INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for their anti-inflammatory, analgesic and antipyretic effects, however their use is associated with the broad spectrum of side effects observed in human as well as the experimental animals. Despite damaging activity of NSAIDs in upper gastrointestinal (GI) tract, these drugs exert deleterious influence in lower GI tract, including colon. The role of GI microflora in the pathogenesis of NSAID-induced experimental colonic damage is not completely understood. The aim of this study was 1) to evaluate the relative importance of the GI microflora on the experimental colonic damage in the presence of caused by NSAID, and 2) to assess the efficacy of antibiotic treatment with ampicillin on the process of healing of colitis. We compared the effect of vehicle, ASA applied 40 mg/kg intragastrically (i.g.) or the selective cyclooxygenase (COX)-2 inhibitor, celecoxib (25 mg/kg i.g.) without or with ampicillin treatment (800 mg/kg i.g.) administered throughout the period of 10 days, on the intensity of TNBS-induced colitis in rats. The severity of colonic damage, the alterations in the colonic blood flow (CBF) and myeloperoxidase (MPO) activity, the mucosal expression of TNF-α, IL-1β, COX-2, VEGF and iNOS in the colonic tissue. ASA and coxib also resulted also in a significant increase of E. coli counts in the stool at day 3 and day 10 of observation compared with the intact rats. Moreover, E. coli translocation from the colon to the blood and extraintestinal organs such as liver and spleen in the group of rats treated without or with ASA and coxib. E. coli was the most common bacteria isolated from these organs. Treatment with ampicillin significantly attenuated the ASA- or celecoxib-induced increase in plasma levels of IL-1β and TNF-α and suppressed the mucosal mRNA expression for IL-1β and TNF-β, COX-2, VEGF and iNOS in the colonic tissue. ASA and coxib also resulted also in a significant increase of E. coli counts in the stool at day 3 and day 10 of observation in ASA- and coxib-treated rats with colitis. Antibiotic therapy markedly reduced bacterial translocation to the colonic tissue and the extraintestinal organs such as the liver and spleen. We conclude that administration of ASA and to lesser extent of celecoxib, delays the healing of experimental colitis and enhances the alterations in colonic blood flow, proinflammatory markers such as IL-1β, TNF-α, COX-2, iNOS and VEGF and increased intestinal mucosal permeability resulting in the intestinal bacterial translocation to the blood, spleen and liver. Antibiotic treatment with ampicillin is effective in the diminishing of the severity of colonic damage, counteracts both the NSAID-induced fall in colonic microcirculation and bacterial E. coli translocation to the extraintestinal organs.

Key words: aspirin, colonic blood flow, coxib, cyclooxygenase, cytokines, Escherichia coli, inflammatory bowel disease, ulcerative colitis, vascular endothelial growth factor
given naproxen for 2 weeks (5). Small intestinal ulcerations were detected in 8.4% of NSAIDs users, comparing with 0.6% of non users (6). The clinical consequences of NSAIDs use within small intestine include reduced hematoctrit elevated epithelial permeability and diaphragm-like strictures (7). Colonoscopy studies have also shown, that NSAIDs therapy is associated with the risk in formation of colonic ulcers, diffuse colitis, bleedings or even intestinal perforation (8). NSAID treatment has also been shown to exacerbate the other GI tract chronic diseases such as inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn’s disease (4, 9). Although the inhibition of mucosal prostaglandins (PG) synthesis during NSAIDs therapy represents the major event in NSAID-induced gastropathy, the pathogenesis of NSAIDs-induced intestinal lesions is multifatorial and involves combination of pathogenic factors, such as NSAID-induced enhancement in gastric acid secretion and the exaggeration of the mucosal response to the variety of luminal aggressors such as bile and/or bacteria. Moreover the enterohepatic circulation of NSAIDs may trigger NSAIDs-related pathogenic mechanisms in both, the upper and lower GI tract, with consequent inflammatory reactions and the macroscopic and microscopic alterations (1, 4, 9, 10).

The pathogenesis of IBD is complex and includes a genetic predisposition, specific immunological properties of the GI tract mucosa and the type of GI microflora (11). It is assumed that the GI microflora is necessary for the development of NSAIDs-induced intestinal damage and may enhance the activity of IBD (12). It has been shown, that NSAIDs therapy may impair the homeostasis of GI microflora causing the overgrowth of Gram-negative and anaerobic bacteria, thus playing an important role in exacerbation of injury caused by NSAIDs (13). Moreover, the experimental colitis could not be developed in germ-free animals indicating that GI tract microflora plays an essential role in initiating and perpetuating of the colonic inflammation (14).

None of the treatment including the administration of 3-fatty acids, glucose, sucralate, misoprostol, NO donors (CINODs), phosphodiesterase inhibitors (theophylline, pentoxifylline) had been proven to be effective in counteracting NSAID-induced upper and lower GI tract side effects associated with NSAIDs therapy (15). Evidence in animal studies have indicated that antimicrobials, such as metronidazole, tetracycline and kanamycin may attenuate NSAID-induced enteropathy (4, 16). However, the efficacy of antibiotics in the healing of experimental colitis in the presence of NSAID administration has not been fully elucidated.

We compared the effect of ampicillin on the healing of colitis induced by the intrarectal administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in rats treated with conventional NSAID such as ASA or the selective COX-2 inhibitor, celecoxib. We evaluated the effect of ASA and celecoxib on treatment of mucosal damage, the alterations in the colonic blood flow (CBF), myeloperoxidase (MPO) activity and the plasma concentration of proinflammatory cytokines TNF-α and IL-1β as well as the expression of proinflammatory markers IL-1β, TNF-α, COX-2, iNOS and VEGF in the colonic mucosa of rats with colitis. An attempt was made to determine the G- and G+ microflora in animals subjected to the treatment with ASA and celecoxib perpetuating the colitis and undergoing the concomitant treatment with vehicle of antibiotic ampicillin.

**MATERIAL AND METHODS**

Animal studies were carried out on Wistar male rats weighing 180-220 g. The animals had free access to water and food and were adapted to laboratory conditions and 12 h day/night cycles for 10 days after TNBS administration. The study was approved by the local Ethical Committee at the Jagiellonian University Medical College in Cracow, Poland and run in accordance with the Helsinki declaration.

Colitis in rats was induced by rectal administration of TNBS (Sigma, Slough, UK) with a dose of 10 mg/kg, dissolved in 50% solution of ethanol as reported in our previous study (17). 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 so as to avoid loss of the TNBS solution via the rectum. The animals of control group were given 0.9% saline in the same volume corresponding to the rats who were administered TNBS.

Animals with TNBS-induced colitis were randomized into the four experimental groups (A-D), consisting of 10 rats per group and they received i.g. the following daily treatments: A- vehicle (saline), B- ASA (40 mg/kg), C- celecoxib (10 mg/kg), D- ASA (40 mg/kg) and E. celecoxib (10 mg/kg) administered concomitantly with ampicillin (800 mg/kg i.g.).

After 3, 10 and 14 days from induction of colonic lesions with TNBS, the animals were anaesthetized to determine CBF using the H2 gas clearance technique (17, 18). The abdominal cavity was opened and after separation of the colon, the CBF in the areas of the mucosa not affected by inflammatory lesions were measured. CBF was expressed as a percentage of the CBF in the vehicle-control rats without TNBS administration.

At the termination of the experiment, the removed 8 cm segment of the colon was opened along the longer axis. The areas of colonic damage were evaluated planimetrically (Morphomat, Carl Zeiss, Berlin, Germany) by two independent researchers. Following that, the removed segment of the colon was weighed. Subsequently, the fragments of the colon (2-10 mm) involving colonic lesions were sampled, fixed with formaldehyde, embedded in paraffin and routinely stained with hematoxilin and eosin for histological assessment.

The presence and intensity of histological changes were evaluated due to the following criteria: presence, area and depth of ulceration, presence and intensity of inflammatory infiltrations, ulcerations and fibrosis (19).

**Determination of plasma IL-1β and TNF-α levels and gastric mucosal MPO activity**

Immediately after the CBF measurements, a venous blood sample was drawn from the vena cava and placed into EDTA-containing vials and used for the determination of plasma IL-1β and TNF-α. Blood was collected and placed into sterile, plastic syringes, kept in ice till centrifugation. The blood samples were centrifuged with the speed 1000 G for 10 minutes in 15°C temperature and the sera were stored in -80°C. The serum level of proinflammatory cytokines IL-1β and TNF-α were evaluated with high sensitive ELISA (Quantikine HS, R&D Systems, Minneapolis, Minn., USA) according to manufacturer’s instructions. Intensity of the colorful reaction was estimated in the spectrophotometer Stat Fax 2100 (Awareness Technology Inc, Palo City, FL, USA) at 490 nm. The intra-and inter-assay coefficients of variation were 8.5% and 10.6%, respectively, for TNF-α, and 10.2% and 10.4%, respectively, for IL-1β.

The fragments of colonic tissue weighing about 200 mg were collected and frozen in -70°C for the determination of MPO activity by ELISA as reported recently (17).

**Expression of COX-2, IL-1β, TNF-α, VEGF and iNOS transcripts in the rat colonic mucosa determined by reverse transcriptase-polymerase chain reaction (RT-PCR)**

The mRNA expression for COX-2, IL-1β, TNF-α and iNOS were determined by RT-PCR in the unchanged colonic mucosa of intact rats or those with TNBS colitis given vehicle,
ASA and celecoxib without or with ampicillin treatment. Samples of the colon mucosa (about 200 mg) were scrapped off into ice using glass slides and then immediately snapped frozen in liquid nitrogen and stored at -80°C. The total RNA was isolated from the colon mucosa according to the technique described by Chomczynski and Sacchi (1987) using Trizol Reagent (Invitrogen, Carlsbad, USA) and the manufacturer’s protocol. The first strand of cDNA was synthesized from total cellular RNA (2 µg) using Reverse Transcription System (Promega, Madison, USA). The PCR was carried out in an automatic DNA thermal cycler, using 1 µg cDNA and Promega PCR reagents. For amplification of COX-2, TNF-α, IL-1β, VEGF and iNOS cDNA, and gene-specific primers (Sigma-Aldrich St. Louis, MO, USA) were used. The COX-2 primer sequences were as follows: upstream: 5’-ACA ACA TTC CCT TCC TTC-3’, downstream: 5’-CCT TAT TTC CTT TCA CAC-3’. The expected length of this PCR product was 201 bp. The IL-1β primer sequences were as follows: upstream, 5’-GCT ACC TAT GTC TTG CCC GT-3’, downstream, 5’-GAC CAT TGC TGT TTC CTA GG-3’ (the length of expected PCR product - 543 bp). The TNF-α primer sequences were as follows: upstream, 5’ TAC TGA ACT TCG GGG TGA TTG GTC C-3’, downstream, 5’-CAG CCT TGT CCC TTG AAG AGA ACC-3’ (the length of expected PCR product - 295 bp). The iNOS primer sequences were as follows: upstream: 5’-CCA CAA TAG TAC AAT ACT ACT TGG -3’, downstream: 5’-ACG AGG TGT TCA GCC GGC TGC TCC ACC-3’ (the length of expected PCR product - 397 bp). The VEGF primer sequences were as follows: upstream: 5’-GGT CCT CTG CCA TTC T-3’, downstream: 5’-TTG AAC ACC GAG CAG T-3’ (the length of expected PCR product - 271 bp). Primer annealing was carried out as follows: at 56°C, 60°C, 60°C, 59°C and 58°C for COX-2, IL-1β, TNF-α, VEGF and iNOS, respectively. Amplification of the control rat α-actin was performed on the same samples to verify the RNA integrity. The β-actin primer sequences were as follows: upstream, 5’-TTG TAA CCA ACT GGG ACG ATATGG-3’, downstream, 5’-GAT TCT GAT CTT CAT GGT GCT AAGG-3’ and primers annealing temperature was 54°C. The expected length of PCR product was 764 bp. PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bromide and then visualized under UV light. Location of predicted PCR product was confirmed by using O’Gene Ruler 50 bp DNA ladder (Fermentas, Life Sciences, San Francisco, USA) as standard marker. Comparison between different treatment groups was made by determination of the COX-2, IL-1β, TNF-α, VEGF and iNOS/β-actin ratio of the immunoreactive area by densitometry (Gel-Pro Analyser, Fotodyne Incorporated, MI, USA).

Microbiological evaluation of the colonic microflora

Faeces samples (about 1 g) have been collected and weighed and immediately sent to microbiological laboratory where they were homogenized and subjected to bacteriological examination assessed both, qualitatively and quantitatively. The number of bacteria in feces was calculated as CFU (Colony Forming Units) per gram of tissue using the following formula: CFU/g tissue = (n x dilution of culture x 5) x W where n is the number of colonies on the plate, 5 relates to the 5 fold dilution of the 1 g homogenized feces and W is the weight of specimen in g (1 g).

A further 10 fold dilution in physiological saline homogenized faeces were sown onto following microbiological media: Columbia agar with 5% sheep blood, MacConkey Agar and chrom ID CPS medium (bioMerieux) for rapid direct identification of Escherichia coli and other bacteria. For the accuracy of identification of Escherichia coli (E. coli), the API system (API20E bioMerieux) was employed. The culture

![Fig. 1. Effect of administration of ampicillin (800 mg/kg i.g.) on the area of colonic lesions and the alterations in CBF in animals treated daily with vehicle, ASA (40 mg/kg i.g.) or celecoxib (10 mg/kg i.g.) at day 10 after colitis induction. Mean±S.E.M. of 6-8 animals. An asterisk indicates a significant change (p<0.05) as compared to the values obtained in vehicle (control). An asterisk and cross indicate a significant change (p<0.05) as compared to the respective values obtained in ASA and vehicle group. Cross indicates a significant change (p<0.05) as compared to the respective values obtained in vehicle, ASA and celecoxib group.](image-url)
specimens were incubated for 48 h in an aerobically atmosphere at 37°C and bacterial colonies were counted. All liver, spleen, intestine and blood samples were examined qualitatively. Microorganisms were identified by standard conventional microbiological procedures as described above in order to determine translocation. Interpretation of the results was based on the presence of Gram-negative (G-) E. coli and G-positive (G+) Enterococcus faecalis in the material collected.

Statistical analysis

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

RESULTS

The influence of non selective COX inhibitor ASA or selective COX-2 inhibitor celecoxib with or without ampicillin treatment on the healing of colonic lesions

Intrarectal administration of TNBS resulted in the inflammation of colonic mucosa characterized by the acute colonic lesions with extensive mucosal ulcerations. The area of these lesions reached the maximum at day 3 and then it significantly declined at day 10 and day 14 of observation, respectively (data not shown). The CBF was decreased by about 40% on day 3, but it was significantly increased at day 10 and day 14 as reported by our group recently (20).

Based on this time-dependent assessment of colitis, we decided to present in this paper data collected at day 10 after the TNBS administration. Treatment with ASA caused the increase of intensity of the colonic lesions in the colon in comparison with the group of animals given vehicle (Fig. 1). Celecoxib also resulted in the increase of area of the inflammatory lesions in the colon but this increase in lesion incidence was smaller comparing with that observed in animals given ASA (Fig. 1). Concurrent i.g. treatment with ampicillin administered with ASA or celecoxib significantly decreased the area of colonic lesions comparing with animals treated with vehicle, ASA or celecoxib administered alone (Fig. 1).

Severity of inflammatory lesions induced ASA was accompanied by the significant decrease of CBF as compared with the groups of rats administered with vehicle or celecoxib (Fig. 1). Ampicillin added to vehicle, ASA or celecoxib produced the significant increase in CBF by about 19%, 22% and 34%, respectively, comparing with vehicle, ASA or celecoxib-treated animals without concurrent treatment with this antibiotic (Fig. 1).

Effect of vehicle, ASA and celecoxib with or without ampicillin on the weight of colon and MPO activity

The weight of examined colonic tissue was significantly increased in rats with TNBS-induced colitis treated with vehicle, ASA and celecoxib in comparison with that measured in rats without colitis and treated with vehicle, ASA or celecoxib (Fig. 2). The weight of colonic tissue of rats administered vehicle, ASA or celecoxib was significantly increased at 10 day of observation, in the group with TNBS induced colitis, in comparison with the animals without induction of colitis (Fig. 2). Concurrent treatment with ampicillin at dose 800 mg/kg resulted in the decrease of the weight of colonic tissue in rats given vehicle, ASA and celecoxib by about 19%, 25% and 33%, respectively (Fig. 2).
MPO activity, as the marker of neutrophil infiltration of colonic mucosa reached the value of 27±2.5 ng/ml and this remained unchanged in the intestine of rats treated with vehicle or those subjected to ASA or celecoxib (Fig. 2). MPO activity, which corresponded with the intensity of inflammation, was significantly increased in the colon mucosa. Both ASA and celecoxib caused significant elevation of MPO activity though the increase in the MPO content induced by celecoxib was significantly smaller as compared to that recorded in ASA-treated animals (Fig. 2). Administration of ampicillin caused a significant fall (p<0.05) of MPO activity in the colonic mucosa comparing with group of rats receiving vehicle, ASA or celecoxib alone (Fig. 2).

Macroscopic and microscopic appearance of colonic mucosa in rats without or with colitis

The intact colonic mucosa showed the normal macroscopic appearance (Fig. 3A). On day 10 upon colitis induction in ASA-treated rats (Fig. 3C) the area of colonic damage assessed macroscopically was larger than that in rats with colitis treated with vehicle (Fig. 3B). The area of colonic damage was significantly smaller in celecoxib-treated animals as compared with that observed in rat treated with ASA (Fig. 3D). Pretreatment with ampicillin resulted in the significant decrease of the area of colonic damage in the rats, given ASA or celecoxib comparing with the vehicle group and ASA- or celecoxib-treated animals without antibiotic treatment (Fig. 3E, F).

By histology, intact colonic mucosa showed regular colonic architecture and continuity with no signs of inflammation (Fig. 4A). Histopathological examination of the inflamed colon mucosa at day 3 after induction of colitis revealed swelling of the submucosa, necrotic lesions, micro-clothes as well as hemorrhagic lesions and acute neutrophil infiltrates in the region of colon mucosa and submucosa and were similar in all examined groups. In vehicle-treated TNBS rats on day 10, the widespread ulceration and an intense chronic infiltrate with the presence of numerous neutrophils penetrating the muscularis mucosa, submucosa with some features of mucosal regeneration were observed (Fig. 4B). In ASA-treated rats with colitis (Fig. 4C) there was a deep ulceration with necrosis and an intense inflammation followed by severe neutrophil infiltration and with epithelial regeneration of lesser intensity comparing with vehicle group. The partially healed epithelium and abnormal crypt architecture, possessing a more pronounced regeneration were observed in colonic mucosa of the rat with colitis treated with celecoxib (Fig. 4D) when compared to that receiving ASA or vehicle. In rats treated with combination of ampicillin and ASA, the more pronounced decrease of chronic infiltrate intensity with features of spread regeneration in group of animals given ASA was observed comparing to animals treated with ASA alone (Fig. 4E). In rats administered this antibiotic along the treatment with celecoxib, the small damage and a moderate infiltrate in the muscularis propria were observed. Process of healing of colonic mucosa was evidently more advanced in comparison with rats treated with celecoxib alone (Fig. 4F).

**Effect of ampicillin treatment on the intensity of inflammation of the colon mucosa and plasma proinflammatory cytokine levels**

Plasma levels of proinflammatory cytokines IL-1β and TNF-α were negligible in intact rats (for IL-1β: 7.3±0.4 pg/ ml and TNF-α: 1.2±0.6 pg/ ml, respectively) was markedly elevated (p<0.05) in
animals with TNBS induced colitis administered with ASA or celecoxib as compared to those recorded in vehicle-treated animals (Fig. 5). Concurrent treatment with ampicillin co-administered with vehicle, ASA or celecoxib significantly attenuated plasma the IL-1β and TNF-α levels as compared to the values of these cytokines achieved with vehicle, ASA and celecoxib alone (Fig. 5). The reduction in the plasma of cytokine level was associated with a significant decrease (p<0.05) of the weight of colonic tissue in rats treated with ampicillin (Fig. 5).

Effect of administration of ampicillin on the intensity of inflammation in the colonic mucosa and the mucosal expression of COX-2, iNOS, IL-1β, TNF-α, and VEGF in rats with colitis

As shown in Figs. 6 and 7, the signal for the expression of TNF-α, IL-1β and COX-2, iNOS and VEGF was significantly increased in vehicle-treated colonic mucosa of rats with colitis compared to that in the intact mucosa. The ratio of TNF-α, IL-1β and COX-2, iNOS and VEGF mRNA over β-actin mRNA confirmed that expression of these proinflammatory markers was significantly elevated in TNBS-treated animals comparing to intact rats. Treatment with ASA or celecoxib resulted in a strong signal of mRNAs for TNF-α, IL-1β, COX-2, iNOS and VEGF (Figs. 6 and 7, left panel). The semi-quantitative ratio of TNF-α and IL-1β, COX-2 and iNOS, confirmed that the expression of these inflammatory markers were significantly increased in the colonic mucosa of rats treated with ASA in comparison with animals treated with celecoxib (Figs. 6 and 7, right panel). The ratio of VEGF mRNA over β-actin mRNA revealed increased expression of mRNA for VEGF, which is an important factor in the regulation of process of angiogenesis and inflammation in the colon mucosa of rats with colitis receiving vehicle, ASA or celecoxib in comparison with intact rats (Fig. 6, right panel).

Treatment with ampicillin suppressed the signal of mRNA expression for TNF-α, IL-1β, COX-2, iNOS and VEGF in the colonic tissue in rats treated with ASA or celecoxib (Figs. 6 and 7). The ratio of TNF-α, IL-1β, COX-2, iNOS and VEGF mRNA over β-actin mRNA confirmed that their expression was significantly decreased in vehicle-, ASA- and celecoxib-treated animals as compared to animals without concurrent ampicillin treatment.

Bacteriological evaluation of the microflora in the colon

We evaluated bacterial stool concentration of G- bacteria E. coli, which is considered as predominant species in the rat intestinal microflora. Its number in the intact rats accounted for ~10^7 CFU/g of the colon content (Table 1). In vehicle-treated animals at day 3 and day 10 after induction of colitis, the

\[ \text{Bacteriological evaluation of the microflora in the colon} \]
Fig. 5. The effect of ampicillin (800 mg/kg i.g.) administration on the weight of colonic tissue and the plasma levels of proinflammatory cytokines IL-1β and TNF-α in rats treated with vehicle, ASA (40 mg/kg i.g.) or celecoxib (10 mg/kg i.g.) at day 10 after colitis induction. Mean±S.E.M. of 6-8 animals. An asterisk indicates a significant change (p<0.02) when compared to the values obtained in rats with colitis treated with vehicle (control). An asterisk and cross indicate a significant difference (p<0.05) as compared to the respective values obtained in colitis rats administered with ASA. The cross indicates a significant change (p<0.02) when compared to the respective values obtained in colitis rats administered with vehicle, ASA and celecoxib without cotreatment with ampicillin.

Fig. 6. The effect of ampicillin (800 mg/kg i.g.) on the expression of mRNA for TNF-α and IL-1β in colonic mucosa of intact rats those treated with vehicle, ASA (40 mg/kg i.g.) or celecoxib (10 mg/kg i.g.) at day 10 upon induction of colitis. Mean±S.E.M of 4 determinations in 4 animals. An asterisk indicates a significant change (p<0.05) when compared to the values obtained in intact colonic mucosa. The cross indicates a significant change (p<0.05) when compared to the respective values obtained in the rats treated with vehicle, ASA and celecoxib without the combination with ampicillin.
significant rise in the number of \textit{E. coli} was observed by approximately 10-100-fold (1.3x10^4±0.34 CFU/g - 3.05x10^5±0.65 CFU/g, respectively), in comparison with the intact rats (Table 1).

Administration of ASA or celecoxib resulted in significant 100-10,000-fold increase of \textit{E. coli} counts in the stool at the day 3 and day 10 of observation compared with respective bacterial counts in the intact rats (Table 1). Concurrent treatment with ampicillin administered with ASA or celecoxib caused a significant fall in the number of \textit{E. coli} in the stool at day 3 of observation followed by non significant increase in this bacterial count at day 10 not exceeding 8x10^3±0.68 CFU/g and 5.85x10^4±0.23 CFU/g, respectively (Table 1). At the same time, 10-10 000 fold decrease in bacterial counts for G+ bacteria inhabiting the GI tract such as \textit{Enterococcus faecalis} was observed at day 3 and day 10 in rats with colitis treated with vehicle, ASA or celecoxib compared with the intact animals (Fig. 8). Administration of ampicillin before ASA or celecoxib treatment augmented the fall of \textit{Enterococcus faecalis} number in the stool in comparison with intact rats and animals not treated with ampicillin (Fig. 8).

The presence of bacteria \textit{E. coli} was detected in the colonic tissue as well as in the extraintestinal organs such as blood, liver and spleen suggesting the intestinal bacteria passing through the impaired colonic mucosa due to inflammation. Induction of colitis caused bacterial translocation from the colonic mucosa to blood.
and the bacterial growth was also observed in the spleen and the liver (10^4-10^3 CFU/g, respectively). In the animals treated with ASA or celecoxib, single colonies of *E. coli* were cultured from the blood, however, in the colon tissue, liver and spleen of ASA and celecoxib-treated animals, the confluent growth of this bacteria in both groups (10^8 CFU/g) in all animals (100%) was observed (Fig. 8). The combined treatment with ampicillin and ASA or celecoxib markedly inhibited (by about 1000-10 000 fold) *E. coli* culture counts in the colonic tissue as well as the blood, liver and spleen as compared to ASA and celecoxib alone (Fig. 8).

**DISCUSSION**

In the present study we determined the influence of ASA which is a nonselective COX-1 and COX-2 inhibitor and celecoxib, the highly selective COX-2 inhibitor, on the process of healing of the inflammatory lesions in the experimental colitis and the possibility that antibiotic treatment will be effective in the healing of colonic damage due to limiting the bacterial aspect associated with the harmful influence of this NSAID and coxib on the process of healing of colitis. While in the stomach, gastric acid plays a major role in the pathomechanism of gastric mucosal damage induced by NSAID, the intestinal perturbation caused by these class of drugs seem to be acid-independent and the presence of bacteria, bile and neutrophil in the lower GI tract are considered as important pathologic factors in the NSAIDs-induced perturbations in the intestine (13). The mechanism of the damage and interactions between NSAIDs and the bacteria inhabiting lower GI tract is not clear. Inhibition of PGs biosynthesis seems to act as the critical component of the colon-damaging manifestation of NSAIDs ingestion in humans and experimental animals. The NSAID-induced damage of the intestinal mucosa is a pH-independent phenomenon and the antisecretory drugs as well as mucosal protective agents such as sucralfate and misoprostol even exerting some degree of intestinal protection are not able to counteract the NSAIDs-induced increased intestinal permeability in the GI tract (21). It has been demonstrated that the intestinal damage induced by various NSAIDs could be attenuated by antimicrobial treatment (22).

In the present study we found that TNBS-induced experimental colitis manifested by the presence of macroscopic and microscopic lesions corresponding with an increase in the colonic weight is accompanied with a significant fall in the CBF, increase of MPO activity in the colon mucosa and significant increase in the plasma proinflammatory cytokines IL-1β and TNF-α levels. Administration of ASA or celecoxib significantly enhanced the area of colonic damage, produced an increase in colonic tissue weight, elevated the mRNA expression of these proinflammatory cytokines in the colonic mucosa as well as produced the rise in the plasma cytokine IL-1β and TNF-α levels. Results of our studies are in keeping with those presented by Watanabe et al. (13) because inflammatory cytokines were thought by them to play a major role in the pathogenesis of NSAID-induced enteropathy. Moreover, it was reported that TNF-α was involved in the triggering of inflammatory response followed by chemokines overexpression and mucosal neutrophil infiltration in the rats administered with indomethacin (23). Bertrand et al. (24) concluded that TNF-α plays critical role in NSAID-induced small intestinal enteropathy. Our results documented that NSAID-induced colonic mucosal lesions involve an increase the iNOS expression in the colonic mucosa which is in keeping with previous observation that the iNOS-NO system is involved in the pathomechanism of the intestinal
lesions induced by indomethacin (10). Changes in the microcirculation were considered as important in the context of NSAIDs-induced enteropathy depending on the selectivity of COX-inhibition (25). In our present study, the TNBS-induced colitis that was associated with the fall in the CBF was apparently worsened by concomitant treatment with ASA or celecoxib.

Vascular endothelial growth factor (VEGF) plays important role in the process of angiogenesis and was considered to mediate the inflammatory reaction in the course of human and experimental colitis in rodents (26). We provided for the first time evidence that NSAID and coxib may influence the mucosal expression of VEGF in the colon because the administration of ASA or celecoxib caused an overexpression of mRNA for VEGF in the colonic mucosa of rats with colitis comparing with intact animals. These results suggest usefulness of VEGF as the inflammatory marker in the evaluation of intensity of inflammation during time course of colitis. We believe that factors inhibiting the VEGF expression and possibly the activity of this peptide could constitute a new strategy for the anti-inflammatory treatment of colitis but this issue requires further studies.

In our study, the increased activity of MPO, the indicator of the intensity of neutrophil infiltration of inflamed colonic mucosa was observed. Moreover, the administration of ASA or celecoxib significantly enhanced MPO activity suggesting an important role of activated neutrophils in the pathogenesis of NSAIDs-induced colonic damage. Neutrophils have previously been considered to play a role in the pathomechanism of NSAIDs-induced gastroenteropathy (27).

The major finding of our present study is the observation that that induction of the colitis caused a significant increase of the growth of \( E. \) coli in the stool and that the intraintestinal \( E. \) coli concentration was increased following ASA or celecoxib administration. This \( E. \) coli overgrowth in response to classic NSAID and coxib was accompanied by the exaggeration of colonic damage and the overexpression of mRNA for TNF-\( \alpha \), IL-1\( \beta \) and iNOS in the colonic mucosa. Our results are corroborative with that of Konaka et al. (10), who also demonstrated the involvement of TNF-\( \alpha \) and iNOS activation followed by the mucosa infiltration of neutrophils with subsequent increased permeability of the intestinal mucosa. Herein, we provided the evidence that the pathogenic mechanism of ASA and to lesser extent of celecoxib during colitis involves increased expression and release of TNF-\( \alpha \) and IL-1\( \beta \) and other proinflammatory markers such as iNOS and VEGF. Moreover, the overgrowth of intestinal bacteria observed after treatment with NSAID and coxib could contribute to the increased colonic permeability. This notion is supported by the fact that the presence of cultures of \( E. \) coli was detected in the blood of ASA- and coxib-treated rats with colitis as well as the confluent growth of that bacteria was confirmed in the colonic tissue and extraintestinal tissues including liver and spleen. Interestingly, we failed to find any differences in the induction of bacterial growth between groups of rats treated with ASA or celecoxib suggesting that overgrowth of bacteria might be independent of the selectivity of COX-inhibitor employed in our study.

The important role of bacterial microflora in the pathogenesis of NSAIDs-induced enteropathy is supported by previous evidence that germ-free rats were resistant to NSAIDs-induced enteropathy (16). Recent studies revealed the growth of Gram negative bacteria in the ileum of the NSAIDs-treated animals (16). In another experimental studies, the protective activity of some antibiotics such as tetracycline (28), kanamycin (29) or antimicrobial metronidazole (12) in attenuation of NSAIDs-induced enteropathy were emphasized.

It is of interest that the combined treatment of ampicillin with ASA or celecoxib, significantly decreased the bacterial growth resulting in partial resolution of colonic lesions and improvement of CBF in rats with colitis. Ampicillin also decreased the plasma concentration of IL-1\( \beta \) and TNF-\( \alpha \) and the mucosal expression of mRNAs for iNOS and VEGF in colitis suggesting that this antibiotic exerts a beneficial antiinflammatory effect possibly due to inhibitory effect on the bacterial endotoxins. Indeed, Konaka et al. (10) documented that the suppression of the enterobacterial counts and iNOS activity contributed to the inhibition of the severity of intestinal lesions induced by indomethacin. Our study imply that ampicillin could be highly efficient in the mechanism of protection and recovery of colonic mucosa from NSAID-induced enteropathy in animals with experimental colitis. The mechanism of this beneficial activity of ampicillin in addition to the amelioration of proinflammatory markers such as COX-2, iNOS and cytokines may include that the suppression of the bacterial overgrowth in the colon and bacteria, in particular \( E. \) coli translocation to extraintestinal organs. This protective activity of ampicillin includes its profitable influence on the mucosal microcirculation in the colon since we have observed the improvement of CBF and inhibition of VEGF in the colonic mucosa. Our study emphasizes the importance of enterobacterial factors, possibly required for triggering effect of proinflammatory factors and cytokines responsible for the impaired microcirculation in augmentation of colonic damage caused by NSAIDs as originally suggested (30). The hypothesis was proposed that with the repeated exposures of the intestine to the NSAID, as it is excreted in the bile, further epithelial injury occurs and this is exacerbated by the elevated number of luminal bacteria (30). The elevation of the bacterial numbers occurs only with NSAIDs that undergo enterohepatic recirculation suggesting that systemic suppression of PG synthesis by the NSAIDs might be not the primary pathogenic mechanism of intestinal ulcerations. Therefore, the better tolerability of moderately selective COX-2 inhibitors such as etodolac and nabumetone could be attributable to the lack of enterohepatic circulation rather than to COX-2 selectivity (30).

In summary, we demonstrated that the conventional NSAIDs such as ASA or the selective COX-2 inhibitor, celecoxib, delayed the healing of colonic damage in experimental colitis and this effect was accompanied by the fall in the CBF, the enhancement in plasma levels of proinflammatory cytokines IL-1\( \beta \) and TNF-\( \alpha \) and up-regulation of COX-2 and iNOS expression in inflamed colonic mucosa. Treatment with ampicillin in rats administered with ASA or celecoxib had beneficial effect on the process of healing of mucosal damage, associated with suppression of plasma proinflammatory cytokines IL-1\( \beta \) and TNF-\( \alpha \) and colonic tissue as well as iNOS expression in the colonic tissue. Beneficial effect of ampicillin seems to be associated with improvement of the colonic microcirculation and downregulation of expression of VEGF mRNA in the colonic tissue. Antibiotics and factors inhibiting the COX-2 and VEGF expression could account for the new strategy of the anti-inflammatory treatment of colitis. Control of intestinal microflora appears to be important in prophylaxis of ulcerative colitis in patients subjected to NSAID or coxib therapy (31). Ampicillin counteracts the ASA or celecoxib induced delay in healing of colitis suggesting that monitoring of intestinal microflora by antibiotics play an important role in prophylaxis of colitis exaggerated by NSAIDs and in inducing remission in relapsing disorder such as ulcerative colitis in humans (31). Recent systematic review of randomized controlled trials in humans suggest that further research into antibiotic therapy of ulcerative colitis and Crohn’s
disease should be addressed to establish which antibiotic or antibiotic combination is the most effective in treating these disorders. Finally, our microbiological studies indicated that the decrease of bacterial count by antibiotic treatment in the colonic microflora may facilitate the control of the process of inflammation in the colonic mucosa and contributes to the resolution of inflammation resulting in the acceleration of colonic healing.

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