Preventive and therapeutic possibilities for inhibition of the inflammatory consequences of experimental colitis

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2. **Kovács T**, Varga G, Érces D, Tőkés T, Tiszlavicz L, Ghyczy M, Boros M, Kaszaki J: Dietary phosphatidylcholine supplementation attenuates inflammatory mucosal damage in a rat model of experimental colitis. *Shock* 2012 (Epub ahead of print]). **IF=3.203**
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2. **Kovács T**, Fazekas B, Varga G, Érces D, Kaszaki J, Vécsei L, Boros M: Az NMDA-receptorgátlás vizsgálata bélgyulladásos patkánymodellben. *Magyar Sebészet* 2009; 62: 154.
3. Varga G, **Kovács T**, Kaszaki J, Ghyczy M, Boros M: A foszfatidil-etanolamin gyulladáscsökkentő hatása kísérletes colitis modellben. *Magyar Sebészet* 2009; 62: 145-146.
4. Fazekas B, Varga G, Érces D, **Kovács T**, Kaszaki J, Vécsei L, Boros M: Az NMDA-receptor-aktiváció jelentősége kísérletes bélgyulladásban. *Magyar Sebészet* 2009; 62: 153.
5. Varga G, Érces D, Fazekas B, Fülöp M, **Kovács T**, Kaszaki J, Fülöp F, Vécsei L, Boros M: N-Methyl-D-aspartate receptor inhibition decreases motility and inflammatory activation in experimental colitis. *Shock* 2009; 32 (S1): 14.

6. Varga G, **Kovács T**, Tőkés T, Érces D, Kaszaki J, Ghyczy M, Boros M: Effects of oral phosphatidylcholine on the inflammatory activation in early and late phases of experimental colitis. *Acta Physiologica* 2011; 202 (S684): 124-125.

CONTENTS

List of abbreviations

CLSEM	confocal laser scanning endomicroscopy
CNS	central nervous system
CO	cardiac output
ENS	enteric nervous system
FITC-dextran	fluorescein isothiocyanate-dextran
GI	gastrointestinal
HR	heart rate
IBD	inflammatory bowel disease
IL-6	interleukin-6
KynA	kynurenic acid
MAP	mean arterial pressure
NAD	nicotinamide-adenine dinucleotide
NMDA	N-methyl-D-aspartate
PC	phosphatidylcholine
PMN	polymorphonuclear granulocyte
TNBS	trinitro benzene sulfonic acid
TNF-α	tumour necrosis factor-alpha

SUMMARY

Inflammatory bowel diseases are accompanied by a severe morphological impairment. Conservative treatment involves anti-inflammatory, immunomodulant and biological therapy. All of the currently used medicaments have side-effects with significant morbidity, especially as concerns their long-term use. New therapeutic possibilities are therefore being sought, which are efficacious and have fewer side-effects.

Our aim was to follow the time course of the inflammatory and morphological changes in the large intestine during the acute and subacute phases of experimental colitis in the rat. We also examined the possible anti-inflammatory effects of phosphatidylcholine (PC) pre-treatment and treatment with the N-methyl-D-aspartate (NMDA) receptor antagonist kynurenic acid (KynA) and its synthetic analogue SZR-72. The effects of these therapies on morphological changes were observed with the help of conventional and a novel *in vivo* histological method, confocal laser scanning endomicroscopy.

PC, a major component of biomembranes, exerts its effects in colitis in different ways. On the one hand, it helps to restore the mucin layer of the colon, which is diminished in colitis, and which has a very important role in preserving the integrity of the mucosa. This is facilitated by the finding that PC increases the number of mucus-producing goblet cells in trinitro benzene sulfonic acid colitis. On the other hand, choline, a metabolite of PC, has a direct anti-inflammatory effect too. Through blockade of the production of the pro-inflammatory cytokines tumour necrosis factor-alpha and interleukin-6, it inhibits the extravasation and infiltration of leukocytes in the wall of the colon, and thereby prevents consequent morphological damage. In our studies, these changes were observed in the acute and subacute phases of colitis.

The NMDA receptors, a subtype of glutamate receptors, are upregulated in experimental colitis. Treatment with the NMDA antagonist KynA and the blood-brain barrier-permeable SZR-72 can block the inflammatory process in both phases of colitis, by decreasing cytokine levels and tissue leukocyte accumulation. On the other hand, the single administration of NMDA antagonists was unable to treat the morphological impairment.

In conclusion, it may be assumed that PC pretreatment and NMDA antagonist therapy can successfully attenuate the inflammatory process in colitis, manifested in the rising levels of pro-inflammatory cytokines and the significant leukocyte infiltration in both phases of colitis. The severe morphological impairment that evolves in colitis can be effectively prevented by PC pretreatment, but NMDA antagonism does not influence these changes.

1. INTRODUCTION

1.1. Characteristics of human inflammatory bowel diseases

Inflammatory bowel diseases (IBDs) (Crohn's disease and ulcerative colitis) are chronic, remitting diseases with unknown aetiology. The incidence of IBDs has increased in recent decades, and in Crohn's disease the onset of symptoms is occuring at younger ages (Ekbom 1991, Armitage 2001). The morphological changes of the intestines resulting from the inflammation differ in the two main types. Ulcerative colitis affects only the mucosa; always evolves in the rectum, and the inflammatory process continually progresses orally. Consequently, pseudopolyps and ulcerations will develop. Crohn's disease is a transmural, granulomatous disease, which can affect any segment of the gastrointestinal (GI) tract. Segmental involvement of the intestines usually occurs. In Crohn's disease, interintestinal and perianal fistulas, abscesses and strictures are characteristic. Chronic inflammation will raise the risk of a malignant transformation (Munkholm 2003).

Endoscopy plays the main role in the diagnostics: not only can the severity of inflammation be assessed, but biopsies can be taken, and the histological analysis can facilitate the diagnosis, control the efficacy of the medical treatment and check on areas suspicious for malignancy.

In recent years IBD research has focused on cytokines, and especially on tumour necrosis factor-alpha (TNF-α). This mediator originates from different types of leukocytes: lymphocytes, macrophages and polymorphonuclear (PMN) granulocytes. TNF-α is a proximal cytokine generated during an inflammatory response and is capable of activating other cytokines and by staying at the focus of inflammation, inducing the development of the inflammatory cascade in IBDs (Zhou 2006a, 2006b).

1.2. Current medical therapy in IBDs

In the therapeutic repertoire of IBDs, the main emphasis is on conservative treatment. With the help of local and systemic anti-inflammatory and immunomodulant agents, an effort is made to block the activation of inflammation and relapses. The medical therapy involves anti-inflammatory mesalamin compounds, corticosteroids and immunomodulators (Braus 2009). The great innovation in the past decade was biological therapy, which targets TNF-α signalling with the use of monoclonal anti-TNF-α antibody (Rutgeerts, 2004). Surgical intervention is needed only in the event of the ineffectiveness of this conservative treatment or to treat complications. In ulcerative colitis, restorative proctocolectomy with ileoanostomy is

a possibility, while in Crohn's disease limited intestinal resections, or the curing of fistulas and abscesses can be performed.

The side-effects of long-term medication with corticosteroids and other anti-inflammatory drugs are well known: osteoporosis, hypertension, diabetes, cataract, skin striae, pathological fractures, etc. Opportunistic infections or even tumour development are the main risks of immunomodulant drugs. Biological therapy has much less severe and less frequent side-effects: infusion reactions, serum sickness, opportunistic infections, rarely demyelinating disease and a worsening of congestive heart failure (Rutgeerts, 2004). Although biological therapy allows a decrease of the steroid or immunomodulant intake, it can rarely replace it. Since most IBDs start in the second or third decade of life, very long medical treatment is needed. New therapeutic possibilities with much milder side-effects than those of the currently used ones would therefore be of great significance.

1.3. Experimental models of human IBD

The most widely used experimental method used to model human IBD is the application of 2,4,6-trinitro benzene sulfonic acid (TNBS) to rodents. A single intracolonic enema of TNBS causes transmural colitis, which lasts longer than 8 weeks, and reproduces several symptoms of human IBD (Morris 1989). This model is characterized by a weight loss (Morris 1989) with visceral hyperalgesia (Zhou 2008), significant elevations of the pro-inflammatory cytokines, including TNF-α (Neurath 1997) and interleukin-6 (IL-6) (Hove 2001), extensive ulcerations, morphological changes (Tatsumi 1996) and tissue leukocyte accumulation (Kiss 1997).

1.4. Methods of investigating morphological impairment

The consequence of colitis is a variable extent of colonic morphological damage. By means of conventional histological investigations, taking full-thickness colon biopsies, the depth and severity of tissue damage can be determined. Nevertheless, with the help of novel histological techniques, dynamic analyses can also be achieved (McLaren 2002). With fluorescence confocal laser scanning endomicroscopy (CLSEM), sectioning, fixation and embedding artefacts can be avoided. With CLSEM, 3-dimensional optical biopsies providing good-quality images of the epithelial layer can be obtained *in vivo* without physical disruption of the epithelial integrity (Kiesslich 2007). By means of this approach, the cellular and subcellular structures of the colonic epithelium (surface epithelium and crypts) and connective tissue can be examined. Another advantage of this method is that the changes in the microvascular structure and damage in the microvessels can be outlined.

1.5. Therapeutic possibilities in colitis: The potential role of phosphatidylcholine

Phosphatidylcholine (PC) is a ubiquitous phospholipid, a major component of biomembranes, and a number of experimental and clinical studies have demonstrated that it alleviates the consequences of inflammation and ischaemia in different organs and experimental models (Stremmel 2005, Erős 2006, 2009, Gera 2007, Ghyczy 2008, Tőkés 2011). Furthermore, it has been proved to inhibit the mucosal damage and morphological impairment caused by acids and other noxious agents in the GI tract (el-Hariri 1992, Mourelle 1996, Demirbilek 2002, Erős 2006). Treede *et al* have provided compelling *in vitro* evidence that exogenous PC significantly inhibits TNF-α-induced inflammatory responses (Treede 2007).

In vivo, PC is produced via two major pathways. Two fatty acids undergo addition to glycerol phosphate, to generate phosphatidic acid. This is converted to diacylglycerol, after which phosphocholine (the head group) is added to cytidine 5-diphosphocholine. The second, minor pathway involves the methylation of phospatidylethanolamine, in which three methyl groups are added to the ethanolamine head group of the phospholipid, converting it into PC. Orally taken PC serves as a slow-release blood choline source. The choline component of PC participates in a wide range of responses, including interference with the mechanism of activation of the polymorphonuclear (PMN) leukocytes (Monje 2003) and this pathway become important under inflammatory stress conditions. Choline itself is anti-inflammatory; PC is taken up by phagocytic cells, and it may accumulate in inflamed tissues (Kuehl 1957, Cleland 1979). On the other hand, the hydrolysis of PC by phospholipase D generates choline in cholinergic neurones (Blusztajn 1983), and this choline is used for the synthesis of the principal vagal neurotransmitter, acetylcholine. Previous studies have shown that some of the choline is stored in the form of membrane PC, and this pathway may become particularly important when extracellular circulating choline concentrations are low (e.g. during a dietary choline deficiency) or when acetylcholine synthesis and release are accelerated by high neuronal activity (Ulus 1989, Lee 1993).

1.6. Therapeutic possibilities in colitis: the potential role of NMDA antagonism

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS), but it is present in the enteric nervous system (ENS) too (Liu 1997, Giaroni 2003). Among the several types of glutamate receptors, the N-methyl-D-aspartate (NMDA) type is

expressed on a high proportion of the ENS neurones (Wiley 1991, Sinsky 1998, Kirchgessner 2001, Giaroni 2003).

Kynurenic acid (KynA) is an antagonist of the NMDA receptors, with high affinity for the glycine co-agonist site (Kessler 1989, Stone 1993). It is a product of an alternative tryptophan pathway, the major route for the conversion of tryptophan to nicotinamide-adenine dinucleotide (NAD) and NAD phosphate, leading to the production of a number of biologically active molecules with neuractive properties.

L-Tryptophan is an important essential amino acid used for protein synthesis and a precursor of bioactive molecules. Approximately 1-2% of the intake is metabolized through serotonin synthesis, but most enters the kynurenine pathway, synthesizing L-kynurenine, quinolinic acid, KynA and NAD.

Two major products of the tryptophan - L-kynurenine pathway, quinolinic acid and KynA, act on the glutamate receptors. Quinolinic acid is an agonist of the NMDA glutamate receptors with pro-inflammatory properties, while KynA is an endogenous NMDA receptor antagonist. KynA is able to reduce the excitotoxic damage to the CNS both *in vivo* (Simon 1986, Faden 1989) and *in vitro* (Choi 1988) and can be considered neuroprotective in neurodegenerative disorders (Klivényi 2004).

A number of clinical data suggest that the metabolism of tryptophan along the kynurenine pathway is altered in inflammatory GI disorders. The plasma level of L-kynurenine is elevated in IBD patients, and the level is likewise increased in coeliac disease (Forrest 2002, Torres 2007). Increased serum levels of free tryptophan have also been reported in patients with diarrhoea-predominant irritable bowel syndrome (Christmas 2010). These observations clearly suggest a role for NMDA-glutamate receptors and kynurenine modulation in the symptoms of inflammatory GI conditions.

A synthetic analogue of KynA has recently been prepared: SZR-72 (2-(2-N,N-dimethylaminoethylamine-1-carbonyl)-1H-quinolin-4-one hydrochloride, synthetized by the Institute of Pharmaceutical Chemistry, University of Szeged (Patent No. 104448-1998/Ky/me). The main difference from KynA is that it is blood-brain barrier-permeable. It was originally developed to influence the NMDA receptor overexcitation in the CNS, but its role and peripheral effects on the ENS are still largely unmapped (Fülöp 2009).

2. AIMS

The main goals of our study were to evaluate the degree of inflammatory activation and morphological changes in the distal colon in experimental colitis and to design new methods via which to influence such events. Medical therapy typically starts after the onset of signs and symptoms, and experimental studies involving delayed treatments are therefore arguably more relevant to the clinical scenario. Thus, as a novel approach, we planned to estimate the efficacy of a potentially antiinflammatory treatment delayed until 6 days after the initiating insult. In this line, our aims were:

• to investigate the inflammatory changes in the acute and subacute phases of experimental colitis;

• to analyse the morphological changes in colitis with conventional, static and novel, dynamic histological methods;

• to observe the possible modulatory effect of dietary PC pretreatments in the acute and subacute phases of colitis on the inflammatory changes, with special emphasis on morphology changes;

• to investigate the effects of acute and delayed NMDA antagonism on the inflammatory process and morphological impairment in this model.

3. MATERIALS AND METHODS

3.1. Animals

The experimental protocol was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged and followed the NIH guidelines for the care and use of experimental animals. The experiments were performed on 96 (Study I: 48, Study II: 48) male Sprague-Dawley rats (280-320 g) housed in plastic cages in a thermoneutral environment (21±2 °C) with a 12-h dark-light cycle. The animals, fed on a normal diet with tap water *ad libitum*, were divided into two studies.

3.2. Induction of colitis

Colonic inflammation was induced by the intracolonic administration of TNBS (40 mg/kg in 0.25 mL of 25% ethanol) through an 8-cm-long soft plastic catheter under transient diethyl ether anaesthesia. In the sham-operated groups, only the vehicle for TNBS was administered. The animals were deprived of food, but not water, for 12 h prior to the enemas.

3.3. Surgical preparation

The animals were anaesthetized with sodium pentobarbital (50 mg/kg bw ip) 1 or 6 days after the enema and placed in a supine position on a heating pad. Tracheostomy was performed to facilitate spontaneous breathing, and the right jugular vein was cannulated with PE50 tubing for fluid administration and Ringer's lactate infusion (10 mL/kg/h) during the experiments. A thermistor-tip catheter (PTH-01; Experimetria Ltd., Budapest, Hungary) was positioned into the ascending aorta through the right common carotid artery to measure the cardiac output (CO) by a thermodilution technique. The right femoral artery was cannulated with PE40 tubing for mean arterial pressure (MAP) and heart rate (HR) measurements.

3.4. Haemodynamic measurements

Pressure signals (BPR-02 transducer; Experimetria Ltd., Budapest, Hungary) were measured continuously and registered with a computerized data-acquisition system (Experimetria Ltd., Budapest, Hungary). The HR was calculated from the MAP curve. CO was detected by a thermodilution technique, using a SPEL Advanced Cardiosys 1.4 computer (Experimetria Ltd., Budapest, Hungary). Gases in arterial blood samples were measured with a blood gas analyser (AVL Compact 2, Graz, Austria).

3.5. Measurement of plasma TNF-α and IL-6

Blood samples (0.5 mL) were taken from the inferior caval vein into precooled, heparizined (100 U/mL) polypropylene tubes, centrifuged at 1000 g at 4 °C for 30 min and then stored at -70 °C until assay. Plasma TNF- α concentrations were determined in duplicate by means of a commercially available enzyme-linked immunosorbent assay (Quantikine ultrasensitive ELISA kit for rat TNF-α; Biomedica Hungaria Kft, Budapest, Hungary). The minimum detectable level was less than 5 pg/mL, and the interassay and intra-assay coefficients of variation were less than 10%.

Plasma IL-6 concentrations were measured with a commercially available enzyme-linked immunosorbent assay (Quantikine ultrasensitive ELISA kit for rat IL-6; Biomedica Hungaria Kft, Budapest, Hungary). The minimum detectable dose of rat IL-6 was in the range 14-36 pg/mL.

3.6. In vivo detection of tissue damage

The extent of mucosal damage of the distal colon was evaluated by means of fluorescence CLSEM (Five1, Optiscan Pty. Ltd., Melbourne, Victoria, Australia) developed for *in vivo* histology. The analysis was performed twice, separately by two investigators. The mucosal surface of the distal colon 8 cm proximal to the anus was surgically exposed and laid flat for examination. The microvascular structure was recorded after the iv administration of 0.3 mL of fluorescein isothiocyanate-dextran (FITC-dextran, 150 KDa, 20 mg/mL solution dissolved in saline, Sigma Chem.). The objective of the device was placed onto the mucosal surface of the descending colon, and confocal imaging was performed 5 min after dye administration (1 scan/image, 1024 x 512 pixels and 475 x 475 μm per image). The changes in the mucosal architecture were examined following topical application of the fluorescent dye acridine orange (Sigma-Aldrich Inc, St. Louis, MO, USA). The surplus dye was washed off the mucosal surface of the colon with saline 2 min before imaging.

Non-overlapping fields of active areas of disease were processed in TNBS-treated animals and compared with samples from PC-treated or control groups by using a semiquantitative scoring system. We employed four criteria: I. the structure of the microvessels (0 = normal, 1 = dye extravasation, but the vessel structure is still recognizable, 2 = destruction and the vessel structure is unrecognizable); II. crypt denudation (0 = no denudation, 1 = at least one area without a recognizable crypt structure per field of view, 2 = more than one area without a recognizable crypt structure per field of view, or more than 30% of the field covered by denuded crypts); III. oedema (0 = no oedema, 1 = moderate epithelial

swelling, 2 = severe oedema); and IV. epithelial cell outlines (0 = normal, clearly, well-defined outlines, 1 = blurred outlines, 2 = lack of normal cellular contours). In each field of view, the number of goblet cells was counted and the ratio relative to the number of visualized glands was calculated (number of goblet cells / number of glands).

3.7. Conventional histopathological analysis

Full-thickness colon biopsies taken at the end of the experiments were analysed in each group. The tissue was fixed in 6% buffered formalin, embedded in paraffin, cut into 4-μm-thick sections and stained with haematoxylin and eosin. The coded sections were evaluated by an independent specialist in histopathology. The infiltration of leukocytes was detected and the severity of tissue damage was compared with that in the control group by using a modification of the semiquantitative scoring system of Riley *et al.* (Riley 1991). The grading was performed with the following criteria: 1. acute inflammatory cell infiltration of the lamina propria with PMN cells (0 – 3); 2. a crypt abscess (0 – 3); 3. mucin depletion (0 – 3); 4. surface epithelial integrity (0 – 3); 5. a chronic inflammatory cell infiltrate, round cells in the lamina propria (0 – 3); 6. crypt architectural irregularities (0 – 3); 7: a transmural lesion or lesions affected the lamina propria only (1 - 0); 8: diffuse or focal lesions(1 – 0).

3.8. Experimental protocols

Study I

In the first part of Study I, 24 animals (series I) were randomly allocated into 3 groups (n=8 in each group). Group 1 served as sham-operated controls, while in group 2 colitis was induced, but the animals received standard laboratory diet. In group 3, the animals were fed with a special diet (Ssniff Spezialdiäten, Ssniff GmbH, Soest, Germany) containing 2% PC (1,2-diacylglycero-3-phosphocholine, R45, Lipoid GmbH, Ludwigshafen, Germany) for 6 days prior to the TNBS enema. In these groups, the experiments were started with baseline haemodynamic measurements 1 day after TNBS induction.

The experimental set-up was identical in series II, in the subacute phase of colitis. Group 4 (n = 8) was the sham-operated group, while in groups 5 and 6 (n=8 in each) colitis was induced 6 days before the measurements. Group 5 received no treatment, while group 6 received a 2% PC-enriched diet for 3 days before and 3 days after the TNBS enema. The haemodynamic measurements were started 6 days following the TNBS enema.

In series I, the animals were anaesthetized 1 day after the TNBS enema, and in series II, 6 days after colitis induction. Surgery was performed to allow registration of the haemodynamic parameters at 1-h intervals (CO data were measured only at the end of the

experiments). Additionally, fluorescence CLSEM was performed to examine the morphological changes in the mucosa of the distal colon, venous blood samples were taken to determine the biochemical changes in the plasma, and colon biopsies were taken for histological analysis.

Study II

In the first part of Study II, the animals were assigned to one or other of 4 groups (series III). Group 1 was the sham-operated group, while colitis was induced in groups 2-4. On the following day, the animals were anaesthetized and surgery was performed to monitor the haemodynamic parameters for 6 h, starting 17 h after the enemas. Group 2 served as untreated control, while in group 3 the animals received 25 mg/kg of KynA (Sigma-Aldrich Inc, St. Louis, Missouri, USA) dissolved in 0.1 M NaOH in a total volume of 1 mL with the pH adjusted to 7.2-7.4. Group 4 was treated iv with 10 mg/kg of SZR-72 in a 1 mL/h infusion for 60 min. SZR-72 was dissolved in 1 mL of saline and the pH was adjusted to 7.2-7.4. The infusion of KynA or SZR-72 started 60 min after the end of surgery and lasted for 60 min. At the end of the examinations, venous blood samples were taken to determine the biochemical changes in the plasma.

In the second part of Study II (series IV), the animals were also allotted to 4 groups (n=6 in each group). Group 5 was the sham-operated group, while colitis was induced in groups 6-8. The haemodynamic measurements were started 6 days following the TNBS enema. The animals in groups 7 and 8 received KynA or SZR-72, respectively. In each group, fluorescence CLSEM was performed, venous blood samples were taken to determine cytokine levels and colon biopsies were obtained for histological analysis.

3.9. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Friedman repeated measures analysis of variance on ranks was applied within groups. Time-dependent differences from the baseline for each group were assessed by Dunn's method. Differences between groups were analysed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures, median values and 75^{th} and 25^{th} percentiles are given; p values < 0.05 were considered statistically significant.

4. RESULTS

4.1. Results of Study I

4.1.1. Estimation of the severity of colitis

The severity of colitis after TNBS induction was estimated between day 1 and day 6 with observations of the stools, and measurement of the body weight of the rats. The colitis was manifested by diarrhoea, the appearance of blood in the stools and loss of weight in the colitis groups, in contrast with the control group. In the first phase (1-3 days) of acute colitis, the animals demonstrated a significant weight loss relative to the baseline (body weight on day 0) and to the control group. A significant weight gain was observed in the control group, and also in the PC-pretreated group, in contrast with the baseline values (Figure 1; Table 1).

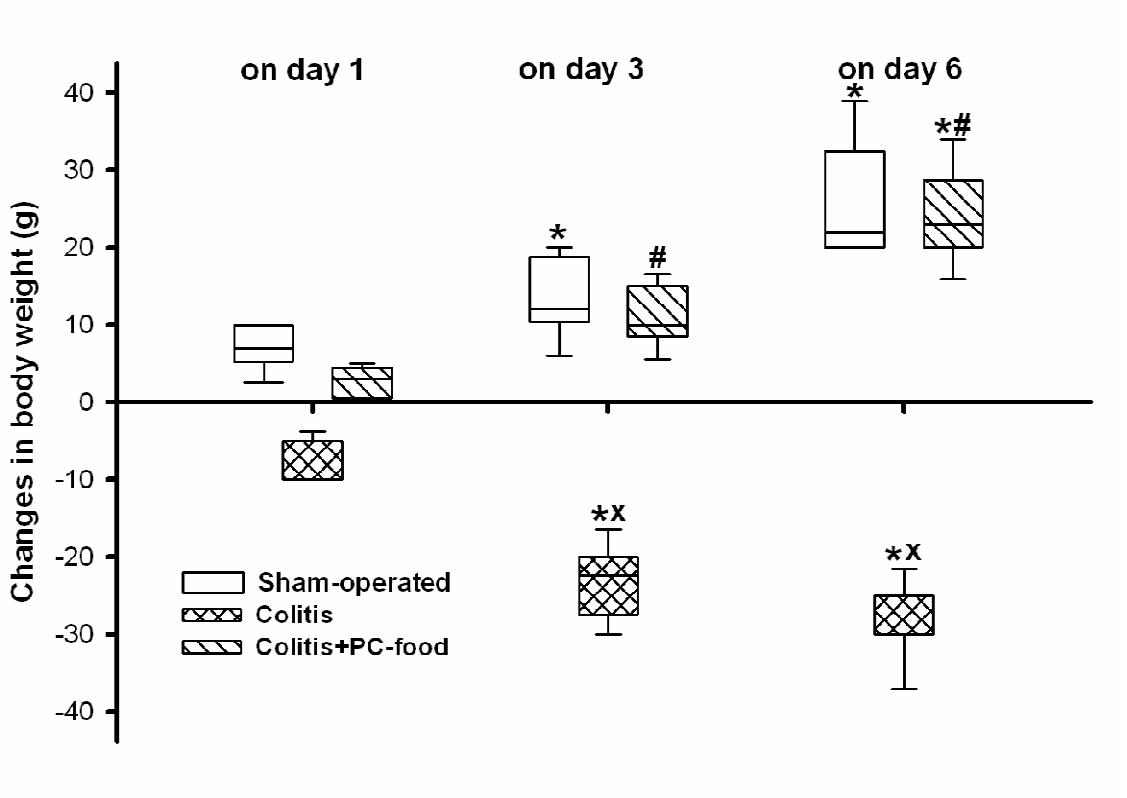

Figure 1. Changes in body weight between days 1 and 6 in the sham-operated (white box), colitis (checked white box) and PC-pretreated colitis (striped white box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. * p<0.05 within groups *vs* baseline values, ^{x} p<0.05 between groups and sham-operated group, $^{\#}$ p<0.05 between PC-pretreated groups and colitis group.

Table 1. Estimation of the severity of colitis

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blood in the faeces - colitis group	+	+	+	-	-	-
Blood in the faeces - control group	-	-	-	-	-	-
Blood in the faeces in colitis+PC group	-/+	-	-	-	-	-
Loss of weight in colitis group	~ 10 g	~ 10 g	~ 5-10 g	~ 5 g	~ 5 g	-
Gain of weight in control group	~ 5 g	~ 5 g	~ 5 g	~ 5 g	~ 5 g	~ 5 g
Gain of weight in colitis+PC group	~ 2 g	~ 2 g	~ 4 g	~ 5 g	~ 5 g	~ 5 g
Diarrhoea in colitis groups	+	+	-	-	-	-
Diarrhoea in control group	-	-	-	-	-	-
Diarrhoea in colitis+PC group	+	-	-	-	-	-

4.1.2. Haemodynamics

There were no significant changes in the haemodynamic parameters as compared with the baseline values in the sham-operated groups during the observation periods. In series I, the MAP was significantly lower in the colitis group than in the sham-operated group. PC feeding normalized this elevation (Figure 2A). CO was significantly higher than in the sham-operated group and the PC feeding did not influence the colitis-induced changes in CO as compared with the colitis group (Figure 2B). There were no significant changes in HR in any of the 3 groups (data not shown). In series II, there were no significant differences between the groups in the MAP, CO (Figure 2A,B) or HR (data not shown) changes 6 days after the vehicle enema or colitis induction.

4.1.3. Changes in plasma TNF-α and plasma IL-6 levels

In series I, the plasma level of TNF-α was significantly increased after colitis induction as compared with the control group. In series II, the plasma level of TNF-α was still significantly increased 6 days after colitis induction as compared with the control group. PC treatment effectively decreased this change, on day 1 and also on day 6 (Figure 3A). The plasma IL-6 concentration in the non-treated colitis group was unchanged on day 1 after colitis, but in the PC-pretreated group an elevated IL-6 level was found. In series II, the IL-6 concentration was significantly increased 6 days after colitis induction relative to the sham-operated group on day 1, to the sham-operated group on day 6 and to the colitis group on day

1. PC pretreatment 6 days after colitis induction significantly reduced the plasma IL-6 level in comparison with the colitis group on day 6 (Figure 3B).

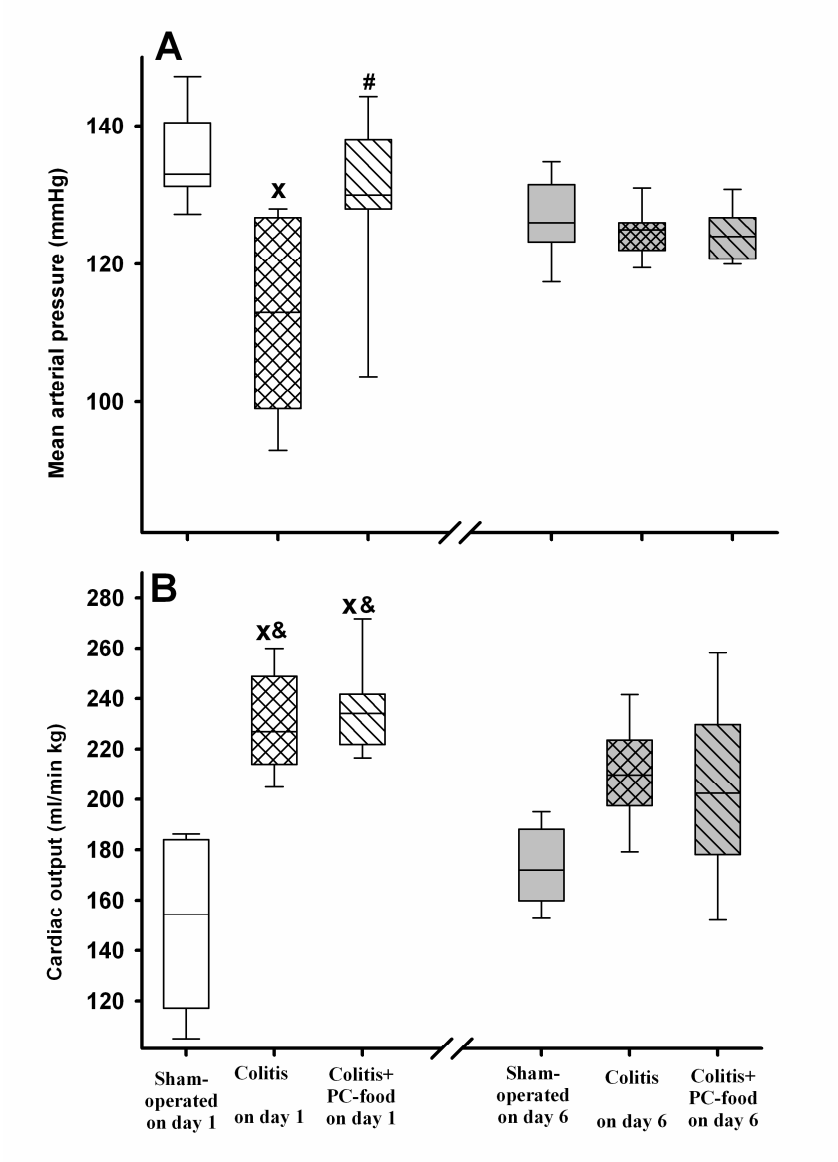

Figure 2. Changes in mean arterial pressure (**A**) and cardiac output (**B**) on day 1 in the sham-operated (white box), colitis (checked white box) and PC-pretreated colitis (striped white box) groups; and on day 6 in the sham-operated (empty grey box), colitis (checked grey box) and PC-pretreated colitis (striped grey box) groups. The plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. ^{x} $p<0.05$ between groups and sham-operated group on day 1, $^{\&}$ $p<0.05$ between groups and sham-operated group on day 6, $^{\#}$ $p<0.05$ between PC-pretreated group and colitis group on day 1.

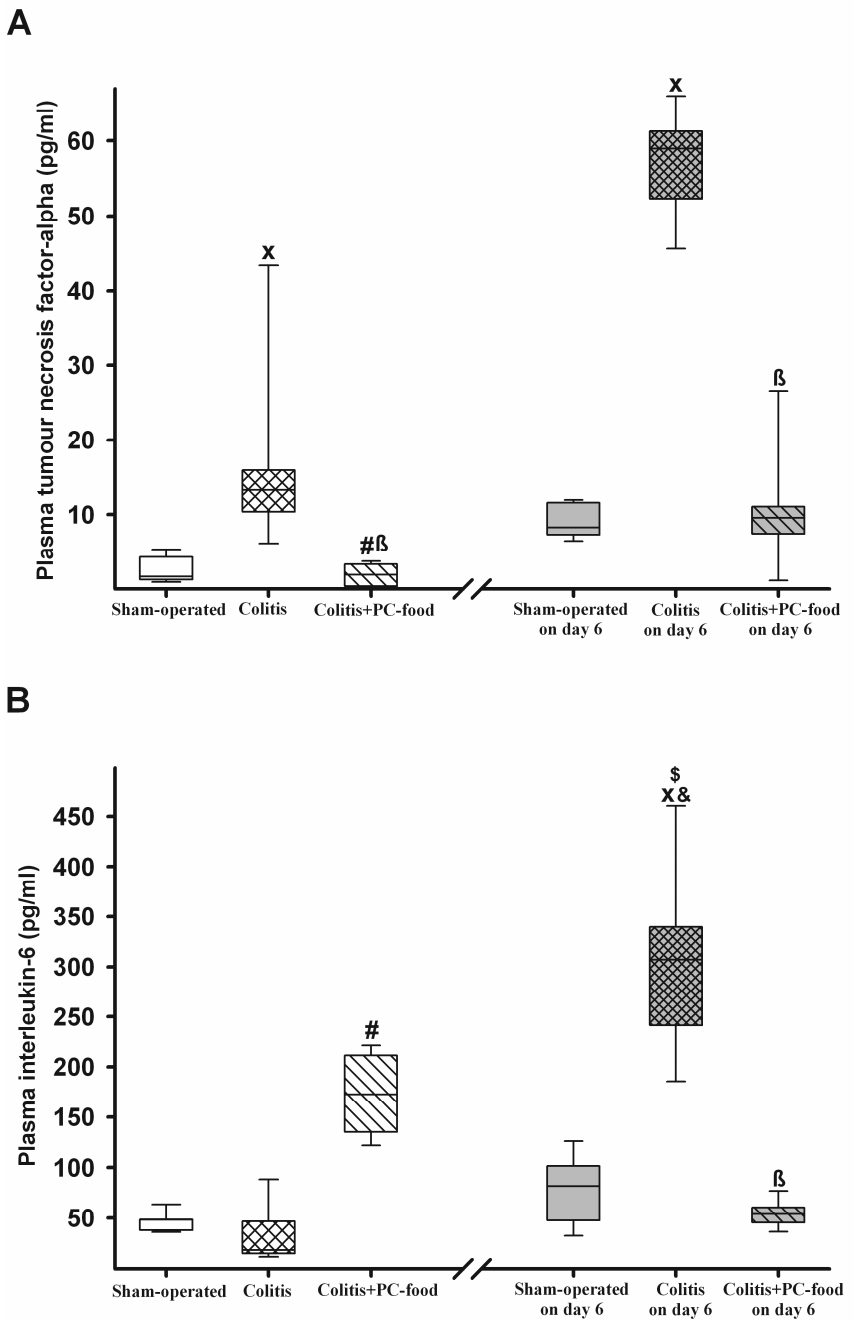

Figure 3. Changes in plasma TNF- α (**A**) and IL-6 (**B**) levels on day 1 in the sham-operated (white box), colitis (checked white box) and PC-pretreated colitis (striped white box) groups; and on day 6 in the sham-operated (empty grey box), colitis (checked grey box) and PC-pretreated colitis (striped grey box) groups. ^{x} $p<0.05$ between groups and sham-operated group on day 1, $^{\&}$ $p<0.05$ between groups and sham-operated group on day 6, $^{\#}$ $p<0.05$ between PC-pretreated groups and colitis group on day 1, ^{ß} $p<0.05$ between PC-pretreated groups and colitis group on day 6, $^{\$}$ $p<0.05$ between colitis group on day 1 and colitis group on day 6.

4.1.4. Leukocyte infiltration

There was no apparent leukocyte infiltration in the control groups, whereas significant leukocyte infiltration was seen in the non-treated colitis groups. The level of leukocyte infiltration decreased significantly in both PC-treated groups (Figure 4).

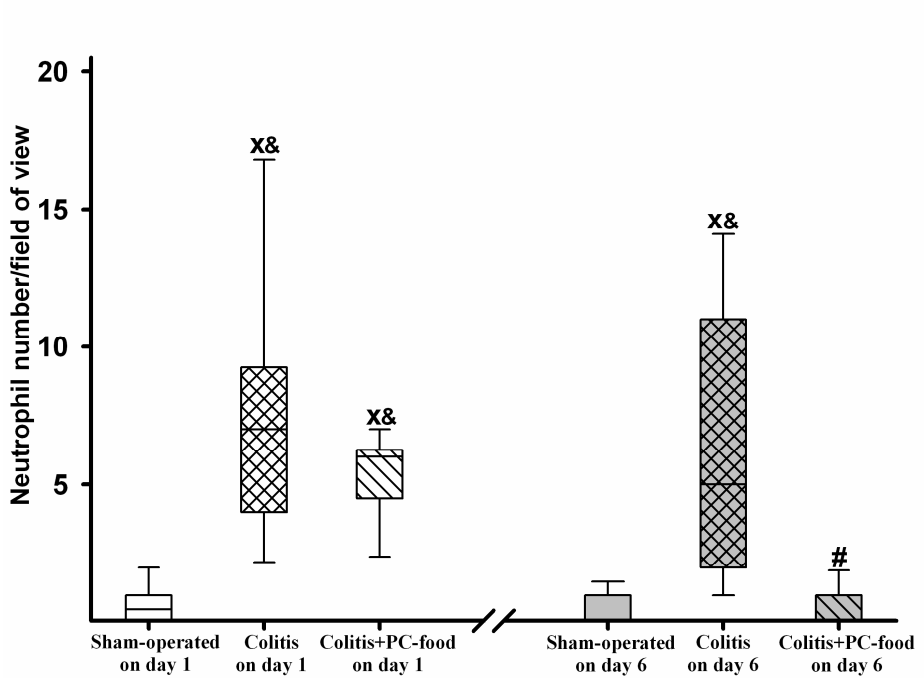

Figure 4. Changes in neutrophil leukocyte number/field of view on day 1 in the sham-operated (white box), colitis (checked white box) and PC-pretreated colitis (striped white box) groups; and on day 6 in the sham-operated (empty grey box), colitis (checked grey box) and PC-pretreated colitis (striped grey box) groups. ^{x} $p<0.05$ between groups and sham-operated group on day 1, $^{\&}$ $p<0.05$ between groups and sham-operated group on day 6, $^{\#}$ $p<0.05$ between PC-pretreated groups and colitis group on day 1, ^{ß} $p<0.05$ between PC-pretreated groups and colitis group on day 6.

4.1.5. Conventional light microscopy

The grading of histopathological sections revealed a normal colonic mucosal structure in the control group on day 1 and on day 6 (Figure 4B). No lack of mucin was observed in the colon sections. Colonic mucosal damage was found in both non-treated colitis groups. The mucosal injury on day 1 of colitis involved diffuse transmural damage. 6 days after colitis induction with TNBS, the damage affected only the lamina propria layer and the injury was focal. In both non-treated colitis groups, mucin depletion was observed. After pretreatment with PC-food, the level of injury was markedly decreased. In both pretreated groups, PC

significantly attenuated the morphological changes in the inflamed colon and mucin covered the mucosa (Figure 5).

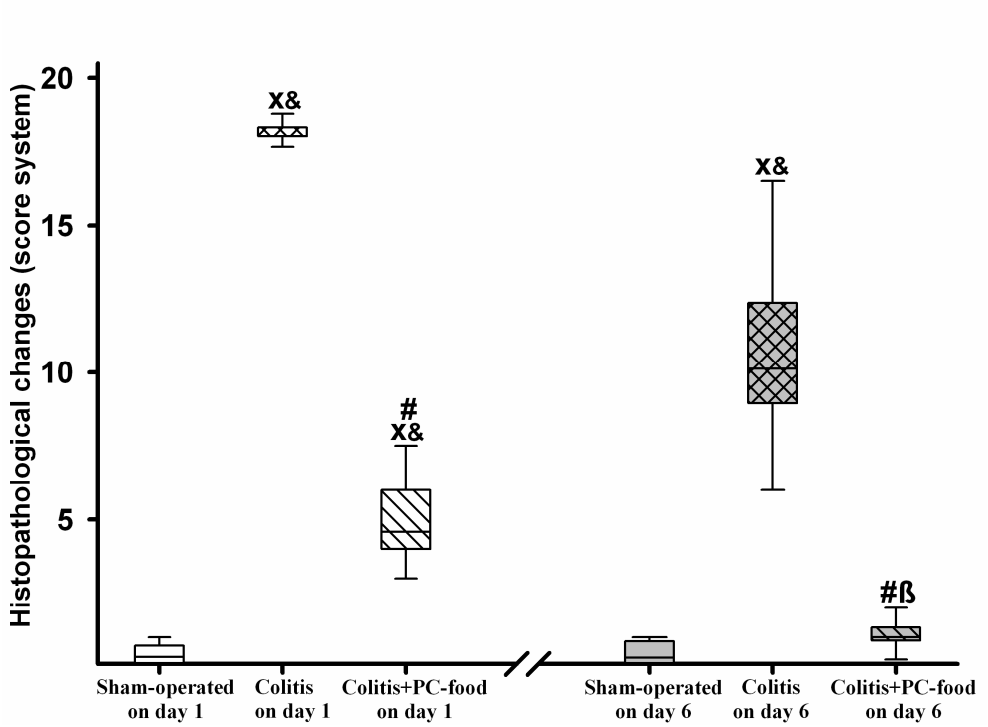

Figure 5. Changes in the grade of histopathological changes on day 1 in the sham-operated (white box), colitis (checked white box) and PC-pretreated colitis (striped white box) groups; and on day 6 in the sham-operated (empty grey box), colitis (checked grey box) and PC-pretreated colitis (striped grey box) groups. ^{x} $p<0.05$ between groups and sham-operated group on day 1, $^{\&}$ $p<0.05$ between groups and sham-operated group on day 6, $^{\#}$ $p<0.05$ between PC-pretreated groups and colitis group on day 1, ^{ß} $p<0.05$ between PC-pretreated groups and colitis group on day 6.

4.1.6. Tissue damage - in vivo detection

The morphology of the colonic mucosa was examined with the aid of acridine orange staining. In the control group, the luminal openings of the crypts were covered with a continuous layer of epithelial cells, which appeared as black holes opening onto the surface of the mucosa (M=0; p25=0; p75=0.83; Figures 6A and 7). In series I, the confocal microscopic evaluation demonstrated significant tissue damage in the acute phase of colitis (M=7.0; p25=7.0; p75=7.8; Figure 6A) in contrast with the control groups. The acridine orange staining of the surface of the glands of the mucosa revealed complete loss of the epithelium (Figures 6B and 7). PC feeding significantly influenced the structural changes in the epithelial morphology of the inflamed colonic mucosa on day 1 of colitis. These changes were still

higher than in the control group, but the degree of injury was decreased (M=4.0; p25=4.0; p75=4.8; Figure 3A), and the loss of epithelium was prevented (Figures 6C and 7).

The tissue damage was also pronounced in the case of subacute colitis, (M=4.3; p25=2.5; p75=5.7; Figures 6D and 7), in contrast with the sham-operated groups. Thinning and loss of the epithelium with denuded crypts were seen on the surface of the glands in each case, and the spaces between the glands were greatly enlarged (Figures 6E and 7). By day 6, PC pretreatment prevented the structural changes in the morphology of the inflamed colonic mucosa. PC feeding protected the mucosa from reduction, thinning and loss of epithelium (Figure 5J). The changes were significantly lower than those in the sham-operated group on day 1 (M=1.3; p25=1.0; p75=2.0; Figures 6F and 7).

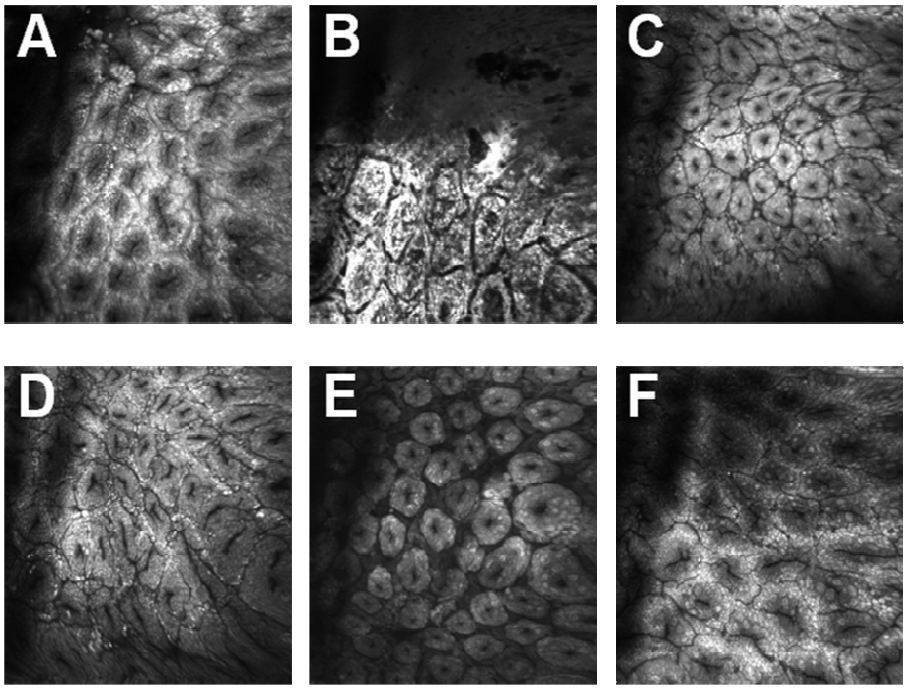

Figure 6. *In vivo* histology images of the mucosal surface of the distal rat colon recorded following the topical administration of acridine orange. **A:** Normal structure of the mucosa; the luminal openings of the crypts appear as black holes opening onto the surface. **B:** Total loss of epithelium on the surface of the glands in the colitis group 1 day after colitis induction. **C:** The spaces between the glands are enlarged in the PC-pretreated colitis group 1 day after colitis induction. **D:** Normal structure of the mucosa; the luminal openings of the crypts appear as black holes opening onto the surface. **E:** Thinning and loss of the epithelium on the surface of the glands; the spaces between the glands are enlarged 6 days after colitis induction. **F:** The PC-pretreated group with many goblet cells. The luminal openings of the

crypts appear as black holes opening onto the surface of the mucosa 6 days after colitis induction.

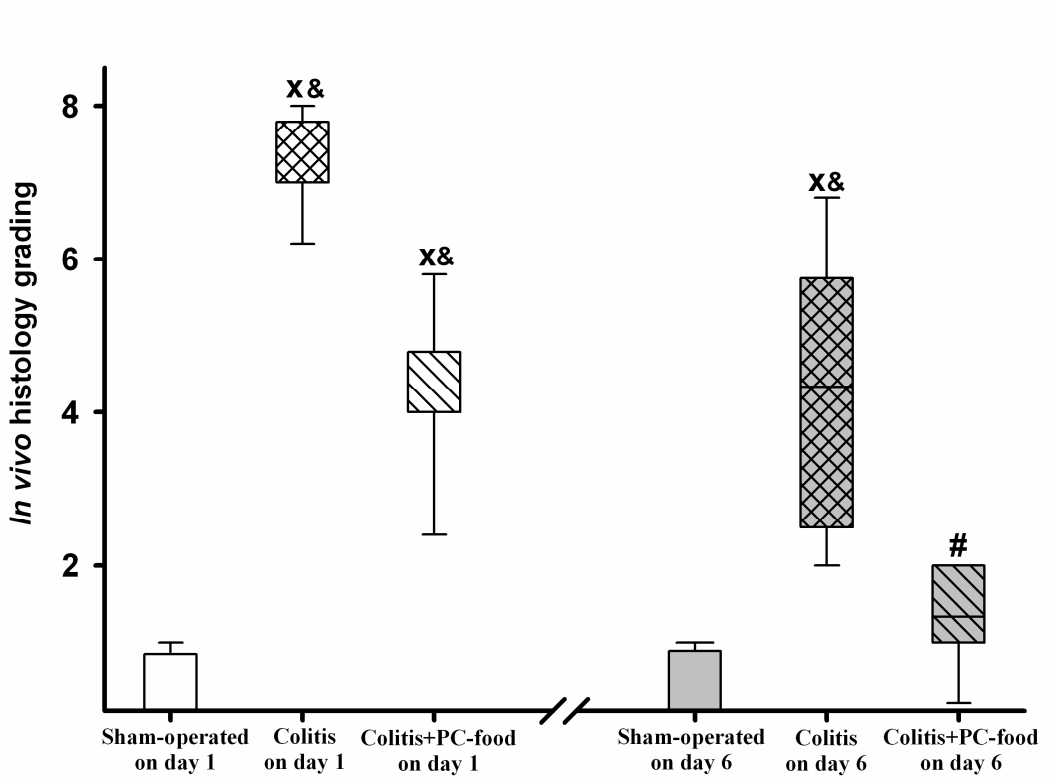

Figure 7. Grading of *in vivo* histology on day 1 in the sham-operated (white box), colitis (checked white box) and PC-pretreated colitis (striped white box) groups; and on day 6 in the sham-operated (empty grey box), colitis (checked grey box) and PC-pretreated colitis (striped grey box) groups. ^{x} $p<0.05$ between groups and sham-operated group on day 1, $^{\&}$ $p<0.05$ between groups and sham-operated group on day 6, $^{\#}$ $p<0.05$ between PC-pretreated groups and colitis group on day 1, $^{\$}$ $p<0.05$ between colitis group on day 1 and colitis group on day 6, $^{\%}$ $p<0.05$ between PC-pretreated group on day 1 and PC-pretreated group on day 6.

4.1.7. Changes in the number of goblet cells

In the control group, the average number of goblet cells was 1.3 (p25=1.2; p75=1.4). In series I, in the acute phase of colitis resulting in severe tissue damage, their number could not be determined. As a result of PC feeding, the number of goblet cells remained at the control level (Figure 8).

In the subacute phase of colitis in the untreated TNBS group, the number of goblet cells was significantly higher relative to day 1 of colitis. In the PC-pretreated animals, the number of goblet cells was significantly increased as compared not only with the sham-operated groups, but also with the PC-pretreated colitis group on day 1 (Figure 8).

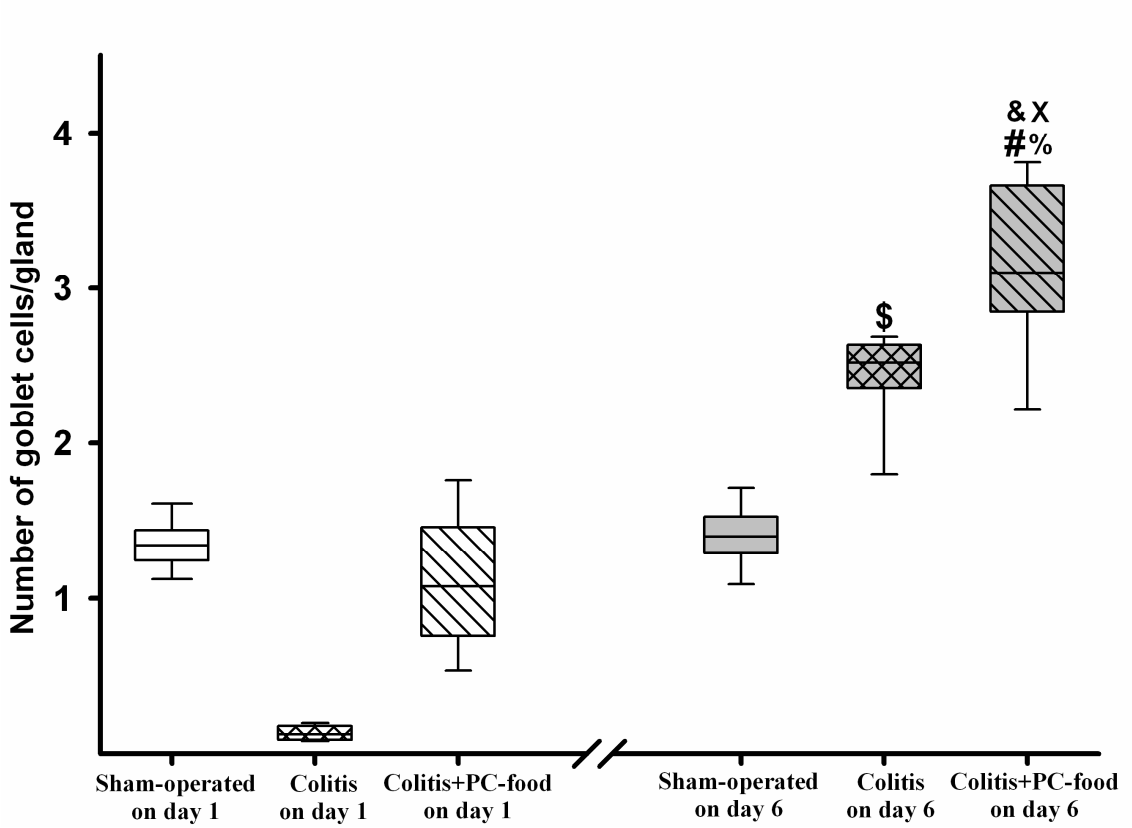

Figure 8. Number of goblet cells on day 1 in the sham-operated (white box), colitis (checked white box) and PC-pretreated colitis (striped white box) groups; and on day 6 in the sham-operated (empty grey box), colitis (checked grey box) and PC-pretreated colitis (striped grey box) groups. ^{x} $p<0.05$ between groups and sham-operated group on day 1, $^{\&}$ $p<0.05$ between groups and sham-operated group on day 6, $^{\#}$ $p<0.05$ between PC-pretreated groups and colitis group on day 1, $^{\$}$ $p<0.05$ between colitis group on day 1 and colitis group on day 6, $^{\%}$ $p<0.05$ between PC-pretreated group on day 1 and PC-pretreated group on day 6.

4.2. Results of Study II

4.2.1. Haemodynamics

In series III, there were no significant changes in the haemodynamic parameters as compared with the baseline values in the sham-operated group during the observation period (17 h) (Table 2). The CO was significantly higher in the colitis groups than in the sham-operated group, while the MAP was significantly lower in the TNBS-treated groups than in the sham-operated group (Table 2). Treatment with KynA did not influence the colitis-induced changes in MAP or CO as compared with the colitis group (Table 2).

Table 2. The effects of colitis and NMDA antagonism on mean arterial pressure (mmHg) and cardiac output index (mL/min/kg) on day 1 day after TNBS colitis induction.

Series III	Hours after colitis induction	17 h	19 h	21 h	23 h
		Mean arterial pressure			
Sham-operated	**Median**	**134**	**129**	**129**	**126**
	25p; 75p	*131; 137*	*128; 134*	*128; 131*	*125; 129*
Colitis	**Median**	**112 x**	**113 x**	**108 x**	**106 x**
	25p; 75p	*98; 117*	*98; 123*	*91; 119*	*98;119*
Colitis + KynA	**Median**	**119 x**	**118**	**113 x**	**110 x**
	25p; 75p	*110; 122*	*106; 123*	*105; 125*	*106; 116*
Colitis + SZR-72	**Median**	**116**	**113**	**115 x**	**111 x**
	25p; 75p	*103; 125*	*107; 124*	*109; 122*	*105; 114*
		Cardiac output index			
Sham-operated	**Median**	**153.8**	**147.2**	**159,6**	**164.8**
	25p; 75p	*120.2; 188.2*	*120.2; 185.2*	*128.1; 178.9*	*132.5; 188.2*
Colitis	**Median**	**215.2 x**	**224.4 x**	**225.8 x**	**234.5**
	25p; 75p	*197.4; 244*	*210.6; 274.8*	*196.1; 248.6*	*208.1; 255.1*
Colitis + KynA	**Median**	**204.9**	**196.5**	**221.7**	**238.6**
	25p; 75p	*183.8; 219.7*	*184.5; 240.8*	*201.1; 244.1*	*225.9; 244.9*
Colitis + SZR-72	**Median**	**215.3**	**185**	**192.8**	**193.2**
	25p; 75p	*183.2; 226.6*	*173.1; 202.8*	*180.5; 203.7*	*180.6; 223.4*

x $p < 0.05$ between groups *vs* sham-operated group

In series IV, 6 days after colitis induction there were no significant between-group differences in CO or HR under the baseline conditions. The administration of the NMDA receptor inhibitors did not influence these haemodynamic parameters (Table 3).

4.2.2. Changes in plasma TNF-α and IL-6 levels

The TNBS colitis group manifested moderate, but statistically not significant increases in the plasma levels of TNF-α at the end of the observation period. The TNF-α concentrations in the NMDA antagonist-treated groups did not differ significantly from those in the colitis group (Annex I, Table 2).

In series IV, 6 days after colitis induction, the plasma level of TNF-α was significantly increased as compared with the control group. KynA treatment significantly reduced the plasma TNF-α level, as did SZR-72 treatment, but less effectively (Figure 9A).

Six days after colitis induction, the IL-6 concentrations were significantly increased relative to the sham-operated group. NMDA antagonist treatment significantly reduced this change (Figure 9B).

Table 3. The effects of colitis and NMDA antagonism on mean arterial pressure (mmHg) and cardiac output (mL/min/kg) on day 6 after TNBS colitis induction.

Series IV	Hours after colitis induction	144 h	146 h	148 h	149 h
		Mean arterial pressure			
Control	**Median**	**121**	**123**	**118**	**122**
	25p; 75p	*117; 128*	*108; 130*	*113; 129*	*119; 134*
Colitis	**Median**	**123**	**120**	**118**	**116**
	25p; 75p	*107; 123*	*107; 123*	*106; 126*	*110;121*
Colitis + KynA	**Median**	**129**	**127**	**121**	**119**
	25p; 75p	*123; 130*	*120; 130*	*115; 127*	*117; 124*
Colitis + SZR-72	**Median**	**123**	**118**	**124**	**120**
	25p; 75p	*122; 126*	*116; 126*	*121; 129*	*115; 123*
		Cardiac output index			
Control	**Median**	**190**	**194**	**177**	**167**
	25p; 75p	*164; 212*	*170; 223*	*160; 217*	*158; 183*
Colitis	**Median**	**221**	**226**	**213**	**201**
	25p; 75p	*213; 229*	*197; 246*	*193; 223*	*140; 262*
Colitis + KynA	**Median**	**205**	**211**	**222**	**190**
	25p; 75p	*186; 232*	*183; 236*	*210; 232*	*176; 203*
Colitis + SZR-72	**Median**	**220**	**218**	**192**	**220**
	25p; 75p	*143; 269*	*160; 287*	*151; 237*	*190; 255*

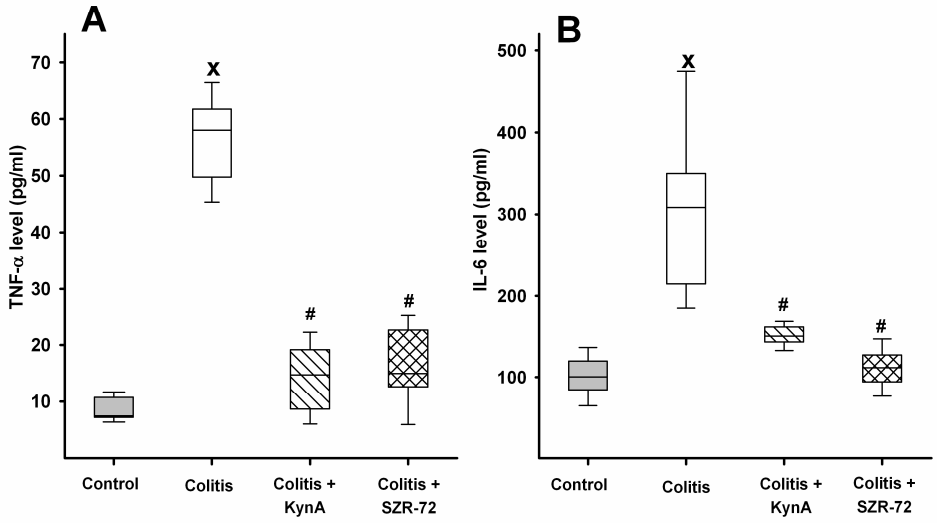

Figure 9. Changes in plasma TNF-α levels (A) and IL-6 levels in the sham-operated (shaded box), colitis (empty box), KynA-treated colitis (striped box) and SZR-72-treated (checked

box) groups on day 6. [x] $p<0.05$ between groups and sham-operated group values, [#] $p<0.05$ between colitis+NMDA antagonist-treated group and colitis group values.

4.2.3. Leukocyte infiltration

In the subacute phase of colitis, leukocyte infiltrations were not seen in the control group, but were consistently present in the samples from the non-treated colitis group. The extent of leukocyte infiltration was significantly decreased in both NMDA antagonist-treated groups (Figure 10).

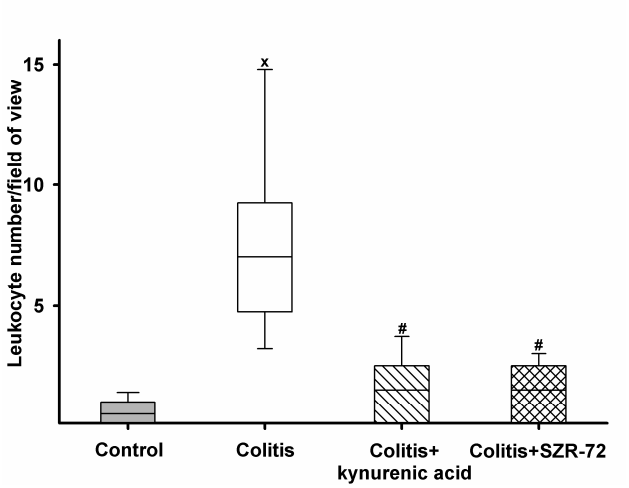

Fig.10. Changes in leukocyte number/field of view in the sham-operated (shaded box), colitis (empty box), KynA-treated colitis (striped box) and SZR-72-treated (checked box) groups. [x]$p<0.05$ for the difference between the colitis group and the sham-operated group values, [#]$p<0.05$ for the differences between the NMDA antagonist-treated group and the colitis group values.

4.2.4. Conventional light microscopy

Six days after colitis induction, a normal colonic mucosal structure was seen in the control group, whereas focal mucosal damage was present in each colitis group, affecting the lamina propria. After treatment with KynA or SZR-72, the level of injury did not differ markedly between the groups and the NMDA antagonist treatment did not influence the grade of morphological changes of the inflamed colon (Figures 11 and 12).

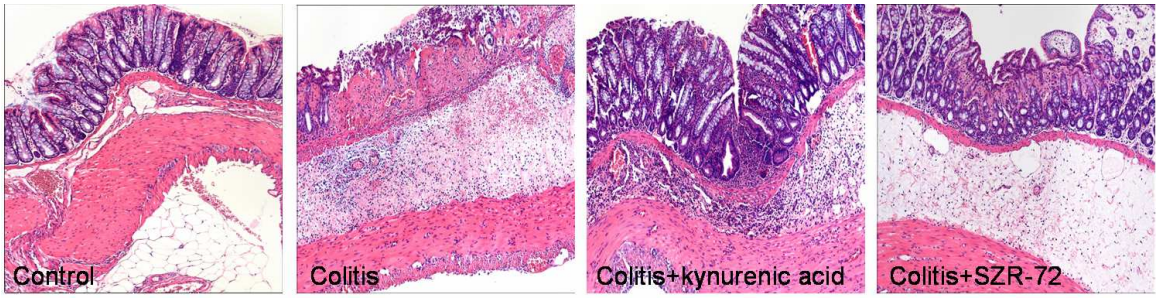

Figure 11: The colonic mucosa was normal, with no apparent leukocyte infiltration in the control group. In the colitis group, colonic mucosal damage was found, with a large number of leukocyte cells infiltrating into the mucosa or submucosa. Colonic mucosal damage was found with moderate leukocyte infiltration in both NMDA antagonist-treated groups.

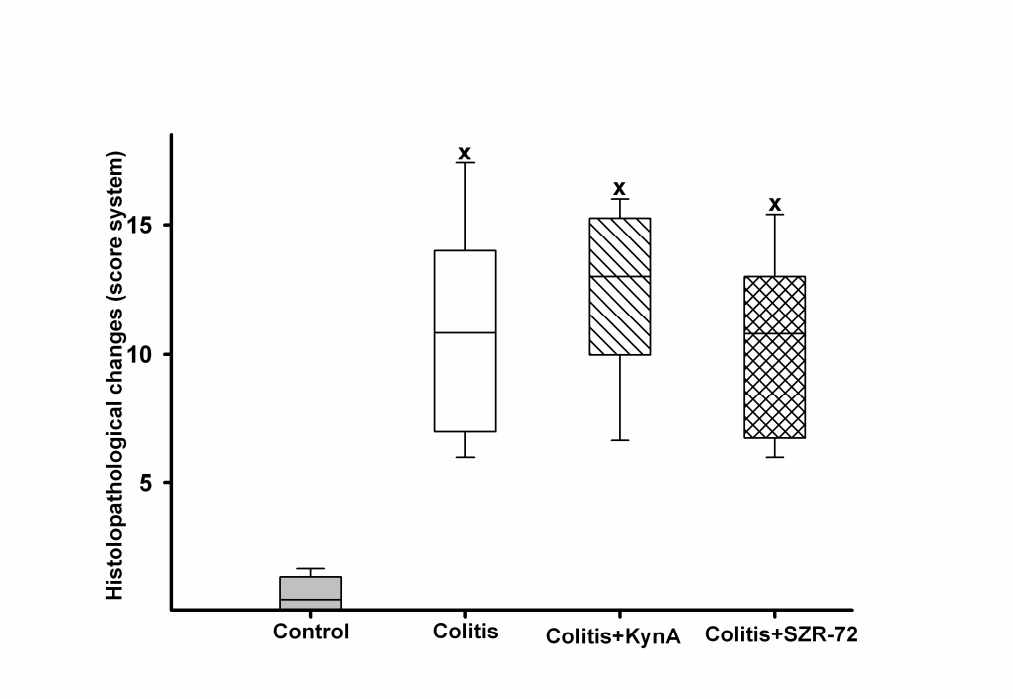

Figure 12. Histopathological changes in the sham-operated (shaded box), colitis (empty box), KynA-treated colitis (striped box) and SZR-72-treated (checked box) groups. $^{x}p<0.05$ between groups *vs* sham-operated group values.

4.2.5. In vivo microscopy

The morphology of colonic mucosa was examined by *in vivo* acridine orange staining. In the control group, the luminal openings of the crypts were covered with a continuous layer of epithelial cells, which appeared as black holes on the surface of the mucosa (median (M) score 0; p25=0; p75=0.8; Figure 13A). Six days after the TNBS enema, the confocal microscopic evaluation demonstrated the development of significant tissue damage (M=4.33;

p25=2.5; p75=5.75). Reduction, thinning and complete loss of the epithelium (Figure 13B) were observed on the surface of the glands in each case, and the spaces between the glands were greatly enlarged. After treatment with KynA or SZR-72, the level of injury did not differ markedly between the groups and the NMDA antagonist treatment did not influence the structural changes in the morphology of the inflamed colonic mucosa significantly (KynA treatment: M=4.1; p25=2.5; p75=5.0; SZR-72 treatment: M=3.83; p25=2.25; p75=4.75; Figure 13C,D).

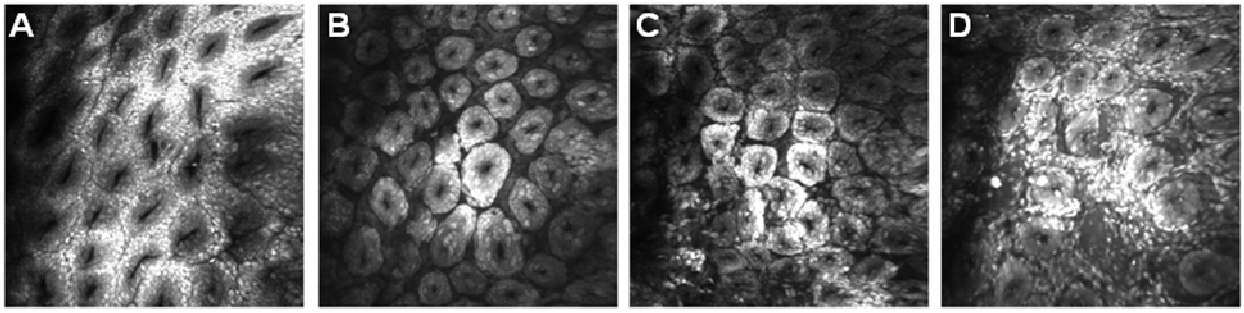

Figure 13. *In vivo* histology images of the mucosal surface of the distal rat colon, recorded following the topical administration of acridine orange. **A:** Normal structure of the colonic mucosa. The luminal openings of the crypts appear as black circles on the surface of the mucosa. **B:** Six days after colitis induction, thinning and loss of the epithelium are observed on the surface of the glands, and the spaces between the glands are enlarged. **C:** Six days after colitis induction, thinning and loss of the epithelium are observed on the surface of the glands, and the spaces between the glands are enlarged in the KynA-treated group. **D:** Six days after colitis induction, thinning and loss of the epithelium are observed on the surface of the glands, and the spaces between the glands are enlarged in the SZR-72-treated colitis group.

4.2.6. Changes in the number of goblet cells

In the subacute phase of colitis in the untreated TNBS group, the number of goblet cells was significantly higher relative to the sham-operated group. KynA treatment did not have any effect on the number of goblet cells; it remained as high as in the colitis group (data not shown).

5. DISCUSSION

The results of our experiments demonstrated the significant inflammatory and morphological responses during the acute and subacute phases of TNBS-induced experimental colitis, and the efficacies of pretreatment with oral PC and treatment with the NMDA receptor antagonist KynA and its synthetic analogue SZR-72 in this model.

5.1. Characterization of acute and subacute phases of TNBS- induced colitis

The onset of TNBS-caused colonic inflammation shares many similarities with fulminant episodes of human IBD. The TNBS enema causes extensive, long-lasting transmural colitis in the rat. The model mimics well not only the symptoms of IBD (e.g. diarrhoea, bloody stools and loss in body weight) (Morris 1989), but also the changes in inflammatory mediator levels (Neurath 1997), and morphological alterations (e.g. local tissue damage, necrosis and colonic leukocyte infiltration) (Tatsumi 1996, Kiss 1997). Typical macro- and microcirculatory changes develop, with mucosal leukocyte recruitment and extravasation. Different degrees of morphological impairment evolve, which are time-dependent. Indeed, the inflammatory or morphological changes in the acute and subacute stages are not directly relevant to later-phase events (Vermeulen 2011).

In our studies, the intracolonic TNBS administration induced manifest colitis with a hyperdynamic macrocirculatory reaction in the acute phase, and increased cytokine levels, with PMN leukocyte accumulation in the proximal colon. The extent of tissue damage was well characterized by conventional and *in vivo* histology: a loss of epithelium, denudation of crypts, and mucin depletion were seen. Most importantly, the number of goblet cells decreased to an extreme low level.

The subacute phase of colitis is characterized by increased plasma levels of the proinflammatory cytokines TNF-α and IL-6 (Hove 2001), with normal systemic haemodynamics, and marked leukocyte infiltration in the intestinal tissue. The results of light microscopic and *in vivo* histological analyses revealed that the affected mucosa is still far from being repaired (McLaren 2002). Our results fit in well with these observations; still elevated cytokine levels, normalized haemodynamics, a huge number of extravasated neutrophils, and as a consequence severe tissue damage was observed. However, the number of goblet cells was increased as compared with that in the acute phase, which may be a sign of a regenerator mechanism.

5.2. Significance of PC therapy in experimental colitis

In Study I, we examined the potentially preventive dietary effects of a PC regimen on the haemodynamic, inflammatory and *in vivo* morphological changes in the acute and resolving phases of TNBS-induced colitis. The results demonstrated that a PC-enriched diet reduced the signs of colitis-induced local inflammatory activation, and prevented the epithelial damage to the colonic mucosa. These beneficial changes were already evident after 1 day, and also 6 days after the inflammatory challenge. Our data lend support to previous findings and provide new evidence of the beneficial effects of PC supplementation in the GI tract (Lichtenberger 1995, Stremmel 2005, Erős 2006). The protective effects of phospholipids in the colon were first outlined after intraluminal application in acetic acid-induced murine colitis (Fabia 1992). Later, polyunsaturated PC mixtures were successfully used in TNBS-provoked colitis (Tatsumi 1996) and in a double-blind, randomized, placebo-controlled human study. The majority of chronically active IBD patients treated for 3 months with delayed-release PC without concomitant steroid treatment achieved clinical remission or exhibited an improvement of the clinical activity (Stremmel 2005).

The mucosal erosions, the morphological damage caused by TNBS-induced colitis, and especially the cellular mechanisms of epithelial cell destruction and repair can be conveniently examined (Grisham 2008). The recent development of a fiberoptic confocal endomicroscope allows potential applications for the non-invasive monitoring of dynamic processes *in vivo* (McLaren 2002) and offers a possibility for acquiring more precise *in vivo* data for histological analysis.

Indeed, intravital CLSEM revealed the lack of epithelium and denudation of the crypts in the early phase, and the morphological damage was still pronounced in the subacute phase of colitis; a thinned epithelium and enlarged interglandular spaces were present by day 6. The *in vivo* and real-time histology data revealed the time-dependent differences in epithelial damage and regeneration of the damaged colonic mucosa. By means of this approach, the cellular and subcellular structures of the colonic epithelium (surface epithelium and crypts), connective tissue and vasculature could be examined. Conventional histological analysis confirmed our findings, and demonstrated the significant leukocyte infiltration of the colon in colitis. PC therapy was successful in decreasing the infiltration of the intestinal wall, which contributes to less morphological damage.

Although the increased PC input clearly conferred protection against excessive epithelial damage in the colon, it is not easy to distinguish the local and systemic effects of

the compound. Endogenous PC is an important component of mucosal hydrophobicity and thus contributes to the barrier properties of the epithelium (Ehehalt 2004, 2010, Stremmel 2010). Indeed, it has been shown that the mucus PC concentration and the hydrophobicity of the mucosal surface are significantly reduced after intracolonic TNBS administration (Tatsumi 1996). More importantly, the total PC and lysophosphatidylcholine concentrations may be significantly reduced in the mucus of the rectum, colon and terminal ileum of patients with ulcerative colitis (Ehehalt 2004, Braun 2009). The thinner mucus layer can not separate microorganisms from the mucosa effectively, and thus commensal bacteria will come into direct contact with the epithelial cells (Swidsinski 2002). Since PC comprises the bulk of the phospholipids in the mucus, a selective transport process into the epithelial layer is hypothesized (Stremmel 2010). This theory is supported by the results of rat experiments with radiolabelled PC, which demonstrated translocation into the mucus (Ehehalt 2004, Dial 2008). Our observations relating to the increased number of mucus-producing goblet cells in the PC-pretreated colitis groups underline the significance of the PC-induced recovery process in the resolving phase of colitis. As the mucin network is strongly negatively charged, the PC head group is bound electrostatically to the mucin network, forming a monolayer with the fatty acid chains extending luminally. This establishes a hydrophobic surface on top of a hydrated gel, which prevents the adherence and penetration of bacteria (Lichtenberger 1995).

Other data suggest that the proportion of more saturated PC species increases under inflammatory conditions, as a consequence of the elevated phospholipase A2 activity. Decreased PC secretion or an increased breakdown of PC due to elevated epithelial and/or bacterial (Mauch 1993) phospholipase activity can cause a critically low mucus PC concentration (Swidsinski 2002). On the other hand, a lower adherence of PC to the glycoprotein network due to an altered mucin composition (Einerhand 2002) can also lead to an extremely low mucus PC level and decreased hydrophobicity, especially in the rectum and distal colon (Braun 2009). This will allow bacteria to adhere directly to the epithelial cells, and provoke a mucosal immune response. The non-occurrence of the protective, anti-inflammatory effect of PC (Treede 2007), the bacterial colonization and the activated mucosal immune reaction lead to further deterioration of the mucus.

Another explanation is provided if orally administered PC serves as a slow-release blood choline source and the choline component of PC is able to influence the inflammatory process through the cholinergic anti-inflammatory pathway (Tracey 2007), including interference with the activation of PMN leukocytes. Nevertheless, the mediators formed during the hydrolysis of PC (e.g. betaine and dimethylglycine) may also influence cell-cell

interactions in a favourable manner (Ghyczy 2003). It was recently found that the multistep extravasation cascade of leukocytes (rolling, adhesion and transmigration) was reduced by PC in the post-ischaemic periosteum (Gera 2007). Our present results reveal that PC treatment also decreases the colitis-induced tissue granulocyte accumulation. An elevated PMN accumulation is characteristic of GI inflammation, and the inhibition of PMN leukocyte activation may result in less tissue damage. In this line, PC metabolites with an alcoholic moiety in the molecule inhibit the reactive oxygen species-producing activity of PMN leukocytes (Ghyczy 2008, 2003). PC is readily taken up by phagocytic cells and, accordingly, it may accumulate in inflamed tissues (Miranda 2008). Other *in vitro* data have shown that dipalmitoyl-PC modulates the inflammatory functions of monocytic cells (Tonks 2001) and that a mixture of PC and phosphatidylglycerol inhibits the respiratory burst and superoxide generation of human PMN granulocytes (Chao 1995). Early reports demonstrated that immunization with PC drastically reduces upregulated TNF-α production in parasitaemic mice, in correlation with a shift from a Th1-type to a protective Th2-type immune response (Bordmann 1998). Indeed, evidence of a TNF-alpha-linked mechanism of action for PC was provided by recent *in vitro* anti-TNF-α findings, and specific inhibition of the TLR-4-dependent inflammatory pathway (Treede 2009, Ishikado 2009).

TNF-α, originating from lymphocytes, macrophages or PMNs, can induce the production of reactive oxygen species and thereby amplify and prolong inflammation through the activation of oxidative stress-responsive genes (Neurath 1997). Moreover, TNF-α can induce the production of nuclear factor kappa B and other cytokines, in this way initiating the inflammatory cascade of IBDs (Zhou 2006a, Zhou 2006b). Direct evidence that TNF-α plays a role in the pathogenesis of experimental colitis has been obtained in many animal models, in which blockade of the action of cytokines delayed the onset of or suppressed inflammation and ameliorated colon destruction (Neurath 1995, Ferrante 2007). By interacting with its receptors I and II, TNF-α recruits PMN leukocytes to inflammatory sites, stimulates monocytes and vascular endothelial cells to release cytokines, induces the cascade effects for other cytokines, and finally results in inflammatory lesions in the tissues (Murthy 2002, Myers 2003). IL-6 can also stimulate PMN chemotaxis and is related to the presence of necrosis in the colon.

According to recent reports, TNF-α additionally exerts a direct effect on mucin production, as it induces downregulation of the expression of mucin genes in TNBS colitis. Treatment with a TNF-α neutralizing antibody prevented the depletion of goblet cells and adherent mucin, and through this mechanism the extent of epithelial cell damage was reduced

(Dharmani 2011). Our data further suggest that PC pretreatment protects against elevation of the TNF-α level, and increases the count of mucus-producing goblet cells, facilitating the recovery of a protective mucin layer in both the acute and subacute phases of TNBS-induced colitis.

5.3. The role of NMDA receptors in experimental colitis

In Study II, besides the examination of the inflammatory and morphological changes in TNBS colitis, the study design additionally allowed us to investigate the functional role of the NMDA glutamate receptors. The glutamate receptors are known to be distributed in the intestinal tract, and the NMDA receptor subtype is functional in the myenteric plexus (Wiley 1991). It has also been established that the NMDA receptors play a role in the modulation of the enteric cholinergic function (Liu 1997, Giaroni 2003). More importantly, other *in vivo* data have shown that the expression of the NMDA receptors increases in peripheral inflammatory reactions (Tan 2008) and the receptor upregulation is present on the neurones of the myenteric plexus in TNBS-induced colitis too (Zhou 2006b).

The pathomechanism of IBDs is still largely unmapped, but the available clinical and experimental data suggest that it is associated with an abnormal immune response in the GI tract. More importantly, the recent study by Welters *et al*. (2010) disclosed the relationship between NMDA receptor activation and leukocyte cell lines. It has further been demonstrated that NMDA receptors are expressed not only on neurones, but also on a number of non-neuronal cell types, including endothelial cells and immune-competent cells, and this points to the action of a common regulatory mechanism (Boldyrev 2004, Hinoi 2004, Miglio 2005, Reijerkerk 2010). NMDA receptors are slightly expressed on inactive lymphocytes; the expression is induced only after an appropriate stimulus (Mashkina 2010). Moreover, it has been reported that NMDA receptor expression and a normal function are required for activation of the respiratory burst of PMN granulocytes (Kim-Park 1997). NMDA activation by glutamate can alter the ion balance between the intra- and extracellular spaces through elevation of the intracellular Ca^{2+} level, and the increased Ca^{2+} influx may result in excitotoxicity. It is therefore suggested that a regulatory interplay between the neuronal and cellular immune systems is highly likely in the GI tract through NMDA receptor activation.

In our study, NMDA antagonist treatment significantly reduced the inflammatory response induced by colitis, and decreased the TNF-α and IL-6 levels 6 days after TNBS administration, while in the acute phase of colitis there was no detectable effect of NMDA antagonist therapy on the TNF-α level. The explanation of these results may be the different

temporal expression patterns of the cytokines in experimental colitis: it has been demonstrated that TNF-α induction is time-related and the plasma levels are not usually changed significantly in the early phase of this colitis model (Barbier 1998).

Although conventional histology showed that NMDA antagonist therapy successfully decreased the leukocyte infiltration of the colon, the beneficial protective effect of NMDA antagonist treatment on morphological damage was not seen on *in vivo* CLSEM, as it was following PC pretreatment. The explanation for that may be the longer duration of PC therapy, as the animals received PC before the induction of colitis and also in the acute phase. On the other hand, KynA was given only once, to treat the already evolved inflammation. As the reduction of the plasma TNF-α level is at the focus of the mechanism of both treatments, and anti- TNF-α antibodies can prevent the decrease of mucin producing goblet cells (Dharmani 2011), it may be assumed that the repeated administration of KynA through decreasing the TNF-α level, may result in favourable morphological changes.

In conclusion, the inflammatory process induced by TNBS in the colon was successfully influenced by both PC pretreatment and KynA treatment. In this regard, the reduction of the plasma TNF-α level is at the focus of the mechanism of both treatments. We have presented *in vivo* evidence that the potentially beneficial effects of oral PC supplementation may be linked to the acceleration of the recovery processes of the impaired mucosal barrier. It may additionally be suggested that blockade of the enteric NMDA receptors might provide greater therapeutic efficacy by targeting local and systemic inflammatory changes in the later phases of acute colitis.

6. SUMMARY OF NEW FINDINGS

1. TNBS administration induced clinically relevant, manifest colitis with a hyperdynamic macrocirculatory reaction in the acute phase, increased cytokine levels, and PMN leukocyte accumulation in the proximal colon. In the subacute phase, the haemodynamics was normalized, but the levels of cytokines and the extent of leukocyte infiltration were still elevated.

2. Our *in vivo* histological analysis with CLSEM technique characterized the time-dependent morphological changes in the different phases of TNBS colitis.

3. We have provided evidence that PC pretreatment is capable of preventing the morphological damage to the intestinal wall in both phases of colitis in this model. Through the blockade of pro-inflammatory cytokine production and the inhibition of tissue leukocyte accumulation, PC pretreatment leads to an alleviation of the inflammatory process in both the acute and the subacute phases of colitis. As a consequence of PC pretreatment, the number of goblet cells increases significantly and therefore an enhanced mucus level might contribute to a lower degree of tissue damage in the mucosa of colon.

4. A single treatment with an NMDA antagonist is not capable of overcoming the morphological consequences of TNBS colitis. However, NMDA antagonist treatment successfully attenuated the inflammatory reaction in the later phases of colitis through decrease of the cytokine levels, and consequently the extent of colonic leukocyte infiltration was reduced. Since the patholology is multifactorial, multifunctional therapy is clearly required to address the various pathological aspects of the inflammatory changes in the later phases of colitis.

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9. ANNEX