inhibition leads to low levels of GSH, letting the cells susceptible to oxidative damages caused by H2O2, resulting in a state of oxidative stress [36,39]. In TNBS-induced colitis we observed that GSH-Rd activity was increased in colitic group when compared with non-colitic group. This data suggest that the system, in an attempt of restoring the reduced state of intestinal mucosa, generates more GSH. On the other hand, Isatin pre-treatment showed a similar activity to that of the non-colitic group at 6 mg/kg dose, however 25 mg/kg dose showed no significant difference with either colitic or non-colitic group (Table 2). The data obtained from SOD activity, GSH levels and GSH-Px and GSH-Rd suggest that preventive and protective effects of Isatin could be due, at least in part, to its antioxidant properties.

Still on inflammatory process, some immune cells are recruited to the inflamed site, including neutrophils and macrophages. These cells secrete inflammatory cytokines and the most abundant proinflammatory enzyme; myeloperoxidase (MPO). Thus, MPO activity is an indirect indication of inflammatory activity. MPO and pro-inflammatory cytokine levels are directly proportional to tissue neutrophils/macrophages content and therefore inflamed tissues present a higher activity of this enzyme [40]. The present study showed that both Isatin 6 mg/kg and 25 mg/kg prevented the increase of MPO, when compared with colitic group, showing no difference with non-colitic group. Moreover colitic animals showed a higher activity of the enzyme when compared with non-colitic group (Table 2). Its levels were kept close to that found in non-colitic group, suggesting a lower infiltration of immune cells at the inflamed site in treated animals.

Pro-inflammatory cytokines secreted by immune cells include tumor necrosis factor alpha (TNF-a) and interferon gamma (IFNc). The secretion of these inflammatory cytokines is an important event in immune response and its deregulation plays an important role in the pathophysiology of IBD, by orchestrating the immune inflammatory process that leads to epithelial barrier dysfunction and, subsequent, tissue damage [41,42].

TNF-a is one of the most important pro-inflammatory cytokines that directly influences some events such as proliferation, differentiation, cell death, modulation of gene transcription and inflammation [43–45]. Its expression results in epithelial barrier damage, initiation of apoptosis in epithelial cells, and initiation of chemokine secretion by colonic epithelial cells [44]. It's an important cytokine early released during the inflammatory response that plays an important role in TNBS-induced colitis and it is likely to be the key regulator of the inflammatory cascade in IBD [45]. Our results showed that TNF-a levels are increased in colitic group when compared with non-colitic group. On the other hand, Isatin pretreatment (6 and 25 mg/kg) prevented the increase of TNF-a expression when compared to colitic group.

It's important to notice that this dose does not exhibit significant difference with the noncolitic group, indicating a potential anti-inflammatory action (Fig. 3). TNF-a and MPO results indicate that Isatin pre-treatment may be effective in protecting the gut mucosa by preventing immune cell infiltration, and consequently, avoiding MPO activation and pro-inflammatory cytokine release.

This cytokine, together with IFN-c, are reported to mediate inflammatory conditions by increasing the permeability of the epithelial tight junction [46–48]. IFN-c modulatory effect over wounds is mediated by TNF-a and these two inflammatory cytokines are critical for wound repair [49]. In our experiment TNBS-induced colitis shows a higher concentration of IFN-c in colitic tissues, significant when compared with non-colitic tissues. However, animals that received pre-treatment with Isatin 6 mg/kg or 25 mg/kg showed lower concentrations of the cytokine, similar to those found in non-colitic group, indicating, again, the protective potential of this alkaloid (Table 3).

COX is a key enzyme in the conversion of arachidonic acid into prostaglandins. COX-1 is constitutively and is a critical housekeeping enzyme, whereas COX-2 is rapidly up-regulated by growth factor and cytokines, and thus responsible for inflammation [50]. TNF-a acts as a modulator of the expression of COX-2, enhancing it during inflammation. Studies have shown that COX-2 is predominantly expressed in experimental colitis [37,45]. In this work, we observed that COX-1 presented no alterations across the inflammatory process in all groups (Fig. 4a), corroborating with literature [37,51]. On the other hand, colitic group showed high COX-2 expression, when compared with non-colitic group. However, Isatin 6 mg/kg and 25 mg/kg prevented the expression of the enzyme, corroborating with data found in TNF-a assay (Fig. 4b).This data suggest that the protection of the intestinal mucosa, attributed to Isatin, against the severe inflammatory process, results in reduced levels of TNF-a and consequently reduced COX-2 expression. It is important to notice that the pre-treatment with the alkaloid showed no significant difference with non-colitic group, showing that this alkaloid is effective in gut protection against the hapten.

During inflammation, proinflammatory cytokines, such as TNFa, are released into the inflamed site, enhancing COX-2 expression and, subsequently, the synthesis of prostaglandin E2 (PGE_2) [52,53]. This prostanoid is a key mediator in IBD [54] and it appears to have a dual effect [55]. High levels of this prostanoid are told to exacerbate inflammatory process, while its signaling is required for suppression of colitis symptoms and protection of intestinal mucosa against damage by maintaining the integrity of epithelial wall [54–56]. Here we observed that colitic tissues showed increased expression of COX-2 and, consequently, increased levels of PGE_2 against non-colitic tissues. Isatin pre-treatment animals (6 and 25 mg/kg) showed a reduced inflammatory process and, as result, a lower COX-2 expression subsequently lower PGE_2 levels, indicating that these doses were effective in prevent PGE_2 overproduction. It is important to notice that the Isatin pre-treatment showed very similar PGE_2 levels when compared with non-colitic tissues (Table 3).

Another cytokine released during infiltration process is interleukin (IL)-10, which is well known as a strong immunosuppressive, mainly through its negative effects on antigen presentation [57]. IL-10 play an important role in some models of colitis, by down-regulating the inflammatory process and restoring gut mucosa to a state which negative regulation is dominant [57,58]. During IBD process, we observed that IL-10 levels are suppressed in colonic tissues, when compared with non-colitic group, confirming findings of other authors [59,60]. On the other hand, Isatin pretreatment with 6 mg/kg or 25 mg/kg was able to prevent IL-10 suppression (Table 3), suggesting that this results in a better modulation of inflammatory process and protection of gut mucosa.

In summary, Isatin pre-treatment with 6 mg/kg or 25 mg/kg can significantly prevent the outcome of TNBS-induced colitis, through an intimate correlation between antioxidant and anti-inflammatory properties of this indole alkaloid. These results open new perspectives and also evidence the need for further investigations of the possible action mechanism involving this alkaloid and IBD pathophysiology.

Conflict of interest

None declared.

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2.5. Conclusões

Quando administrado previamente, tanto a dose de 6 mg/Kg quanto a dose de 25 mg/Kg do alcaloide indólico isatina protegeu de forma efetiva a mucosa intestinal contra o processo inflamatório induzido pelo ácido 2,4,6-trinitrobenzeno sulfônico (TNBS), por meio de uma relação intrínseca entre propriedades anti-inflamatórias e antioxidantes. Verificamos que a dose de 6 mg/Kg foi eficaz em prevenir o aumento dos níveis de da atividade da enzima mieloperoxidade (MPO), bem como o aumento dos níveis de TNF-α, IFN-γ e COX-2, e a queda dos níveis de IL-10. Tal efeito também foi verificado na dose de 25mg/Kg. Entretanto, esta dose não foi eficaz em prevenir a formação de certos mediadores, tendo como consequência uma proteção menos efetiva quando comparada com a dose de 6mg/Kg. Estes resultados abrem novas perspectivas, assim como demandam estudos mais detalhados sobre os mecanismos de ação deste alcaloide indólico durante os processos inflamatórios, como é o caso do protocolo experimental utilizado neste trabalho.

2.6. Considerações

O pré-tratamento com isatina elenca importantes questões sobre este grupo de alcaloides. Como demonstrado neste trabalho, o alcaloide exerce efeitos protetores, evitando o aumento dos níveis de certos mediadores pró-inflamatórios, bem como evitando a queda significativa de IL-10, citocina de caráter anti-inflamatório. O composto foi ainda efetivo na proteção contra o estresse oxidativo, estado este atrelado a severidade do processo inflamatório instaurado. Desta forma, estes resultados demandam estudos mais aprofundados sobre os efeitos protetores deste alcaloide não somente em modelos de colite ulcerativa, mas também em outros modelos experimentais.

3. Artigo: “Anti-inflammatory properties of isatin: An inhibitory mechanism of tumor necrosis factor alpha and interleukin-1 beta”.

3.1. Objetivos

Verificar as características macroscópicas e bioquímicas do processo inflamatório induzido pelo ácido 2,4,6-trinitrobenzeno sulfônico, bem como os efeitos do tratamento com o alcalóide indólico isatina sobre a mucosa inflamada após três e sete dias de desafio.

Avaliar macroscopicamente os efeitos da dose única de 6 mg/Kg de isatina em modelo agudo de colite ulcerativa induzida pelo ácido 2,4,6-trinitrobenzeno sulfônico (TNBS).

Avaliar a produção de mediadores pró-inflamatórios, bem como os efeitos benéficos do tratamento com isatina sobre a mucosa intestinal em modelo inflamatório instaurado pelo TNBS.

Verificar a participação de mediadores do processo de estresse oxidativo sobre a inflamação da mucosa intestinal, bem como os possíveis efeitos benéficos do tratamento com o alcalóide indólico.

3.2. Periódico submetido

International Journal of Colorectal Disease (fator de impacto 2014 – 2,449)

3.3. Correspondência da revista

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3.4. Artigo

Abstract

Aims: To investigate the effects of isatin on the intestinal mucosa damaged by 2,4,6- trinitrobenzene sulfonic acid (TNBS).

Main methods: Colitis was induced by TNBS in 50% ethanol (40 mg/mL; 0,25mL) given intrarectally to male Unib-Wh rats. Isatin (6 mg/Kg) was administered orally two hours after the induction of colitis. Non-colitic and colitic control group received saline orally simultaneously. Animals were then sacrificed on the third and seventh day after induction. Macroscopic analysis and biochemical assays were realized to evaluate the action mechanism involving this alkaloid in the treatment of TNBS inflammation.

Key findings: Macroscopic results showed that animals treated once with the alkaloid had reduction of diarrhea ($p<0,01$ and $p<0,05$ respectively), adhesion ($p<0,001$ for both days), colon weight/length ratio ($p<0,01$ and $p<0,001$ respectively) and inflammation score index ($p<0,01$ and $p<0,001$ respectively). In biochemical assays, we noticed that isatin treatment was effective in reducing the inflammatory process on the third day and the antioxidant and anti-inflammatory activity were maintained until seventh day. The alkaloid was effective in increasing GSH content ($p<0,001$ for both days), increasing GSH-Px activity ($p<0,001$ and $p<0,05$ respectively) and reducing MPO activity ($p<0,001$ and $p<0,05$ respectively). Isatin single dose also reduced TNF-α levels ($p<0,001$ for both days), IFN-γ ($p<0,01$ and $p<0,001$ respectively), IL-1β ($p<0,001$ for both days), IL-12 ($p<0,01$ for both days), PGE2 ($p<0001$ for both days) and increasing IL-10 levels ($p<0,01$ and $p<0,001$ respectively).

Significance: These results shows that isatin, when administered on the first day after induction of the colitis, reduced successfully the inflammatory process.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic intestinal manifestation that has two major forms: Crohn's disease (CD) and ulcerative colitis (UC). These inflammatory conditions are characterize by damage to epithelial layer and deeper layer, which is caused by a mix of genetic and environmental factors into an susceptive immune system(Monteleone, Caruso, & Pallone, 2014). Due to this multifactorial etiology a safe therapy has not been developed yet(Ko & Auyeung, 2014).

The current clinical guidelines (Regueiro, Loftus, Steinhart, Cohen, & Inflammatory Bowel Disease, 2006)for IBD recommend different therapeutic approaches such as glucocorticoids, aminosalicylates, immunosuppressors and biological therapies (Bernstein et al., 2010; Carter, Lobo, Travis, & Ibd Section, 2004; Travis et al., 2006). However, this variety of therapeutic approaches does not cure the disease. They only reduce the inflammatory process, letting the disease in a remissive phase (Bernstein, 2014). Together with remission of inflammatory process, these drugs are accomplished with a several number of adverse effects, such as paresthesias, hypertension, hypertrichosis and headache(Kornbluth, Sachar, & Practice Parameters Committee of the American College of, 2010). In this context, there is a need to find new compounds with anti-inflammatory activity and less side effects that contributes to IBD prevention and/or treatment.

Plant-derived molecules are considered to possess anti-inflammatory properties and therefore were proposed as an alternative natural approach to prevent or treat chronic inflammatory diseases (Chen, 2011; Kma, 2014).

Isatin, 1H-indole-2,3-dione, is a heterocyclic compound found in various medicinal plant species(Candido-Bacani et al., 2013). This compound has significant importance in medicinal chemistry by its wide variety of biological activities and its relevance in a broad range of drug therapies(Pakravan et al., 2013; Silva, 2013). Among these activities, the modulation of pro-inflammatory mediators such as cytokine production, reduction of leukocyte infiltration and also inhibition, expression and activity of inducible isoforms of NOS and COX(Kandasamy et al., 2010; Matheus, Violante Fde, Garden, Pinto, & Fernandes, 2007)were studied. Based on these studies, we hypothesized that the inhibition of these mediators by Isatin mightbe a useful therapy target for IBD treatment. In the present study, we evaluated the ability of Isatin to inhibit intestinal inflammation in a TNBS-induced colitis model in rats.

2. Materials and methods

2.1. Animals

Male Unib: WH rats (n = 7, 150–250 g) from Central Animal House of the Universidade Estadual de Campinas (CEMIB-UNICAMP; São Paulo, Brazil) were used. The animals fed a

certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark—12 h light, humidity (60 ± 1.0%) and temperature (21 ± 1 °C). The experimental protocols were approved by the Institutional Animal Care and Use Committee (CEUA/IB/UNICAMP, No. 2773-1).

2.2. Induction of colitis

Colitis was induced using the method originally described by Morris (1989). Briefly, animals were fasted overnight and then anaesthetized with halothane. Under anesthesia, they were administered 10 mg of trinitrobenzenesulphonic acid (TNBS) dissolved in 0.25 ml 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm into the anus. During and after TNBS administration, the rats remained in a head-down position until they recovered from the anesthesia. Rats from the non-colitic (normal) group received 0.25 ml of saline instead of TNBS.

2.3. Acute colitisand assessment of the inflammatory process

To evaluate the inflammatory process and the behavior of isatin against TNBS colon inflammation, we conducted two different protocols. In the first protocol animal were treated 2, 24 and 48 hours after TNBS challenge, being sacrificed after 72 hours. On the second protocol, animals were treated 2, 24, 48, 72, 96, 120 and 148 hours after induction, being sacrificed after 172 hours after TNBS challenge.

Therefore, rats from colitic untreated group and colitic treated group received the hapten while rats from non-colitic group received saline solution intra-rectally, as described above. Two hours after induction colitic rats from treated group received a 6 mg/Kg dose of isatin, while colitic untreated animals received the vehicle. The drug and vehicle were administered by means of an oroesophageal catheter (volume: 10 ml/kg). An additional group was included for reference: a non-colitic group that received saline intracolonically and the oral vehicle and a colitic group that received only the first dose of TNBS and the vehicle orally. The body weight of the animals, the occurrence of diarrhea (as detected by perianal fur soiling) were recorded daily. Animals from all groups (n = 10) were killed by CO2 gas three and seven after colitis induction, according protocols.

The colonic segments obtained after laparotomy, and the occurrence of adhesions between the colon and adjacent organs was note. The colonic segments were place on an ice-

cold plate, cleaned (fat removed) and then blotted on filter paper. We measure the weight and length of the colon under a constant load (2 g). The colon was then open longitudinally and scored for macroscopically visible damage on a 0–10 scale by two observers blinded to the treatments, according to the criteria described by Bell(Bell, Gall, & Wallace, 1995). The colon was then divide longitudinally into different pieces to be use for the following biochemical assays.

2.4. Glutathione (GSH) content

Total GSH content was quantified with the recycling assay described by Anderson (1985). Samples were thawed, minced, diluted to a concentration of 1:20 (w/v) in ice-cold 5% (w/v) trichloroacetic acid and homogenized. The homogenates were centrifuge at 9000g for 15 min at 4 °C, and the supernatants used to quantify glutathione content. The results were express as nmol per mg of protein.

2.5. Glutathione peroxidase activity (GSH-Px)

GSH-Px activity was quantified by following the decrease in absorbance at 365 nm induced by 0.25 mM H2O2 in the presence of reduced glutathione (10 mM), NADPH, (4 mM), and 1 U enzymatic activity of GSH-Rd(Yoshikawa et al., 1993). Results were express as nmol per min per mg of protein.

2.6. Myeloperoxidase (MPO) activity

MPO activity was measured according to the technique described by Krawisz(Krawisz, Sharon, & Stenson, 1984). Samples were suspend in 1 ml of 50 mM phosphate buffer incorporating 0.5% hexadecyltrimethylammonium bromide (pH 6.0) and minced with scissors for 15 s on an ice-cold plate. The resultant suspension was subsequently dilute to a final 1:20 w/v ratio, homogenized for 1 min with an automatic Heidolphhomogeniser, sonicated for 10 s and subjected to three freeze–thaw cycles. The homogenates were then centrifuge at 7000g and 4 °C for 10 min, and the supernatants were assay for MPO activity. The results were express as MPO units per g of protein.

2.7. IFN-γ, TNF-α, IL-10, IL-1β and IL-12 levels

Colonic samples for the determination of IFN-γ, TNF-α, IL-10, IL-1β and IL-12 levels were weighed, homogenized, minced on an ice-cold plate and suspended in a centrifugation tube with 10mmol/L sodium phosphate buffer (pH 7.4) (1:5 w/v). The tubes were place in a shaker submersed in a water bath (37 °C) for 20 min and then centrifuged at 9000g for 30 s at 4 °C; the supernatants were froze at −80 °C until assayed. IFN-γ, TNF-α, IL-10, IL-1β and IL-12

levels were quantified by ELISA MAX™ Delux Sets to measure natural and recombinant rat enzyme (Biolegend Inc., CA USA). The results were express as pg per mg of protein.

2.8. PGE2 levels

Colonic samples, for the determination of PGE2, were weighed, homogenized, minced on an ice-cold plate and suspended in a centrifugation tube with 10 mmol/L sodium phosphate buffer (pH 7.4) (1:5 w/v). The tubes were place in a shaker submersed in a water bath (37 °C) for 20 min and then centrifuged at 9000g for 30 s at 4 °C; the supernatants were froze at −80 °C until assayed. The prostaglandin E2 levels were quantified with an immune-enzymatic dosage kit from R&D Systems (USA) and the results expressed as pg per micro liter.

2.9. Statistical analysis

Results were express as mean ± standard error of means (SEM). The statistical significance of each test group in relation to the control was calculate using two-way ANOVA followed by Tukey's t-test.

3. Results

3.1. Macroscopic evaluation of colon rats challenged with TNBS

Rats challenged with TNBS revealed chronic diarrhea after three days of inflammation (table 1), been this symptom extended until seventh day (table 2). Macroscopic inspection of the cecum, colon and rectum showed that colitic animals had severe colonic mucosal damage with edema, extensive adhesion to adjacent organs and deep ulcerations, after three days (table 1), when compared with non-colitic animals. We also observed these results after seven days of inflammation (table 2). In addition, we observed a significant increase in the colon weight/length ratio, an indicator of inflammation, in colitic rats. On the other hand, animals treated with a single dose of isatin 6 mg/Kg showed after three days (table 1) reduction of diarrhea and a significant reduction of adhesion to adjacent organs. These animals also had lower colon weight/length ratio and score index showed a significant reduction of inflammation. On the seventh day (table 2) these results were maintained.

3.2. Effect of isatin treatment on glutathione (GSH) content and glutathione peroxidase (GSH-Px) activity.

The first parameter of oxidative stress induced by TNBS in rat colon was measure by assessing glutathione (GSH) levels. As shown in table 3 and 4, TNBS significantly reduced the levels of GSH on the third day, being maintain during all over the treatment. Isatin 6 mg/Kg single treatment, in contrast, was able to restore GSH levels significantly on the third day of

observation, and this result was maintained throughout the treatment, when compared with colitic group.

Isatin was also effective over the GSH-PX activity. On the third day of observation, we saw that the activity of this enzyme was reduce on the colitic untreated group, when compared with non-colitic group, but the animals that received either isatin treatment show a higher activity of the enzyme, when compared with colitic untreated animals. On seventh day, we observed a similar result in non-colitic and colitic untreated animals. Isatin 6 mg/Kg treatment showed a reduction of GSH_Px activity, however, the activity of the enzyme still presents significant difference versus colitic group.

3.3. Effect of isatin treatment on colon myeloperoxidase (MPO) activity during TNBS-induced colitis

The significant increase in MPO activity (table 3 and 4) in colitic group, compared with non-colitic group, was observe on the third day, being maintain all over the treatment. Isatin 6 mg/Kg treatment, similarly to non-colitic group, was able to restore MPO activity near to non-colitic MPO activity on the third day, being maintain throughout the next days.

3.4. Assessment of colonic IFN-γ, TNF-α, IL-12, IL-1β and IL-10 levels

The levels of colonic IFN-γ, TNF-α, IL-10, IL-1β and IL-12 are listed on Table 5 and table 6. The IFN-γ levels have proved significantly increased after three days of challenge with TNBS and remained increased throughout the seven days of procedure. The animals that received a 6 mg/Kg dose of isatin showed a reduction on the IFN-γ levels on the third day, and this result remained during all over the experiment. Still on table 5 and table 6, it can be observe TNF-α level. This cytokine shows to be significantly increase after challenge with TNBS, whereas isatin 6 mg/Kg dose group shows low levels of this substance on third and seventh day of observation. Another investigated cytokine was IL-12. This cytokine showed to be significantly increased in colitic group on third and seventh day of observation, when compared with non-colitic group, whereas treated group showed a significantly reduction on interleukin levels. IL-1β, another interleukin quantified, showed a significantly increase in colitic group after challenge with TNBS, but this increase in the cytokine levels were reverted in isatin group after three days of observation being this result remained over the following days. Finally, we quantified IL-10 levels on colic tissues. In this assay, we observed a reduction on the production of the cytokine on colitic group, when compared with non-colitic group. However animals that received a 6 mg/Kg dose of isatin showed a significantly increase in the production of the interleukin on the third day, and this result prevailed over the following days.

3.5. Evaluation of PGE2 levels in colonic tissues

PGE2 levels was evaluate with non-enzymatic dosage kit from R&D Systems. In this assay, we observed that animals of colitic group produced a significant quantity of the prostanoid after challenge with TNBS (table 5 and 6), when compared with non-colitic group. Animals that received a isatin 6 mg/Kg dose showed significant reduction in the production of PGE2, when compared with colitic group, on the third and the following days.

4. Discussion

Intestinal inflammation is accompanied by recruitment of immune cells and excessive production of pro-inflammatory cytokines and reactive oxygen species (Kruidenier & Verspaget, 2002; Pineton de Chambrun et al., 2010). In the last few years the therapeutic approaches for ulcerative colitis changed from mere treatment of symptoms towards significant alteration of disease behavior and maintenance of colic integrity (Pineton de Chambrun et al., 2010). Classical therapies include corticosteroids, aminosalicylates and immunomodulatory agents. These therapies are effective against gut inflammation evolution, however associated with high dependent-doses and subsequently high range of side effects. The prolongation of these treatments is associated with serious side effects such as adrenal suppression and atrophy, suppression of the secretion of corticotropin, anemia with Heinz body, reversible infertility in men, pancreatitis, susceptibility to infections and others side effects. It is noteworthy that these three therapeutical approaches are prescribed in combination with each other (Ko & Auyeung, 2014; Pineton de Chambrun et al., 2010). Novel investigation agents include anti-inflammatory cytokines therapies, antibiotics and/or probiotics usage, enteral nutrition, and herbal medicine. In this case, these therapies are still under investigation on their effectiveness and dose size. Here we present the indole alkaloid isatin (1H-indole-2,3-dione) as a possible therapeutic approach, with anti-inflammatory effectiveness, presented within seven days, on acute TNBS-induced colitis .

Recently we published a study showing its effects in animals pre-treated with this alkaloid over TNBS-colitis challenge. The study evaluated inflammatory and oxidative parameters and shows that animals pre-treated with 6and 25 mg/Kg isatin doses were had better protect against the action of the TNBS over their colon. The present study focus on one of the better doses of isatin, found on the previous work, to treat animals challenged with TNBS, besides to evaluate the evolution of the disease. Therefore, Unib/WS rats were divide into four groups. The first group contained animals that not received TNBS, being our positive control. The second group contained animals that were challenge with TNBS but not received any treatment. The third group contained animals that were challenge with TNBS and received a single dose of isatin two hours after TNBS induction. To evaluate the evolution of the disease and consequently the effects the treatment, we sacrificed half of each group on third day and the rest on seventh day.

To evaluate the possible anti-inflammatory properties of isatin 6 mg/Kg single dose, a few macroscopic parameters, such as diarrhea, adhesion and inflammation score index were evaluated (table 1 and 2). TNBS challenged animals showed a significant increase of adhesion of the colon to adjacent organs after three days of inflammation, being this symptom maintained until seventh day of inflammation. Animals treated with isatin, in turn, had no adhesion of the colon to adjacent organs. These animals also showed no diarrhea both in the third and seventh day after inflammation induction. Besides, this results are accompanied by a lower weight/length ratio of the colon and a lower score index of inflammation in isatin 6 mg/Kg treated animals.

The first evaluated biochemical parameter was reduced glutathione (GSH). This non-enzymatic tripeptide, is primarily used by glutathione peroxidase (GSH-Px) to reduce hydrogen peroxide (H_2O_2) into H_2O and O_2(Dodda, Chhajed, Mishra, & Padhy, 2014). During acute inflammation established by TNBS, GSH content reduced in the early days of inflammation (table 3), and this depletion is maintained all over the procedure (table 4)(Dodda et al., 2014; Orsi, Seito, & Di Stasi, 2014). It plays an vital role in maintaining the redox state of the cell by acting as a scavenger of reactive oxygen species (ROS) and keeping glutathione peroxidase (GSH-Px) in a reduced state (Ek et al., 2014). Our study reveals a depletion of GSH in colitic untreated group on the third day after challenge, when compared with non-colitic group (table 3), being this result maintained until the end of the treatment (table 4). Animals that received a 6 mg/Kg dose of isatin shows a reestablishment of this tripeptide on the third day (table 3) and its levels kept through all over the treatment (table 4). This data suggest that isatin has the capacity of protecting intestinal mucosa from injuries by reestablishing the antioxidant barrier, and so, avoiding the action of reactive species.

However, GSH itself does not reduce hydrogen peroxide (H_2O_2) levels. This action is mediated by glutathione peroxidase (GSH-Px), an antioxidant enzyme that helps scavenging and inactivating H_2O_2, thereby protecting tissues from deleterious damage caused by the peroxide (Kannan & Guruvayoorappan, 2013; J. Xing et al., 2013). Recently we published a work showing the effects of isatin over inflammatory process established by TNBS (Socca et al., 2014). Our results revealed that isatin 6 mg/Kg was effective in protect intestinal mucosa against TNBS, avoiding GSH depletion and increasing GSH-PX activity. In the present work, TNBS inflammation results in depletion of GSH and GSH-Px activity in colitic group on the third and seventh day (table 3 and 4), when compared with non-colitic group. In contrast, animals that received a single dose of isatin 6 mg/Kg shows an increase of the enzyme activity after three days of inflammation (table 3). After seven days, we analyzed again GSH-Px activity, and the test revealed a significant decrease on enzyme activity (table 4).

The results obtained in GSH-Px activity analysis match with GSH results. Both results shows a depletion of GSH levels and GSH-Px activity in animals that were challenge with

TNBS only. The severe inflammatory process established in these animals possibly results in a severe damage on intestinal mucosal barrier, which in turn resulted in the impairment of the barrier and consequently reduction of glutathione levels. However, GSH-Px activity in the same group was significantly reduced, which may indicates that GSH was not consume but exhausted due to TNBS Inflammation, corroborating data found with literature (J. Xing et al., 2013; J. F. Xing et al., 2012).

During inflammatory process, some immune cells are recruit to inflamed site, including macrophages and neutrophils. These cells secrete inflammatory cytokines and the most pro-inflammatory enzyme, myeloperoxidase (MPO). For this reason, MPO is an indirect indicator of inflammatory activity (Borrelli et al., 2013). The enzyme catalysis the reaction of H_2O_2 into hypochlorous acid (HOCl), a potent oxidant agent that mediate tissue damage (Pullar, Vissers, & Winterbourn, 2000). The present study shows that animals challenged with TNBS had a significate increase in MPO activity (table 3), suggesting increased production of HOCl. This result was then maintain until the end of the treatment (table 4). Animals that were challenge with TNBS and treated with isatin showed a significant reduced activity of MPO when compared with colitic untreated group, after three days (table 3) and the enzyme activity remained low until seventh day of observation (table 4). This data reinforces a possible powerful anti-inflammatory activity of isatin 6 mg/Kg.

Immune cells also secrete pro-inflammatory cytokines into inflamed tissue, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). The secretion of these inflammatory cytokines is an important event in the evolution of IBD and leads to epithelial barrier dysfunction, and subsequent tissue damage (Moussa et al., 2012; Nishitani et al., 2013).

TNF-α is one of the most important pro-inflammatory cytokine that directly influences some events such as proliferation, differentiation and inflammation. This cytokine has an important role in pathogenesis of IBD, by perpetuating inflammatory process in disrupted epithelial barrier, causing Th0 differentiation into Th1 cells and subsequently pro-inflammatory cytokine secretion into inflamed tissue (E. J. Cho et al., 2011; M. S. da Silva et al., 2010; de Almeida et al., 2013). Recently we published a work showing the effects of pre-treatment animals with 6 mg/Kg of isatin in a TNBS acute colitis model. The paper shows that isatin pre-treatment succeeded protect mucosal barrier against TNBS challenge.

In the present study, we show the effects of treatment with isatin 6 mg/Kg after three and seven days of inflammation induced by TNBS. The study reveals an increase in TNF-α level after three days of inflammation (table 5), being this effect maintained until seventh day (table 6). On third day we analyses the colon of animals treated with isatin 6 mg/Kg. The test shows a significant reduce in TNF-α content, in animals challenged with TNBS and treated with the alkaloid, on the third and seventh day of inflammation (table 5 and 6). This data reinforces the hypothesis that isatin has significant anti-inflammatory properties.

TNF-α also mediates the effects of another pro-inflammatory compound, interferon-gamma (IFN-γ). This cytokine is report to mediate inflammatory conditions by increasing epithelial tight junction permeability (Q. Wang et al., 2008; Zou et al., 2014), leading to tissue damage, septic shock and autoimmune diseases (Lim, 2011). In colitic tissues we found high levels of this cytokine, being this data confirmed by TNBS model(Zou et al., 2014). Our data shows high levels of the cytokine after three days of inflammation (table 5), compared with non-colitic group, and this result holds up until the end of the treatment (table 6). Isatin, on the other hand, seems to be benefic by reducing inflammatory conditions and subsequently reducing the production of IFN-γ after three and therefore the seventh day (table 5 and 6), corroborating the hypothesis low plasma concentrations of the alkaloid seems to be benefic.

Interleukin-1 beta (IL-1β), together with TNF-α plays an important role in colon tissues, being involved in the pathogenesis of colitis (Impellizzeri et al., 2014). After epithelial disruption, macrophages and other immune cells penetrate into adjacent tissues, stimulates T lymphocytes differentiation, and secrete pro-inflammatory cytokines, such as IL-1β (Bersudsky et al., 2014; Sahoo, Ceballos-Olvera, del Barrio, & Re, 2011). The present study confirmed a significant increase of IL-1β in inflamed tissue after three days of TNBS challenge (table 5), when compared with non-colitic group and this data was maintained on seventh day (table 6). Rats treated only on the first day with isatin 6 mg/Kg present significant decrease levels of the cytokine either on the third and seventh day of analysis, compared with colitic untreated group.

Interleukin 12 (IL-12) is a pro-inflammatory cytokine secreted by activated antigen presenting cells (APC) in response to sensing of microbial components by *Toll-like receptors* (TLR) (Krummen et al., 2010). Its function involves the induction of T-bet and controlling the differentiation of naïve T cells into IFN-γ-producing Th1 cells (Croxford, Kulig, & Becher, 2014), being this effect of great importance on the maintenance of inflammatory state like in ulcerative colitis (Croxford et al., 2014; Krummen et al., 2010).

The present study shows a significant increase in IL-12 content three days after induction of ulcerative colitis by TNBS (table 5) and the levels of the cytokine do not decreased after seven days of inflammation (table 6). Isatin, however, was able to significantly reduce IL-12 content in inflamed tissues, compared with colitic untreated tissues, already on the third day of procedure and this result maintained until the end of the study.

Another pro-inflammatory compound produced in presence of TNF-α is prostaglandin E2 (PGE2). During inflammatory process triggered by TNBS and consequently immune cells infiltration, TNF-α is release into inflamed tissue and enhancing COX-2 activity and, subsequently, prostaglandin E2 (PGE_2) synthesis(Montrose et al., 2014). The present work shows that inflammatory state, caused by TNBS in the intestinal mucosa, is follow by increase of pro-inflammatory cytokines like TNF-α. This process is responsible for COX-2 activation and PGE_2 synthesis, being in agreement with literature(Nagib, Tadros, ElSayed, & Khalifa,

2013). This prostanoid is an important mediator in ulcerative colitis, having a dual effect(D. Wang & Dubois, 2010). High levels of PGE_2 supposedly exacerbate inflammatory process, while basal levels of it are required for suppression of inflammatory symptoms and protection of intestinal mucosa, maintain epithelial integrity (Nagib et al., 2013; D. Wang & Dubois, 2010).

After three days of TNBS challenge (table 5), colitic tissues shows high levels of PGE_2 against non-colitic tissues, being this result maintained until seventh day (table 6). Isatin 6 mg/Kg instead, was able to reduce significantly the inflammatory state after three days of inflammation, resulting in reduction on the production of PGE_2. We also observed this effect of isatin seven days after TNBS challenge, which indicate that the alkaloid may have influence on NF-κB and subsequently COX-2 ways. This data reinforces the hypothesis that in the presence of the alkaloid the inflammatory process recede and as consequence, the levels of inflammatory cytokines and prostanoids decay.

Another cytokine released during inflammation is interleukin 10 (IL-10). This cytokine is a well known strong immunosuppressive, mainly through its negative effect over antigen presentation. IL-10 also plays an important role in inflammatory bowel diseases, including ulcerative colitis, by down-regulating inflammatory process and restoring intestinal mucosa to a state which negative regulation is dominant. During TNBS inflammation, we observe a suppression of IL-10 content, on the third day, in colonic tissues (table 5), when compared with non-colitic tissues, confirming findings of other authors. This effect of TNBS was also present on seventh day (table 6). On the other hand, isatin 6 mg/Kg single dose was able to reduce inflammatory process and increase IL-10 levels already on third day and this effect maintained until the end of the experiment.

In summary, this paper shows evidences of the beneficial effects of isatin 6 mg/Kg. We present here that a single treatment two hours after TNBS challenge was enough to reduce the inflammatory process instaured by the hapten stablishing an anti-inflammatory environment, helping the immune system to combat pro-inflammatory agents, through an intimate relationship between antioxidant and anti-inflammatory properties of isatin. These results open new perspectives and evidences the need for further investigation of the possible mechanisms involving this indole alkaloid and IBD pathophysiology.

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Table1

Group	Adhesions score (0–2)	Diarrhea score (0–1)	Colon weight/ length ratio (mg/cm)	Lesion Score (0-10)
Non-colitic	0,0 ***	0,0 **	0,101 ± 0,019 ***	0,0 ***
Colitic untreated	1,0	1,0	0,192 ± 0,015	9,0
Colitic + isatin 6 mg/Kg treatment	0,0 ***	0,0**	0,151 ± 0,017 **	6,0 **

Table2

Group	Adhesions score (0–2)	Diarrhea score (0–1)	Colon weight/ length ratio (mg/cm)	Lesion Score (0-10)
Non-colitic	0,0 ***	0,0 *	0,100 ± 0,009 ***	0,0 ***
Colitic untreated	1,0	1,0	0,196 ± 0,013	10,0
Colitic + isatin 6 mg/Kg treatment	0,0 ***	0,0*	0,14 ± 0,013 ***	7,0 ***

Table3

Group	GSH	GSH-PX	MPO
Non-colitic	8,61 ± 3,65 ***	8,52 ± 4,01 ***	7,70 ± 0,97 ***
Colitic untreated	3,64 ± 1,14	3,18 ± 0,87	15,80 ± 1,12
Colitic + isatin 6 mg/Kg treatment	7,82 ± 1,30 ***	8,10 ± 2,13 ***	9,72 ± 0,58 ***

Table4

Group	GSH	GSH-PX	MPO
Non-colitic	11,0 ± 1,6 ***	9,85 ± 2,97 ***	7,39 ± 0,75 **
Colitic untreated	3,2 ± 0,8	3,00 ± 0,98	13,29 ± 3,41
Colitic + isatin 6 mg/Kg treatment	8,6 ± 1,1 ***	5,80 ± 0,75 *	8,81 ± 1,16 *

Table 5

Group	TNF-α	IFN-γ	IL-1β	IL-12	PGE2	IL-10
Non-colitic	1,35 ± 0,61 ***	4,20 ± 1,71 **	13,84 ± 3,33 ***	14,44 ± 3,26 **	19,09 ± 2,62 ***	6,60 ± 0,35 **
Colitic untreated	6,32 ± 1,91	7,34 ± 0,48	35,91 ± 4,11	28,87 ± 8,39	31,07 ± 2,82	4,82 ± 0,26
Colitic + isatin 6 mg/Kg treatment	2,42 ± 0,72 ***	4,26 ± 1,15 **	16,54 ± 4,76 ***	13,38 ± 3,65 **	19,22 ± 3,97 ***	6,41 ± 0,60 **

Table 6

Group	TNF-α	IFN-γ	IL-1β	IL-12	PGE2	IL-10
Non-colitic	1,28 ± 0,42 ***	3,77 ± 1,42 ***	13,67 ± 3,82 ***	14,40 ± 4,26 ***	12,85 ± 2,82 ***	8,01 ± 0,72 ***
Colitic untreated	6,10 ± 1,14	9,69 ± 0,97	31,96 ± 2,90	32,57 ± 4,35	33,31 ± 4,36	5,16 ± 0,91
Colitic + isatin 6 mg/Kg treatment	2,62 ± 0,43 ***	4,98 ± 1,36 ***	16,78 ± 2,71 ***	19,88 ± 5,51 **	13,53 ± 3,39 ***	8,24 ± 0,40 ***

Table 1. Macroscopic results following experimental colitis and treatment with isatin 6 mg/kg after three days. Data expressed by mean ± SD. Weight/length ratio data analyzed by ANOVA followed by Bonferroni post-test. Adhesion, diarrhea and lesion score analyzed by nonparametric Kruskal-Wallis test followed by Dunn's test. ***p<0,001; **p<0,01 significantly different from colitic group.

Table 2. Macroscopic results following experimental colitis and treatment with isatin 6 mg/kg after seven days. Data expressed by mean ± SD. Weight/length ratio data analyzed by ANOVA followed by Bonferroni post-test. Adhesion, diarrhea and lesion score analyzed by nonparametric Kruskal-Wallis test followed by Dunn's test. ***p<0,001;*p<0,05 significantly different from colitic group.

Table 3.Effect of isatin on tissue glutathione content, glutathione peroxidase and myeloperoxidase activity after three days in TNBS-induced colitis rats. Data expressed as mean ± SD ANOVA followed by Bonferroni post-test, ***: p<0,001; **: p<0,01; *: p<0,05 significantly different from colitic group.

Table 4.Effect of isatin on tissue glutathione content, glutathione peroxidase and myeloperoxidase activity after seven days in TNBS-induced colitis rats. Data expressed as mean ± SD ANOVA followed by Bonferroni post-test, ***: p<0,001; **: p<0,01; *: p<0,05significantly different from colitic group.

Table 5. Effect of isatin 6 mg/Kg dose on colonic TNF-α, IFN-γ, IL-1β, IL-12, PGE2 and IL-10 content after three days in TNBS-induced colitis in rats. Data expressed as mean ± SD ANOVA followed by Bonferroni post-test, ***: p<0,001; **: p<0,01; *: p<0,05 significantly different from colitic group.

Table 6. Effect of isatin 6 mg/Kg dose on colonic TNF-α, IFN-γ, IL-1β, IL-12, PGE2 and IL-10 content after seven days in TNBS-induced colitis in rats. Data expressed as mean ± SD ANOVA followed by Bonferroni post-test, ***: p<0,001; **: p<0,01 significantly different from colitic group.

3.5. Conclusões

Efeitos benéficos do alcaloide indólico isatina foram demonstrados neste trabalho, sendo que o tratamento de dose única de isatina, duas horas após o desafio com TNBS, foi suficiente para reduzir a intensidade do processo inflamatório instaurado pelo ácido sobre a mucosa intestinal. O alcaloide auxiliou o sistema imune contra os agentes pró-inflamatórios, devido a uma relação intrínseca entre suas propriedades anti-inflamatórias e antioxidantes, previamente levantadas e demonstradas por este grupo de pesquisa. No entanto, os mecanismos pelos quais este alcaloide atua no processo inflamatório ainda não foram totalmente elucidados, fato este que demanda estudos direcionados às suas vias de atuação.

3.6. Considerações

O tratamento com isatina em modelo agudo de inflamação intestinal induzida por TNBS mostrou-se eficiente sobre os efeitos deletérios do ácido sobre a mucosa intestinal. Após três dias da indução, já verificamos um processo inflamatório local intenso, no qual é possível notar o espessamento do tecido bem como a severidade do processo inflamatório. Tal efeito é evidenciado novamente após sete dias da indução, demonstrando a evolução do processo instaurado.

Os efeitos deste alcaloide sobre a mucosa intestinal inflamada foram demonstrados neste trabalho, no qual verificou-se redução significativa nos níveis de alguns mediadores pró-inflamatórios, bem como o aumento dos níveis de um mediador anti-inflamatório, fato este que indica os efeitos anti-inflamatórios deste composto. Apesar de os efeitos e mecanismos de ação deste alcaloide ainda não estarem completamente elucidados, os resultados promissores encontrados, associados ao fato de ser encontrado no reino vegetal, e em tecidos de mamíferos, sugere que investigações mais profundas sejam conduzidas, afim de se abranger e estabelecer todos os efeitos biológicos e farmacológicos deste composto e de seus derivados.

4. Conclusão geral do trabalho

O trabalho aqui apresentado teve como objetivos principais avaliar os mecanismos de ação do alcaloide indólico isatina sobre a mucosa intestinal inflamada. Para tanto, seguimos dois protocolos experimentais de colite aguda, onde o agente indutor é o ácido 2,4,6-trinitrobenzeno sulfônico (TNBS). O primeiro protocolo teve por objetivo avaliar cinco diferentes doses do alcalóide como possíveis agentes protetores da mucosa, prevenindo o tecido cólico contra as ações do ácido. Neste protocolo avaliamos parâmetros macroscópicos, bioquímicos e moleculares no intuito de verificar se uma ou mais doses poderiam apresentar efeitos protetores, antioxidantes e anti-inflamatórios. Após a eutanásia e subsequente laparotomia, verificamos os parâmetros macroscópicos da lesão, onde evidenciamos um índice de lesão inflamatório significativamente elevado no grupo colítico ($p<0,001$). Notamos ainda a presença de diarreia ($p<0,001$) e a adesão do cólon à órgãos adjacentes ($p<0,001$), quando comparados ao grupo não colítico. Dentre os grupos pré-tratados, verificamos que somente os animais tratados com 6 ou 25mg/Kg de isatina apresentaram resultados significativamente diferentes do grupo colítico. Nestes grupos verificamos um menor índice de lesão ($p<0,01$ e $p<0,05$, respectivamente), ausência de diarreia no grupo pré-tratado com a dose de 6 mg/Kg ($p<0,001$) e de adesão do cólon ($p<0,001$), quando comparados ao grupo colítico. Em vista destes resultados, estas doses foram selecionadas para avaliações bioquímicas e moleculares, afim de verificar por quais mecanismos este alcalóide, nas doses selecionadas, exerce suas propriedades preventivas.

Após avaliação macroscópica, o cólon destes animais foi processado para análises bioquímicas e moleculares. Primeiramente, avaliamos os componentes do processo oxidativo, onde quantificamos os níveis de glutationa (GSH), bem como a atividade das enzimas superóxido dismutase (SOD), glutationa peroxidase (GSH-Px), glutationa redutase (GSH-Gr) e mieloperoxidase (MPO). Nestes ensaios, notamos uma queda significativa dos níveis de GSH nos animais colíticos ($p<0,01$), bem como a redução expressiva da atividade da SOD ($p<0,001$) e aumento significativo das atividades da MPO ($p<0,001$), GSH-Px ($p<0,001$) e GSH-Gr ($p<0,05$). Foi observado, ainda, que a dose de 6 mg/Kg de isatina foi eficaz em proteger a mucosa cólica contra os danos causados pelo TNBS. Neste grupo, os animais pré-tratados com esta dose, quando comparados ao grupo colítico, apresentaram níveis mais elevados de GSH ($p<0,05$) e maior atividade da SOD ($p<0,05$), bem como e redução da atividade da MPO ($p<0,001$), da GSH-Px ($p<0,001$) e da GSH-Gr ($p<0,05$). Já os animais pré-tratados com a dose de 25 mg/Kg de isatina, quando comparados aos animais colíticos, apresentaram níveis mais elevados de GSH ($p<0,05$), menor atividade da MPO ($p<0,001$) e da GSH-Px ($p<0,001$).

Após analisar as reações dos processos oxidativos, verificamos a atividade anti-inflamatória deste alcaloide. Nestes ensaios, foram avaliados os níveis de interferon gamma (IFN-γ), prostaglandina E_2 (PGE_2), interleucina-10 (IL-10), fator de necrose tumoral (TNF-α), e das cicloxigenases 1 e 2 (COX1 e COX2). Verificamos um aumento significativo dos níveis de IFN-γ ($p<0,001$), de PGE_2 ($p<0,01$), de TNF-α ($p<0,05$) e de COX2 ($p<0,01$), bem como a queda expressiva dos níveis de IL-10 ($p<0,001$). Os animais pré-tratados com isatina 6 mg/Kg, quando comparados ao grupo colítico, apresentaram menores níveis de IFN-γ ($p<0,001$), PGE_2($p<0,05$), TNF-α ($p<0,01$) e COX2 ($p<0,01$), assim como níveis mais elevados de IL-10 ($p<0,001$). Os animais pré-tratados com a dose de 25mg/Kg do alcaloide, comparados ao grupo colítico, apresentaram níveis menores de IFN-γ ($p<0,001$), PGE_2($p<0,001$), TNF-α ($p<0,01$) e COX2 ($p<0,05$), assim como níveis mais elevados de IL-10 ($p<0,05$). Não foram evidenciadas alterações nos níveis de COX1, devido ao fato desta cicloxigenase ser constitutiva, e por este motivo, não sofrer alterações durante o processo inflamatório.

No segundo protocolo experimental, avaliamos os efeitos da dose com resultados mais promissores em animais já inflamados. Para tanto, os animais foram desafiados por TNBS no primeiro dia e tratados com isatina 6 mg/Kg após 2 horas. Neste protocolo, os grupos avaliados foram compostos por animais não-colíticos, animais colíticos e animais colíticos tratados com isatina 6 mg/Kg. Três dias após o desafio, metade dos animais de cada grupo foi sacrificado, seguidos de retirada do cólon e avaliações macroscópicas. O mesmo procedimento foi realizado sete dias após a indução. Desta forma, foi possível avaliar a evolução do processo instaurado pelo TNBS, bem como os efeitos desencadeados pelo tratamento único com o alcaloide.

Avaliando o cólon dos animais do terceiro e do sétimo dia, verificamos o índice de lesão inflamatória significativamente maior em comparação ao grupo não-colítico ($p<0,001$ em ambos os dias), bem como adesão do cólon à órgãos adjacentes ($p<0,001$ em ambos os dias). Além disso, os animais tratados com 6 mg/Kg de isatina apresentara menores índices de lesão inflamatória em ambos os dias ($p<0,01$ e $p<0,001$ respectivamente) e ausência de adesão do cólon à órgãos adjacentes.

Em seguida o cólon destes animais foi processado para análises bioquímicas. Primeiramente avaliamos alguns parâmetros do processo oxidativo. Nestes ensaios verificamos a queda significativa dos níveis de GSH ($p<0,001$ em ambos os dias) e da atividade da GSH-Px ($p<0,001$ em ambos os dias), bem como o aumento significativo da atividade da MPO ($p<0,001$ e $p<0,01$ respectivamente). Já os animais tratados com isatina 6 mg/Kg apresentaram níveis mais elevados de GSH ($p<0,001$ em ambos os dias) e maior atividade da

GSH-Px ($p<0,001$ e $p<0,05$ respectivamente), além de menor atividade da MPO ($p<0,001$ e $p<0,05$ respectivamente).

Após avaliação da atividade antioxidante do alcaloide, verificamos a atividade anti-inflamatória da isatina. Nestes ensaios, notou-se um aumento expressivo dos níveis de TNF-α após três e sete dias de inflamação ($p<0,001$ em ambos os dias), IFN-γ ($p<0,01$ e $p<0,001$ respectivamente), IL-1β ($p<0,001$ em ambos os dias), IL-12 ($p<0,01$ e $p<0,001$ respectivamente), PGE_2 ($p<0,001$ em ambos os dias) e níveis mais baixos de IL-10 ($p<0,01$ e $p<0,001$ respectivamente). Já os animais tratados com o alcaloide, quando comparados aos animais colíticos, apresentaram níveis mais baixos de TNF-α ($p<0,001$ em ambos os dias), IFN-γ ($p<0,01$ e $p<0,001$ respectivamente), IL-1β ($p<0,001$ em ambos os dias), IL-12 ($p<0,01$ em ambos os dias), PGE_2 ($p<0,001$ em ambos os dias) e níveis mais elevados de IL-10 ($p<0,01$ e $p<0,001$ respectivamente).

Através da compilação dos resultados de ambos os protocolos foi possível concluir que a isatina, um alcaloide indólico endógeno, também encontrado em tecidos vegetais, é um candidato promissor para maiores investigações no tratamento de colite ulcerativa. Como apresentado, o composto apresenta uma relação intrínseca entre propriedades antioxidantes e anti-inflamatórias que culminaram na proteção da mucosa cólica contra os efeitos deletérios do ácido 2,4,6-trinitrobenzeno sulfônico, bem como na amenização de um processo inflamatório já instaurado pelo ácido.

No entanto, algumas análises ainda se fazem necessárias de forma a afirmar a real participação deste alcaloide na prevenção da mucosa intestinal e na redução do processo inflamatório. Para tanto, deve-se avaliar a via de sinalização que este alcaloide ativa ou inativa de forma a exercer seus efeitos farmacológicos na inflamação intestinal. Estes estudos, juntamente com estudos de mecanismos de ação em outros modelos inflamatórios, ajudarão a elucidar com maior precisão os efeitos deste alcalóide sobre os sistemas biológicos humanos, o que porventura poderá selecionar a isatina como um agente farmacológico em potencial no tratamento de certos processos inflamatórios, dentre eles as doenças inflamatórias intestinais.

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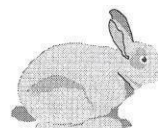
ANEXOS



CEUA/Unicamp

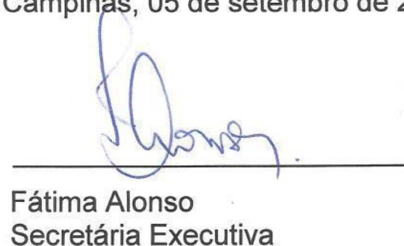
Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto "**Avaliação da atividade anti-inflamatória do alcalóide isatina em ratos submetidos ao modelo de colite ulcerativa induzida por ácido trinitrobenzenossulfônico**" (protocolo nº **2773-1**), sob a responsabilidade de **Profa. Dra. Alba Regina Monteiro Souza Brito / Eduardo Augusto Rabelo Socca**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **05 de setembro julho de 2012**.

Campinas, 05 de setembro de 2012.



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Atividades desenvolvidas

O desenvolvimento deste projeto possibilitou não só o desenvolvimento técnico, mas também uma formação aprofundada e direcionada à área farmacológica. Os estudos realizados neste período focaram a caracterização da atividade farmacológica de produtos naturais, visando sua aplicação no estudo de doenças inflamatórias intestinais. Durante sua execução, diversas atividades foram realizadas, no intuito de enriquecer a formação profissional do aluno, as quais estão descritas nesta seção.

Disciplinas cursadas

CÓDIGO DA DISCIPLINA	DISCIPLINA	DEPARTAMENTO	CONCEITO
MF802	Tópicos com Ênfase em Toxicologia de Sistemas	FCM	A
MF787	Desenho Experimental	FCM	A
MF800	Espécies Reativas e Oxigênio e Nitrogênio: Moduladores da Resposta Celular	FCM	A
MF720	Tópicos de Farmacologia 1	FCM	A
MF725	Tópicos de Farmacologia 6	FCM	A
MF748	Imunomodulação Produzida por Plantas Medicinais	FCM	A
MF791	Interações Droga-Receptor	FCM	A
MF733	Atualidades em Farmacologia	FCM	B
MF744	Temas Avançados em Farmacologia	FCM	B

Artigos publicados

1. MANZO, L. P. ; DE FARIA, F.M. ; DUNDER, R. J. ; **SOCCA, E. A. R**. ; CONSONNI, S. R. ; ALMEIDA, A. C. A ; SOUZA BRITO, A.R.M. ; LUIZ-FERREIRA, A. . Royal Jelly oral treatment and its dual role in TNBS colitis in mice. The Scientific World Journal, v. 2015, p. 956235, 2015. **Qualis B**

2. DUNDER, R. J. ; Luiz-Ferreira, Anderson ; ALMEIDA, A. C. A ; de-Faria, Felipe M. ; TAKAYAMA, C. ; **SOCCA, E. A. R**. ; SALVADOR, M. J. ; MELLO, G. C. ; dos Santos C. ; de Oliva-Neto P ; Souza-Brito, Alba R. M. . Applications of the hexanic fraction of Agave sisalana Perrine ex Engelm (Asparagaceae): control of inflammation and pain screening. Memórias do Instituto Oswaldo Cruz (Impresso), v. 108, p. 263-271, 2013. **Qualis B**

3. **RABELO SOCCA, EDUARDO AUGUSTO** ; Luiz-Ferreira, Anderson ; de Faria, Felipe Meira ; DE ALMEIDA, ANA CRISTINA ; Dunder, Ricardo José ; Manzo, Luis Paulo ; Souza Brito, Alba Regina Monteiro . Inhibition of tumor necrosis factor-alpha and cyclooxigenase-2 by Isatin: A molecular mechanism of protection against TNBS-induced colitis in rats. Chemico-Biological Interactions (Print), v. 209, p. 48-55, 2013. **Qualis A**

4. de Faria, Felipe Meira ; Luiz-Ferreira, Anderson ; **SOCCA, E. A. R.** ; de Almeida, Ana Cristina Alves ; Dunder, Ricardo José ; Manzo, Luis Paulo ; da Silva, Marcelo Aparecido ; VILEGAS, Wagner ; Rozza, Ariane Leite ; Pellizzon, Cláudia Helena ; dos Santos, Lourdes Campaner ; Souza Brito, Alba Regina Monteiro ; BRITO, ARMS . Effects of Rhizophora mangle on Experimental Colitis Induced by TNBS in Rats. Evidence-Based Complementary and Alternative Medicine (Online), v. 2012, p. 1-11, 2012. **Qualis A**

5. Takayama, Christiane ; de-Faria, Felipe Meira ; de Almeida, Ana Cristina Alves ; Valim-Araújo, Deborah de Arantes e Oliveira ; Rehen, Camilla Souza ; Dunder, Ricardo José ; **SOCCA, E. A. R.** ; Manzo, Luis Paulo ; Rozza, Ariane Leite ; Salvador, Marcos José ; Pellizzon, Claúdia Helena ; Hiruma-Lima, Clélia Akiko ; Luiz-Ferreira, Anderson ; Souza-Brito, Alba Regina Monteiro . Gastroprotective and ulcer healing effects of essential oil from Hyptis spicigera Lam. (Lamiaceae). Journal of Ethnopharmacology, v. 135, p. 147-155, 2011. **Qualis A**

6. Araujo, Deborah A. O. Valim ; Takayama, Christiane ; de-Faria, Felipe M. ; **Socca, Eduardo A. R.** ; Dunder, Ricardo J. ; Manzo, Luis P. ; Luiz-Ferreira, Anderson ; Souza-Brito, Alba R. M. . Gastroprotective effects of essential oil from Protium heptaphyllum on experimental gastric ulcer models in rats. Revista Brasileira de Farmacognosia (Impresso), v. 21, p. 721-729, 2011. **Qualis B**

Artigos submetidos

Revista Submetida: International Journal of Colorectal Disease (fator de impacto 2014 – 2,449)

Manuscript ID: IJCD-D-15-00519

Title: Anti-inflammatory properties of isatin: An inhibitory mechanism of tumor necrosis factor alpha and interleukin-1 beta

Authors: Eduardo Augusto Rabelo Socca, Anderson Luiz-Ferreira, Ana Cristina Alves de Almeida, Felipe Meira de Faria, Luis Paulo Manzo, Alba Regina Monteiro Souza Brito

Received Date: 23 June 2015

Participações em eventos, congressos e apresentação de resumos

1. de-Faria, Felipe Meira **; SOCCA, E. A. R**. ; ALMEIDA, A. C. A ; Dunder, Ricardo J. ; Manzo, Luis P. ; CONSONNI, S. R. ; LUIZ-FERREIRA, A. ; BRITO, ARMS . Avaliação preliminar de três extratos orgânicos obtidos a partir das cascas de Rhizophora mangle L. em modelo agudo de colite induzida por DSS em camundongos.. In: Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012, Águas de Lindóia.. Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012.

2. Dunder, Ricardo J. ; Luiz-Ferreira, Anderson ; ALMEIDA, A. C. A ; MANZO, L. P. ; DE FARIA, F.M. ; **SOCCA, E. A. R.** ; CONSONNI, S. R. ; SANTOS, R. C. ; GIACOPINNI, T. J. M. ; Souza-Brito, Alba R. M. . Propriedades analgésicas do alcalóide índigo através através de diferentes modelos de nocicepção.. In: Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012, Águas de Lindóia.. Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012.

3. GIACOPINNI, T. J. M. ; ALMEIDA, A. C. A ; MANZO, L. P. ; **SOCCA, E. A. R.** ; SALVADOR, M. J. ; DE FARIA, F.M. ; LUIZ-FERREIRA, A. ; DUNDER, R. J. ; Souza-Brito, Alba R. M. . Avaliação da composição química e das propriedades terapêuticas do extrato hidroalcólico de Caryocar brasiliensis (pequi) em modelos de inflamação e dor.. In: Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012, Águas de Lindóia.. Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012.

4. **SOCCA, E. A. R.** ; DE FARIA, F.M. ; DUNDER, R. J. ; ALMEIDA, A. C. A ; Manzo, Luis P. ; GIACOPINNI, T. J. M. ; LUIZ-FERREIRA, A. ; Souza-Brito, Alba R. M. . Avaliação da atividade do alcalóide isatina em ratos submetidos ao modelo de colite ulcerativa aguda induzida por ácido trinitrobenzenossulfônico. In: Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012, Águas de Lindóia.. Federação de Sociedades de Biologia Experimental-FESBE, 2012, 2012.

5. ALMEIDA, A. C. A ; DE FARIA, F.M. ; DUNDER, R. J. ; MANZO, L. P. ; **SOCCA, E. A. R.** ; LUIZ-FERREIRA, A. ; Souza-Brito, Alba R. M. . Effect of the alkaloid indigo in dextran sodium salt-induced colitis (DSS).. In: 44th Brazilian Congress of Pharmacology and Experimental Therapeutics,2012, 2012, Foz do Iguaçu.. 44th Brazilian Congress of Pharmacology and Experimental Therapeutics, 2012., 2012.

6. DE FARIA, F.M. ; LUIZ-FERREIRA, A. ; **SOCCA, E. A. R.** ; ALMEIDA, A. C. A ; MANZO, L. P. ; da SILVA, M. A. ; VILEGAS, W. ; Souza-Brito, Alba R. M. . Rhizophora mangle as an anti-inflammatory source of drug: role on cytokines in TNBS-induced colitis in rats.. In: 44th Brazilian Congress of Pharmacology and Experimental Therapeutics, 2012, 2012, Foz do Iguaçu.. 44th Brazilian Congress of Pharmacology and Experimental Therapeutics, 2012., 2012.

7. de-Faria, Felipe M. ; Luiz-Ferreira, Anderson ; **Socca, Eduardo A. R.** ; DUNDER, R. J. ; ALMEIDA, A. C. A ; da SILVA, M. A. ; SOUZA BRITO, A.R.M. . Rhizophora mangle L. ameliorates TNBS-induced colitis in rats through the expression of NF-kappaB.. In: XX - Congresso Italo-Latino-Americano de Etnomedicina -SILAE, 2011, Fortaleza. XX - Congresso Italo-Latino-Americano de Etnomedicina - SILAE, 2011.

8. XXVII-Federação de Sociedades de Biologia Experimental-FESBE. Avaliação da atividade do alcalóide isatina em ratos submetidos ao modelo de colite ulcerativa aguda induzida por ácido trinitrobenzenossulfônico. 2012. (Congresso).

Cursos Ministrados

1. I Simpósio de Ciências Biológicas do Sudeste Goiano. Inflamação e Dor. 2013. (Simpósio).

2. II curso de inverno de Fisiologia.Fisiologia do trato gastrointestinal.II curso de inverno de Fisiologia.Fisiologia do trato gastrointestinal. 2012. (Outra).

3. II Simpósio de Ciências Biológicas do Sudeste Goiano. Doenças Inflamatórias Intestinais. 2014. (Simpósio).