https://doi.org/10.31925/farmacia.2023.5.6 **ORIGINAL ARTICLE**

ANTI-INFLAMMATORY EFFECT OF MONOAMMONIUM GLYCYRRHIZINATE ON EXPERIMENTAL COLITIS

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Abstract

Monoammonium glycyrrhizinate (MAG), as a derivative of glycyrrhizic acid, has anti-inflammatory, anti-allergic, anti-tumour, antimicrobial, antioxidant, anti-diabetic, anti-ulcer and hepatoprotective effects. In this study, we aimed to investigate the anti-inflammatory activity of MAG on hapten-induced experimental colitis. MAG in doses of 30 and 50 mg/kg bw was injected intraperitoneally for 6 days, starting one day before inducing colitis with 2,4-dinitrobenzene sulfonic acid. On day 6, colon segments were scored for macroscopic and histological damage. Blood samples were taken in order to measure levels of cytokines by ELISA methods. Our data showed that MAG at a dose of 50 mg/kg bw improved clinical symptoms and macroscopic and histological damage to the colon in all the rats with colitis. MAG significantly affected the serum concentrations of the inflammatory cytokines TNF-α, IL-1 and IL-6 and the level of the anti-inflammatory cytokine IL-10 on day 6 after induction of colitis. These findings indicate that MAG significantly inhibits colonic inflammatory damage in a rat model of inflammatory bowel disease.

Rezumat

Sarea de monoamoniu a acidului glicirizic (MAG) are efecte antiinflamatorii, antialergice, antitumorale, antimicrobiene, antioxidante, antidiabetice, antiulceroase și hepatoprotectoare. În acest studiu, ne-am propus să investigăm activitatea antiinflamatorie a MAG asupra colitei induse experimental de haptene. MAG în doze de 30 și 50 mg/kgc a fost injectat intraperitoneal timp de 6 zile. Cu o zi înainte de inducerea colitei experimentale s-a administrat acidul 2,4-dinitrobenzen sulfonic. În ziua a 6-a, segmentele de colon au fost notate în funcție de leziunile macroscopice și histologice. S-au prelevat probe de sânge pentru a măsura nivelurile de citokine prin metode ELISA. Datele obținute au arătat că MAG, în doză de 50 mg/kgc, a îmbunătățit simptomele clinice, leziunile macroscopice și histologice ale colonului la toți șobolanii cărora li s-a indus experimental colita. MAG a afectat semnificativ concentrațiile serice ale citokinelor inflamatorii TNF-α, IL-1 și IL-6 și nivelul citokinelor antiinflamatorii IL-10, începând din ziua 6 după inducerea colitei. Aceste rezultate indică faptul că MAG inhibă în mod semnificativ leziunile inflamatorii ale colonului într-un model de boală inflamatorie intestinală la șobolani.

Keywords: inflammatory bowel diseases, experimental colitis, monoammonium glycyrrhizinate cytokines, ELISA

Introduction

Glycyrrhizic acid (GA) is a triterpenoid saponin found in root extracts of the liquorice plant (*Glycyrrhiza glabra*). When administered orally, GA bioavailability is low due to poor solubility and absorption through the intestinal mucosa [26]. The ammonium salt is produced by acid processing of the aqueous extracts, followed by neutralisation of the precipitated material with dilute ammonia, and then further purified by solvent extraction and other separation techniques [9]. Monoammonium glycyrrhizinate (MAG) is extensively transformed into its active principle, aglycone, 18β-glycyrrhetinic, and absorbed in the gut. GA exhibits various beneficial effects, including anti-inflammatory, anti-allergic, anti-tumour, antimicrobial, antioxidant, anti-diabetic, anti-ulcer and hepatoprotective properties [5]. GA demonstrates anti-inflammatory effects by reducing the levels of IFN-γ, IL-12 and IL-17, and increasing the levels of IL-10, and modulating the balance between TNF-α and IL-10 [17]. Inflammatory bowel diseases (IBDs) are characterised by chronic inflammation and relapses. These conditions occur due to an insufficient immune response to the intestinal microflora in individuals who are genetically predisposed or subjected to certain risk factors [4, 13, 20].

There are two forms of IBD: ulcerative colitis and Crohn's disease. The pathogenesis of the disease is not fully understood. It is suggested in the literature that the activation and imbalance between Th1, Th2 and Th17 cells play a role in the genesis of IBD [18]. Macrophage activation and increased production of cytokines such as TNF-alpha, IL-1 and IL-6 also contribute to the inflammatory response. Studies have shown the role of genetic factors in the development of IBD through disruption of the integrity of the epithelial barrier [24], deficiency of autophagocytosis

[23], congenital defects in receptor populations and problems related to the lymphocyte differentiation, especially in Crohn's disease [28, 29].

This study aimed to evaluate the anti-inflammatory activity of monoammonium glycyrrhizinate on experimental colitis induced by 2,4-dinitrobenzene-sulfonic acid hydrate (DNBS) in Wistar rats.

Materials and Methods

The Bulgarian Food Safety Agency approved these experiments. The experimental protocol was conducted following national laws and policies and international guidelines (EEC Council Directive 86/609, IL 358, 1, 12 December 1987).

Colitis was induced according to the method described by Barbara *et al*. [3]. During brief anaesthesia with a combination of ketamine (MSD, Animal Health, dose of 90 mg/kg bw) and xylazine (Bioveta, dose of 10 mg/kg bw), 30 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 mL of 50% ethanol was administered intrarectally through a polyethylene catheter placed 8 cm proximally to the anus. The controls were treated with 0.25 mL of 50% ethanol. Two groups of animals, MAG1 and MAG2, received MAG at doses of 30 or 50 mg/kg bw, respectively (suspended in phosphate buffered saline) intraperitoneally (i.p.) for 6 days. In order to reach a therapeutic plasma concentration of the active principle of MAG for the period of the experiment, we started the administration one day before the induction of colitis, because 18β-glycyrrhetinic acid has a large volume of distribution and a long half-life [2]. Glycyrrhizic acid, mono-ammonium salt (98.1% purity) and DNBS were purchased from Sigma Aldrich Ltd.

Evaluation of the severity of experimental colitis

The following parameters were measured from day 0 to day 6: body weight, food and water intake, stool consistency (0 = normal, 1 = sparse stools, 3 = diarrhoea), presence of blood in the stool (0 = negative, 1 = positive, 3 = profuse bleeding).

On day 6, the animals were sacrificed, and the colonic segments were isolated. The severity of intestinal inflammation was assessed macroscopically and histologically, according to the criteria reported by Antonioli *et al.* [1]. The macroscopic colonic damages were evaluated as follows: the presence of adhesions between the colon and other organs (0 = none, 1 = small, 2 = large adhesions); the presence of ulcers (0 = none, 1 = hyperaemia, 2 = ulcer without inflammatory reaction, 3 = wound with inflammation at one site, 4 = ulcers and inflammation at two sites, 5 = large lesions extending 1 - 2 cm along the length of the colon, 6 = large lesions extending over 2 cm). The score was increased by 1 for each millimetre of colon wall thickness. The length of the colon was measured. Two different people independently evaluated all parameters.

A light microscopy examination of the affected distal colon segment preparations stained with haematoxylin and eosin was performed. Histologic examination aimed to detect changes in mucosal architecture, cellular infiltration, the presence of crypt abscess and goblet cell depletion.

Immunological studies

Blood samples were collected to determine the cytokine levels (IL-1alpha, IL-6, IL-10 and TNF-alpha) by enzyme-linked immunosorbent assay (ELISA) methods. The immunological tests were performed according to methods described in company protocols (R&D Systems). Each sample was tested in duplicate.

Statistical analysis

Results were presented as mean ± S.E.M. and tested by one-way ANOVA, followed by Fisher's least significant difference procedure as a post-hoc test. A p-value lower than 0.05 was considered statistically significant. Analyses were performed using STATGRAPHICS® Centurion XV statistical software.

Results and Discussion

Up to day 6 after DNBS administration, we recorded changes in body weight, reduced food intake, diarrhoea and sometimes blood in the stool. A significant body weight loss was recorded after inducing colitis (Figure 1). On day 6, the rats in the DNBS and the MAG1 groups (30 mg/kg bw) decreased in body weight by 34.2 ± 8.7 g and 32.5 ± 5.3 g, respectively, while rats in the control and MAG2 groups (50 mg /kg bw) gained weight (16.7 ± 3.2 g and 12.0 ± 2.8 g, respectively).

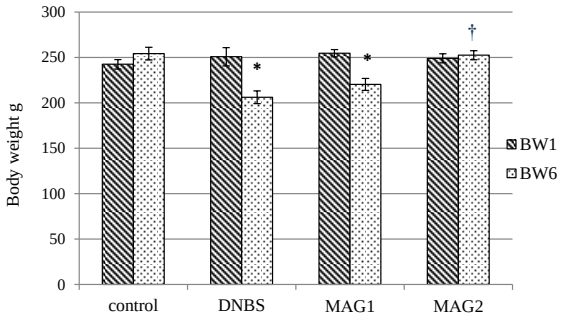

Figure 1.

Body weight on days 1 and 6. Results are presented as mean ± S.E.M. for 8 animals

(* indicates a statistical significance of $p < 0.05$ *vs.* control; † indicates a statistical significance of $p < 0.05$ *vs.* DNBS group)

On day 6 after DNBS administration, the distal colon was thickened, with ulcerative changes and areas of inflammation, expressed by an ulcer index of 3.87 ± 0.61. Intestinal lesions were widespread, but the site of maximum changes was usually located in the distal 4 - 5 cm of the colon. Macroscopic evaluation of preparations in the MAG2 group showed a significant

beneficial effect (ulceration index 1.63 ± 0.25; $p < 0.05$).

The state of diarrhoea was assessed based on the consistency of the stool and the presence of blood. Changes in the diarrhoea index were observed in all animals with induced colitis. The highest diarrhoea index was registered on day 3, with a value of 2.38 ± 0.1 in the DNBS group. A significant difference was observed in the state of diarrhoea compared to the MGA1 and MGA2 groups (index 1.54 ± 0.4 and 1.32 ± 0.18, respectively).

Our data showed that MAG at a dose of 50 mg/kg improved clinical symptoms and macroscopic and histological damage to the colon in all the rats with colitis. DNBS administration resulted in significant weight loss, decreased food intake and alterations in stool consistency, including the observation of blood in all rats with colitis. However, no instances of mortality were recorded. The DNBS-treated rats showed a significant decrease in body weight and food consumption, most pronounced on day 3 ($p < 0.05$). However, these indicators were favourably affected by MAG at a dose of 50 mg/kg bw, and their values were significantly higher, compared to the DNBS group. On day three, after the introduction of the aggressive agent, loose stools were observed in 33% of the animals, diarrhoea in 67% of the animals and the presence of blood in the stools (profuse bleeding) in 40% of the rats with colitis. Out of the rats treated with a dosage of 50 mg/kg bw of MAG, only two exhibited symptoms of diarrhoea and blood in their faeces. Macroscopic evaluation of tissues obtained from animals treated with MAG at a dose of 50 mg/kg bw showed that 50% (n = 4) of the animals had hyperaemia and 3 rats had ulcers with no inflammatory reaction. The animals with colitis experienced notably more severe changes, as 7 out of 8 rats in the group exhibited ulcerative changes characterised by inflammation and large lesions.

Immunological evaluation of the inflammatory cytokines IL-1, IL-6 and tumour necrosis factor-alpha (TNF-α) in the groups treated with MAG showed significantly lower serum levels than those of the DNBS group, while the level of anti-inflammatory IL-10 was markedly increased by MAG (Figures 2 and 3). These effects were more pronounced in the MAG2 group (dose of 50 mg/kg bw).

IBD is considered a multifactorial disease due to the interaction between the immune system, genetic predisposition and responses to environmental and microbial factors [7-9]. A hapten-induced experimental DNBS colitis model was used. The aggressive agent dissolves in alcohol, which improves the penetration of DNBS into the *lamina propria* [3]. DNBS induces haptenization of nearby proteins, leading to their immunogenicity and subsequent activation of the host immune system. This activation results in the recruitment of neutrophils, macrophages and T lymphocytes. DNBS-hapten-induced inflammation involves specific cells, including polymorphonuclear neutrophils, lymphocytes, plasma cells and eosinophils [1].

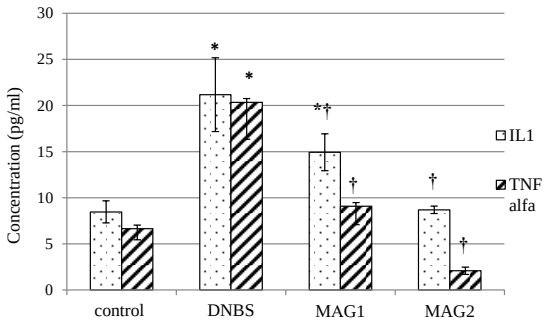

Figure 2.

Level of IL-1 and TNF-α in plasma on day 6. Each column represents the mean ± S.E.M. for 8 animals (* indicates statistical significance $p < 0.05$ *vs.* control; † indicates statistical significance $p < 0.05$ *versus* DNBS group)

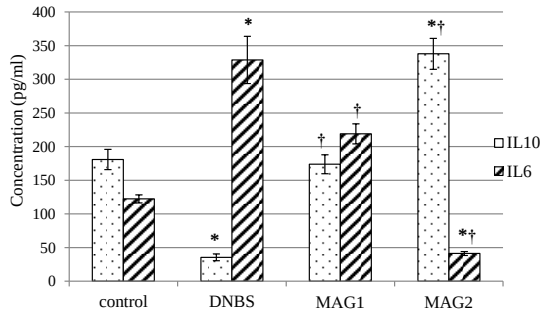

Figure 3.

Level of IL-6 and IL-10 in plasma on day 6. Each column represents the mean ± S.E.M. for 8 rats (* indicates statistical significance $p < 0.05$ *vs.* control group; † indicates statistical significance $p < 0.05$ *versus* DNBS group)

Inflammatory cells can cause tissue damage by releasing oxygen-free radicals, proteases and inflammatory mediators such as cytokines and chemokines. Cytokines are important in the pathogenesis of IBD, and the regulation of their synthesis successfully reduces disease severity and maintains remission. Activated immune cells secrete several cytokines that actively regulate the inflammatory response in CD and UC. After being secreted by antigen-presenting cells, cytokines stimulate and differentiate numerous T-cells, thereby initiating the adaptive immune response. Macrophage expression of TNF-α has been detected in colonic tissues, and serum levels of TNF-α correlate with clinical and laboratory indicators of intestinal disease activity [3]. In addition to TNF-α, IL-1 and IL-6 have pronounced proinflammatory activity. They are secreted by various cell types through the initiation of cyclooxygenase type 2, phospholipase A and inducible nitric oxide synthase, and are involved in immunological responses in the development of IBD. IL-10 has anti-

inflammatory and immunosuppressive properties. It reduces both the presentation of antigens and the subsequent release of proinflammatory cytokines, thereby attenuating mucosal inflammation.

In our experiment, an increase in the serum concentrations of inflammatory cytokines TNF-α, IL-1 and IL-6 and a decreased level of anti-inflammatory cytokine IL-10 on day 6 after induction of colitis were found in DNBS-treated rats. MAG significantly affected the concentration of cytokines, supporting the claim that it produces anti-inflammatory effects.

Microscopic damages were assessed by light microscopy on haematoxylin-eosin-stained preparations. Large areas of mucosal necrosis with completely destroyed glandular architecture were described in the colon of DNBS-treated rats: thickened submucosa with oedema and pronounced infiltration with inflammatory cells, vasodilatation and a thickened muscle layer (Figure 4). Rats treated with a dosage of 50 mg/kg bw of MAG exhibited signs of wound healing, including epithelial regeneration. However, there were still indications of mucin depletion and nuclear changes in the epithelial cells. The goblet cell population in the crypts showed signs of recovery, and the *lamina propria* exhibited a decrease in acute inflammatory infiltrates. Additionally, the adjacent mucosa displayed single crypt-abscesses (Figure 5).

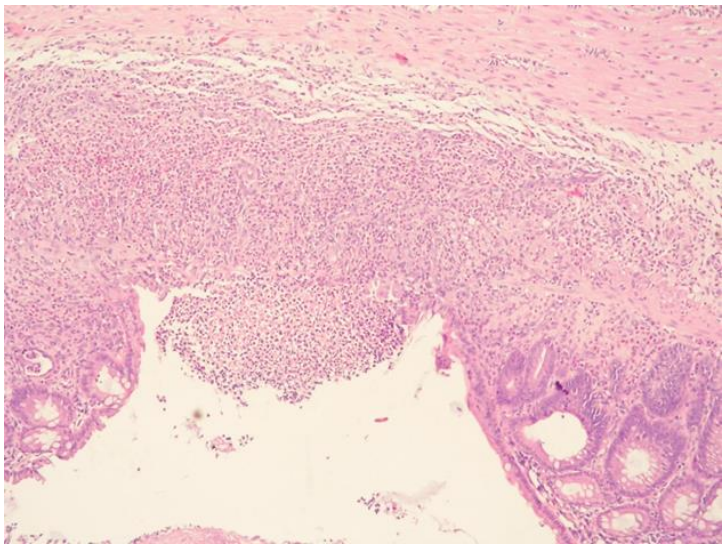

Figure 4.
Severe active inflammation with a surface ulcer, crypt-abscess (the lumen of the crypts is filled with neutrophilic leukocytes), reactive epithelial changes and crypts of different sizes and shapes (H&E; x 200)

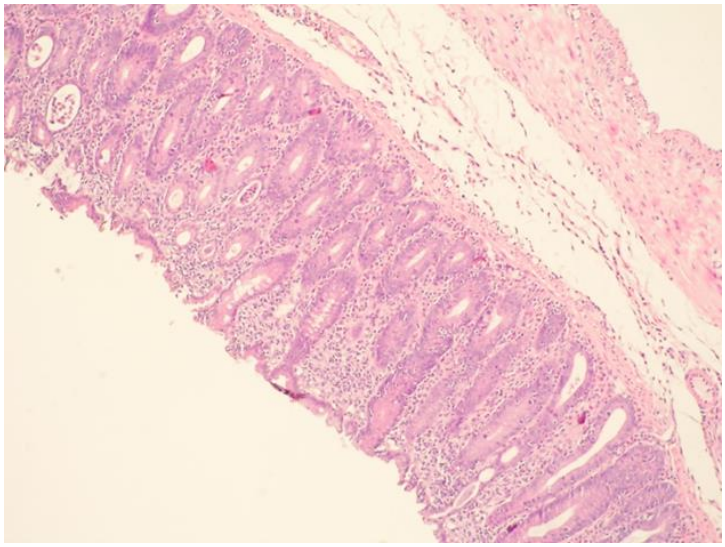

Figure 5.
Chronic inflammation with reverse development (after treatment with MAG 50 mg/kg bw): epithelial regeneration, persistence of mucin depletion and nuclear changes in epithelial cells, recovery of goblet cell population in the crypts, *lamina propria* with reduction of acute inflammatory infiltrates (H&E; x 200)

Signs of wound healing, including the restoration of normal mucosal structure and the presence of granulation tissue characterised by proliferating capillaries and chronic inflammatory infiltrate, were observed in rats treated with a dosage of 50 mg/kg bw of MAG. Other authors have also studied the effects of glycyrrhizin, glycyrrhizic acid and dipotassium salt of glycyrrhizic acid in experimental models of colitis [6, 10, 12, 22, 25, 27]. The administration of these compounds affected the inflammatory responses of the colonic mucosa and oxidative tissue damage, as well as the healing of the mucosal epithelium. The anti-inflammatory effect of glycyrrhizinate extract mediated by NF-κB and TNF-α was evaluated in acetic acid-provoked colitis by Yuan *et al*. [27]. The protective effect of oral administration of glycyrrhizic acid and application of enemas containing glycyrrhizin was investigated in trinitrobenzene sulfonic acid-induced experimental colitis by Sun *et al*. and Liu *et al*. [12, 22]. They found that natural-derived drugs significantly improved macroscopic and microscopic inflammation and myeloperoxidase activity, downregulated the colonic levels of proinflammatory cytokines and increased the anti-inflammatory cytokine IL-10.

When applied locally in experimental dextran colitis, glycyrrhizin preparation reduced the pro-inflammatory cytokines and chemokines interleukin IL-1β, IL-6, TNF-α, CXCL2, CCL2 and inhibited myeloperoxidase activity in the inflamed mucosa [10]. In the same model, GA reduced IL-6 and IL-1β and the expression of COX-2 and PGE2 [6]. Vitali *et al*. have shown that treatment with dipotassium salt of GA reduces the severity of dextran-induced colitis, decreases the release of an early pro-inflammatory cytokine HMGB1, TNF-α, IL-1β and IL-6, and, during inflammation, IL-8 has a protective effect by inhibiting oxidative stress [25]. The results from these experiments support the hypothesis that *Glycyrrhiza glabra*-derived compounds have anti-inflammatory and antioxidant activity in intestinal disorders through different mechanisms: regulating the production of major cytokines, interleukins and genes involved in inflammation and oxidative stress. Using glycyrrhizic acid and other related compounds has been expanding in the manufacturing of different types of herbal products for a wide range of diseases [14]. At appropriate doses, anti-inflammatory, antidiabetic, antioxidant, hepatoprotective, antitumor, antimicrobial and anti-viral properties have been reported in many studies [8, 14], but there is no clinical evidence showing the effect of liquorice in patients with intestinal disorders [11]. Other relevant questions are related to their pharmacokinetics and safety. A promising perspective to overcome some limiting physicochemical parameters is the development of nanoplatforms, micellar carriers and o/w emulsions containing propylene glycol for skin penetration [7, 15, 19, 21]. Ammoniated glycyrrhizic acid is utilised in the confectionery and cosmetic sectors, as well as being added to animal feed, thereby potentially increasing consumer exposure [4]. According to Nazari *et al*., glycyrrhizin salts are moderately toxic. The most significant adverse effects are reversible elevations of blood pressure and hypokalaemia-induced secondary disorders [16].

Conclusions

DNBS-induced colitis is a model of intestinal inflammation that exhibits symptoms such as weight loss, decreased food intake, diarrhoea (including bloody stool), ulceration and distinct histological alterations. Our findings showed that glycyrrhizic acid monoammonium salt significantly improves the general condition and reduces the inflammatory damage of the colon in rats with an experimental model of inflammatory bowel disease.

Clinical trials covering a large number of patients are needed to clarify the benefits of *Glycyrrhiza glabra*-derived compounds in the therapy of IBD and as a natural therapeutic strategy to prevent complications in patients with long-term IBD.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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