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H.Z. TOKLU[1], L. KABASAKAL[1], N. IMERYUZ[2], B. KAN[3], C. CELIKEL[4], S. CETINEL[5], O. ORUN[6], M, YUKSEL[7], G.A. DULGER[1]

A STUDY COMPARING THE EFFICACY OF ANTIMICROBIAL AGENTS VERSUS ENZYME (P-GP) INDUCERS IN THE TREATMENT OF 2,4,6 TRINITROBENZENESULFONIC ACID-INDUCED COLITIS IN RATS

[1]Department of Pharmacology, Marmara University School of Pharmacy, Istanbul, Turkey; [2]Department of Gastroenterology, Marmara University School of Medicine, Istanbul, Turkey; [3]Department of Biophysics, Acibadem University School of Medicine, Istanbul, Turkey; [4]Department of Pathology, Marmara University School of Medicine, Istanbul, Turkey; [5]Department of Histology and Embryology, Marmara University School of Medicine, Istanbul, Turkey; [6]Department of Biophysics, Marmara University School of Medicine, Istanbul, Turkey; [7]Marmara University Vocational School of Health Related Sciences, Istanbul, Turkey

The intestinal microflora is an important cofactor in the pathogenesis of intestinal inflammation; and the epithelial cell barrier function is critical in providing protection against the stimulation of mucosal immune system by the microflora. In the present study, therapeutic role of the antibacterial drugs rifampicin and ciprofloxacine were investigated in comparison to spironolactone, an enzyme inducer, in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis of the rats. Drugs were administered for 14 days following induction of colitis. All drug treatments ameliorated the clinical hallmarks of colitis as determined by body weight loss and assessment of diarrhea, colon length, and histology. Oxidative damage and neutrophil infiltration as well as nuclear factor κB (NF-κB) and tumor necrosis factor α (TNF-α) expressions that were increased during colitis, were decreased significantly. Rifampicin and ciprofloxacin were probably effective due to their antibacterial and immunomodulating properties. The multidrug resistence gene (MDR1) and its product p-glycoprotein (P-gp) has been implicated in the pathogenesis of inflammatory bowel disease (IBD). In the present study, findings of the P-gp expression were inconclusive but regarding previous studies, it can be suggested that the beneficial effects of rifampicin and spironolactone may be partly due to their action as a P-gp ligand. Spironolactone has been reported to supress the transcription of proinflamatory cytokines that are considered to be of importance in immunoinflammatory diseases. It is also a powerful pregnane X receptor (PXR) inducer; thus, inhibition of the expression of NF-κB and TNF-α, and amelioration of inflammation by spironolactone suggest that this may have been through the activation of PXR. However, our findings regarding PXR expression were inconclusive. Activation of PXR by spironolactone probably also contributed to the induction of P-gp, resulting in extrusion of noxious substances from the tissue.

Key words: *colitis, ciprofloxacine, rifampicin, nuclear factor -κB, P-glycoprotein, spironolacton*

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory conditions of the gastrointestinal system. Increasing evidence suggests that the intestinal microflora is an important cofactor in the pathogenesis of intestinal inflammation, and that the epithelial cell barrier function is critical to providing protection against the extensive stimulation of the mucosal immune system by the microflora (1, 2). Detoxification and biotransformation of the luminal agents by the cytochrome P450 superfamily (CYP) enzymes as well as efflux of the compounds back to the gut lumen by the ATP binding cassette (ABC) family of transporters is also a function of these cells (3). Disturbance in the integrity of the epithelium leads to the development of mucosal inflammation associated with intestinal disorders such as inflammatory bowel disease (IBD). Although the etiology of IBD remains unknown, there is circumstantial evidence to link IBD to the failure of the mucosal immune system to attenuate immunity to luminal antigens (4).

Intestinal epithelial cells have been shown to express multiple drug resistance (MDR) genes belonging to the ABC family of transporters. It has been reported that the gene product of the multidrug resistance pump plays a critical role in the host-bacterial interactions in the gastrointestinal system and the maintenance of intestinal homeostasis. One of them, the ATP-dependent drug efflux protein, P-glycoprotein (P-gp) encoded by the multidrug resistance gene (*MDR1*) is responsible for the active excretion of a variety of liphophilic cationic drugs as well as other harmful molecules from the intestines (1, 2). Polymorphisms in the human MDR1 gene have been associated with reduced intestinal P-gp expression in patients with ulcerative colitis and Crohn's disease (5, 6).

The role of the P-glycoprotein (P-gp), the ATP-dependent drug efflux protein, encoded by the multidrug resistance gene (*MDR1)* is probably more complex and includes the deference of the bacterial products (7). Panwala *et al*. (2) reported that *mdr 1a-/-* knockout mice are susceptible to developing a severe, spontaneous intestinal inflammation that has a pathology similar to that of human inflammatory bowel disease and that treating *mdr 1a-/-* mice with oral antibiotics can both prevent the progression of the disease and resolve active inflammation. If this is the case, either defective or stimulated function of the efflux pump may alter the course of the disease models. In fact, Iizasa *et al*. (4) have reported that following induction of colitis with DDS (dextran sodium sulfate) in mice, the expressions of *mdr 1a* and PXR were reduced in the large intestine and P-gp function was decreased; this was accompanied with sever inflammation.

Based on a gene expression profiling in patients with UC and CD, Langmann *et al*. (3) have suggested that the disease- and tissue-specific decrease in the expression of detoxifying enzymes and ABC transporters may be explained by a loss of pregnane X receptor (PXR) expression. They also concluded that dysregulation of xenobiotic metabolism and PXR activity in the gut is likely to contribute to the pathophysiology of UC (3). Similar observations were reported by Mencarelli *et al*. (8) and Shah *et al*. (9) in human cell lines and in the mouse model of dextran sulfate sodium (DSS)-induced colitis, respectively. Pregnane X receptor (PXR) is a member of the nuclear receptor family of ligand-activated transcription factors, and is an integral component of the body's defense mechanism involved in the detoxication of xenobiotics. PXR activation regulates the expression of biotransformation (CYP) enzymes and transport proteins involved in the metabolism and elimination of harmful chemicals and organisms from the body (3).

It is also reported that NF-κB target genes are upregulated and small bowel inflammation is significantly increased in mice lacking the steroid X receptor (SXR) ortholog PXR, thereby demonstrating a direct link between SXR and drug-mediated antagonism of NF-κB (10). In fact, a reciprocal inhibition was reported between PXR and NF-κB signaling pathways; PXR activation inhibited the activity of NF-κB and the expression of its target genes. This inhibition was shown to be PXR dependent and potentiated by PXR ligands (10). Thus, PXR or its human version SXR is expressed at high levels in the liver and intestine and is reported to be a master regulator of drug and xenobiotic metabolism, and inflammation in these tissues (8, 11). In fact, rifaximin, an analogue of rifampicin and an activator of human PXR, has been demonstrated to ameliorate the clinical hallmarks of colitis in DDS- and TNBS- colitis of the hPXR (humanized PXR) mice (12).

On the other hand, since the intestinal microflora has been demonstrated to be an important cofactor in the pathogenesis of intestinal inflammation, various probiotics and antibacterial agents have been tried in patients with IBD and were observed to be beneficial (13, 14). Metronidazol, rifaximin and ciprofloxacin are among the antibiotics that are currently used in the treatment of IBD. In both animal models and humans with inflammatory diseases, Lahat *et al*. (15) have demonstrated the immunomodulating effect of ciprofloxacin in addition to its antimicrobial properties. They have suggested that antibiotic agents may act by eradicating or reducing the burden of bacterial immune targets, thus they may be useful in controlling septic complications and may exert an immunomodulatory effect.

The purpose of the present study was to compare the efficacy of antibacterials versus enzyme (P-gp) inducing agents in the treatment of IBD. We chose rifampicin as the antibacterial agent because it is also known to induce transport proteins (16, 17). We aimed to evaluate its antiinflammatory and immunomodulating effects in TNBS induced colitis of the rats in comparison to ciprofloxacin, another antibacterial drug with immunomodulatory effects, and to spironolactone, a diuretic with strong P-gp and PXR inducing activity (18, 19).

MATERIALS AND METHODS

Animals

Male Spraque-Dawley rats (300–350 g) were housed in a room at a constant temperature of 22±2°C, with 12:12-h light/dark cycles, and fed on standard pellet chow and water *ad libitum*. The study was approved by the Marmara University School of Medicine, Animal Care and Use Committee (No: 08.2007.MAR).

Rats were assigned randomly to two different groups, namely, the control group and the colitis group. Each group was divided into 4 treatment subgroups, with 6 rats in each.

Experimental groups

- Control (vehicle) group (Cont) (rectal alcohol (0.50 ml of %50 (v/v) ethanol) + p.o % 0.4 methylcellulose (MC);
- TNBS (2,4,6-trinitrobenzenesulfonic acid) group (colitis group) (T) (rectal TNBS + p.o MC);
- Rifampicin group (R)(rectal saline + p.o. rifampicin);
- TNBS + rifampicin group (treatment group) (TR) (rectal TNBS + p.o. rifampicin);
- Ciprofloxacin group (C) (rectal saline + p.o. ciprofloxacin);
- TNBS + ciprofloxacin (treatment group) (TC) group (rectal TNBS + p.o.ciprofloxacin);
- Spironolactone (S) (rectal saline + p.o. spironolactone);
- TNBS+spironolactone (treatment group) (TS) (rectal TNBS + p.o. spironolactone);

Induction of colitis

The rats were fasted for 18 h before the experiments, with free access to water. Colitis was induced as previously described by Morris *et al*. (20). Rats were lightly anesthetized by ether, and 30 mg of TNBS (Fluka Chemie, Deutchland) in a volume of 0.25 ml, dissolved in 0.50 ml of %50 (v/v) ethanol, was administered into the colon. For this purpose a medical-grade polyurethane cannula with an external diameter of 2.0 mm was inserted intrarectally into the rat so that the tip was 8 cm proximal to the anus. After instilling the TNBS, the cannula was left in place for a few seconds, then gently removed. Rats in the control group received only 0.25 ml of saline intracolonically. Rifampicin (15 mg/kg) (active ingredient kindly supplied by Kocak Pharmaceuticals, Istanbul, Turkey), ciprofloxacin (50 mg/kg) (Cipro tablets, Biofarma Pharmaceuticals, Turkey) or spironolactone (80 mg/kg) (Aldactone tablets, Ali Raif Pharmaceuticals, Turkey) were suspended in % 0.4 MC and initiating 24 h after TNBS administration, were administered to rats in the treatment groups as oral gavage for 14 days; matched control groups received only % 0.4 MC. The rats were checked daily for behaviour, and stool consistency. At the end of the treatment periods (on the 15th day) rats were sacrificed by cervical dislocation. Abdomen was opened, and after macroscopic evaluation of the intestines and the colon, tissue specimens were taken and frozen at –80°C for the measurement of biochemical and histochemical parameters or stored in formol for histological examination. Samples for electron microscopy, electroforesis and Western blot analysis were immediately sent to the Departments of Histology or Biophysics.

Assessment of colitis

Macroscopic inspection of the colon, ileum and jejunum were evaluated as described by Wallace *et al*. (21). For each animal, at postmortem laparotomy, 6 cm of colon, extending proximally from 2 cm above the anal orifice, as well as ileum and jejunum tissues were removed. Tissues were first split longitudinally, slightly cleaned in physiological saline to remove fecal residues, weighed and pinned out onto a card. An independent observer who was blinded to the treatment scored the macroscopic appearance of the intestinal mucosa.

Mucosal injury was evaluated macroscopically also according to Wallace *et al*. (21).

(1) Focal hiperemia, no ulceration;
(2) No ulceration or hyperemia, thickening of the intestinal wall;
(3) Ulceration and inflamation at a single site;
(4) Ulceration and inflamation at two or more sites;
(5) Major damage >1cm,
(6–10) Major damage >2cm

Histopathological evaluation

Formalin-fixed paraffin embedded tissue blocks were prepared from representative tissue samples of jejenum, ileum and colon. Histopathologic evaluation was performed in HE stained tissue sections according to Amedo *et al*. (22). For statistical analysis, grade 1 and 2 were grouped as mild colitis and grade 3–6 were grouped as severe colitis.

Immunohistochemistry

1. Tumor necrosis factor-α immunohistochemistry

Snap-frozen tissue samples were cut into 5 µm thick cryosections and fixed in cold acetone for 10 minutes at room temperature. To destroy endogenous peroxidase activity, blocking serum (goat ABC Staining System, SC-2023; Santa Cruz Biotechnology) was used for 60 minutes at room temperature. The sections were incubated with primary antibody TNF-α (SC-1351; Santa Cruz Biotechnology) in 1/100 concentration for 60 minutes at room temperature and rinsed in PBS. Biotinylated secondary antibody was applied for 30 min, at room temperature; and incubated in avidin-biotin peroxidase for 30 minutes. Diaminobenzidine (DAB) was used as chromogen. Nuclear counterstaining was performed with Mayer's hematoxylin.

2. Necrosis factor-κB immunohistochemistry

IHC was performed on the sequential tissue sections, 4 µm in thickness, prepared from formalin fixed paraffin embedded tissue blocks. Immunohistochemical stains were performed by the streptavidin-biotin peroxidase technique using DAB as the chromogen and run in parallel with known positive and negative controls. Briefly, the sections were dewaxed with xylene, rinsed in graded ethanols, and rehydrated in water before blocking endogenous peroxidase activity with 3% H_2O_2 for 10 minutes. Antigen retrieval was achieved by heating the sections in citrate buffer, pH 6.0, using a commercial microwave oven. The sections were washed with phosphate buffer saline (PBS) and ultra V block (UltraVision Detection System; Lab Vision) was used to prevent nonspesific staining. Then, the primary antibody, NFκB/p65 (Rel A) Ab-1; Lab Vision) was applied according to manufactuerer's instructions. Thereafter, the biotinylated secondary antibody streptavidin peroxidase (UltraVision Detection System; Lab Vision) was applied at room temperature. Counterstain was performed by Mayer's heamatoxylin.

3. Scoring immunohistochemical expression of tumor necrosis factor-α and nuclear factor-κB

Evaluation of IHC staining for TNF-α nd NF-κB were done semiquantitatively according to both the intensity and quantification of the positively stained areas (0–4): 0 = no staining, 1(+) = rare stained cells, 2(+) = small groups of stained cells, 3(+) = prominent staining in large groups of cells, 4(+) = diffuse prominent staining. For statistical analysis, 0 and 1(+) staining were accepted as TNF-α (or NF-κB) negative, and 2(+), 3(+), 4(+) staining as TNF-α (or NF-κB) positive. TNF-α or NF-κB expressing cells were also counted in 2 high power fields (×400). The ratio of TNF-α (or NF-κB) positive cells to the total number of cells counted gave us the percentage of TNF-α (or NF-κB) positivity.

Scanning electron microscopy

For scanning electron microscopic (SEM) examination, tissue samples were fixed for 4 hours at 4°C in a 0.1 M phosphate- buffered 2.5% glutaraldehyde solution (0.1 M, pH 7.2), postfixed in a 1% phosphate-buffered osmium tetroxide solution (0.1 M, pH 7.2), and passed through an increasing alcohol and amyl acetate series. After drying the tissue samples with a Bio-Rad critical point dryer and gold coating with a Bio-Rad SC 502, tissue samples were examined under a Jeol 5200 JSM (Tokyo, Japan) scanning electron microscope.

Western blot analysis

Tissue specimens (0.3–0.5 g) were quickly removed, washed with Krebs buffer and stored at –80°C until the day of use. For immunblot analysis, tissues were minced and homogenized in 2 ml ice-cold homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, with 1 mM PMSF and 0.1 U/ml aprotinin as protease inhibitors) using an Ultra-Turrax homogenizer at 9500 rpm (3×30 s intervals) and then a Potter S homogenizer with 10 strokes at 400 rpm. Part of the homogenate was used for detection of PXR proteins. The remaining homogenate was centrifuged for 10 min at 3000 g, and the supernatant was again centrifuged for 30 min at 15,000 g. The pellets containing the crude membranes were resuspended in 0.2 ml suspension buffer (50 mM mannitol, 50 mM Tris-HCl, pH 7.4, 1 mM PMSF and 0.1 U/ml aprotinin), aliquoted and used for P-gp detection.

Protein concentration was quantified according to Lowry *et al*. (23). 100–250 µg protein was solubilized in electrophoresis sample buffer containing beta-mercaptoethanol and separated on an 8% SDS-PAGE gel. Transfer of proteins to nitrocellulose filter was carried out using the Novex system (Invitrogen). Blots were blocked with TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) contaning 5% nonfat dry milk and 0.05% Tween-20 for 1 hour and washed once with TBST (TBS + % 0.05 Tween-20) at 25°C. Antibodies against mouse P-glycoprotein (Calbiochem), PXR (GeneTex) and beta-actin (Sigma) were diluted with this blocking solution (dilution 1:40 , 1:250 and 1:1000, respectively) and reacted with the membranes overnight at 4°C. The blots were washed three times with TBST and incubated with an alkaline-phosphatase conjugated secondary antibody. Immunoreactive proteins were detected by NBT/BCIP reagents. The intensities of the bands were quantified using the Image J software (NIH).

Malondialdehyde and glutathione assays

Tissue samples were homogenized with ice-cold 150 mM KCl for determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (24). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}, and results are expressed as nmol MDA per gram tissue. Glutathione was determined by the spectrophotometric method which was based on the use of Ellman's reagent (25). Briefly, after centrifugation at 3000 rpm for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 M Na_2HPO_4.2H2O solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg ml^{-1} 1% sodium citrate) was added, and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of 13600 M^{-1} cm $^{-1}$. Results are expressed in mmol GSH per gram tissue.

Myeloperoxidase assay

MPO activity was measured in tissues in a procedure similar to that documented by Hillegas *et al*. (26). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. Absorbance was measured at 460 nm and MPO activity was expressed as unit per gram tissue.

Chemiluminescence (CL) assay

Lucigenin (bis-N-methylacridiniumnitrate) and luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) were obtained from Sigma (St. Louis, MO). Measurements were made at room temperature using Mini Lumat LB 9509 luminometer (EG & G Berthold, Germany). Tissue samples were put into two vials and CL counts were obtained after the addition of luminol (selective for O_2^-) or lucigenin (measures hydroxyl radical, H_2O_2, hypochloride, $ONOO^-$), 0.2 mM l-1 each. All counts were obtained at 15 s intervals for 5 min and corrected for mg of the tissue sample. Data were expressed as area under the curve of relative light units (rlu) (27).

Statistical analysis

All values in the figures and text are expressed as arithmetic mean ±standard error of the mean (S.E.M.).The data were evaluated with Graph Pad Prism Version 4.0 software (GraphPad Software, San Diego; CA; USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Tukey test. In the experiments involving histology or immunohistochemistry data were expressed as median (range) and either Kruskal Wallis test or Chi-square test was used for the comparison of the groups. P values of <0.05 were considered statistically significant.

RESULTS

Two weeks after intracolonic administration of TNBS, rats had anorexia with a marked loss in body weight (p<0.001). Colitis caused severe diarrhea in the majority of animals. There were inflammatory changes in the intestinal tract that were associated with a significant increase of weight/length of the rat colon (p<0.001) (*Table 1*), an indicator of inflammation. Macroscopic inspection of the colon showed a flaccid appearence, loss of normal morphology and evidence of bowel wall thickening, inflammation and ulcers. Lesions in the distal colon were quantified using a macroscopic damage score of Wallace *et al*. (21) (data not shown). In the present study, colon, ileum and jejunum tissues were evaluated, but no apperent damage was observed in the jejunum.

Treatment of TNBS-rats with rifampicin, ciprofloxacin or spironolactone for 14 days after induction of colitis prevented the loss in body weight significantly and also the morphological changes in the intestines (*Table 1*). Treatment with these drugs also attenuated the extent and severity of the colonic injury and reduced the macroscobic damage score.

Biochemical studies

Tissue MDA and GSH levels were determined in order to evaluate the oxidative damage induced by TNBS colitis. As is evident in *Figs. 1a* and *2a* MDA levels were increased significantly (p<0.001) in both tissue (colon and ileum) segments of the TNBS treated rats, indicating that there was lipid peroxidation. Treatment of colitis with rifampicin, ciprofloxacin or spironolactone decreased tissue MDA levels significantly. However, tissue MDA levels after treatment with ciprofloxacin or spironolactone were still significantly high compared to the control levels, and spironolactone appeared to be the least effective in this respect. In parallel to the development of colitis, levels of GSH, a physiologic antioxidant, were decreased significantly in both tissue segments (p<0.001) (*Figs. 1b* and *2b*). Rifampicin and ciprofloxacin treatments increased GSH levels significantly in the ileum and colon (p<0.05) but levels were not back to the control levels. On the other hand, treatment with spironolactone did not have a significant effect on GSH levels in either tissue.

As can be seen from *Figs. 1c* and *2c*, there was a marked increase in MPO activity, an indicator of infiltration of the tissue with neutrophils, in both ileum and colon tissues and this was also a characteristic of the TNBS induced colitis (p<0.001)(*Fig. 2c*). Treatment with rifampicin, ciprofloxacine or spironolactone decreased the polymorphonuclear neutrophil infiltration in both intestinal segments significantly (p<0.001), rifampicin being the most efficient and spironolactone the least.

To demonstrate accumulation of free radicals in the tissues, luminol and lucigenin tests were carried out. Superoxide radical was determined with lucigenin, hydroxyl and hydroperoxide radicals were determined with the help of luminol. Results of these tests demonstrate that as a result of colitis, free radicals were increased significantly in both tissues (p<0.001) (*Figs. 1d*, *1e*, *2d* and *2e*). Treatment of colitis with rifampicin, ciprofloxacin or spironolactone decreased free radicals significantly in both tissues, back to the control levels.

Table 1. Changes in the volume (weight /length) of the colonic tissue of the rats.(C: control; T: colitis; R: rifampicin, 15 mg/kg; Cip: cyprofloxacine, 50 mg/kg; S: spironolactone, 80 mg/kg po). Data are expressed as mean ±S.E.M. ***p <0.001 significantly different from control; $^{++}$p <0.05, $^{++}$p <0.01, $^{+++}$p <0.001 significantly different from colitis.

Group n: 6	Control	R	Cip	S	T	TR	Tcip	TS
Body weight changes (g), 14 days	**10 ±0.68**	**9.7 ±0.71**	**10 ±0.58**	**10 ±0.63**	**–12 ±1.03*****	**–7 ±0.82***,$^{++}$**	**–7.3 ±0.80***,$^{++}$**	**–7.8 ±0.95***,$^{+}$**
Colon weight: length ratio (mg/cm), 14 days	**133 ±9.99**	**133 ±9.88**	**134 ±9.96**	**141 ±6.47**	**278 ±7.38*****	**198 ±16.49**,$^{+++}$**	**200 ±16.04**,$^{+++}$**	**221 ±10.56***,$^{+}$**

All of these results demonstrate that TNBS induced colitis causes significant oxidative damage in the intestinal tissues and this effect can be reversed by treatment with any of these drugs.

Histomorphology

Paraffin blocks stained with hemotoxylin eosine were evaluated morphologically. Wide spread ulcerations were observed all through the intestinal wall (degree 6) in the TNBS colitis group. After 7 days treatment, statistically significant amelioration was observed only in the ciprofloxacin group. In the rifampicin treatment group, 3rd degree damage was observed only in one case where as there were minimal focal inflamation in the lamina propria in 4 cases. In the ciprofloxacin treatment group, there were minimal inflamatory reactions in 4 cases. Complete recovery after drug treatment was achieved in 4 cases in the rifampicin reatment group, 2 cases in the ciprofloxacine group and in all cases of the spironolactone treatment group. There were no significant tissue alterations in any of the control groups. In the ileum tissue samples, increases were observed in focal inflamatory cell counts in 2 of the TNBS colitis group, 2 in the rifampicin control group, 1 in spironolactone control group, 4 in the rifampicin treatment group and 4 in the ciprofloxacin treatment group. Other tissue samples were within the normal values.

Recovery was observed in all of the 14 days treatment groups in comparison to the TNBS colitis group. Severe colitis

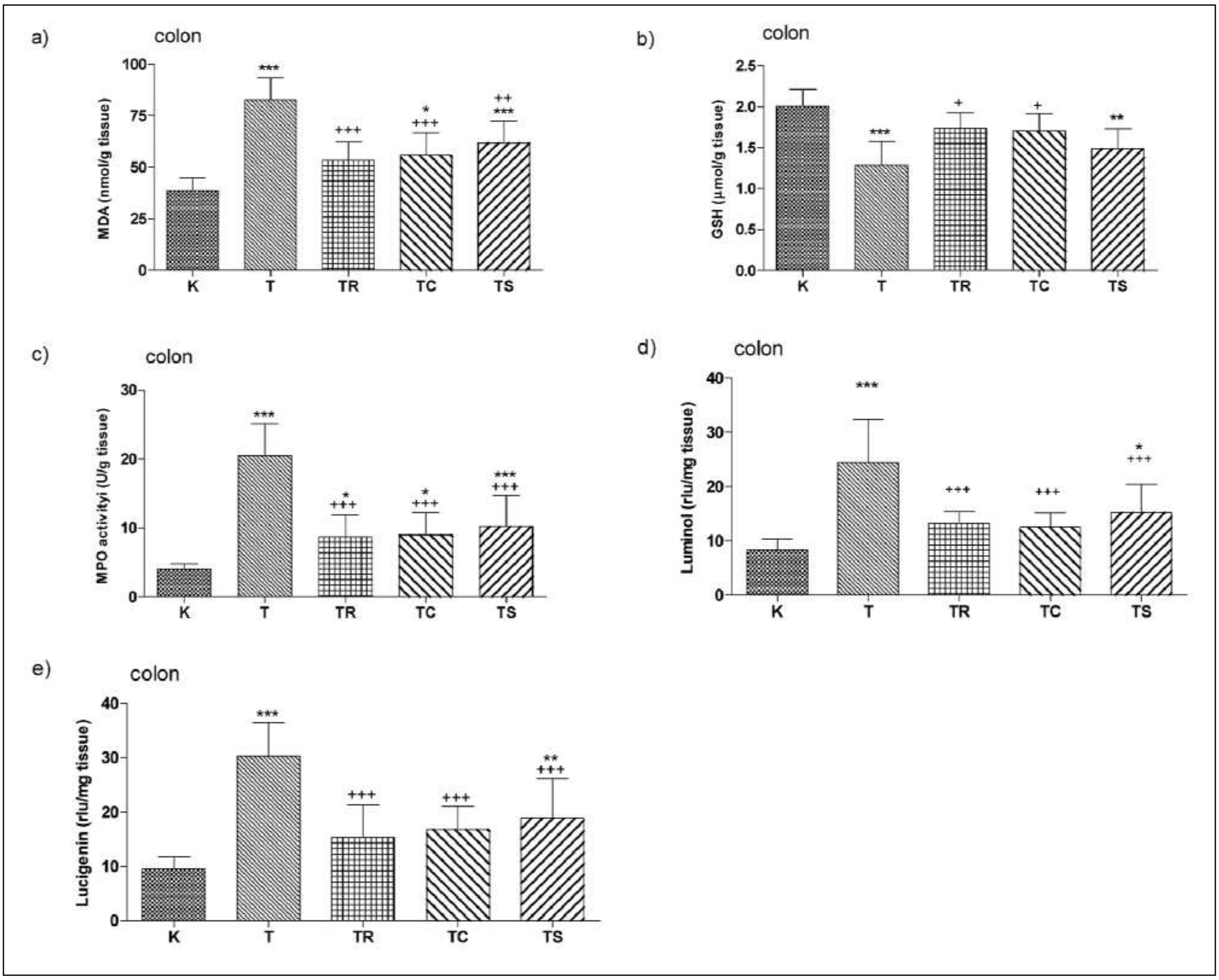

Fig. 1. Oxidative damage in the colon tissue caused by TNBS colitis and the effect of treatment with rifampicine (R), ciprofloxacine (C), or spironolactone (S) on various parameters of oxidative damage and on neutrophil infiltration which is represented by myeloperoxidise levels (MPO) of the colon tissue.

a) MDA level, an indication of lipid peroxidation, were increased in TNBS colitis significantly ($p<0.001$), but this effect was prevented by treatment with rifampicine ($p<0.001$), ciprofloxacin ($p<0.001$) or by spironolactone ($p<0.01$). Rifampicine was the most efficient in this respect.

b) GSH levels that were decreased significantly during colitis were increased after treatment with rifampicine and ciprofloxacin but not after treatment with spironolactone.

c)MPO levels were increased significantly in colitis, but neutrophil infiltration was prevented significantly by all three drugs, but spironolactone was again the least efficient.

d) Luminol and *e*) lucigenin levels that were increased in colitis were also decreased significantly by drug treatment. In all of these measurements, spironolactone appeared to be the least efficient in preventing oxidative damage.

$^{+++}$ ($p<0.001$) *vs*. control (Cont), ***($p<0.001$) *vs*. colitis; $^{++}$ ($p<0.01$) *vs*. control (Cont), **($p<0.01$) *vs*. colitis; $^{+}$ ($p<0.05$) *vs*. control (Cont), *($p<0.05$) *vs*. colitis.

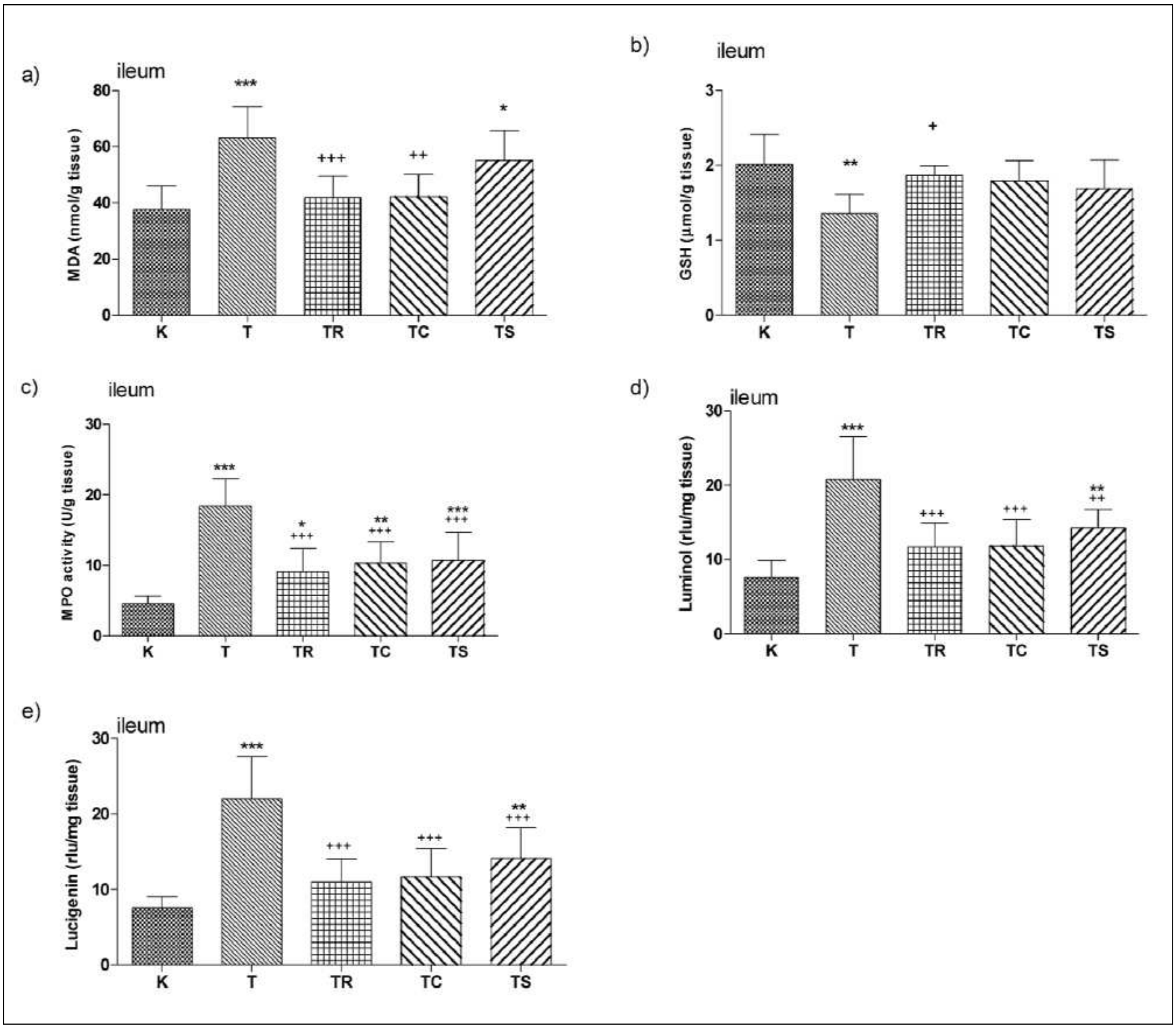

Fig. 2. Oxidative damage in the ileum tissue caused by TNBS colitis and the effect of treatment with rifampicine (R), ciprofloxacin (C) or spironolactone (S) on various parameters of oxidative damage and on neutrophil infiltration which is represented by myeloperoxidise levels (MPO) of the colon tissue.
a) MDA level, an indication of lipid peroxidation, were increased in TNBS colitis significantly ($p<0.001$), but this effect was prevented by treatment with rifampicine ($p<0.001$), or ciprofloxacin ($p<0$). But after spironolactone MDA levels were still higher than the control group.
b) GSH levels that were decreased significantly during colitis were increased after treatment with rifampicine, but not after treatment with ciprofloxacin or spironolactone.
c) MPO levels were increased significantly in colitis, but neutrophil infiltration was prevented significantly by all three drugs. However, MPO levels were still significantly higher than the control.
d) luminol and *e*) lucigenin levels that were increased in colitis were also decreased significantly by drug treatment. In all of these measurements, spironolactone appeared to be the least efficient in preventing oxidative damage.
$^{+++}$ ($p<0.001$) *vs*. control (Cont), ***($p<0.001$) *vs*. colitis; $^{++}$ ($p<0.01$) *vs*. control (Cont), **($p<0.01$) *vs*. colitis; $^{+}$ ($p<0.05$) *vs*. control (Cont), *($p<0.05$) *vs*. colitis.

was observed in 2 cases in the rifampicin treatment group, whereas there was minimal focal inflamation in the lamina propria of 2 cases in the same group. Signs of mild colitis was observed in 4 cases of the ciprofloxacin group. There were no signs of inflammation in control groups. In the ileum tissue samples, increase in focal inflammatory cell count was observed in only 2 cases of TNBS colitis group. Other tissue samples were graded as normal (*Fig. 3*). Histomorphological evaluations of the colon tissues are presented respectively in *Table 2*.

Histochemical studies
1. Immunohistochemical grading

Histochemical evaluations of the colon and ileum tissues are presented in *Table 3* and *Table 4*, respectively; representative histomorphological pictures are given in *Fig. 3*.

In the colon tissue samples, TNF-α immunoexpression was high in the TNBS colitis group (TNF-α score = 4, TNF-α ratio = 47). When compared to the colitis group, there were

Table 2. Histomorphological evaluation of the colon tissue.

COLON 14 days (n=6)	Normal colon	Mild colitis (1-2)	Severe colitis (3-6)	P chi- square†	P chi- square *
Control	6				
TNBS colitis			6		
Rifampicin control	6			Ns	0.0022
Spironolactone control	6			Ns	0.0022
Ciprofloxacine control	6			Ns	0.0022
Rifampicin treatment	2	2	2	0.45	0.0667
Spironolactone treatment	2	3	1		0.1
Ciprofloxacine treatment	2	4		1	0.0022

* in comparison to TNBC colitis, † in comparison to control; normal and mild to medium was evaluated together for chi-square test.

Table 3. Histochemical evaluation in the colon (n=6). Range of scores or percentages for 6 animals are given in parenthesis.

COLON 14 days (n=6)	TNF score	TNF %	NF-κB score	NF-κB values
Control	0.5 (0–1)	1.5 (0–3)	0.5 (0–1)	1 (6–14)
TNBS colitis	2.5 (2–3)	47 (31–55)	3 (2–3)	68.5 (33–78)
Rifampicin control	0.5 (0–1)	3 (0–12)	0 (0–1)	4.5 (2–15)
Spironolactone control	0 (0–1)	0 (0–5)	0 (0–1)	2 (0–7)
Ciprofloxacine control	1 (0–3)	3.5 (0–32)	1 (0–3)	6 (0–13)
Rifampicin treatment	0.5 (0–3)	1.5 (0–17)	1 (0–1)	9.5 (4–13)
Spironolactone treatment	1.5 (0–3)	11 (0–42)	1 (0–3)	13 (0–39)
Ciprofloxacine treatment	1 (0–3)	3.5 (0–32)	1 (1–2)	15 (2–33)
Kruskal Wallis	0.0128	0.0032	0.0002	0.0022

Table 4. Histochemical evaluation in the ileum.

ILEUM 14 days	TNF score	TNF %	NF-κB score	NF-κB values
Control	0	0.5 (0–1)	0 (0–0)	2 (1–3)
TNBS colitis	1 (0–2)	5 (1–27)	0 (0–1)	2 (1–4)
Rifampicin control	0 (0–1)	0 (0–1)	0 (0–1)	2.5 (1–5)
Spironolactone control	0 (0–1)	0 (0–3)	0 (0–1)	2 (0–7)
Ciprofloxacine control	0.5(0–1)	0.5 (0–2)	1.5 (0–3)	2 (0–4)
Rifampicin treatment	0 (0–1)	0 (0–2)	0.5 (0–1)	2 (0–6)
Spironolactone treatment	0 (0–1)	0 (0–6)	0 (0–1)	2 (0–4)
Ciprofloxacine treatment	0.5 (0–1)	1 (0–7)	0 (0–2)	2 (0–7)
Kruskal Wallis	0.29	0.0216	0.3	0.5

significant decreases in TNF-α immunoexpression in all of the treatment groups (*Fig. 4*). NF-κB immunoexpression (NF-κB score = 4) was significantly high in the TNBS colitis group compared to all of the treatment groups (*Fig. 5*).

2. Electron microscopic studies

There was no difference observed among the control rifampicin, spironolactone and ciprofloxacin groups when compared with the morphology of the control group (*Fig. 6a*). Induction of colitis led to devastating changes in the morphology of the tissues. The degeneration was apparent on the 7th day whereas on the 14th day the changes were much more prominent with the finding of severe desquamation of epithelium besides the presence of aggregates erithrocytes (*Fig. 6b*). Treatment with rifampicin, ciprofloxacin or spironolactone displayed a significiant reversal of degeneration in the order of spironolactone, rifampicin and ciprofloxacin (*Figs. 6c-6e*).

3. Western blot analysis

The levels of P-gp and PXR proteins in all treatment groups were determined in comparison to their controls by Western blot analysis. Sample blots illustrating P-gp and β-actin expression are shown in *Fig. 7*. Band intensities were determined using the Image J software (NIH). Signals were normalized with respect to β-actin.

P-gp levels appeared to be decreased after induction of colitis and this effect was reversed after all drug treatments. Rifampicin and spironolactone are known to be enzyme and P-gp inducers and this observation was not unexpected. On the other hand, ciprofloxacin is an antibiotic with no known P-gp inducing effect, and the increase in P-gp levels may be due to the healing of the tissue. Differences between the treatment groups and their respective controls were not significant. However, considering the fact that P-gp levels were decreased during colitis, it can be concluded that all of these treatments brought the P-gp levels back to the control values.

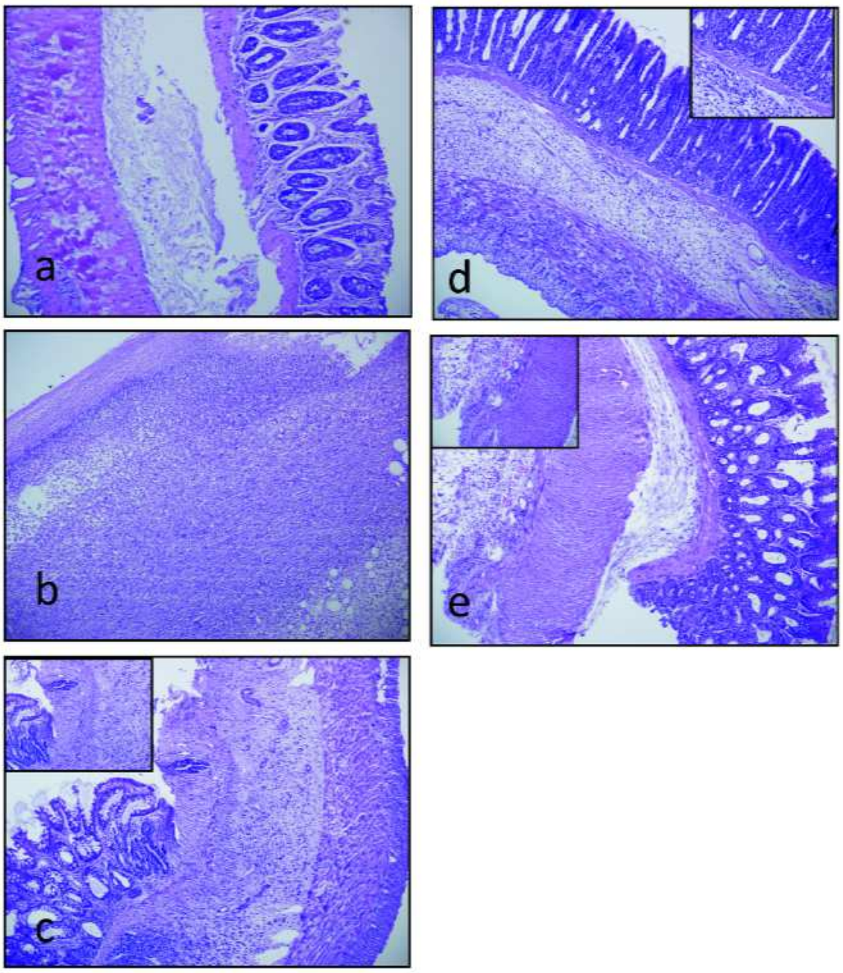

Fig. 3. Histomorphology.
a) The regular appearence of the colon wall (0 degree) H-E × 2000 (paraffin section of distal colon from control rats.);
b) Colon section from TNBS treated rat. Extensive ulceration is evident in all layers of the tissue (grade 6). H-E × 200;
c) Rifampicin treatment group. Focal ulceration scar on the colonic wall with regenerating epithelial cells on the surface, and fibrosis and increased lymphoplasmositer cells on the surface (grade 3) H-E × 200, inset × 400;
d) Ciprofloxacin treatment group. Clonic mucosa is intact, in submocosa there is an incresed level of lymphoplasmositer cells (grade 1) (H-E × 200 (inset × 400);
e) Spironolactone treatment group. There is an increased level of lymphoplasmositer cells in the lamina propria of the basal mucosae and in submucosae, as well (grade 2) (H-E × 200, inset × 400.

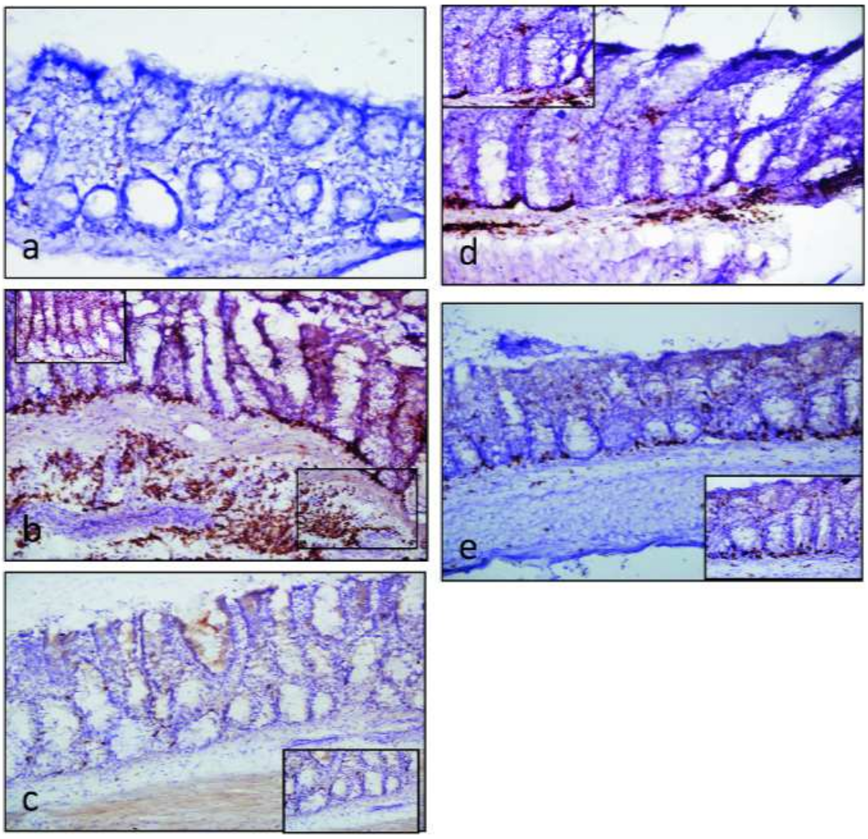

Fig. 4. TNF-α immunohistochemistry.
a) normal colonic mucosa in the control group, score = 0;
b) extensive immunoexpression in the colitis (TNBS) group, score =4;
c) focal immunoexpression in the surface and basal mucosa in TNBS + rifampicin group, score=1;
d) focal immunoexpression in basal mucosa in the TNBS + ciprofloxacin group, score =2;
e) moderate immunostaining in the TNBS + spiranolactone group, score =1.

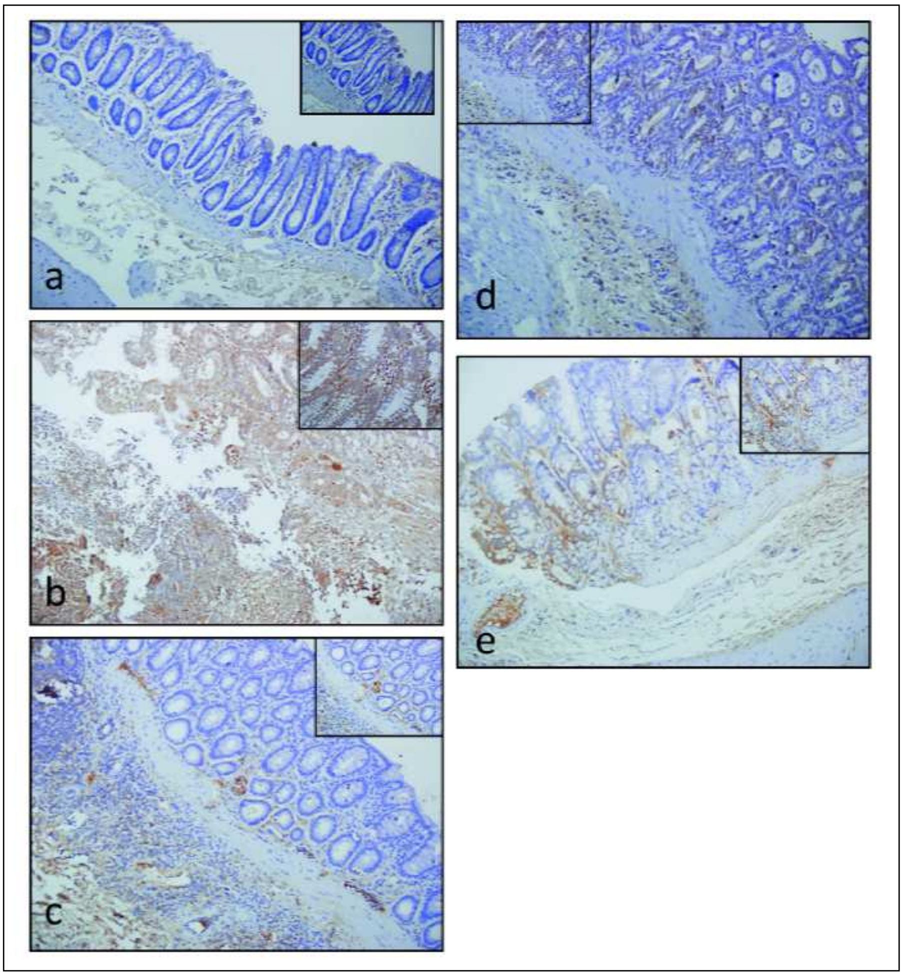

Fig. 5. NFκB immunohistochemistry.
a) NF κB expression in the control group. (NFκB score 0) × 200, inset × 400;
b) TNBS colitis group. Except for the ulcerative areas, a significant NF κB expession is apparent in the colon epithelial cell cytoplasm and inflammatory cells. NFκB (score 4) × 200, inset ×400;
c) Rifampicin treatment group. A weak NFκB immunoexpression (NFκB score 1) × 200, inset × 400;
d) Ciprofloxacine treatment group. A significant NFκB immunuexpression (NFκB score 1) × 200, inset × 400;
e) Spironolactone treatment group. NFκB immunuexpression is decreased compared to the colitis group (NFκB score 2) × 200, inset × 400.

DISCUSSION

Nuclear factor κB (NF-κB) is a key transcription factor of lymphocytes and macrophages with important regulatory functions in the immune system and inflammatory processes (10, 28). These functions are at least partially based on its ability to regulate the promoters of a variety of genes whose products, such as cytokines, adhesion molecules and acute phase proteins, are critical for inflammatory processes. It has been demonstrated that a strong relationship exist between the level of nuclear NF-κB and the positive expression of TNF-α and ICAM-1, as well as mucosal damage indices and MPO activity, a marker of mucosal damage in colitis. In the present study colon tissues obtained from rats with TNBS colitis exhibited significantly more TNF-α and NF-κB expression as well as MPO activity than the control groups, demonstrating the participation of proinflammatory molecules in the development of IBD. MPO activity, TNF-α and NF-κB levels were decreased significantly in all treatment groups.

Rifampicin, an antibacterial drug, is also known to have strong enzyme inducing properties. Cytochrome 450 (CYP) enzymes responsible of drug metabolism and drug transport proteins of ABC casette family, including P-gps are among the enzymes induced by rifampicin (29). Rifampicin was also reported to act as an immunosuppressant to suppress humoral and cellular immunological responses in liver cells, and its immunusuppressant role has been well described in humans (29). In the present study, there may be several mechanisms contributing to its beneficial effects in the treatment of TNBS colitis. First of all, as an antibacterial drug it may have acted by reducing the antigenic exposure of the epithelial and antigen presenting cells. Since it is accepted that the mucosal microflora plays an essential role in initiating and perpetuating the colonic inflammation in colitis, a reduction in the bacterial population may have prevented the extensive stimulation of mucosal immune system by the microflora (1). In fact, Panwala (2) reported that treatment of *mdr1a* knockout (*mdr-1a-)* mice with oral antibiotics can prevent the development of disease and resolve active inflammation. More recent observations indicating that probiotics or antibacterial drugs such as ciprofloxacin, metronidazole or antibiotic combinations are beneficial in the treatment of IBD support this view; these drugs are believed to act by decreasing the antigenic stimulation (13, 30-34).

Zwolinska-Wcislo *et al*. (32) have demonstrated that treatment with the antibiotic ampicillin accelerates the healing of colonic damage impaired by aspirin and celecoxib in TNBS-induced colitis in rats. In the mentioned study, treatment with ampicillin decreased markedly the damage caused by the antiinflammatory agents. It also decreased the cytokine levels as well as the bacterial *E. coli* translocation to the extraintestinal organs. Ghasemmi-Niri *et al*. (33) have studied the effect of treatment with whey-cultured *Lactobacillus casei* in TNBS induced colitis of the rats, and observed that it was very effective in ameliorating the biochemical and histopathological markers of colitis, decreasing lipid peroxidation and MPO activity. It was also observed to decrease TNF-α by modifying intestinal

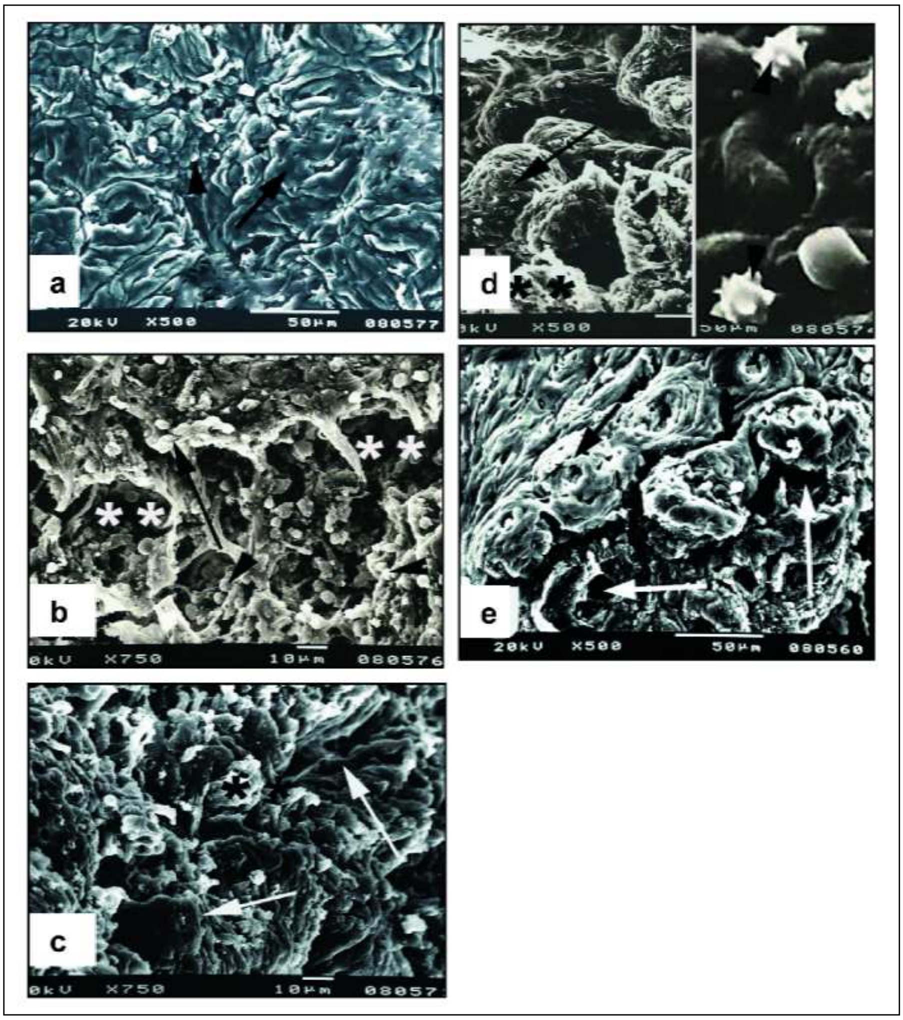

Fig. 6. Electron microscopy.
a) Control group, regular epitelium (arrowhead) and crypts (arrow);
b) 14-day colitis group, severe desquamation of epithelium (arrowhead), nude lamina propria (arrow) and hollow crypts of colonic tissue (**) note the density of erythrocytes;
c) 14-day colitis and rifampicin group, the regenerating epithelium (arrows) and mucus structures (*);
d) 14-day colitis and ciprofloxacin group, the newly formed epithelium (arrow) and mucus structures (**) with the acanthotic erythrocytes (arrowheads-inset);
e) 14-day colitis and spironolactone group, the regenerating epithelium (black arrow) and crypts (white arrows).

antiinflammatory activity. Saksena *et al*. (34) have reported that probiotics like *Lactobacilli* or their soluble factors stimulate P-gp expression and function under normal and inflammatory conditions and thus ameliorate DDS induced colitid of mice. Likewise, considering its enzyme inducing property, rifampicin may have exhibited its beneficial effects by an additional mechanism that involves the induction of P-gps in the epithelial cells, thus increasing the extrusion of bacterial products from the mucosa. Induction of P-gps was reported to be independent of PXR induction in the gut wall (35).

Rifampicin is also a potent ligand for SXR or PXR in humans (10) and recent studies have demonstrated that the activation of the SXR by commonly used drugs (rifampicin) in humans inhibits the activity of NF-κB (36). However, it has been reported that rifampicin is not a ligand for rodent PXR (12, 36), thus, in the present study, it is unlikely for rifampicin to have shown its antiinflammatory effect in rats through induction of PXR.

In the current study, we evaluated the effects of two other drugs, ciprofloxacin and spironolactone. Ciprofloxacin, a fluoroquinolon antibacterial was also very efficient in ameliorating the TNBS colitis. It is used in clinical practice in the treatment of Crohn's disease, and is reported to have beneficial effects (14). It was recently demonstrated that in addition to its antibacterial effect, ciprofloxacin may also have an immunoregulatory effect on intestinal inflammation (37). In another study, antiinflammatory effect of ciprofloxacin was compared to that of seftazidim in TNBS colitis of mice, and it was observed to significantly decrease interleukin-1β, interleukin-8, TNF-α and NF-κB levels, in contrast to seftazidim which had no effect on cytokine levels (15). In the same study, the effect of ciprofloxacin was also compared with dexamethasone and reported that it was acting like an immunomodulator rather than just an antibiotic. Our observations that ciprofloxacin decreases oxidative damage, neutrophil infiltation, TNF-α and NF-κB levels are in accordance with this report, suggesting that it may also have antiinflammatory effects in addition to its antibacterial property. However, the possibility that the decrease in antigenic stimulation has prevented the triggering of the inflammatory cascade cannot be excluded.

Spironolactone is a diuretic with strong enzyme inducing activity. It has been reported to induce intestinal P-gps in rats, thus decreasing the absorption of digoxin (19) and has been demonstrated to activate PXR and to induce CYP enzymes (18). In hepatocyte membranes from spironolactone treated animals, *mrp2* expression was reported to be increased, resulting in choleresis (38) and it has also been demonstrated to induce intestinal Mrp2 transcriptionally, PXR being a potential mediator (39). It is believed that the induction of various CYP 450 enzymes and transport proteins by spironolactone is regulated *via* activation of PXR, farnesoid X receptor (FXR) and constitutive androstane receptor in humans and rodents (18). These findings suggested that spironolactone could be of potential therapeutic application particularly in situations of down-regulation of intestinal *Mrp2* (39). Spironolactone has also

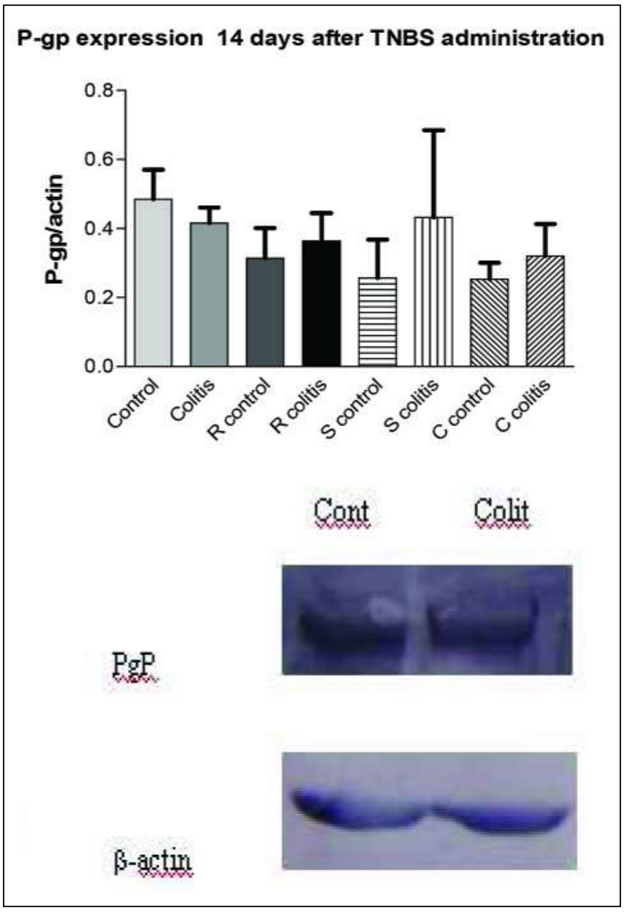

Fig. 7. The levels of P-gp proteins in all treatment groups were determined in comparison to their respective controls by Western blot analysis. Sample blots illustrating P-gp and β-actin expression are shown. Band intensities were determined using the Image J software (NIH). Signals were normalized with respect to β-actin.

been reported to have a supressive effect on the transcription of several proinflamatory cytokines that are considered to be of pathogenic importance in many immunoinflammatory diseases, thus, it was shown to have an antiinflammatory effect in patients with chronic arthritis (40). It was also demonstrated to reduce cytokine production in human peripheral blood mononuclear cells stimulated with angiotensin II (41). In the present study, it decreased the TNF-α and NFκB levels significantly, supporting these previous reports; it might be suggested that it ameliorated the TNBS colitis of the rats by suppressing the transcription of proinflammatory cytokines.

On the other hand, Johnson *et al*. (42) have reported that spironolactone treatment may increase mortality in patients with Crohn's disease and *Clostridium difficile* infection, and also in the rats with TNBS colitis and infected with *Salmonella typhimurium*. These finding are contrary to our observations. It is most probable that in the study of Johnson *et al*. mortality was increased due to the superimposed infection, whereas in the present study there was no specific infection and amelioration of inflamation and oxidative damage due to the induction of colitis was evaluated. Besides, they used a much smaller dose of the drug, 20 mg/kg, and claimed that decreasing the dose increased the survival rate. In the present study however, the dose was 80 mg/kg, which is similar to the dose used in the previous studies that investigated the enzyme inducing effect of spironolactone which was 200 μg/kg b.wt., and this corresponds to about 83 g/kg. Considering the dosage difference, it may be suggested that the high dose of spironolactone used in the present study might have had a stronger inducing effect on PXR and/or suppressive effect on cytokine transcription, thus suppressing the inflamation.

Unfortunately, in the present study results of the Western blot analysis for PXR and P-gp were inconclusive, and it could not be demonstrated that the therapeutic value of spironolactone in the treatment of IBD (and some other inflammatory conditions) that are linked to down regulation of PXR and/or P-gp (43) is due to its PXR activating property. Nevertheless, in view of the previously demonstrated reciprocal inhibition between PXR and NF-κB signaling pathways (10), our observation that the NF-κB and TNF-α levels were significantly decreased suggests that, besides having an immunosuppressive effect, spironolactone may also have been effective through the activation of PXR.

In conclusion, based on our findings, we propose that the antibacterial drugs and enzyme inducing agents like spironolacton are almost equipotent in ameliorating the TNBS colitis in the rats. While rifampicin ameliorated TNBS colitis with its antibacterial properties, most probably it also acted by increasing the expression of P-gps. Ciprofloxacin, the other antibacterial drug, also appears to have a strong immunomodulatory-antiinflammatory effect when used in the treatment of IBD. Spironolactone has been reported to be beneficial in the treatment of colitis for the first time in this study. The significant decrease in TNF-α and NF-κB levels in spironolactone-treated animals suggests that besides having a suppressive effect on cytokine transcription, spironolactone may also have been effective through activation of PXR. Thus, PXR ligands may have a therapeutic value in the treatment of IBD, but further studies are required.

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Author's address: Prof. Dr. Gul Ayanoglu Dulger and Assoc. Prof. Hale Zerrin Toklu, Department of Pharmacology, Marmara University School of Pharmacy, Tibbiye Cad. No: 49 Haydarpasa (34668) Istanbul/ Turkey
E-mail: dulgergul@yahoo.com; haletoklu@yahoo.com