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Research article

USE OF THE PREBIOTIC INULIN IN THE PREVENTION OF ADVERSE SIGNS OF ACUTE COLITIS

HIJOVÁ Emília*, ŠOLTÉSOVÁ Alena, SALAJ Rastislav, KUZMA Jozef, STROJNÝ Ladislav, BOMBA Alojz

Institute of Experimental Medicine, Medical Faculty, University of P. J. Šafárik, Košice, Slovak Republic

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The aim of this study was to investigate the influence of prebiotic inulin diet intervention on the activity of β-glucuronidase and counts of coliforms and lactobacilli in fresh caecal digesta, cytokine levels (IL-6, IL-8) and transcription nuclear factor kappa beta (NFkB) activities in the colon tissue and blood samples of rats with dextran sulphate sodium (DSS) induced acute colitis. Male Sprague-Dawley rats (8 per group) were randomly divided into three groups: Control, Acute colitis and Prebiotic. Colitis was induced using 5% DSS in drinking water for 7d. DSS application significantly increased the activity of β-glucuronidase ($p<0.001$), increased counts of coliform bacteria and decreased lactobacilli count ($p<0.05$) in comparison to the control group. Serum and tissue levels of IL-6 and IL-8 as well as tissue NFkB activities showed an increased expression in the acute colitis group. These results correspond to the average value of the disease activity index score (DAI) and revealed the maximum DAI score (6.5) in the acute colitis group. A decrease in the DAI score (4.13) was observed after application of the prebiotic inulin. Inulin diet intervention positively modified the number of microorganims and decreased β-glucuronidase activity. Colon tissue activities of NFkB were significantly suppressed ($p<0.001$). The synthesis of proinflammatory cytokines IL-6 ($p<0.01$) in the serum and in the colon tissue, as well as tissue IL-8 ($p<0.05$) in the prebiotic group were downregulated. These findings indicate that the dietary intake of inulin suppressed the expression of the observed markers, which play an important role in the inflammatory process, which predisposes to the use of inulin in the prevention or treatment of acute colitis in human and veterinary medicine.

Key words: colitis, Sprague-Dawley rats, inulin, inflammation

INTRODUCTION

Inflammatory bowel diseases (IBD) are well known gastrointestinal disorders in humans; however, similar disorders are also present and often investigated in several animal species [1]. As a nosological unit within inflammatory bowel diseases ulcerative colitis (Colitis ulcerosa - CU) is a chronic, nonspecific relapsing disease characterised by diffuse mucosal inflammation limited to the colon. It is a serious medical and

Corresponding author: e-mail: emilia.hijova@upjs.sk

socio-economic problem with an increasing incidence and prevalence in humans. The aetiology of ulcerative colitis is not yet understood. Failure of immunoregulatory mechanisms due to external environmental factors in genetically predisposed individuals suggests the current concept of the pathophysiology of inflammatory diseases. The course of CU is characterized by an impact frequency of unpredictable occurrence of relapse (acute inflammation) and remission (inflammation in decline). In genetically susceptible individuals, an abnormal communication between the intestinal microbial flora and mucosal immune system forms the basis of the defect, which is accompanied by mucosal inflammatory lesions of the gastrointestinal tract. Studies on animal models of colitis indicated that dysregulation of host/microbial interactions, which included the loss of epithelial and cell barrier functions, were the pre-requisite for the development of IBD [2,3]. Risk factors for colorectal carcinoma development in CU include the duration of the disease, early age of onset, family history of sporadic colorectal carcinoma, and persistent inflammation of the colon [4]. Patients with long-standing CU have a higher risk of developing colitis-associated cancer (CAC) approximately 8-10 years after the initial diagnosis when compared to the general population [5]. In addition to genomic instability that underlies the process of tumorigenesis, continuous inflammation in the intestine seems to be a key factor in CAC development since chronic inflammation is associated with an overexpression of proinflammatory cytokines, chemokines, growth factors as well as their receptors, and reactive oxygen species [6,7].

A high morbidity, significant early and late complications, and decreased quality of life are the reason for seeking new possibilities for the prevention, rational diagnosis and treatment. It has been suggested that modifying the bacterial flora with probiotics and prebiotics may attenuate the inflammatory process and prevent relapses and maintenance of remission in ulcerative colitis [8].

The goal of the presented study was to obtain informations about the effects of the prebiotic inulin on the activity of β-glucuronidase and on the counts of coliforms and lactobacilli in fresh caecal digesta, cytokine levels (IL-6, IL-8), and transcription nuclear factor kappa beta (NFkB) activities in colon tissue and blood samples of rats with dextran sulphate sodium (DSS) induced acute colitis.

MATERIAL AND METHODS

Animals and experimental design

All animals in the experiment were kept in accordance with the principles outlined in Law No. 377/2012 and No. 436/2012 of the Slovak Republic for the Care and Use of Laboratory Animals, and were approved by the Ethical Committee of the Faculty of Medicine of P.J. Šafarik University and State Veterinary and Food Administration of the Slovak Republic (Ro 1136/14-221). Male Sprague-Dawley rats (n=24, 8 per group, 7 weeks old, 220 - 290 g body weight) were housed at the Laboratory of Research Bio-

models of the Faculty of Medicine, P. J. Šafárik University, Slovak Republic with a 12-h light/dark cycle. The room was maintained at 21°C ± 1°C with 50% to 60% humidity.

The rats were randomly assigned to the following groups: C**ontrol** (control group) received the conventional feed (Snina, Slovak Republic) for 14 days, **Acute colitis** (acute colitis group) received conventional feed without DSS for 7 days followed by 7 days of feed with DSS, and **Prebiotic** (acute colitis + prebiotic) group received conventional feed supplemented with prebiotic inulin (BeneoSynergy 1, ORAFTI, Tienen, Belgium) at a dose of 80g/kg feed without DSS for a period of 7 days, followed by 7 days of the same feed with DSS.

All animals had free access to water and feed. Animal weights and clinical monitoring of the health status were recorded daily. After 14 days of consuming the experimental diets, the animals were euthanized under anesthesia (Zoletil, Virbac S.A., France) administered at a dose of 50mg/kg body weight with Xylazin (Riemser, Germany) at a dose of 15 mg/kg body weight, intramuscular). Blood samples were taken by cardiac puncture. Caecal and tissue samples from the colon were recovered for microbial, biochemical and immunological analysis.

Induction of colitis

Colitis was induced using DSS (molecular weight 40 000, TdB Consulting AB, Uppsala, Sweden) added to drinking water at a final concentration of 5% (wt/vol) for 7d. Controls were all time-matched and consisted of rats receiving normal drinking water only. The DSS solution was replenished daily and mean DSS consumption was noted per cage at the end of 7d treatment.

Disease activity index

Disease activity index (DAI) is the combined score of animal weight loss, stool consistency and bleeding present in the stool as described in Table 1. Stool probes were tested to evaluate rectal bleeding by using HemoFEC test (Roche Diagnostics, Slovak Republic). All parameters were assessed on a scale from 0 to 3, or 4, the maximum score was 10. These parameters were each assigned a score and utilized to calculate an average daily disease activity index score for each rat as previously described [9].

Table 1. Disease activity index of acute ulcerative colitis

Score	Stool consistency	Bleeding	Weight loss	Maximum score
0	Formed	Normal color stool	No weight loss	10
1	Mild soft	Brown color stool	5-10% Weight loss	
2	Very soft	Reddish color stool	11-15% Weight loss	
3	Watery stool	Bloody stool	16-20% Weight loss	
4			>20% Weight loss	

Bacteriological examination

Microbial analysis (total counts of lactobacilli and coliforms) of the fecal samples was carried out after completion of the experiment. Feces (1g) were placed in a sterile polyethylene Stomacher Lab Blenders bag (Seward, France) with 9 mL sterile 0.9% NaCl and mixed in BagMixer 400 (Interscience, France). A series of 10-fold dilutions (10^{-2} to 10^{-8}) were made with the same sterile diluent. From each dilution, 0.1 mL aliquots were spread onto selective McConkey agar plates (Merck, Germany) for coliforms and Rogosa agar plates (Biokar Diagnostics, France) for lactobacilli. The plates for lactobacilli culturing were maintained under anaerobic conditions (BD GasPak, Becton, Dickinson and Company, USA) and incubated at 37ºC for 48 h. Plates used for coliform culturing were incubated aerobically at 37ºC for 16-18 h. The numbers of colony forming units (CFU) are expressed as $\log_{10}$ CFU per gram of feces.

Measurement of caecal β-glucuronidase activity

The activity of β – glucuronidase (β-GLUCUR) bacterial enzyme was measured in fresh caecal digesta taken after completion of the experiment by determining the rate of *p*- or *o*-nitrophenol as previously described by Juskiewicz et al. [10]. The reaction contained 0.3 mL of substrate solution (5 mM) *p*-nitrophenyl-β-D-glucuronide for β-glucuronidase (Sigma Aldrich, USA) and 0.2 mL of 1:10 (v/v) dilution of the caecal digesta in 100 mM phosphate buffer (pH 7.0) centrifuged at 10,000 g for 15 min at 4ºC. Incubation was carried out at 37ºC for 10 min, and *p*- or *o*-nitrophenol was quantified after addition of 0.25 M cold sodium carbonate. Absorbtion was measured at 400 nm. Enzymatic activity is expressed as μmol of *p*–nitrophenol per min per gram digesta.

Cytokine levels (IL-6, IL-8), and NFkB in serum and colon homogenates

Blood samples were left to clot for two hours at room temperature before centrifugation for 15 mimutes at 1000xg. The removed serum samples were stored at -20ºC. Colon tissue samples were rinsed in ice cold PBS (pH 7.0-7.2) to remove excess blood thoroughly, cut longitudinally and homogenized in PBS with a homogenizer on ice (Disperser T10 Basic Ultra Turrax, Germany) and stored overnight at -20ºC. Thereon two freeze-thaw cycles were performed to break the cell membranes. After that the homogenates were centrifuged for 5 min at 5000xg at 2 - 8ºC, the supernatant was removed and stored at -20ºC. All enpoints were measured by ELISA method as follows: NFkB in tissue by USCN Life Science, Inc., USA; IL-6 in blood and tissue by eBioscience, USA; IL-8 in tissue by Cusabio Biotech Co., Ltd. China. The final values of each parameter were measured on Synergy H4 multiplate reader (BioTek Instruments, Inc. USA).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) with p values ($p<0.05$) considered to be statistically significant.

RESULTS

During the experimental trial, the clinical changes observed in rats did not lead to death. The mean body weight of the rats at the beginning of the experiment and at the end of the experiment in the control group increased by 30.92% (242.50 g ± 22.69 g vs. 317.50 g ± 21.69 g), in acute colitis by 9.90% (252.50 g ± 10.90 g vs 277.5 g ± 20.72 g), in acute colitis combined with the prebiotic by 14.28% (253.75 g ± 21.76 g vs 290.0 g ± 27.08 g). The average value of all daily disease activity index score showed that the maximum DAI score (6.5) was in the acute colitis group in comparison to the control group (0.3). A DAI score decline was observed after application of the prebiotic (4.13). The applied DSS significantly increased the activity of β-glucuronidase ($p<0.001$) in comparison to the control group and applied prebiotic non-significantly decreased the activity of this enzyme as shown Table 2. Changes of total counts of coliforms and lactobacilli in different experimental groups are summarized in Table 2. In Table 3 are shown changes in cytokines levels and NFkB activities in the serum (s) and tissue (t) in the control group, acute colitis group and in the prebiotic treated group.

Table 2. Activity of β-glucuronidase and total counts of lactobacilli and coliforms

Parameters	Control	Acute colitis	Prebiotic
β-GLUCUR, μmol/min/g	0.14 ± 0.02	0.54 ± 0.05 ***	0.49 ± 0.18
Lactobacilli, $\log_{10}$CFU/g	7.78 ± 0.17	7.15 ± 0.90 *	7.31 ± 0.45
Coliforms, $\log_{10}$CFU/g	5.18 ± 0.56	5.74 ± 1.03	5.51 ± 0.77

Values are expressed as mean ± SD. Statistical significance is between Control/Acute colitis: *$p<0.05$; ***$p<0.001$

Table 3. Serum and tissue changes in cytokine levels, NFkB activities

Parameters	Control	Acute colitis	Prebiotic
NFkB t, ng/mL	41.73 ± 7.41	60.21 ± 10.31 ***	11.65 ± 1.32 +++
IL-6 s, pg/mL	49.31 ± 15.83	61.89 ± 15.33	45.83 ± 9.99 ++
IL-6 t, pg/mL	47.00 ± 8.53	62.65 ± 12.19 **	48.77 ± 5.31 ++
IL-8 t, pg/mL	37.78 ± 7.42	50.12 ± 7.32 ***	47.67 ± 8.49 +

Values are expressed as mean ± SD. Statistical significance is between * Control/Acute colitis and + Acute colitis/Prebiotic: + $p<0.05$; **/++ $p<0.01$; ***/+++ $p<0.001$

DISCUSSION

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders that affects individuals throughout life. The role of interactions between genetic, immunologic, microbial and environmental factors is expected in UC, but the exact

etiology and pathogenesis still remains unclear. Long-standing UC has an increased risk of developing CAC/CRC (colon carcinogenesis) [11]. It is also widely accepted that chronic inflammation promotes carcinogenesis by inducing the production of a variety of cytokines and chemokines that propagate a localized inflammatory response by activating transcription factors such as NF-kB which is accompanied by increased COX-2, iNOS and pro-inflammatory cytokines. These findings have important implications for the possible development of anticancer treatments and offer an opportunity to devise strategies that supports the opinion that probiotics and prebiotics can provide an alternative or adjuvant approach to conventional therapy by modulating the intestinal microflora and host immune system. The goal of this study was to obtain information about the effect of a diet with added inulin in DSS-induced acute colitis rat model.

Prebiotics are a family of molecules which meet the classification criteria for being considered a prebiotic, as defined Gibson and Roberfroid [12]; i.e. resistance to hydrolysis or absorption in the upper gastrointestinal tract, fermentation by the intestinal microbiota, and selective stimulation of growth and/or activity of beneficial intestinal bacteria, such as *Lactobacillus* species and *Bifidobacterium* species which have a positive impact on the health of the host [13-15].

In our study DSS-induced acute colitis increased (not significantly) the number of coliforms and significantly ($p<0.05$) decreased the number of lactobacilli in the acute colitis group in comparison with the control group. Within a short experimental period inulin dietary supplementation positively modified the values observed for microorganisms and activity of β-glucuronidase. Unlike for lactobacilli and coliforms, the activity of β-GLUCUR is believed to be a biomarker of neoplasms and is also perceived as harmful due to the associated release of carcinogens from hepatically derived glucuronic acid conjugates. β-GLUCUR was significantly increased ($p<0.001$) in the acute colitis group.

Chronic inflammation is known to lead to the derangement in signaling processes and to a local microenvironment described as lying somewhere between pre-cancerous stromal cells and cancer cells, even as the details of the steps in the transformation to cancer cells are incompletely understood [16]. In the signaling pathways involved in colonic inflammation NFkB plays a key role. NFkB regulates the expression of various cytokines and modulates the inflammatory processes characteristic of IBD [17]. Further, NFkB controls apoptosis, cell-cycle progression, cell proliferation, and differentiation. Although NFkB activation has been shown to be involved in CRC development, normally functioning NFkB is essential for the maintenance of epithelial cell homeostasis in the gut. Translocation of activated NFkB into the nucleus induces the expression of cytokines such as TNFα and IL-6, and chemokines, all of which contribute to the development of inflammation-related tissue damage. The activity of NFkB in colon tissue samples was markedly increased in acute colitis ($p<0.001$) and may provide a sensitive mean of assessing the state of activation of the mucosal immune response. Inulin treatment suppresed the activity of the critical transcription

factor in mucosa cells ($p<0.001$). Activated NFkB in the acute colitis group significantly activated serum and colon tissue levels of pro-inflammatory cytokines (IL-6 and IL-8) compared to the control group. Dietary intervention of acute colitis with inulin significantly downregulated the synthesis of proinflammtory cytokines IL-6 ($p<0.01$) in the serum and colon tissue and IL-8 ($p<0.05$) in the tissue compared to the acute colitis group. Preclinical and experimental research on the complex IBD puzzle coupled with an active and vibrant research agenda in recent decades might reveal patterns of pharmacological interactions instead of potential single drug targets. The increased collaboration between pharmacological companies, basic researchers and clinical researchers has the potential to bring us closer to developing an optimal pharmaceutical and nutritional approach for the treatment of IBD. The results of the present experiment demonstrate the ability of elected food supplements, such as the prebiotic inulin to intervene and affect the pathophysiological processes in acute colitis. The exact etiology and pathogenesis of ulcerative colitis is not yet known, and in that regard the use of prebiotics is a suitable form of prevention in human and veterinary medicine.

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UPOTREBA PREBIOTIKA INULINA U PREVENTIVI ŠTETNIH MANIFESTACIJA AKUTNOG KOLITISA

HIJOVÁ Emília*, ŠOLTÉSOVÁ Alena, SALAJ Rastislav, KUZMA Jozef, STROJNÝ Ladislav, BOMBA Alojz

Cilj ovog rada bio je da se ispita uticaj dijetarne intervencije sa prebiotikom inulinom na aktivnost β-glukuronidaze i broja koliformnih bakterija i laktobacila u svežem sadržaju cekuma, nivoa citokina (IL-6 i IL-8) i aktivnosti transkripcionog nuklearnog faktora kapa beta (NFkB) u tkivu kolona i uzorcima krvi pacova sa kolitisom indukovanim dekstran-sulfat natrijumom (DSS). Muški Sprague-Dawley pacovi (8 u grupi) su bili nasumično raspoređeni u tri grupe: kontrolna, akutni kolitis i prebiotik grupa. Kolitis je indukovan pomoću 5% DSS u pijaćoj vodi tokom 7 dana. Dodavanje DSSa je signifikantno povećalo aktivnost β-glukuronidaze ($p<0,001$), povećalo broj koliformnih bakterija i smanjilo broj laktobakcila ($p<0,05$) u poređenju sa kontrolnom grupom. Serumski i tkivni nivoi IL-6 i IL-8, kao i tkivna NFkB aktivnost su pokazali povećanu ekspresiju u grupi sa akutnim kolitisom. Ovi rezultati odgovaraju prosečnim vrednostima indeksa aktivnosti bolesti (DAI) i pokazali su da su maksimalne vrednosti DAI bodovanja (6,5) postignute u grupi sa akutnim kolitisom. Smanjenje DAI vrednosti (4,13) je uočeno nakon aplikacije prebiotika inulina. Dijetarna intervencija inulinom pozitivno je modifikovala broj mikroorganizama i smanjila aktivnost β-glukuronidaze.

Tkivne aktivnosti NFkB u kolonu bile su signifikantno ($p<0,001$) suprimirane. Sinteza proinflamatornog citokina IL-6 ($p<0,01$) u serumu i kolonu, kao i tkivni IL-8 ($p<0,05$) u grupi tretiranoj prebiotikom bila je smanjena. Nalazi ukazuju da dijetarni unos inulina suprimira ekspresiju praćenih markera koji imaju bitnu ulogu u inflamatornim procesima, što predisponira upotrebu inulina u prevenciji i terapiji akutnog kolitisa u humanoj i veterinarskoj medicini.