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Pentoxifylline attenuates mucosal damage in an experimental model of rat colitis by modulating tissue biomarkers of inflammation, oxidative stress, and fibrosis

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Background/aim: This study was designed to identify the effect of pentoxifylline on trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats.

Materials and methods: Forty-two female Wistar rats were randomly divided into 7 groups: group A, TNBS + intraperitoneal (IP) pentoxifylline; group B, TNBS + IP saline; group C, TNBS + intrarectal (IR) pentoxifylline; group D, TNBS + IR saline; group E, IP pentoxifylline + TNBS; group F, IP saline + TNBS; group G, IR saline. Pentoxifylline was given daily for 3 days before or 6 days after the induction of colitis. Rats were killed after 6 days.

Results: IP and IR pentoxifylline similarly and significantly reduced damage and histopathological scores. Pentoxifylline attenuated the accumulation of malonyldialdehyde and transforming growth factor β1 and the activities of myeloperoxidase, matrix metalloproteinase-3, and tissue inhibitor of metalloproteinases-1, and it also restored superoxide dismutase activity. The IP route was more effective than the IR route in this regard. Administration of IP pentoxifylline before or after induction did not influence all parameters.

Conclusions: Pentoxifylline showed a therapeutic effect in this experimental colitis model. IP administration seemed to be better. This effect may occur as a result of inhibition of oxidative stress and metalloproteinase activity.

Key words: Fibrosis, inflammatory bowel diseases, matrix metalloproteinases, pentoxifylline, transforming growth factor beta, tumor necrosis factor-alpha

1. Introduction

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the colon and small intestine. Crohn disease (CD) and ulcerative colitis (UC) are the principal types of IBD. There is evidence that IBD is result of a genetic predisposition that leads to a mucosal immune regulatory cell defect and susceptibility to environmental triggers, particularly luminal bacteria, including specific antigens and pathogen-associated molecular patterns. An abnormal relationship is present between innate immune responses to bacterial structures mediated via toll and other receptors and the adaptive immune response (1).

Inflammatory cytokines are important mediators during the development and perpetuation of intestinal inflammation in IBD. Among these cytokines, tumor necrosis factor (TNF)-alpha deserves special attention. Increased concentrations of TNF have been demonstrated in the feces and intestinal biopsies of patients with IBD (2,3). The relationship between TNF-alpha and IBD has been further supported by the fact that anti-TNF agents have been a major advance in the management of acutely ill or corticosteroid-dependent individuals with CD or UC and in individuals with CD with fistulizing disease (4,5). On the other hand, the high cost, risk of side effects, and concern about long-term effects can limit the use of anti-TNF-alpha biological agents in the general IBD population. In this regard, attempts to define other anti-TNF-alpha agents that could be used as potential treatments are still ongoing.

Various therapeutic interventions can inhibit the synthesis or the action of TNF-alpha. Among them, cyclic AMP (cAMP)-elevating agents have been shown to suppress TNF-alpha synthesis in murine macrophages. This observation led to a growing interest in phosphodiesterase inhibitors, which inhibit the degradation of cAMP to 5'-AMP, as candidate molecules

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for the treatment of IBD (6,7). In this study, we assessed the ability of pentoxifylline to attenuate colon damage and inflammation in a trinitrobenzene sulfonic acid (TNBS) rat model, an experimental model of colitis that is characterized by transmural inflammation, ulceration, and fibrosis resembling CD.

2. Materials and methods

2.1. Animals

Forty-two female Wistar rats weighing 200-250 g were kept under constant temperature (22 °C) and humidity with 12-h dark/light cycles and were allowed standard laboratory animal chow and water ad libitum throughout the experimental period.

2.2. Induction of colitis and experimental groups

TNBS was used to induce experimental colitis according to the procedure described by Morris et al. (8). Briefly, rats fasted for 24 h were anesthetized with ketamine hydrochloride and an 8-F polyethylene catheter was inserted rectally until splenic flexure (8 cm from the anus). Then 30 mg of TNBS (Sigma, France) dissolved in a volume of 0.15 mL of 50% ethanol was administered through the catheter. TNBS was retained in the colon for 1 min, after which the fluid was withdrawn. Pentoxifylline (Hemopene, İbrahim Etem, Turkey) ampoules (100 mg, 5 mL) were mixed with physiological saline solution and subsequently administered via intraperitoneal (IP) or intrarectal (IR) route at 100 mg/kg once daily for 3 days before or 6 days after the induction of TNBS colitis. Control animals received only a vehicle (0.9% saline).

The rats were randomly divided into 7 groups of 6 rats each: group A, TNBS + IP pentoxifylline; group B, TNBS + IP saline; group C, TNBS + IR pentoxifylline; group D, TNBS + IR saline; group E, IP pentoxifylline + TNBS; group F, IP saline + TNBS; group G, IR saline.

Rats were killed 6 days after induction of colitis and the distal 10 cm of the colon was excised, opened by longitudinal incision. Tissue samples were prepared for histopathological examination and the remaining mucosa was immediately snap-frozen in liquid nitrogen and stored at –80 °C for determination of superoxide dismutase (SOD), myeloperoxidase (MPO), matrix metalloproteinase-3 (MMP-3), and tissue inhibitor of metalloproteinases-1 (TIMP-1) activities and malonyldialdehyde (MDA) and TGF-β1 levels.

The study protocol was approved by the Gazi University Ethics Committee (03/07/20013; G.Ü.ET-13.048).

2.3. Assessment of colitis

Morphological examination was performed by an experienced pathologist unaware of the experiments being performed. The macroscopic appearances of the colonic mucosa were scored on a scale adapted from Morris et al. ranging from 0 to 4: 0- no macroscopic change; 1- mucosal erythema alone; 2- mild mucosal edema, slight bleeding, or small erosions; 3- moderate edema, bleeding ulcers, or erosions; 4- severe ulceration/erosions, edema, and tissue necrosis (8). For microscopic examination tissue samples were fixed in phosphate-buffered formaldehyde and embedded in paraffin, and routine 5-µm sections were prepared. Tissues were routinely stained with hematoxylin and eosin and were evaluated by light microscopy. The microscopic appearances of the colonic mucosa were scored on a scale adapted from Ackerman et al. (9):

A: Depth of necrosis: none = 0; mucosal = 1; mucosal and submucosal = 2; mucosal, submucosal, and muscularis propria = 3; full thickness = 4.

B: Extent of necrosis: none = 0; small area = 1; moderate area = 2; large area = 3; extensive = 4.

C: Inflammation: none = 0; minimal = 1; mild = 2; moderate = 3; severe = 4.

D: Extent of inflammation: none = 0; mucosal = 1; mucosal and submucosal = 2; mucosal, submucosal, and muscularis propria = 3; full thickness = 4.

The scores for each category examined were calculated for each specimen in the different groups. These were then added to obtain the total score, which was then divided by the number of rats' colons examined in each group to obtain the average histologic score of induced colitis for the group.

2.4. Assessment of tissue biomarkers

The sample tissues were homogenized (50 g/L) in 50 mmol/L ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The homogenate was frozen and thawed thrice, then centrifuged at 15,000 rpm for 15 min at 4 °C. The MPO activity in the supernatant was measured by assay kit (Eastbiopharm, China) according to the provider's instructions. The MDA contents and SOD activities in the supernatant were also measured by assay kit (Eastbiopharm) according to the provider's instructions. TGF-β1 levels and TIMP-1 and MMP-3 activities (Eastbiopharm) were assessed by ELISA technique according to the provider's instructions.

2.5. Statistical analysis

All statistical analyses were performed with SPSS 11.5 for Windows (SPSS Inc., USA). Normality was assessed using the Shapiro–Wilk test. Accordingly, data were expressed as median (min–max). Because data were not normally distributed and the number of tested subjects were less than 30 in each group, nonparametric tests were preferred: the Kruskal–Wallis test for comparison of multiple groups and the Mann–Whitney test to compare two groups. Correlation of tissue biomarkers with each other and histological scores were assessed using Spearman rank correlations. A two-sided P-value of 0.05 or less was considered statistically significant.

3. Results

There was no mortality in animals with colitis. Severe macroscopic inflammation was observed 6 days after intrarectal application of TNBS. Administration of intraperitoneal or intrarectal pentoxifylline similarly and significantly reduced the macroscopic damage severity (Table 1). The colon from sham-treated rats had a normal microscopic appearance. The colon from rats that had been treated with TNBS had inflammatory findings as follows: mucosal edema, necrosis, abnormal crypt architecture, mural thickening of the colon, and intense polymorphonuclear reaction. Intraperitoneal or intrarectal pentoxifylline similarly and significantly attenuated the microscopic damage severity (Table 1; Figure 1).

Significantly increased MPO activity was observed 6 days after TNBS application. Treatment with pentoxifylline attenuated the accumulation of MPO in the colons of rats exposed to TNBS (Table 2; Figure 2).

Compared with those of the sham-treated rats, the contents of MDA, TGF-β1, MMP-3, and TIMP-1 in colonic tissues were significantly increased in rats with TNBS-induced colitis (Tables 2 and 3). Pentoxifylline attenuated the accumulation of MDA and TGF-β1 and the activities of MMP-3 and TIMP-1 (Tables 2 and 3). Compared with the sham-treated rats, SOD activity was significantly decreased in the colonic tissue of rats exposed to TNBS. SOD activity showed restoration to normal levels after pentoxifylline (Table 2) (Figure 3—7).

Although improvement in macroscopic and microscopic damage was similar between IP and IR pentoxifylline, the IP route was more effective than the IR route at decreasing the accumulation of MPO, MDA, TIMP-1, MMP-3, and TGF-β1 and restoring SOD activity to normal levels in rat colonic tissues (Tables 2 and 3). There was no difference between the pathological scores and tissue biomarkers of inflammation and fibrosis with respect to administration of IP pentoxifylline before or after induction of TNBS colitis (Tables 2 and 3).

4. Discussion

This study described the effect of administration of pentoxifylline on the macroscopic and microscopic damage scores of rat colons. The results of histopathological examination indicated that a significant difference was found between rats that had been treated with TNBS and control rats, and pentoxifylline successfully attenuated inflammation and ulcers caused by intracolonic TNBS. The healing effect was obtained from both intraperitoneal and intracolonic administration. These results suggest that pentoxifylline may find application in the treatment of IBD in humans.

The influence of pentoxifylline on the severity of gut inflammation has been examined previously in several animal models of IBD (10). Pentoxifylline and its metabolite-1 significantly attenuated colon damage and inflammation in an animal model of TNBS-induced

Table 1. Effect of treatment with intraperitoneal or intrarectal pentoxifylline on macroscopic and microscopic pathological scores of colonic tissue from rats with TNBS-induced colitis.

	Macroscopic scores	Microscopic scores	P-value	Groups compared
Group A TNBS + IP pentoxifylline	1 (1–2)	5 (4–8)	<0.001	A vs. B
Group B TNBS + IP saline	4 (3–4)	14 (12–14)	>0.05 >0.05	A vs. C A vs. E
Group C TNBS + IR pentoxifylline	1 (1–2)	7 (6–8)		
Group D TNBS + IR saline	3.5 (3–4)	13 (12–14)	<0.001	C vs. D
Group E IP pentoxifylline + TNBS	2.5 (1–3)	8 (4–8)		
Group F IP saline + TNBS	4 (3–4)	14 (12–14)	<0.001	E vs. F
Group G IR saline	0 (0–0)	0 (0–0)		

Scores are expressed as median (min–max). TNBS: Trinitrobenzene sulfonic acid. $P < 0.05$ when all of the individual groups were compared with group G for macroscopic and microscopic scores.

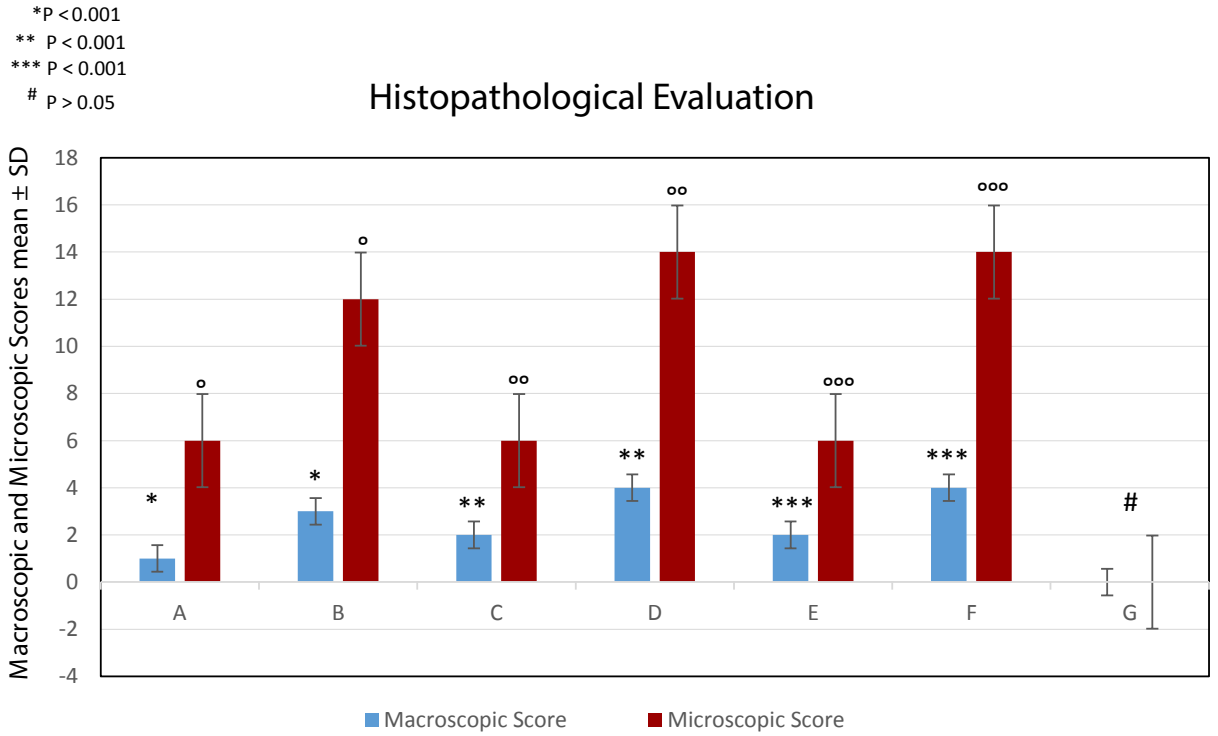

Figure 1. Effect of treatment with intraperitoneal or intrarectal pentoxifylline on macroscopic and microscopic pathological scores of colonic tissue from rats with TNBS-induced colitis.

Table 2. Effect of treatment with pentoxifylline on various tissue biomarkers of inflammation from rats with TNBS-induced colitis.

	MPO (U/g)	MDA (μmol/g)	SOD (U/g)	P-value	Groups compared
Group A TNBS + IP pentoxifylline	0.18 (0.16–0.19)	1.2 (0.9–1.3)	163 (162–164)	<0.001 <0.001 <0.05 <0.05[a] <0.05	A vs. B A vs. C A vs. E A vs. G B vs. G
Group B TNBS + IP saline	0,70 (0.63–0.80)	4.3 (3.4–54)	144 (143–145)		
Group C TNBS + IR pentoxifylline	0.27 (0.26–0.29)	2.3 (1.9–2.5)	157 (155–158)	<0.001 <0.05[b] <0.05	C vs. D C vs. G D vs. G
Group D TNBS + IR saline	0.60 (0.52–0.65)	3.9 (3.2–4.5)	143 (142–144)		
Group E IP pentoxifylline + TNBS	0.36 (0.35–0.41)	2.9 (2.4–3.2)	153 (149–153)	<0.001 <0.05 <0.05	E vs. F E vs. G F vs. G
Group F IP saline + TNBS	0.57 (0.48–0.66)	5.8 (5.1–7)	138 (136–141)		
Group G IR saline	0.17 (0.1–0.21)	1.6 (1–2.8)	159 (150–162)		

TNBS: Trinitrobenzene sulfonic acid, MPO: myeloperoxidase, MDA: malonyldialdehyde, SOD: superoxide dismutase. Scores are expressed as median (min–max). [a]Only SOD showed a significant difference. [b]Only MPO showed a significant difference.

colitis. Metabolite-1 treatment significantly reduced the TNBS-induced increase in colon weight, colon thickness, and total collagen content, supporting its antifibrotic potential (10). In another study, pentoxifylline treatment was not sufficient to reduce the elevation in colonic collagen, although the treatment was sufficient to reduce

Table 3. Effect of treatment with pentoxifylline on various tissue biomarkers of fibrosis from rats with TNBS-induced colitis.

	TIMP-1 (ng/g)	MMP-3 (ng/g)	TGF-β1 (ng/g)	P-value	Groups compared
Group A TNBS + IP pentoxifylline	10.3 (9.3–11.2)	75 (70–89)	1.3 (0.8–1.4)	<0.001 <0.001 <0.05 <0.05[a] <0.05	A vs. B A vs. C A vs. E A vs. G B vs. G
Group B TNBS + IP saline	20.1 (18.3–26.5)	165 (163–168)	4.9 (4.2–5.8)		
Group C TNBS + IR pentoxifylline	13.6 (12.9–14.7)	101 (93–114)	2.4 (2–2.5)	<0.001 <0.05 <0.05	C vs. D C vs. G D vs. G
Group D TNBS + IR saline	19.1 (18.2–21.8)	164 (163–168)	4.8 (4–5.6)		
Group E IP pentoxifylline + TNBS	15 (14.5–16.9)	130 (122–138)	2.8 (2.7–3.8)	<0.001 <0.05 <0.05	E vs. F E vs. G F vs. G
Group F IP saline + TNBS	27 (22.8–28.9)	166 (162–188)	5.7 (5.2–6.8)[c]		
Group G IR saline	7.8 (6.5–10.9)	33 (15–53)	1 (0.6–1.5)		

TNBS: Trinitrobenzene sulfonic acid, TIMP-1: tissue inhibitor of metalloproteinases-1, MMP-3: matrix metalloproteinase-3, TGF-β1: transforming growth factor β1. Scores are expressed as median (min–max). [a]Only TIMP-1 and MMP-3 showed a significant difference.

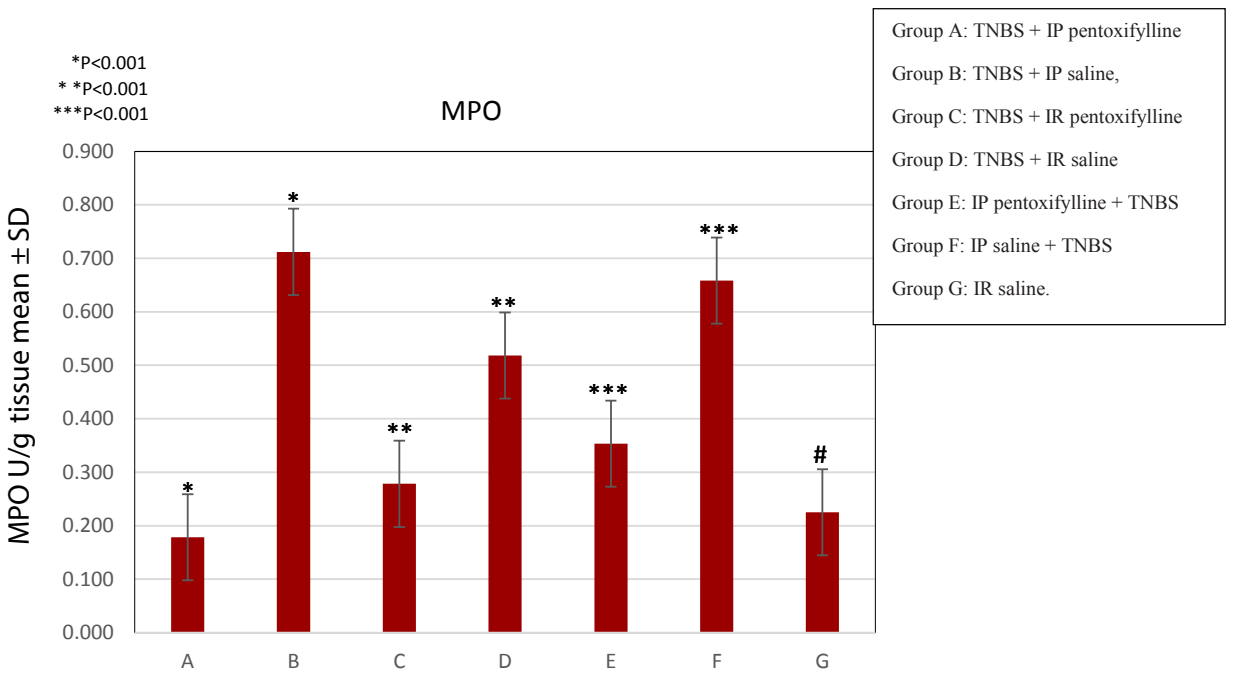

Figure 2. Effect of treatment with pentoxifylline on MPO levels of inflammation from rats with TNBS-induced colitis.

the pathological changes due to TNBS, raising a question about the antifibrotic potential of the treatment (11). Murthy et al. observed that pentoxifylline in combination with a single injection of TNF-alpha monoclonal antibody was significantly more effective in inhibiting the disease severity, ulcer index, and inflammation compared to a placebo or any single monotherapy in dextran sulfate-induced mouse colitis (12).

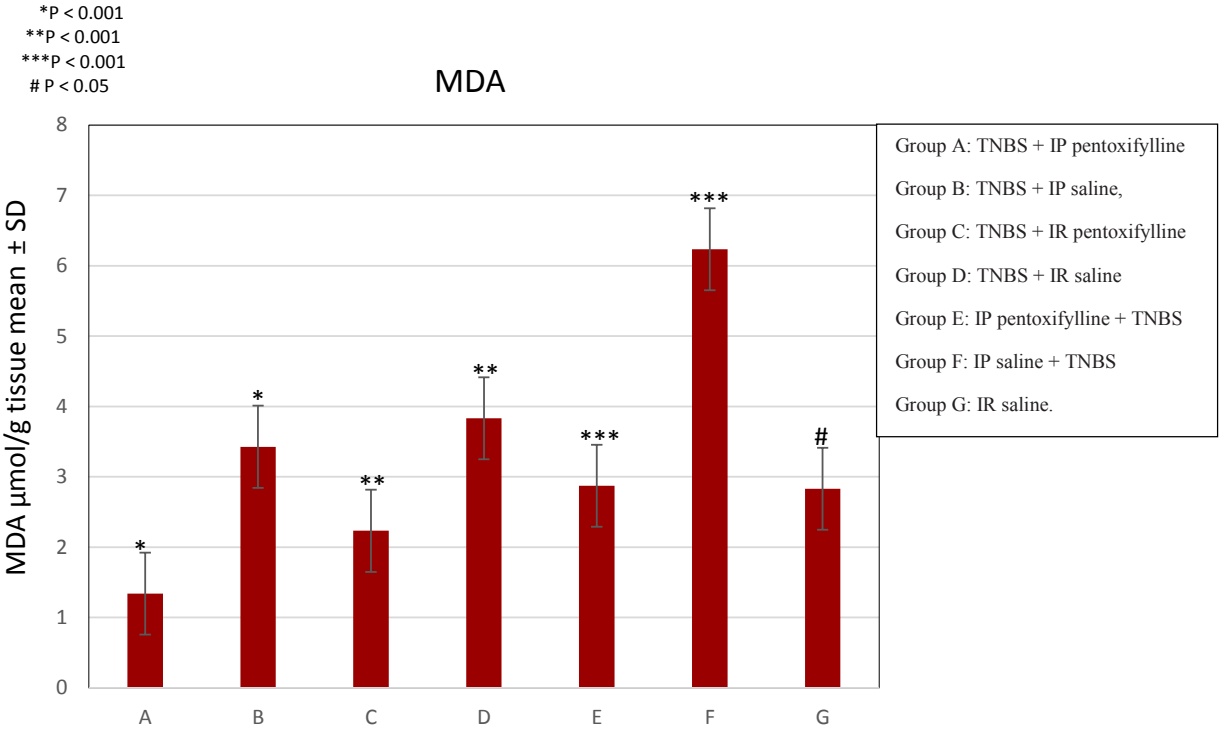

Figure 3. Effect of treatment with pentoxifylline on MDA levels of inflammation from rats with TNBS-induced colitis.

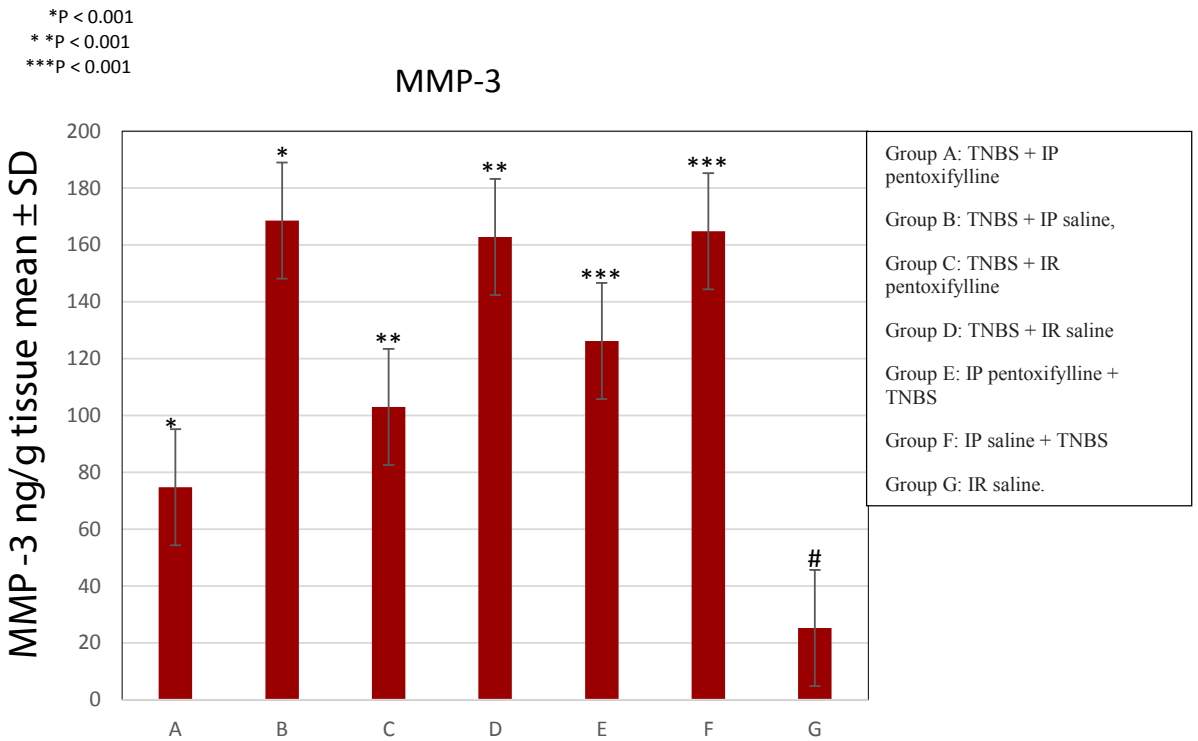

Figure 4. Effect of treatment with pentoxifylline on MMP-3 levels of inflammation from rats with TNBS-induced colitis.

So far, only two studies on this topic have included human subjects (13,14). Peripheral mononuclear cells (PBMCs) and inflamed intestinal mucosa from patients with IBD release various inflammatory mediators (13). The first of the above-mentioned studies carried out in IBD patients showed that pentoxifylline attenuated the

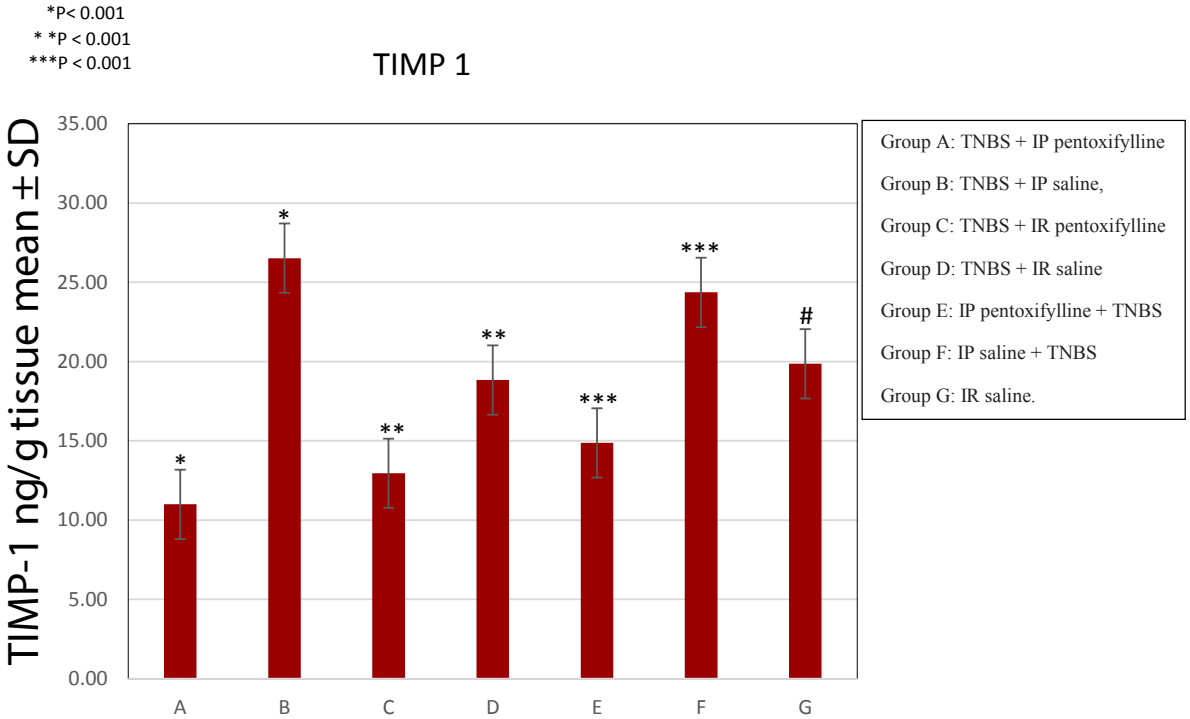

Figure 5. Effect of treatment with pentoxifylline on TIMP-1 levels of inflammation from rats with TNBS-induced colitis.

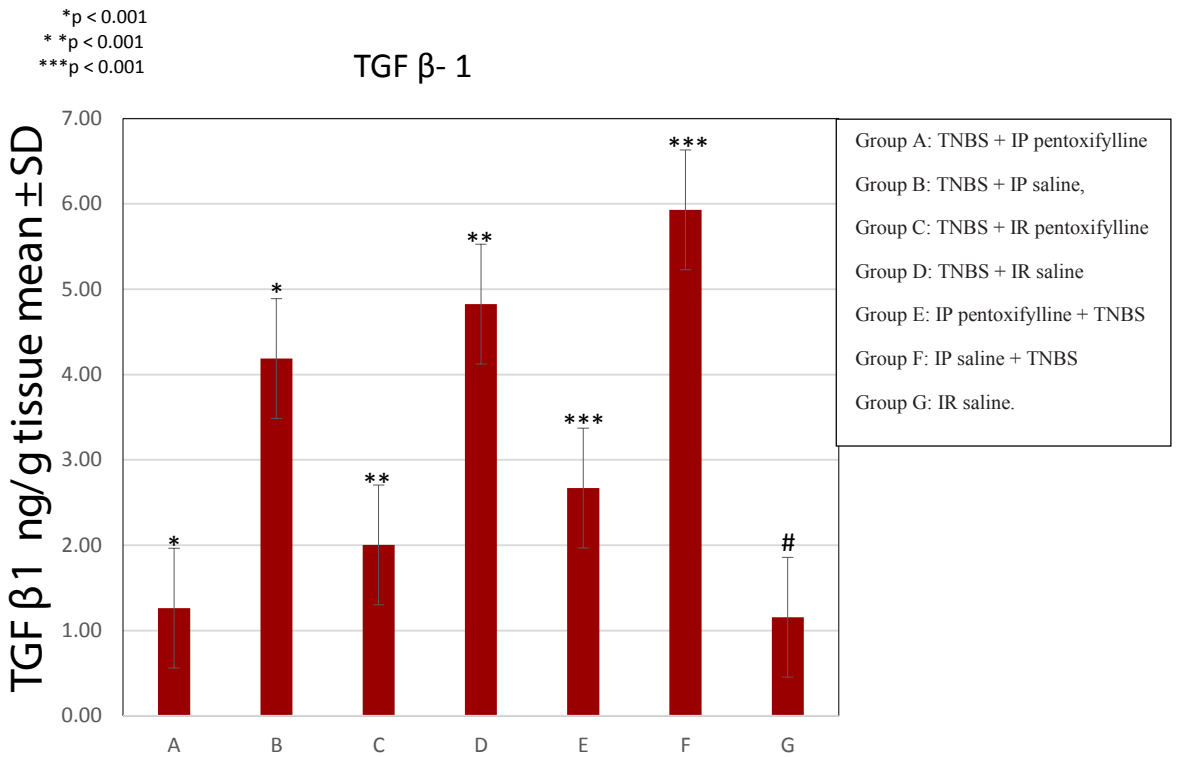

Figure 6. Effect of treatment with pentoxifylline on TGF-β1 levels of inflammation from rats with TNBS-induced colitis.

production of TNF-alpha by PBMCs and the release of TNF-alpha and interleukin-1 by organ cultures of inflamed intestinal mucosa (13). However, inhibition of TNF-alpha production by pentoxifylline in a group of patients with steroid-dependent CD was not successful in improving clinical, endoscopic, or laboratory parameters (14). There

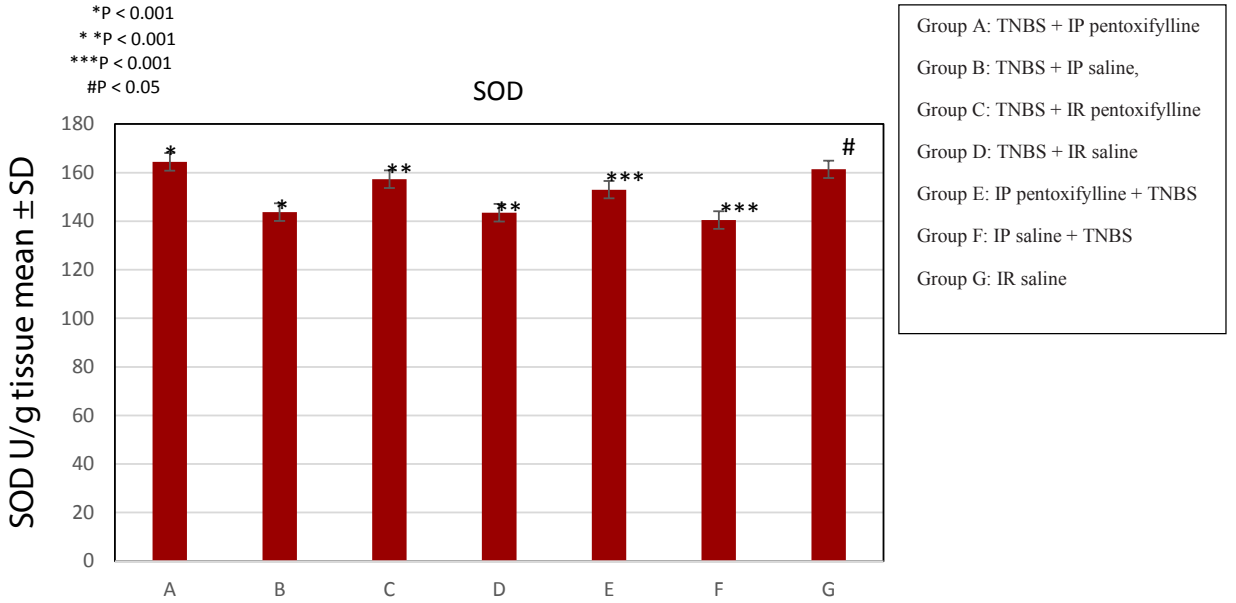

Figure 7. Effect of treatment with pentoxifylline on SOD levels of inflammation from rats with TNBS-induced colitis.

is no satisfactory explanation for this observation but it is already known that blocking TNF-alpha does not turn off the inflammatory cascade in approximately 25% of patients (15). Another consideration is that the patients in the above-mentioned study were mildly active CD patients receiving low-dose steroid therapy. The absence of severe baseline inflammation might have partially accounted for lack of antiinflammatory response to pentoxifylline.

To uncover the mechanisms responsible for the mucosal improvement with pentoxifylline, we measured several tissue biomarkers. MPO is a proinflammatory enzyme present in the azurophilic granules of neutrophilic granulocytes and it serves to quantify neutrophil accumulation in tissues (16). Lipid peroxidation, a type of oxidative degeneration of polyunsaturated fatty acids, is associated with altered membrane structure and decreased activity of antioxidant enzymes. We measured MDA, an end-product of lipid peroxidation, as an indicator of this pathological process (17). Animal studies provide evidence for the critical role of TGF-β1 in triggering and sustaining intestinal fibrogenesis (18). MMPs are involved in the remodeling and degradation of the extracellular matrix and they were increased in inflamed colons of patients with IBD (19). Lastly, SOD was measured in our study because it is a primary defense against oxidative stress, mediating intestinal damage in IBD (20). The finding that pentoxifylline prevented an increase in MPO, MMP-3, and TIMP-1 activities and the accumulation of MDA and TGF-β1 supports the concept of an inhibition of inflammatory response, oxidative stress, and tissue remodeling as pentoxifylline's contribution to the attenuation of macroscopic and microscopic colonic damage. The restoration of SOD activity to normal levels following the administration of pentoxifylline is also consistent with its antioxidant potential.

Another important finding of our study was that administration of pentoxifylline prior to TNBS was partially successful to protect against the development of colitis. This may suggest a role for pentoxifylline in postoperative prophylaxis of CD. Why was intraperitoneal pentoxifylline more effective compared to intrarectal pentoxifylline? TNBS, associated with transmural inflammation, more closely mimics CD. Although topical agents such as 5-ASA may be used in colonic CD, there is reason to question the rationale for using a superficially active antiinflammatory agent in a transmural disease (21). This study suggests that inferior response to intrarectal pentoxifylline might have resulted from a more superficial inflammation in the relevant rats. The efficacy of intraperitoneal pentoxifylline in our study suggests a role for this drug in CD patients with penetrating disease behavior.

In conclusion, the findings of this research indicate that pentoxifylline therapy alters the course of TNBS colitis and results in histological improvement. Decreased oxidative stress and inhibition of cytokine release and inflammatory response may contribute to the therapeutic effects of pentoxifylline. Further research is required to provide evidence of the potential of this drug as an antiinflammatory and antifibrotic agent in IBD.

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