

M. ZWOLINSKA-WCISLO<sup>2</sup>, T. BRZOZOWSKI<sup>1</sup>, A. BUDAK<sup>3</sup>, S. KWIECIEN<sup>1</sup>,  
Z. SLIWOWSKI<sup>1</sup>, D. DROZDOWICZ<sup>1</sup>, D. TROJANOWSKA<sup>3</sup>, L. RUDNICKA-SOSIN<sup>4</sup>,  
T. MACH<sup>2</sup>, S.J. KONTUREK<sup>1</sup>, W.W. PAWLIK<sup>1</sup>

EFFECT OF *CANDIDA* COLONIZATION ON HUMAN ULCERATIVE COLITIS AND THE  
HEALING OF INFLAMMATORY CHANGES OF THE COLON IN THE EXPERIMENTAL  
MODEL OF *COLITIS ULCEROSA*

<sup>1</sup>Department of Physiology Jagiellonian University Medical College, Cracow, Poland;

<sup>2</sup>Clinic of Gastroenterology, Hepatology and Infections Diseases, Jagiellonian University Medical College, Cracow, Poland; <sup>3</sup>Department of Pharmaceutical Microbiology, Jagiellonian University Medical College, Cracow, Poland; <sup>4</sup>Department of Clinical and Experimental Pathomorphology, Jagiellonian University Medical College, Cracow, Poland

The influence of fungal colonization on the course of ulcerative colitis (UC) has not been thoroughly studied. We determined the activity of the disease using clinical, endoscopic and histological index (IACH) criteria in UC patients with fungal colonization and the healing process of UC induced by an intrarectal administration of trinitrobenzene sulfonic acid (TNBS) in rats infected with *Candida*, without and with antifungal (fluconazole) or probiotic (lacidofil) treatment. The intensity of the healing of the colonic lesions was assessed by macro- and microscopic criteria as well as functional alterations in colonic blood flow (CBF). Myeloperoxidase (MPO) content and plasma proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  levels were evaluated. *Candida* more frequently colonized patients with a history of UC within a 5-year period, when compared with those of shorter duration of IBS. Among *Candida* strains colonizing intestinal mucosa, *Candida albicans* was identified in 91% of cases. Significant inhibition of the UC activity index as reflected by clinical, endoscopic and histological criteria was observed in the *Candida* group treated with fluconazole, when compared to that without antifungal treatment. In the animal model, *Candida* infection significantly delayed the healing of TNBS-induced UC, decreased the CBF and raised the plasma IL-1 $\beta$  and TNF- $\alpha$  levels, with these effects reversed by fluconazole or lacidofil treatment. We conclude that 1) *Candida* delays healing of UC in both humans and that induced by TNBS in rats, and 2) antifungal therapy and probiotic treatment during *Candida* infection could be beneficial in the restoration and healing of colonic damage in UC.

Key words: *ulcerative colitis, Candida, proinflammatory cytokines, colonic blood flow, fluconazole, probiotics*

---

## INTRODUCTION

The chronic inflammatory bowel diseases (IBD) comprising of both, ulcerative colitis (UC) and Crohn's disease are characterized by chronic

relapsing inflammation of the gastrointestinal (GI) tract. The pathogenesis of IBD involves four major factors: individual susceptibility, genetic predisposition, microflora of the GI-tract and immunological properties of the gastrointestinal

mucosa, however, microbial aspect plays an important role in the pathogenesis of these disorders (1). It became clear that the microflora of the GI-tract comprises of 400-500 different species, corresponding to  $10^{14}$  of microorganisms, which with their own metabolism represent, an "additional organ" of humans. This has thus far been neglected in the discussions on the pathogenesis of UC (1-4). GI-tract microbiota can be divided into two distinct ecosystems, namely the luminal bacteria, which are either dispersed in liquid feces or bound to food particles, and the mucosa-associated bacteria, bound to the mucus layer adjacent to the intestinal epithelium. Luminal microbiota play an important role in the bloating and flatulence in irritable bowel syndrome (IBS) through carbohydrate fermentation and gas production. However, mucosa-associated microbiota although fewer in number, influences the host *via* immuno-microbial interactions.

The significant role of microbiological factor in the pathogenesis of IBD seems to be confirmed by experimental studies carried out in animals. These studies revealed that colitis could not be induced under conditions of a sterile environment in the GI-tract (5). Until recently microbiological studies have been focused on the pathogenic role of bacteria but the presence and activity of fungi in the GI-tract have not been extensively studied. Under physiological conditions, a dynamic balance between microorganisms present in the intestinal lumen and multifactorial host defense mechanisms exists. As a result of a controlled inflammatory response and eradication of microorganisms by a functional intestinal barrier, a tolerance phenomenon develops (5). Under pathological conditions, especially during the active stage of UC, a decrease in the count of anaerobic bacteria and facultative microorganisms is observed (4, 5).

Recently research has addressed concerns regarding the importance of fungi presence in the lumen of the GI-tract and their effect on the course of non-specific inflammations of the GI system (4, 6). Fungal overgrowth within the gut may be the complication of a bacterial imbalance such as that associated with antibiotic therapy. It can be hypothesized that alteration in the balance between bacterial and fungal species in the mucosal microflora reflects a metabolic imbalance between the microbial ecosystem and the impairment of the mucosal barrier (5). Thus, the GI-tract is the most important reservoir of saprophytic fungi (7).

Another topic of growing interest is the therapeutic role of probiotics, such as food containing microorganisms. This is predominately

due to the favorable effects of probiotics on the course of colitis has been demonstrated using animal models (3, 8). These products are generally delivered into the gut as yogurts, fermented milks, powders, and capsules or as bacterial species such as lactobacilli or bifidobacteria administered in doses ranging from  $10^8$  to  $10^{11}$  bacteria. In humans a beneficial effect of probiotics has unequivocally been proven in prophylactics for pouchitis relapse in patients after colectomy (9). Specific probiotics also appear to directly modulate intestinal pain. The natural course of UC in humans and Crohn's disease patients generally consists of symptomatic periods alternating with remissions. Anti-inflammatory 5-aminosalicylic acid (5-ASA) and the immune suppressive azathioprine are the immune modulators most commonly used to control the symptoms of IBD (10). Azathioprine has progressively replaced 5-ASA in the management of IBD because this drug exhibits superior efficiency for induction and remission in patients with steroid-dependent colitis.

The role of fungal colonization of the GI-tract and its influence on the course of IBD has not yet been fully explored. One of the main reasons for this may be the lack of comparable animal models, which resemble the inflammatory lesions in the colon similar to that observed in humans. Therefore, we attempted to estimate the prevalence of fungi infection in the colon of patients with UC as a function of the duration and activity of the disease in comparison to control group of patients with a diarrhoeal form of IBS. For this purpose, we evaluated the UC activity regarding clinical, endoscopic and histological criteria in comparison with the control IBS group. Moreover, the effect of antifungal therapy with fluconazole or the treatment with probiotic lacidofil was determined in patients with significant fungal colonization of colon mucosa who complained of symptoms such as nausea, appetite problems or weight loss. In addition, part of the patients entered the study based on inflammatory endoscopic and morphologic changes in the intestine. In order to look for the mechanism of fungi effect on UC, we employed animal model of UC that was designed to examine the effects of *Candida* colonization, antifungal as well as probiotic treatment on the healing process of the inflammatory colonic lesions. We also determined the accompanying alterations in colonic blood flow (CBF), histology of the colonic mucosa and the plasma levels of proinflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  in rats with intrarectal administration of trinitrobenzene sulfonic acid (TNBS) infected with *Candida albicans*.

## MATERIALS AND METHODS

### *Clinical studies*

The human study involved 89 UC patients between the ages of 18-72 years, including 56 patients who were in active (symptomatic) and 33 patients at a non-active (non-symptomatic) stage of UC. The control group included 12 patients with diarrhea as a form of IBS (*Table 1*). The patients were informed of the purpose of the study and agreed to participate and were required to sign a document of informed. The local Bioethics Committee at the Jagiellonian University Medical College in Cracow approved both, the clinical and experimental studies. All patients were admitted to the Outpatient Unit of the Department of Gastroenterology and Hepatology of the University Hospital in Cracow with symptoms such as abdominal pain, and typical or bloody diarrhea. The symptoms were presented at the moment of admission or were reported at the time of history taking. The clinical examination included history taking, in particular, current symptoms, duration of UC and the number of the disease relapses during the year. At the beginning of the study and after 4 weeks of follow up, the activity of disease was evaluated. The endoscopic lesions in the colon were assessed with particular respect to the changes indicating UC, the extent and activity of the disease. The assessment of the activity of the UC was based on the index of the disease activity scored using a scale of 0-3, according to the criteria specified for chronic pouchitis by Gionchetti *et al.* (9) as follows: 1) clinical: the number of stool samples (regular: 0 points, 1-2 stools above the norm: 1 point and over 3 stools above the norm: 3 points), the presence of blood in the stool (absence: 0 points, everyday: 1 point), abdominal pain (absence: 0 points, periodic: 1 point, regular: 2 points), and fever (absence: 0 points, presence: 1 point); 2) endoscopic: edema, granularity and friability of the mucosa, exudates, and the presence of ulcerations (absence of each feature: 0 points, presence of each feature: 1 point); 3) histological: presence of polymorphonuclear infiltrates (small grade: 1 point, medium grade with microabscesses: 2 points, intense with microabscesses: 3 points). An index of 0 points, indicated a clinical and endoscopic remission. An index of at least 2 points was classified as a relapse of inflammatory process confirmed by using the clinical, histological and endoscopic criteria. The diarrhoeal forms of IBS were diagnosed by the history of the disease (the Rome criteria II) and by exclusion of organic lesions during colonoscopy performed at the beginning of study. At the time of colonoscopic examinations, biopsies from changed

colonic mucosa were taken for histopathological and mycological examination.

Patients with UC were treated with mesalamine given in a dose of 1g three times daily, azathioprine 2 mg/ kg daily alone or azathioprine given in combination with mesalamine as maintenance therapy. At the time of colonoscopy all examined UC patients received the same treatment for at least 6 month. None of the patients had a history of antibiotic therapy in the last 3 months as well steroid treatment within 1 month. Patients with steroid-dependent disease were excluded from the study. However, the occasional treatment with prednisolone as well as antibiotic therapy in the past years 2 years of disease in patients enrolled to this study was accepted. Part of the patients (N = 20) with significant fungal colonization of the colon ( $10^5$  CFU g<sup>-1</sup>) underwent a 2-week antifungal therapy according to antimycogram confirmatory results and 15 patients were given a single dose of lacidofil three times daily. Patients with diarrhoeal form of IBS were treated with mebeverine applied in a dose of 200 mg twice daily. Cleaning and disinfecting of endoscopic equipment was carried out according to the Guidelines of the European Society of Endoscopy and Polish regulations.

### *Microbial and mucosal histology determination of Candida infection*

The clinical specimens were taken initially on admission of patients, and following 4 weeks, in the case of aggravation of clinical symptoms for the bacteriological and mycological examination of brush smears from inflamed colon mucosa, intestinal content's and stool. The quantitative mycological examination in mucosa with colonic inflammation and stool was performed according to procedure proposed by Muller (11). The cultured bacterial and fungal strains were identified from morphological and biochemical features using ID 32E and IC 32C strips in ATB system (bioMerieux, Warsaw, Poland). Susceptibility of fungi to antifungal therapy was assessed using Fungitest (Sanofi Diagnostics Pasteur, Paris, France). In addition, the MIC for fluconazole was determined with the use of the Etest (AB Biodisk, Stockholm, Sweden).

Biopsies of colon mucosa were stained with H&E and assessed with a 3-point score scale regarding the presence of inflammatory infiltration's, cryptic abscesses and thickening of the muscular layer (9).

### *Studies in animal model of UC*

Animal studies were carried out on 50 Wistar male rats weighing 180-220 g. The animals had free

access to water and food and were adapted to laboratory conditions and 12h day/night cycles for 7 days. UC in rats was induced by rectal administration of TNBS (Sigma, Slough, UK) in a dose of 10 mg/kg, dissolved in 50% solution of ethanol as reported by Reuter and Kennedy (12). Briefly, the animals were anaesthetized with Phenobarbital (60 mg/kg i.p.) and TNBS was administered into the colon in a volume of 0.25 ml per rat at the depth of 8 cm from the rectum with the use of a soft polyethylene catheter. Until the moment of awakening the rats were positioned in the Trendelenburg position in order to avoid loss of TNBS solution *via* the rectum. The animals of control group were given 0.9% saline in the volume corresponding with the TNBS. After awakening and 1h following the end of administration of *C. albicans* rats were given access to water and food again.

#### *Effect of fungal colonization on the course and intensity of inflammatory changes in the colon and CBF*

Animals with TNBS-induced inflammatory changes in the colon were randomized into the four experimental groups (A, B, C and D), with 10 rats in each group. Starting from day 1 of the experiment through the next following 8 days all animals, excluding those of group A, were given intragastrically a suspension of *C. albicans* at  $10^9$  CFU per 1 ml of physiological saline in the following combinations: 1) control group: physiological saline (vehicle); 2) *C. albicans*  $10^9$  CFU/ml applied alone; 3) *C. albicans*  $10^9$  CFU/ml combined with lacidofil  $10^8$  CFU/ml; and 4) *C. albicans*  $10^9$  CFU/ml concomitantly applied with fluconazole given in a dose of 10 mg/kg intramuscularly (i.m.). Fungi strains isolated from biopsy specimens of the colon of patients with UC and Crohn's disease were used in these experiments. In the group with combined administration of *C. albicans* and lacidofil, drinking water was given 2 hrs prior to the administration of *C. albicans* suspensions with a 5% solution of lacidofil in a dose of  $10^8$  CFU/ml, prepared earlier by precipitation for 3 hrs at 37°C. Fluconazole as the antifungal treatment was given with in a dose of 10 mg/kg i.m. The control group received i.g. saline as a vehicle instead of *Candida* fungi suspension or probiotic bacteria.

At day 14 from induction of colonic lesions the animals were anaesthetized for the determination of CBF using the H<sub>2</sub>-gas clearance technique in the stomach, as described originally by our research group (13). The abdominal cavity was opened and after separation of the colon, the CBF was measured in three areas of the mucosa not affected by

inflammatory lesions. CBF was expressed as a percentage of the CBF in control rats without TNBS administration. At the termination of experiment, the removed 8 cm segment of the colon was opened along the longer axis. Determination of the number and the area of colonic damage evaluated the intensity of the macroscopic lesions and degree of inflammation planimetrically by two independent researchers using the criteria specified by Reuter *et al.* (12). Following that, the removed segment of the colon was weighed. Fragments of the inflammatory changed colon (2×10 mm) were sampled, fixed with formaldehyde, embedded in paraffin and routinely stained with haematoxylin and eosin (H&E method) for histological assessment of the mucosal damage and neutrophil infiltration.

The presence and intensity of histological changes were evaluated with the use of a score index, according to Vilaseca *et al.* (14) including the following criteria: presence, area and depth of ulceration, presence and intensity of inflammatory infiltration's, ulcerations and fibrosis. Histological specimens of colonic mucosa of rats were additionally stained using the Giemsa method for the presence of fungi. Both qualitative and quantitative mycological examinations of the colonic biopsies were performed as described previously by Muller (11). Biopsy specimens were homogenized in 4 ml of sterile physiological saline and shaken with 0.25% solution of trypsin to incubate. The solution of the homogenate was inoculated into Sabouraud medium (Difco Lab, Boston, USA) containing 50 µg/ml of chloramphenicol. *Candida* colonies were counted following 48h-incubation at 37°C. Identification of fungi species were carried out based on morphology and enzymatic pattern of a colony using *Candida* ID tests, ID32C stripes (system ATB, bioMerieux, Warsaw, Poland).

#### *Statistical analysis*

Results are expressed as means ± SEM. Statistical analysis was done using Student-t test or analysis of variance and the two way ANOVA test with Tukey *post hoc* test where appropriate. Differences of  $p < 0.05$  were considered significant.

## RESULTS

#### *Clinical studies in patients with fungal colonization*

Within the group of 89 patients with active and non-active phase of UC, whose profile in terms of number, duration of disease and sex is presented in *Table 1*, the significant fungal colonization was

Table 1 Profile of investigated group of patients with active and non-active phase of ulcerative colitis (UC) or with irritable bowel syndrome (IBS)

| Diagnosis             | Number | Duration of disease |        | sex   |               |     |               |
|-----------------------|--------|---------------------|--------|-------|---------------|-----|---------------|
|                       |        | < 5 yr              | > 5 yr | Woman |               | Men |               |
|                       | No     |                     |        | No    | Age           | No  | Age           |
| UC – active phase     | 56     | 21                  | 35     | 28    | 44<br>(18-72) | 27  | 51<br>(18-72) |
| UC – non-active phase | 33     | 21                  | 12     | 24    | 38<br>(18-72) | 9   | 47<br>(18-72) |
| IBS (control group)   | 12     | -                   | -      | 7     | 35<br>(18-48) | 5   | 41<br>(18-45) |

Table 2 Quantitative mycological stool evaluation expressed in percentage in patients with active and non-active UC and the control group (IBS)

| Fungal concentration                    | < 5 years   |              |                  | > 5 years   |              |                  | Control group |
|---|-------------|--------------|------------------|-------------|--------------|------------------|---------------|
|   | No %        | Active phase | Non-active phase | No %        | Active phase | Non-active phase | IBS           |
| > 10 <sup>5</sup> CFU/g                 | 18<br>20,2% | 12<br>13,4%  | 6<br>6,7%        | 33<br>37%   | 27<br>30,3%  | 9<br>10,1%       | 1<br>1,1%     |
| 10 <sup>3</sup> – 10 <sup>4</sup> CFU/g | 24<br>26,9% | 10<br>11,2%  | 14<br>15,7%      | 14<br>15,7% | 10<br>11,2%  | 4<br>4,4%        | 11<br>12,3%   |

found in 37% of patients with a history indicating over a 5-year course of the disease and in 20,2 % of those with a shorter time period ( $p < 0.01$ ). Increased fungal colonization, 10<sup>5</sup> CFU/g was significantly less frequent in the control of the IBS group without UC, reaching only 1.1 % of cases as compared with those patients with over a 5-year course of UC ( $p=0,0005$ ) or shorter history of disease ( $p= 0.0003$ ) (Table 2). As shown in Fig. 1, the most prevalent isolates among fungal strains were: 91 % *Candida albicans* (93 strains), 6.7% *Candida glabrata* (6 strains) and 1.6% *Candida inconspicua* (1 strain).

The total mean activity index of inflammation in UC including clinical, endoscopic and a histological criteria in patients with significant and insignificant fungal colonization of the colon did not reveal differences between those groups ( $13.2 \pm 1.8$  for significant fungal colonization and  $13.6 \pm 1.7$  for non-significant fungal colonization) despite the fact that all patients were treated with mesalamine or azathioprine (Fig. 2). The analysis of the individual clinical and endoscopical activity criteria of UC also failed to exhibit a significant difference between patients with significant (group A) and non-significant (group B) fungal colonization (Fig. 3). A

group of 20 patients (group C) out of the total 51 UC patients with diagnosed significant fungal colonization received the treatment with fluconazole based on the result of antimycogram determination, and 15 patients were given probiotic lacidofil (group E). The remaining 16 patients with significant fungal colonization did not receive antifungal treatment (group D). Following 4 weeks, activity of inflammatory state was evaluated again and a significant difference in inflammatory activity between UC patients conventionally treated with mesalamine or azathioprine without or with the antifungal treatment as well as the group of 15 patients given probiotic was observed (Fig. 2). Antifungal treatment or administration of lacidofil in these patients significantly decreased the total mean activity index of colonic mucosa inflammation as compared with those not treated antifungally ( $8.5 \pm 1.2$  and  $7.7 \pm 1.7$  vs.  $10.6 \pm 1.8$ , respectively) (Fig. 2).

As shown in Fig. 4, the regression of symptoms or a decrease in their intensity at 4 weeks of follow up was observed in patients treated with placebo or in those receiving mesalamine or azathioprine, in conjunction with antifungal treatment or lacidofil. The activity indexes of UC as referred to the clinical

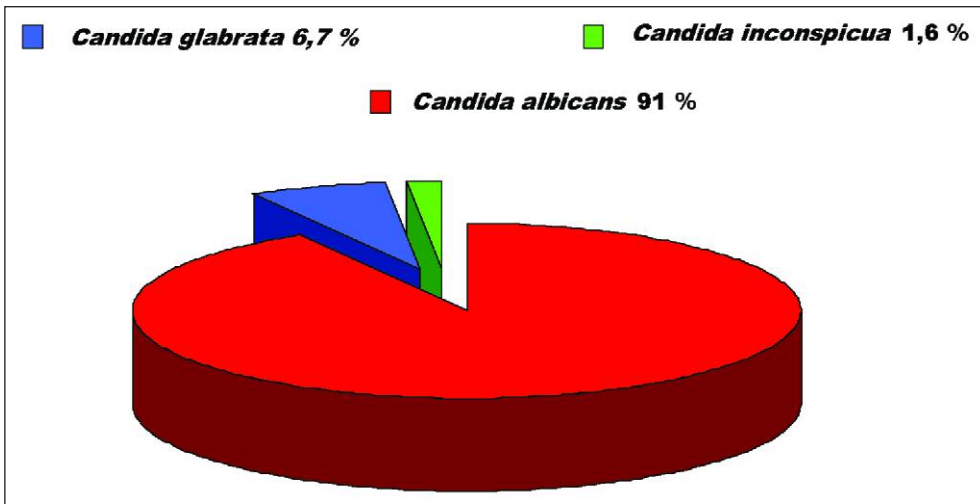


Fig. 1. The frequency of various *Candida* species isolated from the human colonic mucosa.

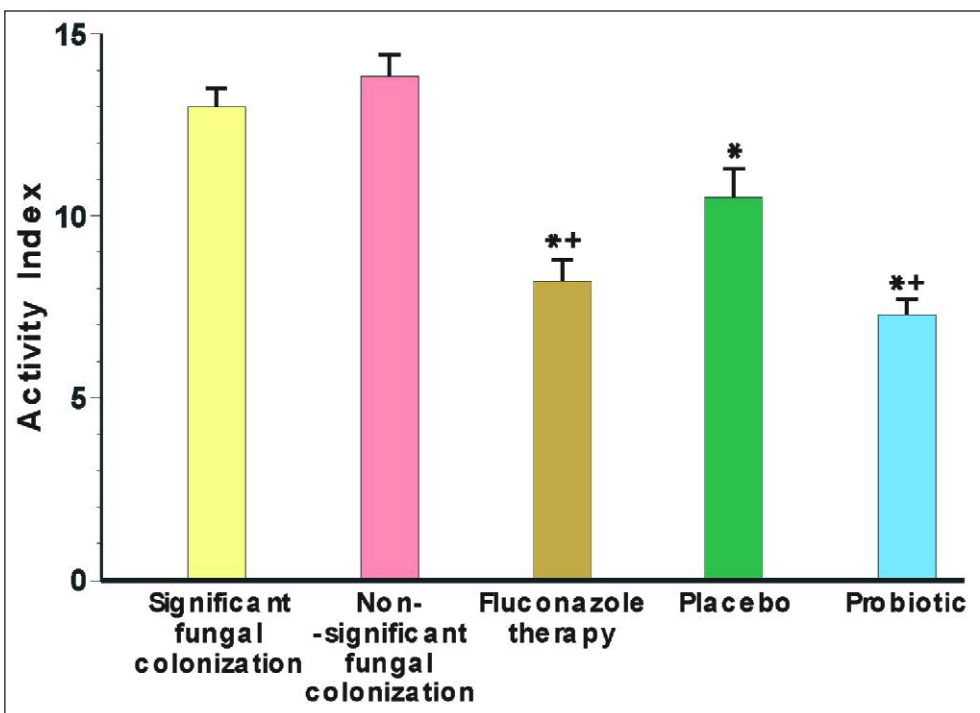


Fig. 2. Total mean activity index of UC in patients with a significant fungal colonization, non significant fungal colonization at their admission to the hospital and after 4 weeks follow up with placebo, fluconazole or probiotic (lacidofil) therapy. Asterisk indicates a significant decrease ( $p < 0.05$ ) as compared to the respective values in patients at admission. Cross indicates a significant decrease ( $p < 0.05$ ) as compared to the values obtained in placebo-treated patients at 4 weeks of follow up.

criteria reached the value  $2.7 \pm 0.7$  for patients not-treated and this was significantly decreased to the values  $1.2 \pm 0.8$  and  $1.7 \pm 0.6$ , for those receiving antifungal treatment and lacidofil, respectively. The endoscopic and histological criteria were significantly decreased in patients treated with fluconazole or lacidofil as compared with those given placebo.

#### Animal studies

The influence of *Candida* infection with or without lacidofil and antifungal treatments on the healing process of colonic lesions, CBF, MPO activity and plasma levels of proinflammatory cytokines  $IL-1\beta$  and  $TNF-\alpha$

Intrarectal administration of TNBS caused severe damage to the colonic mucosa manifested by inflammatory changes in the colon with extensive ulcerations of the mucosa. Macroscopic changes as observed at day 3 from the day of TNBS administration were comparable in *Candida* infected groups with or without lacidofil or fluconazole tested and comprised of widespread necrotic and hemorrhagic lesions. As shown in Fig. 5A and B, the microscopic examination revealed loss of typical colonic crypt structure as compared to that in the intact colon, accompanied by submucosal swelling, necrotic changes, micro clot formations, acute inflammatory neutrophil-induced infiltration's and necrosis of colonic mucosa and submucosa. The fungi hyphae indicating

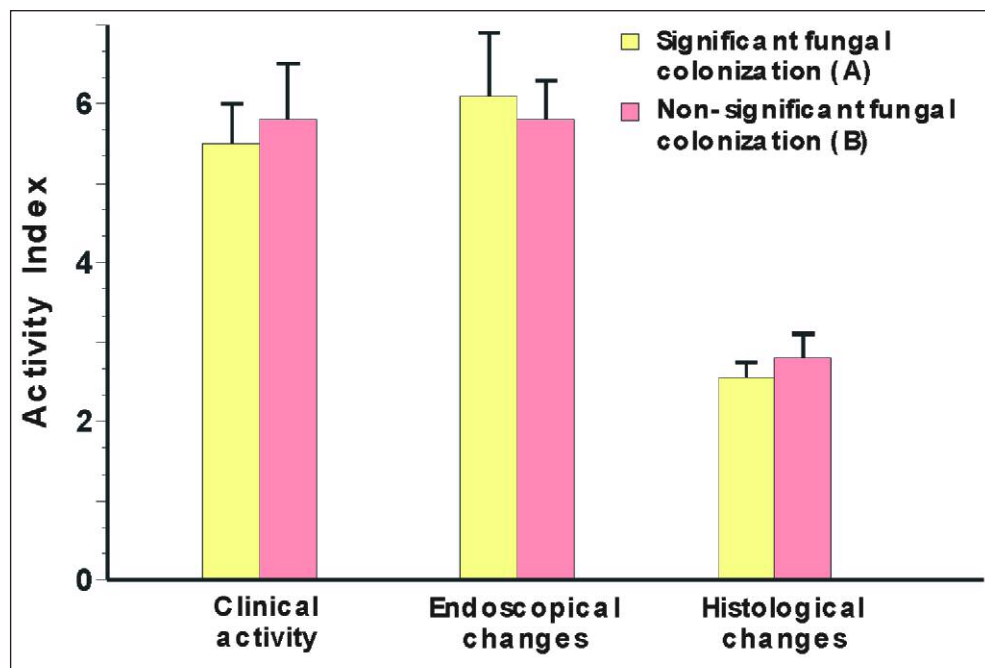


Fig. 3. Initial evaluation of the mean activity of inflammatory lesions in the colon in patients with UC-active phase with significant (group A) and non-significant (group B) *Candida albicans* colonization. No significant changes in activity index for clinical, endoscopic and histological criteria were observed in all patients at their admission to University hospital.

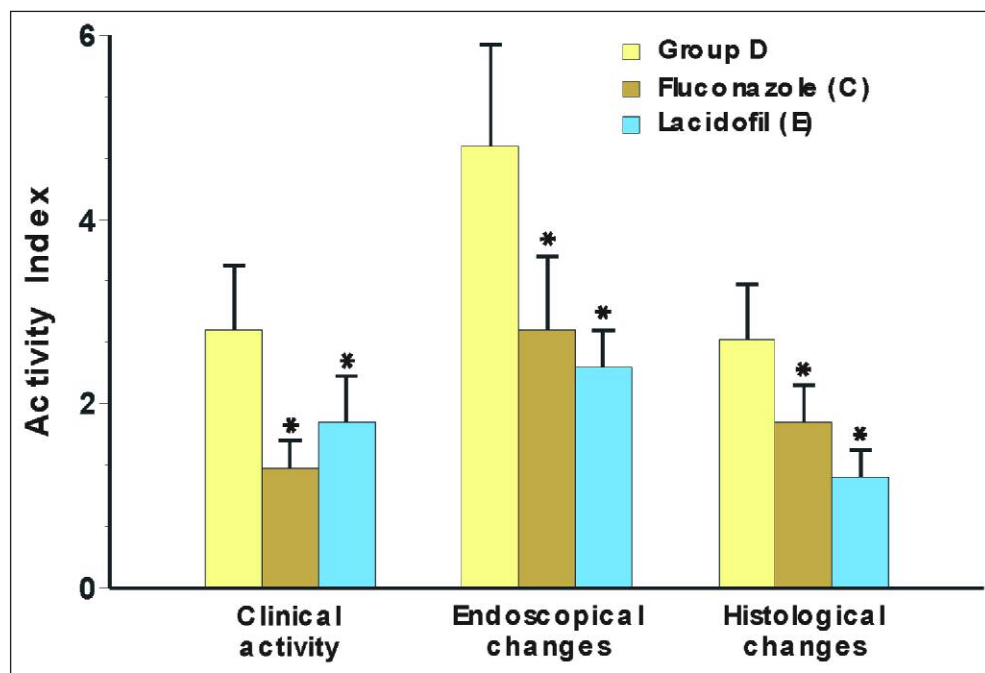


Fig. 4. Evaluation of the mean activity index of inflammatory lesions in patients in the active phase of UC infected with *Candida albicans* treated with placebo (group D), fluconazole (group C) or lacidofil (group E).

colonization by *Candida* of the colonic mucosa were detected in inflamed colonic mucosa of TNBS-induced UC (Fig. 5C).

At day 14 of the experiment, i.e. at the healing stage of the colonic lesions, the significant differences between animal groups were noted. As shown in Fig. 6, TNBS administered animals exhibited a significant decrease in the CBF. The mean area of colonic injury as well as the weight of the tissue was significantly lower and the CBF were significantly higher in lacidofil- or fluconazole-treated animals as compared to the vehicle-control

animals (Figs. 7 and 8). The mean area of colonic ulcerations and the mean weight of the tissue in *Candida*-infected animals were significantly higher than those measured in lacidofil and fluconazole-treated animals (Figs. 7 and 8). Treatment with fluconazole and lacidofil significantly attenuated the mean weight of the colon tissue at day 14 as compared with that obtained in *Candida*-infected rats (Fig. 7). *Candida* fungi titer exceeded  $10^4$  CFU/ml in the group receiving *C. albicans*, while in lacidofil group, the fall of *Candida* titer below a value of  $10^2$  CFU/ml was noted, however this effect

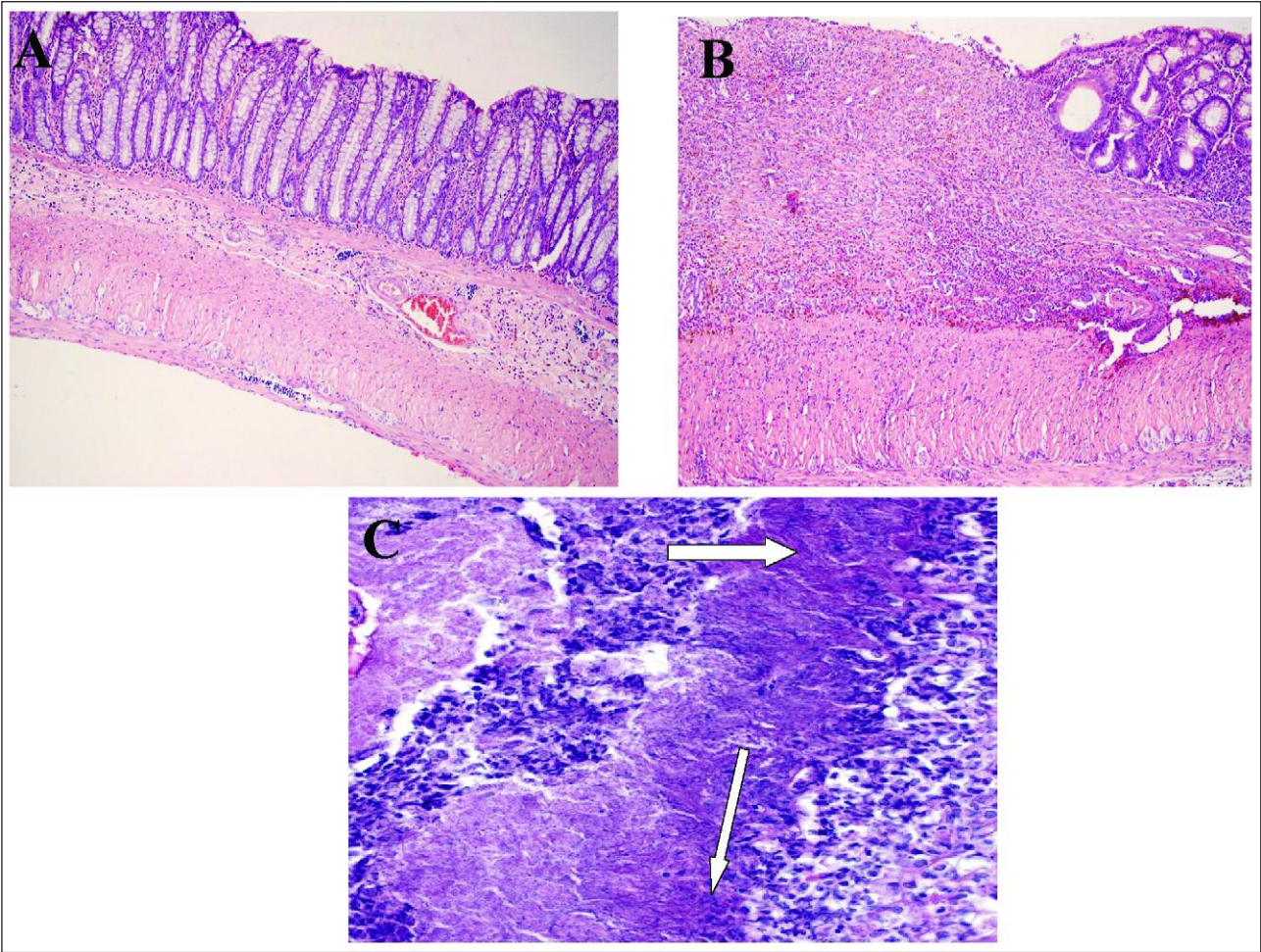


Fig. 5A, B and C. The histological appearance of rat intact colonic mucosa (A) and that with colonic ulceration at day 3 upon intrarectal administration TNBS to induce ulcerative colitis (B). Note, lack of colonic crypts structure and the presence of heavy inflammation associated with extensive neutrophil infiltration in TNBS-treated colonic mucosa. In *Candida*-infected animals with UC, the fungi hyphae are present indicating colonization by fungi of the colonic mucosa (arrows) (C).

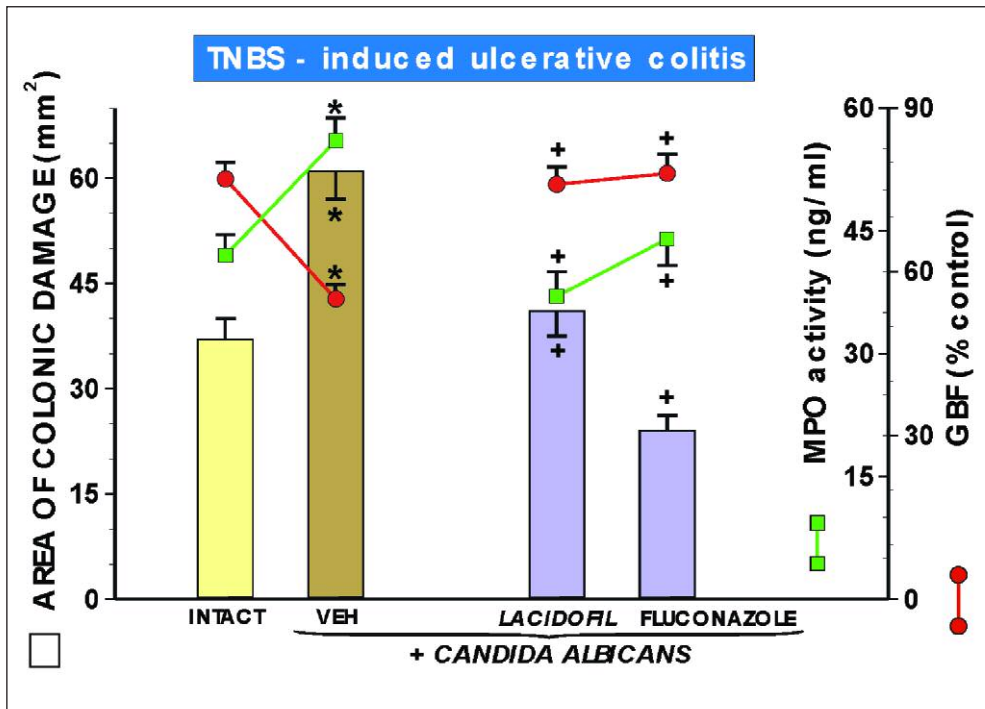


Fig. 6. Area of colonic damage, the changes in colonic blood flow (CBF) and myeloperoxidase (MPO) activity in rats with TNBS-induced ulcerative colitis and those treated with vehicle or inoculated with *Candida albicans* ( $10^9$  CFU/ml-d i.g.) alone with or without lacidofil or fluconazole treatment and determined at day 14 upon colitis induction. Mean  $\pm$  SEM of 6 - 8 rats. Asterisk indicates a significant change as compared to the values obtained rats with colitis without *Candida* infection. Cross indicates a significant decrease below the value obtained in vehicle-control and *Candida* alone inoculated rats.



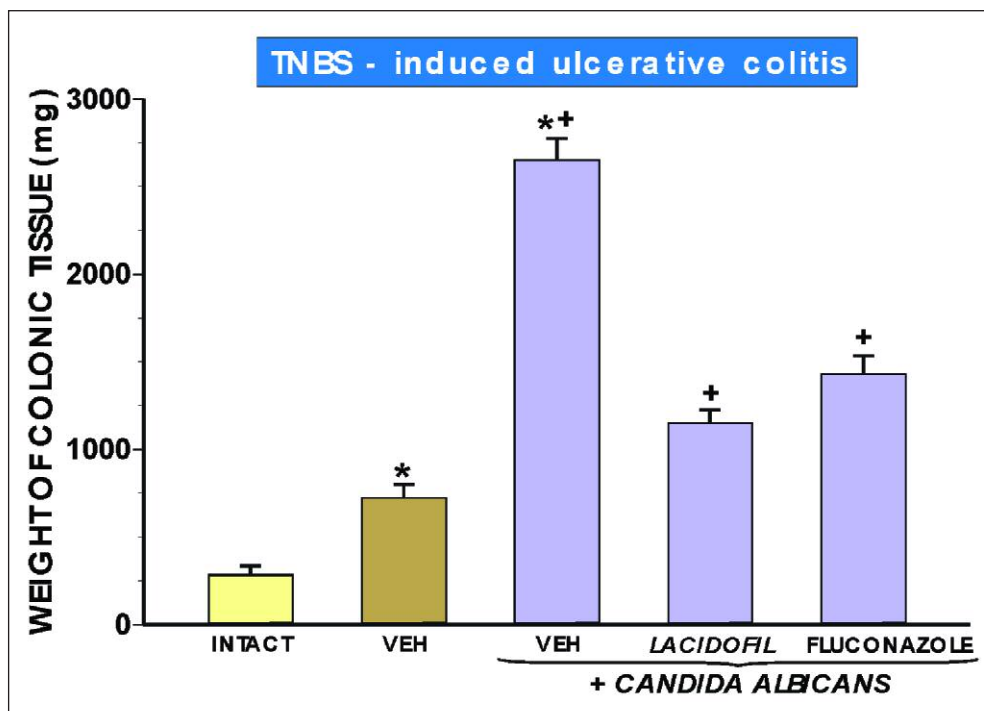


Fig. 7. Weight of colonic tissue in rats with TNBS-induced colitis with or without infection with *Candida albicans* ( $10^9$  CFU/ml-d i.g.) treated throughout the period of 14 days with vehicle, fluconazole or lacidofil. Mean  $\pm$  SEM of 6-8 rats. Asterisk indicates a significant change as compared to the value obtained in intact rats. Asterisk and cross indicate a significant change as compared to the value obtained in vehicle-treated rats without *Candida* infection. Cross indicates a significant change as compared to the value obtained in rats infected with *Candida* only.

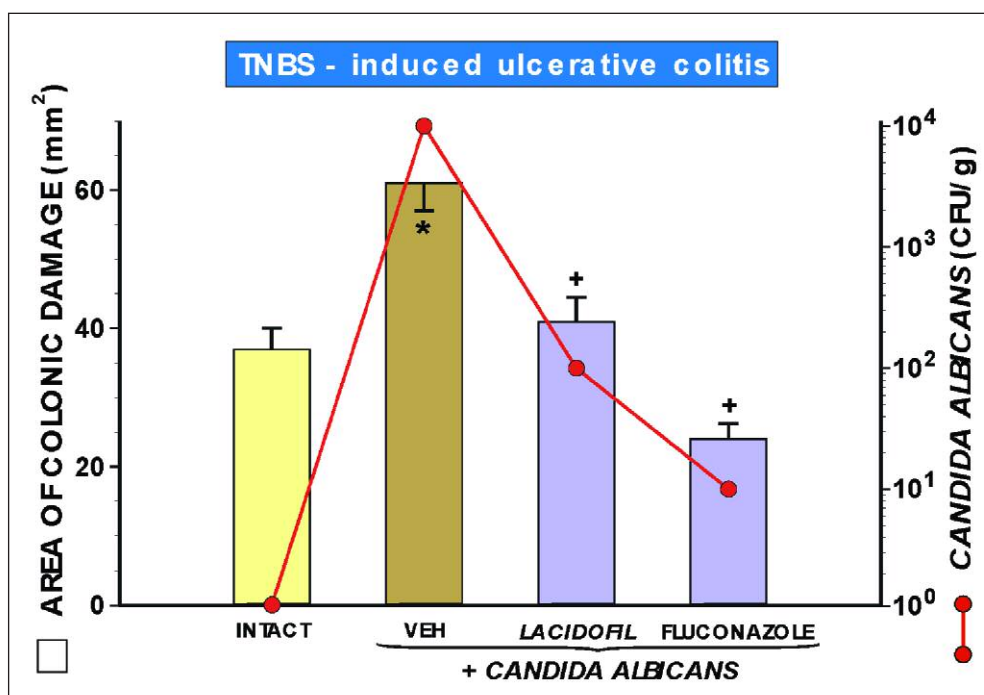


Fig. 8. Area of colonic damage and the titer of *Candida albicans* in colonic mucosa assessed by fungal culture taken from colon biopsies of the rat inoculated by *Candida albicans* with or without concurrent treatment with vehicle (VEH), lacidofil or fluconazole. Mean  $\pm$  SEM of 8 - 10 rats. Asterisk indicates a significant change as compared to the value obtained in rats with colitis without *Candida* infection. Cross indicates a significant change as compared to the values recorded in *Candida*-infected rats without treatment with lacidofil or fluconazole.

was significantly less pronounced in rats treated with lacidofil than in those receiving fluconazole (Fig. 8).

Histological evaluation as reflected by the size of ulcerations, intensity of inflammatory infiltration's, depth of ulcerations, fibrosis and presence of granulomas documented the histological damage index that reached the highest values in *C. albicans* infected animals (Figs 5 B, C and 8). Lacidofil and fluconazole significantly reduced the area of colonic lesions in comparison with the value of this area in rats

inoculated with *Candida*. At day 14, the inflammatory infiltration's consisting of neutrophils, lymphocytes and macrophages were most intense in *C. albicans* infected rats. In lacidofil- and fluconazole-treated animals, the microscopic size of ulcerations was significantly smaller comparing to the respective controls. The fungal colonization was accompanied by the significant elevation of plasma IL-1 $\beta$  and TNF- $\alpha$  levels and this effect was significantly reduced by lacidofil or fluconazole (Fig. 10).

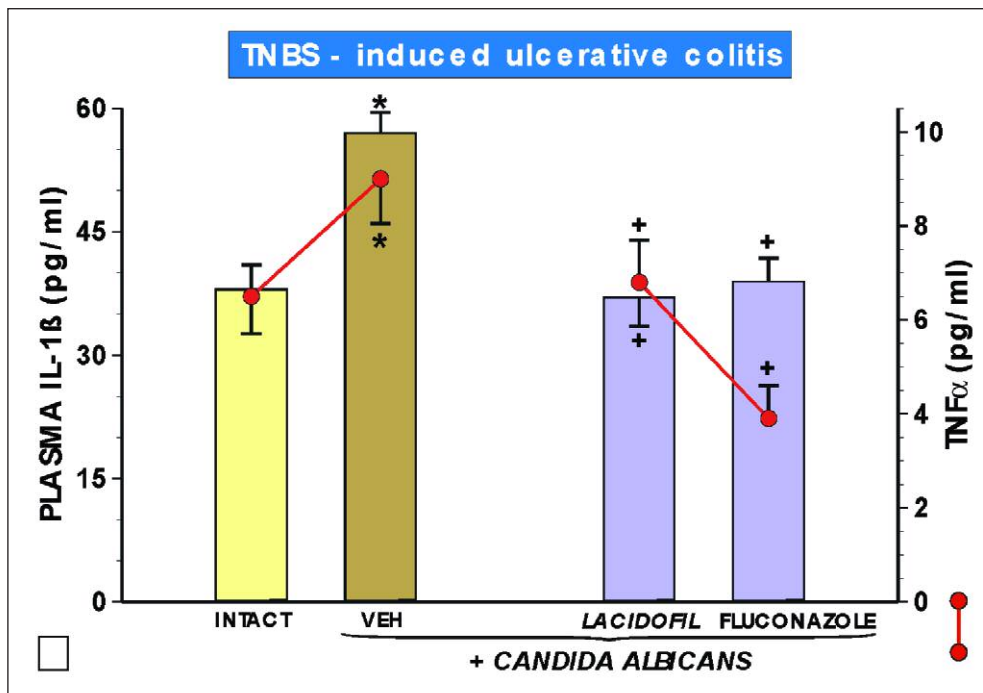


Fig. 9. Plasma IL-1 $\beta$  and TNF- $\alpha$  levels after 14 days of treatment of *Candida albicans*-infected rats with TNBS-induced ulcerative colitis with or without the concurrent treatment with vehicle, lacidofil or fluconazole. Mean  $\pm$  SEM of 8-10 rats. Asterisk indicates significant change as compared to the value obtained in animals with colitis but without *Candida* infection. Cross indicates a significant change as compared to the values recorded in *Candida*-infected rats without treatment with lacidofil or fluconazole.

## DISCUSSION

Results of our clinical observations in humans and experimental studies in rats indicate that a significant fungal colonization of the colon mucosa worsens the UC and delays the healing of inflammatory colonic lesions during the course of this disease as mimicked in rodent model by administration of TNBS. Although fungi were originally considered to represent only a small fraction of total gastrointestinal microbiota, their influence on the course of ulcerative colitis in humans have not been extensively studied (4, 5). Previous studies in humans and animals with chronic experimental ulcerations in the stomach revealed that fungal colonization of the upper GI tract affected the course of chronic gastritis and healing of chronic gastric ulcers (GU). In a previous study, we reported a more frequent, significant fungal colonization (over  $10^4$  CFU/ml) in about 54% of GU patients and 11% of patients with chronic gastritis, when compared with the placebo-control group (15, 16).

Our present human study demonstrates the significantly more frequent *Candida* colonization of the colonic mucosa in patients at active phase and longer duration of UC when compared with control group with IBS without UC. Our finding that *Candida albicans* is the most frequent fungi isolated from UC patients is in keeping with the observation by Bougnoux *et al.* (17) who showed the prevalence of *C. albicans* and *C. glabrata* species in 88% and 9% of carriers, respectively. It is unknown,

however, whether *Candida* colonization is the primary event and thus can be considered a major cause of UC, or if this fungi infection occurred secondary to colitis, which therefore, might predispose this colonic mucosa to the development of a fungal infection. We can only speculate that *Candida albicans* colonization developed as secondary to the colonic damage in UC patients that apparently predisposed this mucosa to fungi overgrowth. This property of *Candida* to colonize the intestine of UC patients seems to be dependent on the time duration of the inflammatory reaction and the individual patient susceptibility to mesalamine and azathioprine treatment. It was documented that azathioprine nearly completely abolishes the leukocyte migration into the mucus while the concentration and the adherence of mucosal flora (i.e. bacteria) dramatically increased. In contrast, mesalamine remains without effect on migration of leukocytes but significantly reduces the concentration and the adherence of mucosal bacteria as compared to untreated UC patients (10). Since in our study, the single or concurrent treatment with 5-ASA and azathioprine was not additive against fungal infection and failed to show further benefit, we decided to use antifungal therapy or probiotic treatment. It is quite possible that both drugs, 5-ASA and azathioprine may neutralize each other's effect on mucosal barrier because the fungi titer and their mucosal adherence seem to not differ significantly from that observed in untreated UC patients.

The initial mean value of the activity index of as well inflammatory changes in the colon of patients in the active phase of UC as individual clinical and endoscopic criteria of disease activity failed to show a differences between patients with significant and non-significant fungal colonization. However, the follow-up examination of the inflammatory status in patients with significant fungal colonization of colon mucosa, as carried out following an initial 4-week observation period, revealed the beneficial effects of antifungal or probiotic therapy. Fluconazole applied to UC patients with significant fungal colonization efficiently accelerated the remission of clinical symptoms and the decrease in their intensity compared to subjects who did not undergo this therapy. It is of interest that a significant improvement of clinical symptoms was also observed in the patients treated with probiotic, when compared with those not treated with fluconazole, but less pronounced than those treated antifungally. Probiotics may be of benefit in managing the symptoms of IBS *via* a number of mechanisms such as increasing mucosal TGF- $\beta$  and IL-10 and attenuating proinflammatory cytokines, for example IL-12 and IFN- $\gamma$  (18). Probiotics have also been shown to alter the integrity of the upper GI mucosa. Our research group has recently demonstrated that the treatment with live probiotic bacteria *L.acidophilus* effectively attenuated the delay in ulcer healing in *Candida*-infected rats with preexisting gastric ulcers (19). In our present study, fluconazole or probiotic decreased the intensity of endoscopic mucosal lesions in human subjects infected with *Candida* following a 4 week period as compared with those with significant fungal colonization not treated antifungally who received mesalamine or azathioprine treatment. Deleterious effects of fungal overgrowth was also considered by Kuhbacher *et al.* (4) as a consequence of the alteration of the balance between bacterial and fungi leading to the impairment of the colonic mucosal barrier.

In experimental part of our study, the rats with ulcerative colitis induced by TNBS infected with *Candida albicans* exhibited a significant increase in the area of gross mucosal colonic ulcerations and the weight of the colon when compared to the vehicle-control group, reflecting a delay in the healing of these ulcerations. This delay was accompanied by the fall in the CBF and a significant rise in the plasma levels of IL-1 $\beta$  and TNF- $\alpha$ . Administration of fluconazole or lacidofil exerted a favorable influence on the colonic ulcer healing in rats infected with *C. albicans* by decreasing the area of colonic ulcerations and the weight of the colon, thus limiting the inflammatory process in the colonic mucosa. These observations were consistent with the

improvement of the colonic microcirculation as reflected by an increase in CBF and a fall in colonic MPO activity with a concomitant decrease in plasma IL-1 $\beta$  and TNF- $\alpha$  levels and the lowest intensity of inflammatory changes after probiotic or fluconazole therapy. This is corroborative with the observation in patients with pouchitis where the probiotic therapy markedly influenced the colonization rate and the diversity of bacterial and fungal microbiota (4). Administration of probiotic bacteria VSL#3 in patients with recurrent or chronic active pouchitis in the phase of remission increased the total number of bacterial cells while decreasing the diversity of fungal cells at the same time (4). Studies in animal model of oral *candidiasis* revealed that feeding live probiotic strain of *Lactobacillus acidophilus* to an infection-prone DBA/2 mice markedly shortened the duration time of fungal colonization of oral cavity following inoculation with *Candida albicans* (20). The probiotic treatment in their study correlated with an early appearance of IL-4 and IFN- $\gamma$  in the cervical lymph node cells and saliva (20). In recent study, cannabinoid receptor agonist, anandamide, exhibited antiinflammatory properties attenuating the development of inflammatory changes and cytokine IL-1 $\beta$  and TNF- $\alpha$  in the mice model of UC (21). In agreement with our present results, the mode of cytokines pattern expression in human peripheral blond mononuclear cells was markedly reduced by probiotic lactic acid bacteria (22). However, it need to be emphasized that the beneficial effect of one probiotic preparation such as *L. acidophilus* in our study, does not imply efficacy of all preparations containing different probiotic bacterial strains, because each individual probiotic strain has its unique biological properties (23).

In summary, we demonstrated that a significant fungal colonization of the colon is more frequent in patients with a long history of UC than in patients with a short history of this disease, and that antifungal treatment attenuates the inflammation of the colon during the course of UC in humans. The detection of significant fungal colonization in the colon of patients in active phase of UC serves as a useful tool in the diagnosis and the treatment of UC patients. Probiotics appear to improve the healing process in UC patients with significant fungal colonization of the colon mucosa treated conventionally with mesalamine or azathioprine. Results of experimental studies indicate that significant *Candida* colonization of the rat colon delays the healing of TNBS-induced colitis and this effect could be due to the impairment of the CBF and enhancement in plasma levels of IL-1 $\beta$  and TNF- $\alpha$ , with these alterations being reversed by antifungal treatment with fluconazole. Probiotic therapy

appears to be beneficial in counteracting the effects of *Candida*-induced delay in the healing of TNBS-induced colitis.

## REFERENCES

1. Shanahan F. Inflammatory bowel disease: immunodiagnostics, immunotherapeutics and ecotherapeutics. *Gastroenterology* 2001; 120: 622- 635.
2. Bengmark S. Ecological control of gastrointestinal tract. The role of probiotic flora. *Gut* 1998; 42: 2-7.
3. Kucharzik T, Maaser C, Luger A *et al.* Recent understanding of IBD pathogenesis: implications for future therapies. *Inflamm Bowel Dis* 2006; 12: 1068- 1083.
4. Kuhbacher T, Ott SJ, Helwig U *et al.* Bacterial and fungal microbiota in relation to probiotic therapy (VSL#3) in pouchitis. *Gut* 2006; 55: 833- 841.
5. Linskens RK, Huijsdens XW, Savelkoul PHM, Vanderbroucke-Grauls CMJE, Meuwissen SGM. The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. *Scand J Gastroenterol* 2001; 12(Suppl 234): 29- 40.
6. Bernhardt H, Knoke M. Mycological aspects of gastrointestinal microflora. *Scand J Gastroenterol* 1997; 32(Suppl 222): 102-106.
7. Cannon RD, Holmes AR, Mason AB, Monk BC. Oral *Candida*, clearance, colonization or candidiasis? *J Dent Res* 1995; 74: 1152-1161.
8. Kennedy RJ, Hoper M, Deodbar K, Kirk SJ, Gardiner KR. Probiotic therapy fails to improve gut permeability in a hapten model of colitis. *Scand J Gastroenterol* 2000; 12, 1266- 1271.
9. Gionchetti P, Rizzello F, Venturi A. *et al.* Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double- blind, placebo-controlled trial. *Gastroenterology* 2000; 119: 305- 309.
10. Swidsinski A, Loening-Baucke V, Bengmark S, Lochs H, Dorffel Y. Azathioprine and Mesalazine-induced effects on the mucosal flora in patients with IBD colitis. *Inflamm Bowel Dis* 2007; 13: 51-56.
11. Muller J. Pathogen identification in indigenous deep mycoses. Basel, La Roche an Co., Co-limited Company, 1986.
12. Reuter B, Asfaha S, Buret A, Sharkey KA, Wallace JL. Exacerbation of inflammation- associated colonic injury in rat through inhibition of cyclooxygenase-2. *J Clin Invest* 1996; 98: 2076-2085.
13. Brzozowski T, Konturek PC, Ptak A *et al.* Nitric oxide releasing aspirin protects the gastric mucosa against stress and promotes healing of stress-induced gastric mucosal damage; role of heat shock protein 70. *Digestion* 2002; 66: 160-172.
14. Vilaseca J, Salas A, Guarner F, Rodriguez R, Malagelada J-R. Participation of thromboxane and other eicosanoid synthesis in the course of experimental inflammatory colitis. *Gastroenterology* 1990; 98: 269-277.
15. Zwolinska-Wcislo M, Budak A, Bogdal J, Trojanowska D, Stachura J. Effect of fungal colonization of gastric mucosa on the course of gastric ulcer healing. *Med Sci Monit* 2001; 7: 266-275.
16. Brzozowski T, Zwolinska-Wcislo M, Konturek PC *et al.* Influence of gastric colonization with *Candida albicans* on ulcer healing in rats: effect of ranitidine, aspirin and probiotic therapy. *Scand J Gastroenterol* 2005; 40: 286-296.
17. Bounoux M-E, Diogo D, Francois N *et al.* Multilocus sequence typing reveals intrafamilial transmission and microevolution of *Candida albicans* isolates from the human digestive tract. *J Clin Microbiol* 2006; 44: 1810-1820.
18. Mach T. Clinical usefulness of probiotics in inflammatory bowel diseases. *J Physiol Pharmacol* 2006; 57 (Suppl 9): 23-33.
19. Zwolinska-Wcislo M, Brzozowski T, Mach T *et al.* Are probiotics effective in the treatment of fungal colonization of the gastrointestinal tract? Experimental and clinical studies. *J Physiol Pharmacol* 2006; 57(Suppl 9): 35-49.
20. Elahi S, Pang G, Ashman R, Clancy R. Enhanced clearance of *Candida albicans* from the oral cavities of mice following oral administration of *Lactobacillus acidophilus*. *Clin Exper Immun* 2005; 141: 29- 36.
21. Engel MA, Kellermann CA, Rau T, Burnat G, Hahn EG, Konturek PC. Ulcerative colitis in AKR mice is attenuated by intraperitoneal anandamide. *J Physiol Pharmacol* 2008; 59: 673-689.
22. Gackowska L, Michalkiewicz J, Krotkiewski M, Helmin-Basa A, Kubiszewska I, Dzierzanowska D. Combined effect of differential lactic acid bacteria strains on the mode of cytokines pattern expression in human peripheral blood mononuclear cells. *J Physiol Pharmacol* 2006; 57(Suppl 9): 13-21.
23. Heczko PB, Strus M, Kochan P. Critical evaluation of probiotic activity of lactic bacteria and their effects. *J Physiol Pharmacol* 2006; 57(Suppl 9): 5-12.

Received: December 16, 2008

Accepted: February 20, 2009

Author's address: Prof. Dr Tomasz Brzozowski, Department of Physiology Jagiellonian University Medical College, 16 Grzegorzeczka Street, 31-531 Cracow, Poland. Phone: (+4812) 6199-631; Fax: (+4812) 421-1578; e-mail: mprbrzo@cyf-kr.edu.pl