LUÍS PAULO BOGNONI MANZO

ESTUDO DOS EFEITOS DA GELEIA REAL E DA PROTEÍNA MRJP3 EM MODELOS DE COLITE INDUZIDA POR TNBS E DSS

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Grande abraço

Contents

1. RESUMO

A retocolite Ulcerativa Inespecífica e a Doença de Chron são as principais doenças inflamatórias intestinais. Apesar dos crescentes esforços, não se sabe ainda suas causas. Os tratamentos ainda não são eficazes, as drogas atuais são eficazes na indução da remissão mas não determinam sua cura. Os efeitos colaterais são severos, o que acarreta em baixa adesão ao tratamento. Os produtos naturais têm sido fonte de compostos usados por todo o mundo em diferentes áreas da medicina e ciência. O tratamento oral com geleia real na dose de 100 mg/Kg mostrou-se capaz de aumentar os níveis de grupamentos sulfidrila (GSH) e também a atividade da enzima glutationa peroxidase (GSH-Px) em camundongos com colite induzida por TNBS. A expressão de COX-2 e NF-κB também foram diminuidos, tais resultados demonstram efeitos antioxidantes e antiiflamatórios da geleia real nesta dose neste modelo de colite experimental. O Tratamento intrarretal com a proteína MRJP3 (50 μg/animal) foi capaz de diminuir os níveis de IL-1β no cólon dos camundongos Balb/c submetidos a colite induzida por DSS. Uma vez que os níveis de IL-6 e IL-10 não foram alterados pelo tratamento intrarretal com MRJP3 50 μg/animal, podemos afirmar que a proteína não atua pela via da IL-10.

Estes efeitos antioxidantes e anti –inflamatórios observados durante o desenvolvimento da colite induzida por TNBS e por DSS em camundongos, podem ser de significativa importância pois abrem portas e encorajam novas pesquisas com a geleia real e sua respectiva proteína MRJP3, motivando novos estudos acerca de outos agentes antioxidantes e outros mediadores anti-inflamatórios envolvidos nestes modelos de colite experimental.

2. ABSTRACT

Ulcerative Colitis and Chron`s Disease are the two major forms of IBD. Despite all efforts, these complications of the GTI are still lacking an effective therapy for their cure. Side effects, high cost and low adhesion to treatment are among the negative aspects. Natural products have been a source of widely used compounds in distinct areas of medicine and research. Royal Jelly (RJ) 100 mg/Kg was capable of augmenting the levels of glutathione (GSH) and the activity of glutathione peroxidase (GSH-Px) in mice undergoing TNBS-induced colitis. Furthermore, COX-2 and NF-κB had their expression decreased by the oral administration of RJ 100 mg/Kg. Intra-rectal MRJP3 treatment (50 μg/animal) was capable of decreasing the levels of IL-1β in mice undergoing DSS-induced colitis, whereas the levels of IL-10 and IL-6 were not altered by MRJP3 50 μg instillation, what indicates that this decrease is not IL-10-induced.

The antioxidant and anti-inflammatory effects observed during the development of DSS and TNBS-induced colitis in mice, may be of crucial importance, since they open and encourage new studies focused on this nourishing substance, especially in experimental models of colitis.

3. INTRODUÇÃO

As doenças inflamatórias intestinais (DII), sobretudo a Doença de Chron (DC) e a Retocolite Ulcerativa Inespecífica (RCUI) são de extrema importância para os sistemas privados e públicos de saúde, não só pela crescente prevalência, atingindo atualmente mais de 1,4 milhões de pacientes nos EUA e 2,2 milhões na Europa (1), sendo que no Brasil, a incidência acompanha a tendência mundial, porém, por não serem de notificação obrigatória, crê-se que o número de casos seja subestimado. Tanto a DC quanto a RCUI acometem o trato gastrintestinal, a sintomatologia também é parecida, incluindo-se: dores abdominais, diarréia severa, sangramento retal, eliminação de muco e, não raro, manifestações extra intestinais, tais como: articulares e dermatológicas (rashes cutâneos) (2). Apesar de suas similaridades, a doença de Chron geralmente manifesta-se ao longo de todo o trato gastrintestinal, desde a boca até o ânus, onde as lesões aparecem como ilhas isoladas de lesões, algo similar a uma colcha de retalhos (3). As lesões atravessam a barreira mucosa e chegam, por vezes a atingir a camada serosa (lesão transmural) (4). A RCUI é mais frequentemente restrita ao reto e ao cólon descendente (colite esquerda)(5), caracteriza-se por um contínuo de lesão, ou seja, não há tecido saudável em meio ao tecido lesionado e as lesões são mais superficiais, não ultrapassando a mucosa (4). Sabe-se que essas duas formas de DII possuem diferentes graus de severidade, em casos extremos leva o paciente a óbito (6), mesmo em casos menos drásticos, constitui-se como fator debilitante. Acometem, majoritariamente, indivíduos de 20 a 40 anos, o que aumenta sua

importância devido ao impacto social e econômico, uma vez que os doentes se afastam de suas atividades rotineiras (trabalho e lazer)(7).

A etiologia da Doença de Chron e da Retocolite Ulcerativa Inespecífica ainda está por ser desvendada em sua plenitude. Sabe-se que são doenças multifatoriais e que portanto, várias causas estão intrincadamente envolvidas no aparecimento de ambas.

Algumas mutações em diferentes genes foram identificadas dentre os portadores de DII. Cho et al (8), relataram que uma disfunção do gene NOD2 (intracellular nucleotide oligomerization domain 2) está associada as DII; modulando a produção de citocinas anti e pró-inflamatórias, aumentando a expressão de moléculas co-estimuladoras levando a uma resposta de células T mais intensa, mediação de efeitos anti-bacterianos induzidos por vários diferentes mecanismos, contudo, não se sabe ao certo se uma mutaçào, isoladamente, pode ser responsável pelo aparecimento da doença. Sabe-se, porém que sua influência determina uma maior predisposição do indivíduo para o aparecimento do quadro inflamatório (9).

A poluição, a exposição a agentes químicos, físicos e biológicos, estilo de vida, tabagismo e dieta perfazem alguns dos fatores ambientais importantes para o desenvolvimento da RCUI e da DC. O tabagismo está relacionado ao desenvolvimento das DII de forma inversa na DC e na RCUI (10), na primeira, este parece ser um agente agressor, aumentando as chances de desenvolvimento, as chances de recidivas e também de cirurgias. Com a interrupção do consumo, ocorre uma diminuição desses parâmetros (11). Para a segunda, o tabagismo parece ter efeito protetor, uma vez que há relatos de pacientes que param de fumar e cursam com recidivas mais frequentemente (12). O tabaco apresenta

centenas de substâncias, destacando-se a nicotina e o monóxido de carbono que agem em diferentes alvos; produção de muco, de citocinas e na micro-circulação. Como estes agentes agem em cada um dos alvos pode variar de indivíduo para indivíduo (13).

Ingesta rica em ácidos graxos de cadeia longa (AGCL), como ω-6, presente em carnes vermelhas, frituras e *fast foods,* em detrimento de ácidos graxos de cadeia curta (AGCC), como o ômega 3 (ω-3), presente em peixes, frutos do mar, vegetais e frutas, está relacionada ao aumento na ocorrência das DII (14). O ácido Linoleico, um AGCL, é um precurssor do Ácido Aracdônico (AA) o qual dá origem a eicosanóides, alguns são importantes mediadores inflamatórios, cujos níveis estão frequentemente aumentados na mucosa intestinal de pacientes com RCUI (15). Vieira de Barros e colegas reportaram que uma dieta enriquecida com ω-3 e ômega 6 (ω-6), balanceadamente, mostrou-se benéfica em um modelo de colite experimental em ratos, evidenciando que um equilíbrio entre as duas formas de ácidos graxos é importante (16), ademais, o ω-3 já foi reconhecido como tendo propriedades anti-inflamatórias (17), uma vez que reduz os níveis de quimiocinas inflamatórias induzidas por ω-6, tais como; prostaglandina E2, tromboxano A2 e leucotrieno B4, envolvidos na permeabilidade vascular e quimiotaxia (18).

É sabido que o estresse afeta diretamente o ritmo e funcionamento do intestino; com impactos na secreção iônica, motilidade, resposta inflamatória e permeabilidade (19);(20). Walker e colegas relataram que pacientes de RCUI sofriam há mais tempo de algum tipo de oscilação de humor em relação a indivíduos controles (21), abrindo espaço para a relação direta entre fatores psicológicos e prevalência das DII. Esta relação é corroborada por experimentos de depressão em camundongos que desenvolveram colite e foram tratados, com

suceso, por terapias anti-depressão (22). De forma contrária, alguns autores afirmam que os elementos psicológicos não são determinantes para o surgimento da doença, estando muito mais relacionados à ocorrência de recidivas (23).

A microbiota intestinal tem papel fundamental na homeostase do intestino e também para o desenvolvimento das DII. Ratos criados em condições *germ free* (ausencia completa de microorganismos) não desenvolvem colite. Sabe que a presença dos microorganismos no intestino é fundamental, sobretudo a de bactérias, não se pode afirmar ainda qual ou quais bactérias são as responsáveis por induzir a inflamação, acredita-se que o conjunto microbiano seja necessário (24). A homeostase do intestino depende da tolerância do mesmo às bactérias ali presentes, assim, o papel da imunidade inata e adaptativa é de suma relevância; evitar uma explosão da população de microorganismos, através de um processo inflamatório contínuo e finamente regulado, evitando assim sua exacerbação. O descontrole da resposta imunológica frente aos microorganismos é um dos pilares da etiologia das DII. A resposta imune inata é tida como a mais importante, tendo em vista que camundongos deficientes de células T e B também desenvolvem colite severa (25). Esta resposta exagerada é desencadeada via, entre outras, receptores do tipo Toll (TLR), que reconhecem padrões moleculares associados a patógenos (PAMPS), como o lipopolissacarideo (LPS), presentes na parede celular de bactérias GRAM (-). A ativação desses receptores leva a produção de citocinas, quimiocinas e moléculas anti-microbianas, importantes no início da resposta imune inata.

O curso da inflamação, sobretudo na RCUI é marcado pelo intenso infiltrado celular, destacando-se a presença de neutrófilos e macrófagos (26). Os macrófagos liberam substâncias quimiotáxicas que atraem os neutrófilos para o

local de inflamação, estes, por sua vez liberam moléculas atrativas de monócitos (27) e outras substâncias moduladoras de inflamação, destacando-se as interleucinas. Por se tratar de um processo extremamente complexo, a inflamação intestinal é circundada por incongruências ainda não elucidadas e os tratamentos disponíbveis ainda não são eficazes para sua cura.

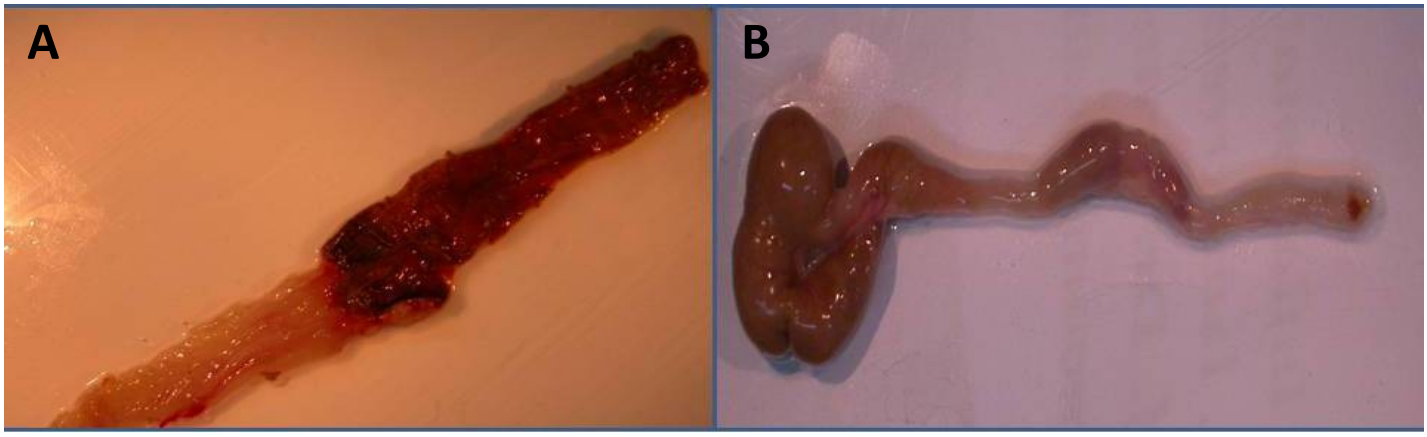
Os tratamentos atualmente disponíveis não curam as DII, induzem eficazmente a remissão e manutenção da inflamação. Na vanguarda estão as terapias biológicas como os anti TNF-α (Infliximab e Adalimumab), geralmente indicados àqueles pacientes que não respondem à terapia convencional; preparações de 5-ASA (Mesalazina e Sulfassalazina), glicocorticóides e imunossupressores (28).

Os produtos naturais sempre figuraram como importante fonte de novos compostos e vêm sendo utilizados para os mais diversos fins; úlcera gástrica (*Anacardium humile)* (29)*,* inflamação (30), e reumatismo (*Salix alba*) (31). Dentre vários outros exemplos, observamos a geléia real, substância produzida nas glândulas hipofarangeais de abelhas *Apis mellifera*. No animal, exerce uma função nutricional pouco compreendida, acredita-se que é fundamental no crescimento e desenvolvimento devido a sua grande concentração de proteínas e amino-ácidos (50% massa seca) (32), sabe-se contudo que seu consumo é determinante para a casta, plasticidade fenotípica e comportamento. Alguns de seus principais componentes bioativos são: *apisimin, royalisin* e as *Major Royal Jelly Proteins* (MRJP) que possuem efeitos imunorregulatórios, anti-bacterianos e anti-inflamatórios (33), (34), (35).

A família das MRJP é subdividida em 5 sub-famílias; 1,2,3,4 e 5, sendo que as mais estudadas são a 1 e a 3. A primeira apresenta atividade pro-inflamatória, aumentando a liberação e produção de TNF-α, um importante mediador

inflamatório em macrófagos de peritôneo de ratos (36). A MRJP 3, segundo Okamoto e colaboradores (37), possui efeito anti-inflamatório, capaz de diminuir a produção de IL-2, IL-4 e IFN-γ em cultura de células de baço e ainda, diminuir a produçào de IL-1β e TNF-α macrófagos ativados *in vitro* (37). Dada a variedade de aplicações e efeitos da Geléia Real, este trabalho teve como objetivo verificar se o tratamento oral com geleia real nas doses de 100, 150 e 200 mg/Kg exerce algum efeito benéfico em modelo de colite experimental induzida por ácido 2,4,6-trinitrobenzenossulfônico (TNBS) em camundongos, bem como se os efeitos da proteina MRJP3 observados *in vitro* poderiam ser extrapolados para um modelo *in vivo* de colite induzida por dextrana sal sodio (DSS) em camundongos. Estes são dois dos modelos de colite experimental mais utilizados pelos cientistas para o estudo das DII em animais, sobretudo, ratos e camundongos. Segundo Wirtz et al., (4) o TNBS é um hapteno que induz uma resposta imunológica direcionada para $Th_{1.}$ Ocorrendo uma lesão contínua e profunda (Figura 1a), ao passo que o DSS induz uma resposta imune polariazada para os linfócitos Th_2, sendo a lesão esparsa e superficial. (Figura 1b).

Figura 1: Lesão induzida por TNBS (A) e lesão induzida por DSS (B).



4. ARTIGO 1

4.1. Objetivos do Artigo 1:

Avaliar os efeitos do tratamento intrarretal com MRJP3 em modelo de colite experimental induzida por DSS em camundongos Balb/c.

Avaliar a produção de algumas citocinas envolvidas no processo inflamatório de colite induzida por DSS em camundongos e testar os possíveis efeitos da MRJP3 sobre essas citocinas.

Avaliar se os efeitos observados in vitro podem ser extrapolados para modelos *in vivo*, através de modelo de colite induzida por DSS em camundongos.

4.2. Título do Artigo 1

Cytokine response to MRJP3 intra-rectal treatment in DSS-induced colitis.

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4.4. Correspondência da revista relativa ao Artigo 1

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Cytokine response to MRJP3 intra-rectal treatment in DSS-induced colitis

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Abstract:

Ulcerative Colitis (UC) and Chron`s Disease (CD) are the two major forms of Inflammatory Bowell Diseases (IBD). Despite all efforts, these complications of the gastrointestinal tract (GIT) are still lacking an effective therapy for their cure. Side effects, high cost and low adhesion to treatment are among the negative aspects. Natural products have been a source of widely used compounds in distinct areas of medicine and research. Intra-rectal Major Royal Jelly Protein 3 (MRJP3) intra-rectal treatment (50 μg/animal) was capable of decreasing the levels of Interleukin 1-β (IL-1β) in mice undergoing (Dextran Sulfate Sodium Salt) DSS-induced colitis. The levels of Interleukin-10 (IL-10) and Interleukin 6 (IL-6) were not altered by MRJP3 50 μg instillation, on the other hand, intra-rectal instillation of MRJP3 (50 μg) exerted a reduction in the levels of IL-1β what indicates an important anti-inflammatory effect in the development of DSS-induced UC and, most importantly, opens and encourages new studies focused on this protein.

Keywords: IBD; ulcerative colitis; MRJP3; Royal Jelly; DSS; mice, interleukins

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1. Introduction

Ulcerative Colitis (UC) and Chron Disease (CD) are the two major forms of the inflammatory bowel diseases (IBD). Both UC and CD are characterized by an exacerbation of the immune system to the commensal microflora of the gut. The ethiology of both diseases are still not entirely elucidated and much effort is still needed. Currently, great importance for the initial development of the disease is given to the imbalance between pro-inflammatory (IL-1β, IL-6) and anti-inflammatory cytokines (IL-4, IL-10) (28). UC presents a continuous ulcerated area, usually restricted to the distal portion of the intestine whereas CD is spread throughout the digestive tract, from the mouth to the rectum in a patchy manner (3). The immunology of both diseases is similar but UC seems to be mediated by a polarized T_h2 response, associated with epithelial barrier dysfunction, on the other hand, CD is likely to be T_h1 polarized, with elevated levels of IL-2 and TNF-α (4). Another fundamental aspect of the IBD is the microflora-dependence for the occurrence of the inflammation, it has already been demonstrated that animals raised in germ free conditions do not develop the uncontrolled inflammation in the gut (38). Another study also investigated the role of the microflora and concluded that animals treated with anti-

TLR4 antibody reduced APC recruitment and consequently chemokines and cytokines production, what, in turn, reduced the severity of DSS-induced colitis (39). The network involving the cytokines is very intricate and complex, in summary, elevated levels of IL-1β and TNF-α are observed in patients with ongoing IBD, as well as, diminished levels of IL-10. It is also known that IL-10 deficient mice undergo spontaneous intestinal inflammation, similarly to humans (40) . The state of the art in IBD treatment is the, so called, biological therapy and among them are the already licensed anti-TNF-α antibody agents (Infliximab, Adalimumab) (28).

Although the therapeutics currently available for the treatment of IBD are effective in inducing remission(41), the causes of the inflammation are not "attacked" what remains as an obstacle to be surpassed. The efficacy of the drugs available in inducing and maintaining remission is, rather, accompanied by severe side effects what directly affects the adhesion to the treatment. In this context, natural products are still a promising niche for researchers to prospect potential natural derived formulation. Royal Jelly (RJ) is consumed worldwide for its myriad properties: antioxidant (32), vasodilative, hypotensive, disinfectant, antitumor and anti-inflammatory (42). Among the major constituents of the RJ, the Major Royal Jelly Protein (MRJP) family is of great interest due to its diverse effects (36). This family is subdivided in 5, MRJP 1-5 (43)and one of the most studied members is MRJP3 whcich has been described to have an immunomodulatory activity *in vitro*, decreasing the levels of IL-1β and TNF-α, pivotal cytokines in the course of inflammation. (37, 44).

Considering the fundamental role of the cytokines in the development of UC and the reported effects of MRJP3 on IL-1β and TNF-α, our group was led to investigate whether this protein could exert similar effects in Balb/c mice undergoing DSS-induced colitis.

2. Results and Discussion

In this study we describe the role of the Major Royal jelly Protein 3, a 70 kDa glycoprotein from Royal Jelly, during the development of acute experimental DSS-induced colitis on the production of pivotal cytokines of the innate immunity (IL-1β, IL-6 and IL-10). Continuous exposure of the mucosa to the commensal microflora triggers an immune response. The defense response induced by the microflora is usually severely controlled but in genetically predisposed individuals this ability seems to be lost and an exacerbated immune response takes place (41, 45). The UC arises from the impairment between pro and anti-inflammatory cytokines in the colon [14]. Since DSS is directly toxic to the epithelial cells of the

gut, an inflammatory reaction is required for further protection and posterior repair of the tissue. This inflammatory process initiated by the contact of the DSS with the mucosa, is added to the natural ongoing and continuous inflammation resulting in a more drastic inflammatory process (4). Interleukins are critical during the acute phase of inflammation, especially those of the innate immune response (TNF-α, IL-1β and IL-6) and according to Wirtz *et al*., 2003 (4) in the DSS-induced colitis, the adaptive immune response is not a major player in the development of the inflammation, at least in the acute phase, but rather, the innate immune system is. These cytokines can have its gene expression induced by the activation of NF-κB through TLR4 (Toll Like Receptor 4) activation, due to PAMP (pathogen-associated molecule pattern)-stimulation (39). Intra-rectal treatment with MRJP3 at three different doses (7.5; 25, 50 µg/ animal) were tested, although the highest dose (50 µg) was the only one that seemed capable of decreasing the weight/length ratio of the colon, when comparing to DSS group (non-treated group) (table 1). This result led us to perform further biochemical analyses using this dose.

Table 1. Weight/ Length ratio of 3 different doses (7,5; 25 and 50 µg) of MRJP3 intra-rectal treatment. Results are presented as mean ± S.E.M. ANOVA followed by Dunnett's t test, *P<0.05, ** p< 0.001, significantly different from DSS group.

GROUPS	Weight/Length (mg/cm)
SHAM	27,01 ± 2,857***
DSS	38,66 ± 3,957
MRJP3 7,5 µg	32,38 ± 2,521
MRJP3 25 µg	33,56 ± 3,747
MRJP3 50 µg	28,02 ± 2,160***

The intra-rectal treatment with MRJP3 at the dose of 50 µg has decreased the levels of IL-1β in the colon of the animals (table 1). Neither IL-6 or IL-10, an anti-inflammatory cytokine (46), was altered by the

instillation of MRJP3, no significant statistical differences were observed for these purposes (figures 2 and 3) respectively, corroborating with the hypothesis of the innate immunity being more important during the initial phase of DSS-induced colitis (4), considering that T-cells are the main source of IL-10 (47) and are mostly involved in the adaptive immune response.

Figure 1. Effect of intra-rectal MRJP3 (50µg) treatment on colonic IL-1β levels in mice undergoing DSS-induced colitis. Results are presented as mean ± S.E.M. ANOVA followed by Dunnett's t test, *P<0.05, significantly different from DSS group.

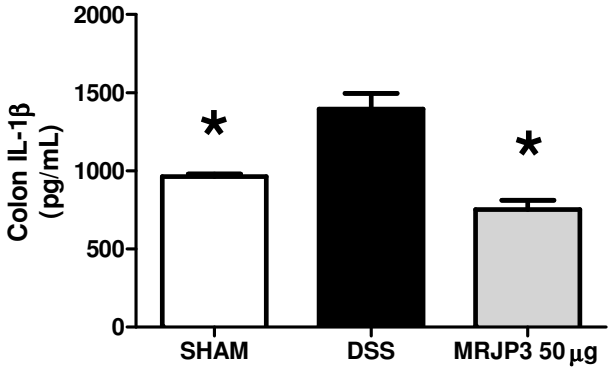


Figure 2. Effect of intra-rectal MRJP3 (50µg) treatment on colonic IL-6 levels in mice undergoing DSS-induced colitis. Results are presented as mean ± S.E.M. ANOVA followed by Dunnett's t test, *P<0.05, significantly different from DSS group.

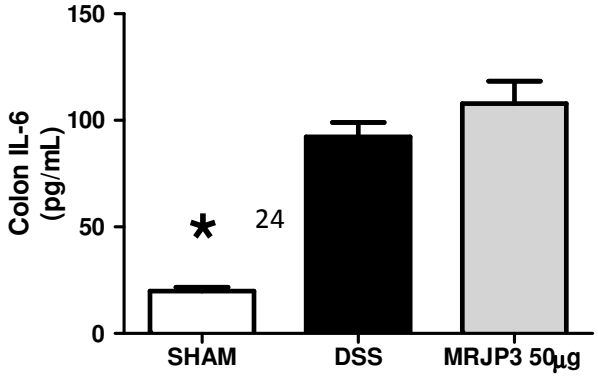
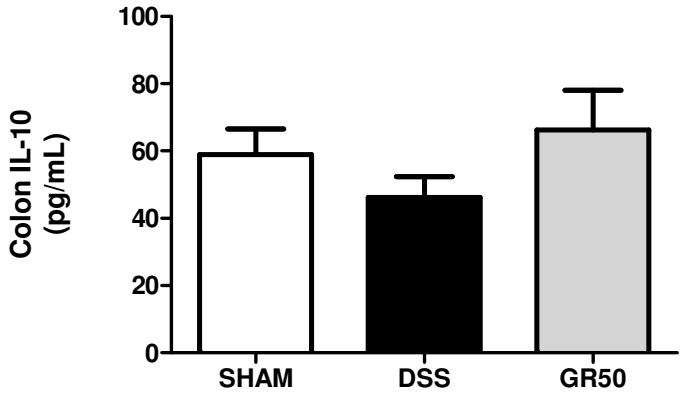


Figure 3. Effect of intra-rectal MRJP3 (50µg) treatment on colonic IL-10 levels in mice undergoing DSS-induced colitis. Results are presented as mean ± S.E.M. ANOVA followed by Dunnett's t test, * $p<0.05$, significantly different from DSS group.



3. Experimental Section

3.1 Animals: male Balb/c, 6-8 week old mice were used. The animals were supplied by CEMIB UNICAMP (Centro Multidisciplinar de Bioterismo) and were kept in a 12 h light/dark cycle at room temperature with free access to food. All the experiments were approved of by the Ethics Committee on the Use of Experimental Animals (CEUA) under the number 2398-1.

3.2 MRJP3 purification: 0.5 g of fresh Royal Jelly was dialyzed in TRIS-HCl at pH 8, passed through a 0.22 µm filter and then centrifuged at 4°C for 30 minutes. The supernatant was collected and followed the methodology described by (44) with modifications.

3.3 DSS-induced colitis: the animals of the DSS and MRJP3 50 received DSS (Dextran Sulfate Sodium Salt – MP Biomedicals, Ohio, USA) (5 % w/v) in the drinking water for 7 consecutive days, according to (4).

3.4 Experimental design: Two groups of animals were set; non-colitic (SHAM) and colitic (DSS, MRJP3 7,5; MRJP3 25 and MRJP3 50). The non-colitic group received only water for 7 days and underwent the 4 days of intrarectal treatment receiving only saline. The colitic groups received DSS 5% (w/v) for 7 consecutive days and also underwent intrarectal treatment with 7,5 , 25 and 50 of MRJP3. The negative control DSS did not receive MRJP3 but, instead, received saline.

3.5 Drugs:

All the drugs and reagents were prepared just before use and stored at appropriate recipients:

Royal Jelly: Baldoni Ind. Com. Prod. Nat. LTDA. – SP, Brazil, DSS (Dextran Sulfate Sodium Salt): MP Biomedicals, Ohio, USA.

3.6 Assessment of colitis:

The weight/length ratio of the colon of the animals was calculated.

3.7 Assessment of IL-1B, IL-6 and IL-10 levels:

Distal colon samples were homogenized and centrifuged at 14000 rpm for 45 minutes at 4°C. The supernatant was collected (cytosolic extract) and used for the cytokine levels assays with IL-1β, IL-6 and IL-10 enzyme immunoassays kits. The values were expressed as pg/mg tissue (R&D Systems, Inc, USA)

3.8 Statistical Analyses:

Results were expressed as mean ± standard error of means (S.E.M.). The statistical significance of each test group in relation to the control was calculated using ANOVA followed by Dunnet's t test.

4. Conclusions

MRJP-3 intra-rectal treatment may be an interesting object for more comprehensive studies since it demonstrated a decrease in the weight/length ratio, one of the parameters used to investigate and analyze the development of the disease. It also downregulated the levels of IL-1β, an important mediator in general inflammatory processes, in this DSS-induced colitis model. The levels of IL-10 and IL-6 were not altered, what suggests a direct effect on either IL-1β production or on its pathway.

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4.6. RESUMO DO ARTIGO 1

Ulcerative Colitis (UC) and Chron`s Disease (CD) are the two major forms of Inflammatory Bowell Diseases (IBD). Despite all efforts, these complications of the gastrointestinal tract (GIT) are still lacking an effective therapy for their cure. Side effects, high cost and low adhesion to treatment are among the negative aspects. Natural products have been a source of widely used compounds in distinct areas of medicine and research. Intra-rectal Major Royal Jelly Protein 3 (MRJP3) intra-rectal treatment (50 μg/animal) was capable of decreasing the levels of Interleukin 1-β (IL-1β) in mice undergoing (Dextran Sulfate Sodium Salt) DSS-induced colitis. The levels of Interleukin-10 (IL-10) and Interleukin 6 (IL-6) were not altered by MRJP3 50 μg instillation, on the other hand, intra-rectal instillation of major royal jelly protein (MRJP3 (50 μg)) exerted a reduction in the levels of IL-1β what indicates an important anti-inflammatory effect in the development of DSS-induced UC and, most importantly, opens and encourages new studies focused on this protein.

5. CONCLUSÃO ARTIGO 1

A proteína MRJP3 (50 μg) foi capaz de diminuir os níveis de IL-1β no cólon dos animais submetidos a colite induzida por DSS quando comparados aos controles negativos. Como os níveis de IL-6 e IL-10 não foram alterados, podemos afirmar que esta inibição se dá diretamente em algum ponto da via de produção da IL-1β e não através de estílmulos da IL-10. Faz-se, então, necessário estudos mais abrangentes e detalhados sobre os mecanismos de ação desta proteína durante processos inflamatórios, sobretudo aqueles em que a resposta imune inata sobrepõe a adquirida, como é o caso da colite induzida por DSS no protocolo utilizado neste trabalho.

6. CONSIDERAÇÕES ARTIGO 1

O tratamento intrarretal com a proteína MRJP3 levanta importantes considerações acerca dessa superfamília de proteínas. Tendo em vista seu efeito sobre a os níveis de Interleucina 1β nesse modelo experimental e o já demonstrado por trabalhos anteriores *in vitro*. Faz-se necessário mais estudos que explorem outros mecanismos e parâmetros envolvidos não só na inflamação intestinal induzida por DSS em camundongos mas também em diferentes modelos e técnicas.

7. OBJETIVOS DO ARTIGO 2:

- Avaliação dos efeitos da Geleia Real em modelo de colite experimental induzida por TNBS, avaliando:

- expressão de COX-2 e NF-κB
- níveis de GSH
- atividade enzimática de GSH-Px.

7.1. Título do Artigo 2

Oral treatment with Royal Jelly protects mice against TNBS-induced colitis.

7.2. Revista ao qual o artigo foi submetido

World Journal of Gastroenterology

7.3. Correspondência da revista relativa ao Artigo 2

Dear Dr. Luis Paulo Manzo,
Thank you very much for your submission to our peer-reviewed, online and open-access journal, World Journal of Gastroenterology.
ESPS Manuscript NO: 307
Title: Oral treatment with Royal Jelly protects mice against TNBS-induced colitis.
Author Name: Luis Paulo Manzo
Received Date: 2012-08-26 05:27:58
If you want to know how we have dealt with your manuscript(s), please track check manuscript(s), it online at our website: http://www.wjgnet.com/esps/ with the registered
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7.4. Artigo 2

Oral treatment with Royal Jelly protects mice against TNBS-induced colitis

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Abstract

Aim: Natural products have been a vast source of compounds, especially interesting for pharmacological, medical and scientific areas. Regarding the importance of the animal-derived natural products, Royal Jelly (RJ) has been increasingly calling researcher's attention due to its innumerous effects; antioxidant, anti tumor, wound healing and vasodilative. TNBS-induced colitis in mice is a reliable model for assessing some resembling characteristics of the human inflammatory bowel diseases.

Materials and Methods: Induction of colitis was performed through a rectal instillation of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) at a total volume of 0.1 mL per mouse. Control group saline received only saline and no TNBS solution. TNBS-colitic group received only TNBS solution and no treatment.

Results: The oral treatment with RJ 100 mg/Kg for 19 consecutive days was capable of reducing inflammatory response due to its free radical scavenging property. The levels of GSH were increased by the treatment, as well as the activity of GSH-Px. Furthermore, RJ 100 mg/Kg downregulated the expressions of both COX-2 and NF-κB, which are two important players in the inflammatory process.

Conclusion: In conclusion, RJ showed to have anti inflammatory and antioxidant properties in TNBS-induced colitis, resulting in the amelioration of the macroscopic score and histological microscopic analyses.

INTRODUCTION

Natural products are very important to humanity and it has been so since very ancient records, thousands of years ago. From food to pharmacology, plant and animal-derived products have made their way providing uncountable molecules used for treating a myriad of problems. Moreover, many contemporary pharmaceuticals are natural products or derivatives thereof (48). Up to current days, more than 10,000 biologically active new molecules have been discovered from marine animals (49) . Some of the most important bioactive compounds isolated from marine animals; spongouridine and spongothymidine from the Caribbean sponge *Cryptotheca crypta* were approved as anti-cancer (cytosine arabinoside, Ara-C) and anti-viral (adenine arabinoside, Ara-A) drugs (50). These examples, although, relevant, are negligible when compared to what is documented and also expected from natural products. The majority of the animal-derived products are terpenes, alkaloids and peptides. These molecules, notably, present several biological activities, which will, in part, be discussed here.

Royal Jelly (RJ) is a nourishing substance secreted by the mandibular and hypopharyngeal glands of worker honey bees *Apis mellifera (51).* The physiological role of the royal jelly in the bees remains to be entirely elucidated, although, it is already known that RJ consumption determines the molecular events of cell differentiation of larvae into queens or workers during the early phases of development (52). Recently, due to the biological effects accredited to RJ, its use has been increasing throughout the world, which, in turn, has been attracting enormous attention from the scientific community (53). Royal Jelly is described to possess various biological activities; antioxidant (32), wound-healing (54), antitumor (55) and vasodilative (56). RJ is a complex mixture of water (60-70%), proteins (12 -15%), carbohydrates (10 – 12%), lipids (3 -7%), minerals, amino- acids and vitamins (57), which will

not be discussed in details here. It is already known that highly reactive free radicals are formed by either exogenous chemicals or endogenous metabolic processes of the body. These free radicals are capable of oxidizing biomolecules, resulting in cell death and consequent tissue damage (57). There are specialized mechanisms to prevent oxidative-cell damage; SOD, catalase, gluthatione peroxidase and reductase.

The ulcerative colitis (UC) and Chron's disease (CD) are the two major forms of inflammatory bowel diseases (IBD). Both UC and CD are of complex etiology and are believed to be due to genetic, environmental and microbial factors (28). These two forms of IBD occur in the intestine, although, CD may affect the whole digestive tract while UC tends to be restricted to the distal portion of the tube (rectum) (3). Either CD or UC present ulcerated areas, the first one is a transmural lesion, affecting the entire wall thickness, from the mucosa to the serosa. The second is shallower, only the mucosal lining is affected. The disposition is also different; the CD lesions are presented in a patchy manner, ulcerated tissue surrounded by healthy tissue and vice and verse, whereas in UC a continuum of lesion is observed (3).

Many authors have proposed that these intestinal conditions are mediated by the activation of both lymphocytes and non-lymphoid cells such as macrophages and neutrophils. Once a vast number of these cells are activated, they migrate to the harmed mucosa of the intestine, leading to an over-production of oxygen free radicals that may damage or even kill the cells in the inflamed area (58). For that reason, the antioxidant defense mechanisms are of crucial importance to the homeostasis and integrity of the tissues. Since scientists have not yet come up with an effective drug for the IBD treatment (28), new and alternative therapies arise not only as a promise but, rather, as a necessity for the patients undergoing such condition. Due to the anti-oxidant properties of royal jelly (32). and the oxidative stress taking place in the intestine of UC

and CD patients, and a study reporting that RJ was effect in attenuating acetic acid-induced colitis in rats (59), a different experimental model, our group decided to investigate whether an oral treatment with RJ would have any effect on mice undergoing TNBS-induced colitis.

MATERIALS AND METHODS

Animals

Unib- SW 6-8 week –old female mice were purchased from CEMIB-UNICAMP. All the protocols used in this study were in accordance with the Ethics Committee on the Use of Experimental Animal (CEUA) from UNICAMP (2398-1). All animals had free acces to tap filtered water and certified rodent chow (Nuvilab®) during the whole experiment. Room temperature was kept constant with 12/12 light dark cycles 60 ± 1% humidity and a temperature of 21 ± 2ºC.

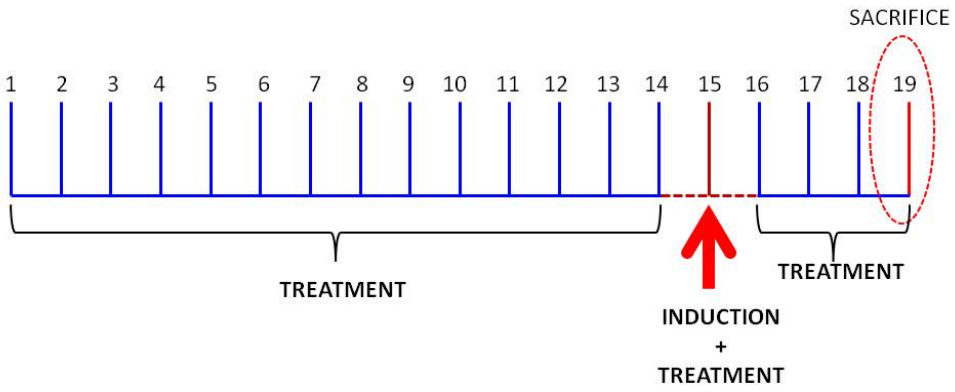
Drugs, reagents and Royal Jelly

All drugs and reagents were prepared right before use and were of chemical grade. 2,4,6-trinitrobenzene sulfonic acid (TNBS), NaCl wasbought from Sigma (MO, USA). Royal Jelly was obtained from Baldoni apiary (São Paulo, Brazil) and solubilized in Saline 0,9% with 5 minute-sonication and heavy agitation in vortex. Following agitation, the solution was centrifuged and supernatant collected and diluted at test doses (100, 150 and 200 mg/Kg of animal).

Experimental protocols

Mice were randomly assigned into four groups (n=10); two of them (non-colitic and control goups) received no treatment and the other 3 received, orally, daily for 19 days, 100, 150 and 200 mg/Kg of Royal Jelly (suspended in saline 10 ml/kg). Both non-colitic and control group (colitic non-treated) were given daily 10 ml/kg of saline. Two weeks after treatment started, mice from the control and treated groups were rendered colitis by the method originally described by

Morris et al, 1989 (60). Briefly, they were anaesthetized with halothane and given 10 mg of TNBS dissolved in 0.1 ml of 50% ethanol (v/v) through a Teflon cannula inserted 4 cm through the anus. Mice from the non-colitic group were administered intracolonically 0.25 ml of phosphate-buffered saline instead of TNBS. Behavior, body weight and stool consistency were recorded daily throughout the experiment. All mice were killed by cervical dislocation 4 days after induction of colitis and the colon was removed aseptically and placed on cold plate and longitudinally cut in two slices.



Macroscopic features of colitis

The severity of colon damage was macroscopically assessed using the criteria previously established for TNBS-induced colitis (61). A score ranging from 0 to 10 was employed, as follows: 0, no damage; 1, hyperaemia without ulcers; 2, hyperaemia and wall thickening without ulcers; 3, one ulceration site without wall thickening; 4, two or more ulceration sites; 5, 0.5 cm extension of inflammation or major damage; 6–10, 1 cm extension of inflammation or severe damage. The score was increased by 1 for every 0.5 cm of damage up to a maximal score of 10; by 0 or 1 for absence or presence of diarrhea and 0, 1 or 2 for absence, presence of mild or severe adhesion respectively.

Histological features of colitis

The colonic was dissected and fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 24 h at 4°C. The tissues of three animals per experimental group were dehydrated in graded concentrations of alcohol, embedded in historesin (Leica Microsystems Heidelberg, Germany) and sectioned transversely at a width of 2 µm. The resulting serial sections were mounted on slides and stained with Hematoxylin & Floxin. The sections were then examined and imaged using a Nikon Eclipse E800 light microscope.

Glutathione level determination (GSH)

GSH levels of colonic tissue of animals were determined by Ellman's reaction using 5′5′-dithio-bis-2-nitrobenzoic acid (DTNB) as described by Anderson (62). The intensity of the yellow colour was read at 412 nm.

Glutathione peroxidase activity (GSH-Px)

GPx 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 GPx (63).

Western blotting analyses

Frozen colon samples were homogenized in 1 mL of cold buffer containing phosphate buffer-PB 0.1 M, pH 7.4 and protease inhibitor cocktail-1% (Sigma-Aldrich® P-8340). Homogenates were centrifuged (12,000 × g, 45 min, 4ºC) and the supernatants were collected and stored at -80ºC. Different centrifugation times and buffers were used for the cytosolic, membrane and nuclear extracts. Protein concentration of the homogenate was determined following Bradford's colorimetric method (64). Then, samples were treated with Laemmili buffer (PB 0.5 M, pH 6.8; glycerol, sodium dodecyl sulfate (SDS) 10%, bromophenol 0.1%, β-mercaptoethanol) in a 1:1 proportion. Equal amounts of protein from samples (70 μg) were separated on 8 % acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: COX-2 (160126), Cayman Chemical, USA) at 1:500 dilution. Each membrane was washed three times for 10 min and incubated with HRP-Goat Anti-Rabbit (Invitrogen® 656120) (COX-2, diluted at 1:5000). To prove equal loading, the blots were analyzed with standard protein ponceau dye (65). Immunodetection was performed using enhanced chemiluminiscence light-detecting kit (SuperSignal® West Femto Chemiluminescent Substrate, Pierce, IL, USA). Densitometric data was performed with G-BOX, Syngene® following normalization to the control (ponceau) (65) by GeneSys® software.

Statistical analysis

Results were expressed as mean ± standard error of means (S.E.M.). The statistical significance of each test group in relation to the control was calculated using ANOVA followed by Dunnet's test.

RESULTS

Macroscopic evaluation of colitis

Mice subjected to Royal Jelley treatment showed an overall lower impact of TNBS-induced colonic damage compared with the TNBS control group. This was observed in the course of the experiment, because treated mice showed a faster weight recovery than those of the control mice (Fig. 1). However, mice from the TNBS control group showed a lower weight gain throughout the experiment in comparison with the mice from the non-colitic group ($P < 0.05$). The anti-inflammatory effect was evidenced macroscopically by a lower colonic damage score than that of TNBS control group ($P < 0.05$), with a significant reduction in the extent of colonic necrosis and/or inflammation induced by TNBS/ethanol (Table 1).

Histological evaluation of colitis

The effective induction of TNBS-induced inflammation was corroborated by the histological damage and inflammatory infiltrate as shown in (Fig. 2; A-E). Figure 2 summarizes the microscopical damage from all groups, colitic and non-colitic (TNBS, RJ100, 150 and 200 mg/kg). The non-colitic group saline showed normal mucosal morphology with intact epithelium, as well as the mucosal lining. The TNBS-colitic control group showed an extensively damaged epithelium, complete absence of goblet cells and no definite mucosal lining as well a great number of inflammatory cells. The RJ 100 mg/Kg group showed a decrease in the number of goblet cells, moderate cell infiltrate, mainly neutrophils and a low intensity tumor when compared with TNBS colitic group. RJ150 group showed a higher number of goblet cells and an apparently healthier epithelium, on the other hand, the number of inflammatory cells is higher

when compared to RJ100 group. The RJ200 group showed a massive infiltrate and no delimitations of either the mucosal lining or epithelium.

Glutathione level determination (GSH)

The GSH levels in the non-colitic group was high and after TNBS colitis induction the GSH levels drastically reduced, on the other hand, RJ 100 mg/Kg was capable of increasing the levels of GSH after colitis induction. Interestingly, the increase exerted by RJ were similar to those of the non-colitic group (Table 2).

Glutathione peroxidase activity (GSH-Px)

There was no signicant statistical difference between the non-colitic and TNBS- colitic regarding GSH-Px activity. RJ at the doses of 100 and 150 mg/Kg were able to increase the activity of GSH-Px, without significant statistical difference between them.

Western blotting analyses

The levels of expression of COX-2 and NF-κB were measured by western blotting from colonic mucosa (Fig. 3). As shown in this figure, exposure of colon to TNBS caused strong expression of COX-2, on the other hand, RJ at all doses (100, 150 and 200 mg/kg) induced down-regulation of COX-2 when compared with TNBS group ($p < 0.001$). The levels of expression of NF-κB p 65 was detected in low quantity in nuclei of normal mucosa whereas a high expression of nuclear factor appeared in colon mucosa from control TNBS colitic group. Nonetheless, upon treatment with RJ (100 mg/kg), the protein expression of NF-κB p65 was decreased (Fig. 4).

DISCUSSION

UC and CD are the two major forms of IBD, both diseases share common features, although, differing in the etiology. These two forms of IBD are characterized by an exacerbated immune response towards enteric microbial population (66). The symptoms are also similar; abdominal pain, severe diarrhea, bloody feces and consequent anaemia and weight loss. Some patients may lose up to 20% of the total weight in very short periods in the acute phase of the disease, which can be observed in animal models of experimental colitis. Animal models of intestinal inflammation are indispensable for our understanding of the pathogenesis of CD and UC (4). These models are used to evaluate new anti-inflammatory strategies(67). One of the most widely used models is colitis induced by the haptinizing agent TNBS. It is thought that this model resembles Crohn's disease because of the resulting mucosal inflammation mediated by a Th1 response (68).

We have demonstrated that all colitic groups lost weight after TNBS induction and that the RJ 100 mg/Kg group restarted weight gain more quickly than those of the TNBS-colitic group. One must consider that this parameter alone does not indicate, by itself, any efficacy of the treatment, although, tells us the general state of the animal. The weight loss can be partially explained by the reduction in chow consumption (data not sown) and water intake due to the damage in the intestine, which, in turn, causes pain and intense diarrhea. This data is supported by the chow consumption and water intake by the non-colitic group (data not shown). The macroscopic evaluation is a very important tool when assessing experimental TNBS-induced colitis since it takes into account the area of ulceration, hence, we can evaluate how damaged the tissue remains. In the TNBS-colitic group the lesion area was large and showed extensive tissue thickening. Both RJ 100 and 150 mg/Kg were effective in reducing the lesion area, although, the

first showed better score results (Table 1). These results show that the RJ oral treatment is capable of reducing the inflammatory process caused by haptening agent. Many aspects are involved in the inflammatory process, such as the Oxygen Reactive Species (ROS), which have already been demonstrated to modulate the immune response.

The respiration process is mandatory for the life of aerobic organisms but, nevertheless, it can be harmful due to the formation of Reactive Oxygen Species (ROS). Despite being considered foes, they play very important roles in living organisms. These molecules may exert an anti-bacterial function through protein, DNA and lipid oxidation and thus, killing the bacteria. On the other hand, when these molecules are over produced, it becomes a threat to cells, due to its great oxidizing capacity (69).. There are extensive data showing that ROS is a player in the pathogenesis of IBD. Increased production of ROS harms the integrity of the epithelial cells, through an initial inflammatory response (70). In order to protect tissues agains ROS-provoked injuries, all cells count on antioxidant enzymes, including glutathione peroxidase (GSH-Px) and radical scavengers such as sulfhydryl compounds GSH (71).

GSH has already been shown to have its level diminished in experimental colitis (72). This is part of the first line of defense against oxidative stress and the treatment with RJ 100 mg/Kg was capable of increasing the levels of GSH (Table 2). These results are in accordance with previous studies showing that the RJ exerts a free radical scavenger activity (32), therefore, ameliorating the ongoing inflammation. The activity of GSH-Px appeared reduced in both non-colitic and TNBS-colitic groups (Table 2), this apparently paradox can be explained by the GSH and H_2O_2 – dependence for the activity of the GSH-Px. For the TNBS group, the limiting factor is the level of GSH (very low), on the other hand, for the non-colitic saline group, the limiting factor is the H_2O_2, that is not augmented, since there is no oxidative stress going on. In both

cases, The RJ 100 mg/Kg was capable of increasing the level of GSH as well as the activity of GSH-Px, demonstrating its antioxidant property (Table 2).

Nuclear Factor κB (NF-κB) is a transcriptional factor and is a pivotal player in the inflammatory cascade. It was initially recognized in B cells only, however, newer studies have demonstrated that it is involved in several biological processes, taking place not only in the adaptive immune response (B cells) but also in the innate immune response (73). Among other functions, it is responsible for the expression of central inflammatory mediators as COX-2. In IBD, the levels of NF-κB have been already showed to be elevated and its expression can be directly linked to the severity of the gut inflammation (74). It has also been reported that the expression of NF-κB is augmented in experimental IBD (75). Due to the importance of NF-κB, not rarely, researchers make huge efforts in understanding its biology for potential pharmacological uses (76). In our experimental model, the NF-κB expression was showed to be augmented int the TNBS-colitic group, corroborating with (74). We have also demonstrated that colonic level of NF-κB was inhibited by the treatment of RJ 100 mg/Kg when compared with TNBS-colitic group (Fig. 4). This NF-κB inhibition is an important parameter for protecting the gut against chronic intestinal inflammation and necrotizing enterocolitis in animal models (75).

Cyclo-oxigenase-2 is one of various inflammatory mediators regulated by NF-κB. COX enzymes are involved in numerous physiological responses including inflammation, where they catalyse the synthesis of prostaglandins (PGs) from arachidonic acid (77). Prostaglandin E_2 (PGE_2) is one of the most important biologically active prostanoids found throughout the gastrointestinal tract. Despite the fact that PGE_2 regulates many physiological functions of the gut including mucosal protection, gastrointestinal secretion and motility, it is implicated in the pathophysiology of IBD (78). COX-2 is an inducible inflammatory enzyme induced by growth

factors, proinflammatory cytokines, tumour promoters and bacterial toxins and is increased in TNBS-induced colitis (77, 79). There is sufficient data to support that the inhibition of this enzyme is beneficial during TNBS-induced colitis (80). Our results showed that the TNBS – colitic group showed an increased expression of COX-2 related to the non-colitic group and that the dose of RJ 100 mg/Kg inhibited the expression of COX-2, when compared to TNBS colitic group. The expression of COX-2 is regulated by the NF-κB and thus, its inhibition may lead to a decrease in the expression of COX-2.

Conclusion

TNBS-induced colitis is a widely accepted model for IBD studies, mainly, UC and CD. According to the results showed and the data published, RJ remains as a prominent field. We demonstrated that oral treatment with RJ showed to be effective in the treatment of TNBS-induced colitis in mice, partly explained by its antioxidant property and the downregulation of pro-inflammatory mediators. It is important, nevertheless, to state that more comprehensive studies need to be carried out in order to evaluate more parameters involved in different models of experimental colitis.

Grants

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Figures and tables legends

Figure 1. Weight means of the 5 different groups on days 1, 6, 13 (pre induction), 14 and 19 (post induction). Data is presented as mean ± S.E.M. ANOVA followed by Dunnett's t test, ^{a} P<0.05, significantly different from colitic non-treated.

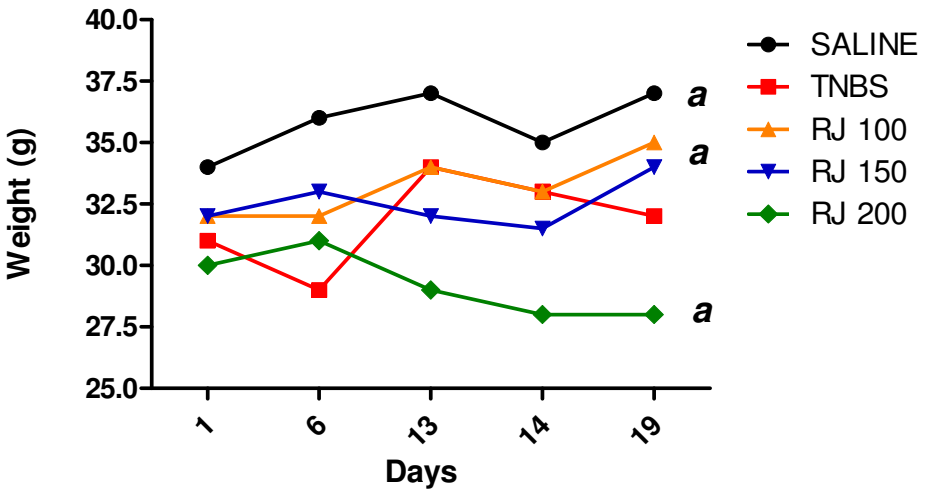
Figure 2. Hematoxylin and Floxin staining of colons from both colitic and non-colitic groups. (A) Histological image from the non-colitic group saline (100x). (B) Colitic group TNBS (not treated) (100x). (C) RJ100 group, received both TNBS and Royal Jelly 100 mg/Kg (100x). (D) RJ150, received both TNBS and Royal Jelly 150 mg/Kg (100x). (D) RJ200 received both TNBS and Royal Jelly 200 mg/Kg (100x).

Figure 3. Representative Western blot analysis COX-2 proteins. Densitometric data were studied following normalization to the control (Ponceau). The results are representative of three experiments performed on different samples and data are expressed as mean ± S.E.M. ANOVA followed by Dunnett's t test, ^{a} P<0.05, ^{b}P<0.01 and ^{c} P<0.001 significantly different from TNBS group.

Figure 4. Representative Western blot analysis NF-κB proteins. Densitometric data were studied following normalization to the control (Ponceau). The results are representative of three experiments performed on different samples and data are expressed as mean ± S.E.M. ANOVA followed by Dunnett's t test, ^{a} P < 0.05 significantly different from TNBS group.

Table 1. Effect of Royal Jelly on the disease activity index of mice undergoing TNBS-induced colitis. Results are presented as mean ± S.E.M. ANOVA followed by Dunnett's t test, [a] $P<0.05$, [b] $P<0.01$ and [c] $P<0.001$ significantly different from colitic non-treated.

Table 2. Effect of Royal Jelly on colonic GSH level, GSH-Px and GR activities in TNBS-induced colitis in mice. Results are presented as mean ± S.E.M. ANOVA followed by Dunnett's t test, [a] $P<0.05$, [b] $P<0.01$ and [c] $P<0.001$ significantly different from colitic non-treated.


40.0
37.5
35.0
32.5
30.0
27.5
25.0
Weight (g)
1
6
13
14
19
Days
a
a
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SALINE
TNBS
RJ 100
RJ 150
RJ 200

Figure 1.

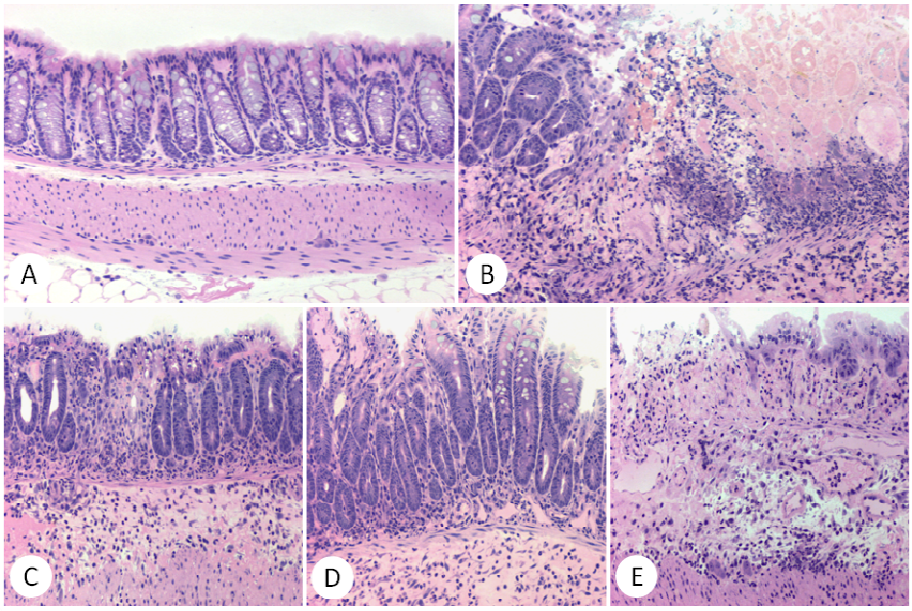

Figure 2

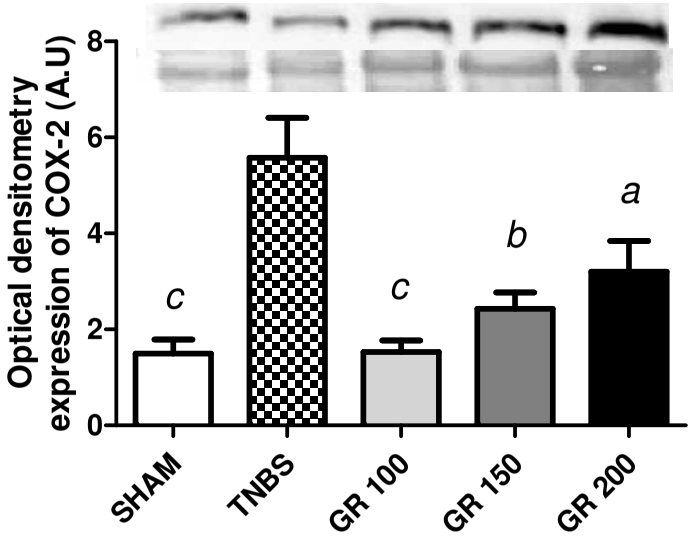
Optical densitometry expression of COX-2 (A.U)
8
6
4
2
0
c
c
b
a
SHAM
TNBS
GR 100
GR 150
GR 200

Figure 3

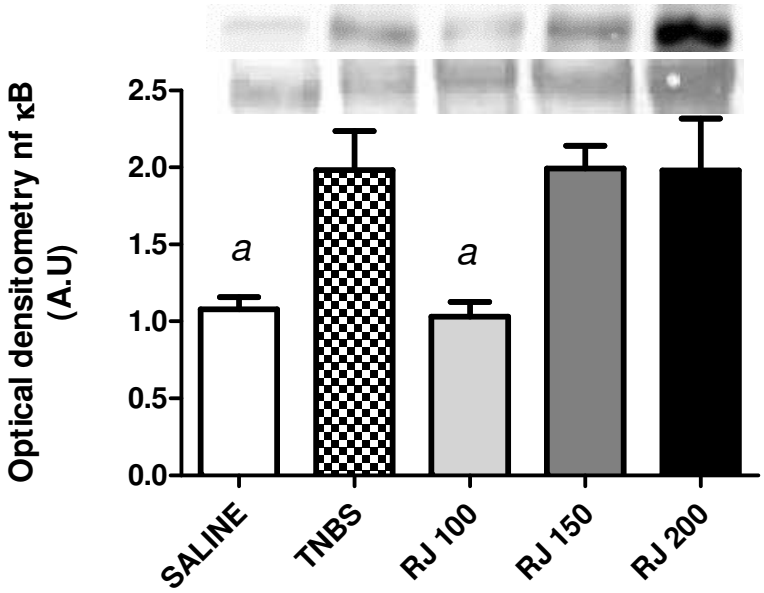
Optical densitometry nf κB
(A.U)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
SALINE
TNBS
RJ 100
RJ 150
RJ 200

Figure 4

Group	Macroscopic assessment	Adhesion	Diarrhea
Non-colitic (saline)	0 ± 0 [c]	0 ± 0 [c]	0 ± 0 [c]
TNBS - colitic	4.5 ± 0.42	1.5 ± 0.54	0.71 ± 0.48
RJ 100 MG/Kg	2.56 ± 0.29 [a]	0.44 ± 0.72 [a]	0.67 ± 0.5
RJ 150 Mg/Kg	2.67 ± 0.236 [a]	0.625 ± 0.91	0.62 ± 0.51
RJ 200 Mg/Kg	3.88 ± 0.515	1.38 ± 0.91	0.750 ± 0.46

Table 1

Group	Colon GSH levels (µmol/mg of protein)	Colon GSH-Px activity (nmol/min/mg protein)
Non-colitic	25.1 ± 5.91 [a]	4.5 ± 0.18
Colitic non-treated	8.6 ± 1.10	5.0 ± 0.319
RJ 100 mg/Kg	22.8 ± 3.11 [a]	11.8 ± 3.18 [a]
RJ 150 mg/Kg	12.6 ± 3.06	12.0 ± 0.82 [a]
RJ 200 mg/Kg	5.6 ± 2.56	9.0 ± 0.712

Table 2

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8. RESUMO ARTIGO 2

Os produtos naturais, em geral, representam uma riquíssima fonte para compostos e moléculas de grande importância para a ciência, medicina e farmacologia. Dada a importância dos produtos naturais de origem animal, a Geléia Real (GR) é promissora devido a suas reconhecidas propriedades anti-oxidantes, anti- tumorais e cicatrizante. Nosso trabalho visou avaliar se tratamentos orais com a GR em 3 diferentes doses (100, 150 E 200 mg/Kg) apresentariam efeitos anti-inflamatórios na colite experimental induzida por TNBS. Os resultados apontaram que a GR 100 mg/Kg foi capaz de aumentar os níveis deGSH e a atividade da GSH-Px em relação ao controle negatico TNBS. O NF-κB e a COX-2 também tiveram suas expressões reduzidas pelo tratamento de GR na dose de 100 mg/Kg, ambos, se comparados ao controle negatico TNBS. Concluímos, então, que a GR na dose de 100 mg/Kg foi capaz de exercer um efeito anti-inflamatório em modelos de colite induzida por TNBS.

9. CONCLUSÃO ARTIGO 2

A Geleia Real na dose de 100 mg/Kg foi capaz de aumentar os níveis de GSH, bem como a atividade da GSH-Px em relação ao controle TNBS, o que representa um significativo poder anti-oxidante que pode explicar a diminuição das área de lesão nesse grupo de animais quando comparados aos do grupo TNBS. Tanto a COX-2 quanto o NF- κB tiveram suas expressões diminuídas no grupo GR 100 mg/Kg quando comparado ao grupo TNBS. Estes resultados traduzem uma ação anti-inflamatória e anti-oxidante da GR em modelos de colite experimental induzida por TNBS.

10. CONSIDERAÇÕES ARTIGO 2

Novas perspectivas se abrem quando um composto apresenta algum tipo de atividade biológica. Sabe-se, porém, que muitos outros estudos devem suceder este nosso inicial, abordando outros aspectos e outros modelos da colite experimental. Os mecanismos de ação, as vias, os efeitos colaterais, a cinética química são parte do imenso trabalho ainda por vir.Os produtos Naturais ainda são uma fonte imensa de novos compostos e drogas. É nosso dever então, estudá-los.

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12. Atividades desenvolvidas

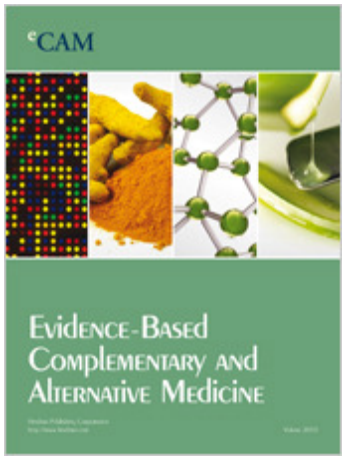
O projeto de mestrado possibilitou a formação específica na área de Farmacologia, com os estudos direcionados para a caracterização de atividade farmacológica de produtos natuirais, sendo o objeto alvo dos estudos as doenças inflamatórias intestinais. Durante a execução do projeto, várias outras atividades foram realizadas, no intuito de enriquecer a formação profissional do aluno, as quais serão descritas a seguir:

Disciplinas cursadas	Departamento
NF 016- Tópicos em fisiologia e biofísica	IB
NH 006 – Animais de Laboratório em Pesquisa biomédica	IB
FP 504 - Bioestatística	FCM
MF 733 – Atualidades em Farmacologia	FCM
NF 122 – Topicos Avan;cados em Fisiologia	IB
NB 161 – Química de Proteínas	IB
NF 133 – Metodologia Científica	IB

13. Artigos publicados:

Impact Factor – 3,014
Qualis: B
Takayama, Christiane ; de-Faria, Felipe Meira ; de Almeida, Ana Cristina Alves ; Valim-AraÃºjo, Deborah de Arantes e Oliveira ; Rehen, Camilla Souza ; Dunder, Ricardo JosÃ ; Socca, Eduardo Augusto Rabelo ; **MANZO, L. P.** ; Rozza, Ariane Leite ; Salvador, Marcos JosÃ ; Pellizzon, ClaÃºdia Helena ; Hiruma-Lima, ClÃ lia Akiko ; Luiz-Ferreira, Anderson ; Souza-Brito, Alba Regina Monteiro . Gastroprotective and ulcer healing effects of essential oil from Hyptis spicigera Lam. (Lamiaceae).

Impact Factor: 3,14
Qualis: B
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.



Impact Factor: 4,774
Qualis: A
This is to confirm the receipt of the electronic files of your Research Article 753971 titled "Effects of Rhizophora mangle on experimental colitis induced by TNBS in rats," by Felipe Meira De faria, Anderson Luiz Ferreira, Eduardo Augusto Rabelo Socca, Ana Cristina Alves Almeida, Ricardo José Dunder, **Luis Paulo Manzo**, Marcelo Aparecido da Silva, Wagner Vilegas, Cláudia Helena Pellizzon, Lourdes Campaner dos Santos, Alba Regina Monteiro Souza-Brito and Ariane Rozza.

14. Artigos Submetidos

International Journal of Molecular Sciences

Impact factor – 2,9
Qualis: B

Manuscript ID: ijms-20916
Type of manuscript: Short Note
Title: **Cytokine response to MRJP3 intra-rectal treatment in DSS-induced colitis**
Authors**: Luis Paulo Manzo** *, Felipe De Faria, Ricardo José Dunder, Ana Cristina Almeida, Eduardo Augusto Socca, Verônica Soares, Marcos Toyama, Luciana De Hollanda, Marcelo Lancelotti, Alba Souza-brito, Anderson Luiz-ferreira
Received: 12 July 2012

World Journal of Gastroenterology

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Thank you very much for your submission to our peer-reviewed, online and open-access journal, World Journal of Gastroenterology.
ESPS Manuscript NO: 307
Title: Oral treatment with Royal Jelly protects mice against TNBS-induced colitis.
Author Name: Luis Paulo Manzo
Received Date: 2012-08-26 05:27:58

15. Participação em Congressos e Apresentação de Resumos

MANZO, L. P. ; A. Luiz-Ferreira ; De Faria, F. M ; Dunder, R.J ; Coope, A. ; SOUZA BRITO, A. R. M . The effect of MRJP3 on the production of cytokines in DSS-induced colitis.. In: Reunião anual das Federaçaoes de Sociedades de Biologia Experimental, 2011, Rio de Janeiro. **Reunião anual das Federaçaoes de Sociedades de Biologia Experimental, 2011.**

ALMEIDA, A. C. A. ; De Faria, F. M ; Dunder, R.J ; **MANZO, L. P.** ; VILEGAS, W. ; Luiz-Ferreira, A. ; SOUZA BRITO, A. R. M . preliminary assessment of the effect of Indigo on Intestinal Inflammation. In: **Congresso Italo-Latinoamericano de ETNOMEDICINA, 2011, Fortaleza. Congresso Italo-Latinoamericano de ETNOMEDICINA, 2011.**

Dunder, R.J ; Luiz-Ferreira, A. ; **MANZO, L. P.** ; ALMEIDA, A. C. A. ; De Faria, F. M ; SOUZA BRITO, A. R. M . Evaluation of Indigo alkaloids in arachidonic acid ear model, a classic model of inflammation by prostaglandinssynthesis. In: **Congresso Italo-latinoamericano de ETNOMEDICINA, 2011.**

De Faria, F. M ; Luiz-Ferreira, A. ; Dunder, R.J ; E.A.R Socca ; **MANZO, L. P.** ; da Silva, M.A ; VILEGAS, W. ; SOUZA BRITO, A. R. M . Rhizophora mangle L. ameliorates TNBS-induced colites in rats through the expression of NF-kappa. In: Congresso Italo-Latinoamericano de ETNOMEDICINA, 2011, Fortaleza. **Congresso Italo-Latinoamericano de ETNOMEDICINA, 2011.**

Dunder, R.J ; Luiz-Ferreira, A. ; **MANZO, L. P.** ; ALMEIDA, A. C. A. ; SANTOS, R. C. ; SOUZA BRITO, A. R. M . Antinociceptive effects of indigo alkaloids from Indigofera truxilensis (Kunth) in classic model of pain. In: Congresso Itelo-Latinoamericano de ETNOMEDICINA, 2011, Fortaleza**. Congresso Itelo-Latinoamericano de ETNOMEDICINA, 2011**.

Dunder, R.J ; Socca, E.A.R ; Luiz-Ferreira, A. ; **MANZO, L. P.** ; Takayama, C. ; ALMEIDA, A. C. A. ; De Faria, F. M ; SOUZA BRITO, A. R. M . Therapeutic properties of indigo. alkalopids derived from Indigofera Truxillensis Kunth in classic models of inflammation. In: **International Society of Ethnopharmacology and Encontro Hispano Português de Etnobiologia (ISE & HPE), 2010**,

De Faria, F. M A.; Luiz-Ferreira; Socca, E.A.R; ALMEIDA, A. C. A; Dunder, R.J; **MANZO, L. P;** da Silva,M.A; Vilegas, W; Pellizzon, C.H; dos Santos, L.C.; SOUZA BRITO, A. R. M . Effects of Rhizophora mangle on experimental colitis induced by TNBS in rats**.** In: **Reunião anual das Federaçaoes de Sociedades de Biologia Experimental, 2012**, Águas de Lindóia-SP.