ROLE OF A SELECTIVE PHOSPHODIESTETASE INHIBITOR IN TREATMENT OF INFLAMMATORY BOWEL DISEASE

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Abstract

This study was done on five groups of rats will be used (NC group), TNBS- untreated colitis, and three groups TNBS-treated colitis rats. Treatments will be oral Sulfasalazine (SS, 500), Roflumilast given by oral route (OR group, 5 mg/kg) or Roflumilast given by rectal route (RR group, 5 mg/kg). Treatments will be given daily for 15 days starting 48 hours after induction of colitis. Serum was collected for measurements of TNF-α, IL-2, IL-12 and cortisol which were done used ELISA kits. Tissues were collected for antioxidant enzymes (GSH-MDA-MPO) and for histopathological and immunohistochemically observations. All treatments significantly improved these changes. Sulfasalazine exerted the greatest effect followed by oral Roflumilast, and then by rectal Roflumilast. The aim of the current study is to evaluate the Roflumilast as a selective phosphodiesterase inhibitor in the treatment of colitis in rats.

Key words: Roflumilast, phosphodiestetase inhibitor, CD, rats

Inflammatory bowel disease (IBD) category is formed mainly of Crohn's disease (CD) and Ulcerative colitis. These diseases are of undefined etiology, most probably immunologically mediated, chronic, and progressive starting at young age and characterized by remissions, relapse and course protracted course (Ananthakrishnan, 2015). CD is a progressive disorder causing permanent intestinal injury and disability (Torres et al., 2017). More demonstration and understanding of IBD pathogenesis will provide appropriate management of the disproportionate and progressive inflammatory response to internal microbes in a genetically susceptible host (Geremia et al., 2014 and Cătană, et al., 2015).

The traditional management of IBD usually consists of the use of immunosuppressive besides anti-inflammatory drugs. For remission induction and maintenance, biological treatment could be used, and finally surgical treatment is introduced in case of failure to response to medical treatment (Gomollón et al., 2017). The anti-inflammatory effect of phosphodiesterase IV (PDE4) inhibitors is rather similar to that of corticosteroids with the advantage of non-interference with the hypothalamo-pituitary-adrenal axis. Roflumilast, a selective PDE-4 inhibitor with antiinflammatory and antifibrotic effects, is FDA-approved as an add-on treatment for COPD (Pauwels 2001, Hatzelmann et al., 2010). In animal models of colitis, when treatments are given before, during, or within one day after induction of colitis, any improvement could express a prophylactic and not a therapeutic effect because it may be simply due to an interference with induction of colitis. Thus, for the animal model to be relevant to human CD, it must be "chronic and immune- mediated" (Goyal et al., 2014). Also to predict clinical efficacy of a treatment in CD, it must be able to reverse "already-established" chronic colitis (Koboziev et al., 2011, Reardon 2016). In experimental models, trinitrobenzene sulfonic acid (TNBS) induces colitis with a Th-1 immune pattern (Randhawa et al., 2014).

Roflumilast is a very selective inhibitor of PDE4 used for severe COPD therapy. Its anti-inflammatory effect is mostly tolerable in comparison with previous inhibitors of PDE4. Such therapy proved dose-dependent enhancement of clinical scoring, length of colon, and production of TNF- α in the colonic mucosa (Rieder et al., 2013). In experimental models, it lowers inflammatory response in different disease. E.g. in COPD associated with bacteria, therapy with Roflumilast decreases lung infiltration by leukocyte, suppress inflammation, and liver damage was prevented. Moreover, it decreased pro-inflammatory cytokines levels in the serum of COPD patients (Feng et al., 2017).

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MATERIAL AND METHOD

Five groups of rats (n=8) will be used (NC group), TNBS-untreated colitis (Chronic colitis was induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), and three groups TNBS-treated colitic rats. Treatments will be Sulfasalazine (SS, 500), Roflumilast given by oral route (RO group, 5 mg/kg) and Roflumilast given by rectal route (RR group, 5 mg/kg). Treatments will be given daily for 15 days starting 48 hours after induction of colitis. Serum was collected for measurements of TNF-α, IL-2 and IL-6 which were done used ELISA kits. Tissues were collected for antioxidant enzymes (GSH- MDA-MPO) and for histopathological and immunohistochemical observations.

Statistical test:

Data were expressed as means ± SEM and analyzed using SPSS version 22. One-way ANOVA and Tukey's post hoc tests were used to test differences among groups. P < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSIONS

Colonic measurements

The TNBS-colitic rats showed significant increases of the contents of MPO (a marker for neutrophil infiltration), and MDA and a significant decrease of GSH level in the colon homogenate. All treatments significantly reversed these TNBS-induced changes with significant differences in-between. Sulfasalazine exerted the greatest changes while oral roflumilast caused more changes than rectal roflumilast. (Tables 1- 3 and Figures 1-3).

Table 1

Effects of Roflumilast on colonic levels of MDA in TNBS-induced colitis (n==8)

Groups	Mean ±SEM
Control healthy	19.31 ± 0.66
TNBS	81.40 ± 2.13
Sulfasalazine	28.93 ± 0.50 [d]
Oral Roflumilast	36.68 ± 1.98 [d, e, f]
Rectal Roflumilast	47.04 ± 1.98 [d]

Data are expressed as mean ± SEM. d: P < 0.001 OR & RR vs. NC and All treatments vs. PC, e: P < 0.01 OR vs. RR (= 0.001), f:P < 0.05 OR vs. SS (= 0.014).

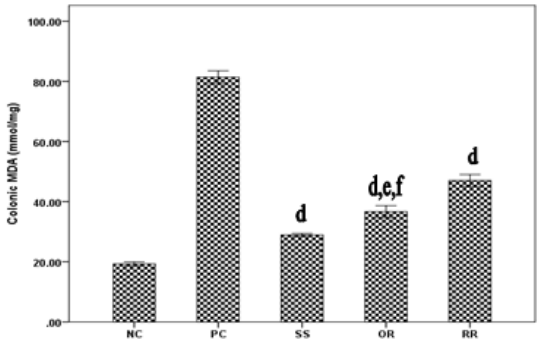

Figure 1. **The MDA content in different rats groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Table 2

Effects of roflumilast on colonic levels of GSH in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	19.60 ± 0.79
TNBS	6.86 ± 0.22
Sulfasalazine	14.73 ± 0.49 [g]
Oral Roflumilast	11.94 ± 0.32 [g, h]
Rectal Roflumilast	9.24 ± 0.28 [g, h]

Data are expressed as mean ± SEM. g : P < 0.001 All treatments vs. NC and SS & OR vs. PC, h : P < 0.01 RR vs. PC (= 0.008), OR vs. SS & RR (= 0.001, 0.002).

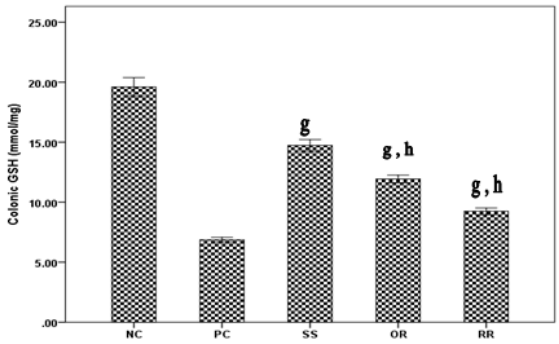

Figure 2. **The GSH content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Table 3

Effects of roflumilast on colonic levels of MPO in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	11.29 ± 0.85
TNBS	50.70 ± 1.05
Sulfasalazine	18.38 ± 0.87 [a]
Oral Roflumilast	23.89 ± 0.92 [a, b, c]
Rectal Roflumilast	28.17 ± 1.00 [a]

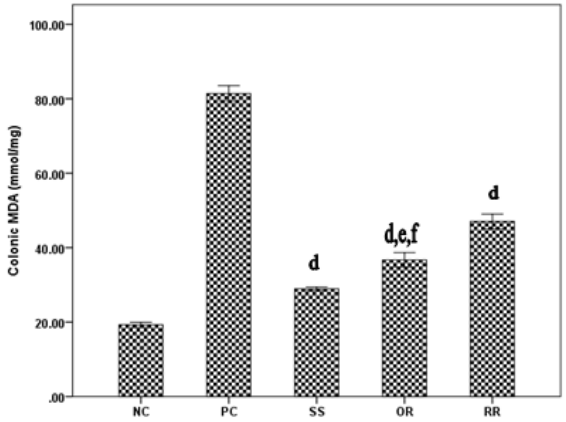

Figure 3. **The MPO content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Serum measurements:

The TNBS colitic rats showed significant increases of the serum TNF-α, IL-2, and IL-6. All treatments significantly reversed these TNBS-induced changes with significant differences in-between. Sulfasalazine exerted the greatest reductions while oral roflumilast caused more reductions than rectal roflumilast (Tables 4- 6 and Figures 4-6).

Table 4

Effects of roflumilast on serum levels of TNF-α in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	17.04±0.67
TNBS	91.18 ± 4.14
Sulfasalazine	28.93 ± 0.5 [a, b]
Oral Roflumilast	38.87 ± 2.33 [a, c]
Rectal Roflumilast	79.37 ± 1.88 [a, b]

Data are expressed as mean ± SEM. a : P < 0.001 Oral roflumilast (OR) & Rectal roflumilast (RR) vs. Normal control (NC) and Sulfasalazine (SS) & OR vs. Positive control (PC), and RR vs. SS & OR. b : P < 0.01 SS vs. NC (= 0.008) and RR vs. PC (=0.008), c : P < 0.05 OR vs. SS (= 0.034).

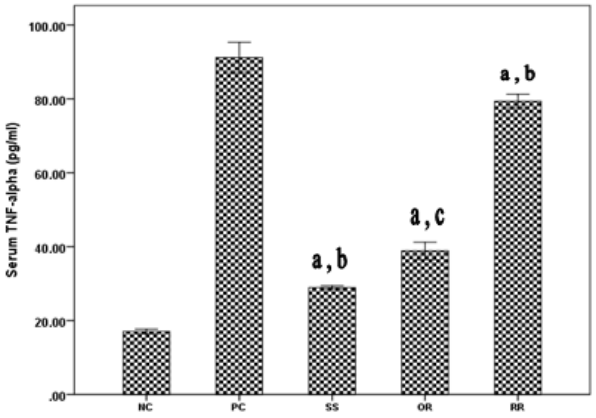

Figure 4. **The TNF content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Table 5

Effects of roflumilast on serum levels of IL-2 in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	196.10 ± 3.19
TNBS	487.00 ± 9.36
Sulfasalazine	257.97 ± 7.72 [d]
Oral Roflumilast	302.75 ± 11.32 [d, e, f]
Rectal Roflumilast	363.87 ± 12.73 [d]

Data are expressed as mean ± SEM. d : P < 0.001 All treatments vs. NC & PC, e : P < 0.01 OR vs. RR (= 0.001), f : P < 0.05 OR vs. SS (= 0.016).

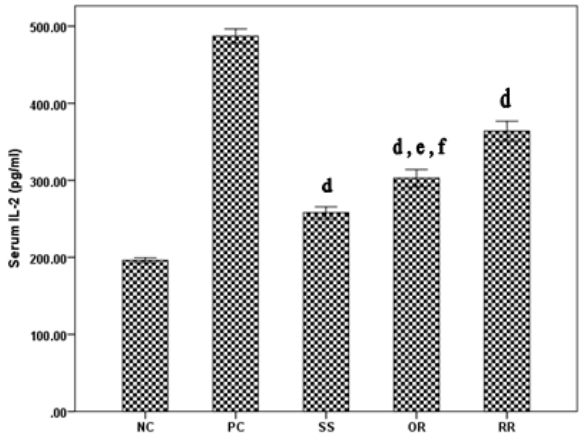

Figure 5. **The IL2 content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test**

Table 6

Effects of roflumilast on serum levels of IL-6 in TNBS-induced colitis rats (n=8)

Groups	Mean ±SEM
Control healthy	10.57 ± 0.42
TNBS	47.04 ± 1.98
Sulfasalazine	16.14 ± 1.13 [g, i]
Oral Roflumilast	21.85 ± 1.00 [g, h]
Rectal Roflumilast	28.93 ± 0.50 [g]

Data are expressed as mean ± SEM. g : P < 0.001 OR & RR vs. NC and All treatments vs. PC, h : P < 0.01 OR vs. RR (= 0.001), i : P < 0.05 SS vs. NC & OR (= 0.013, 0.010).

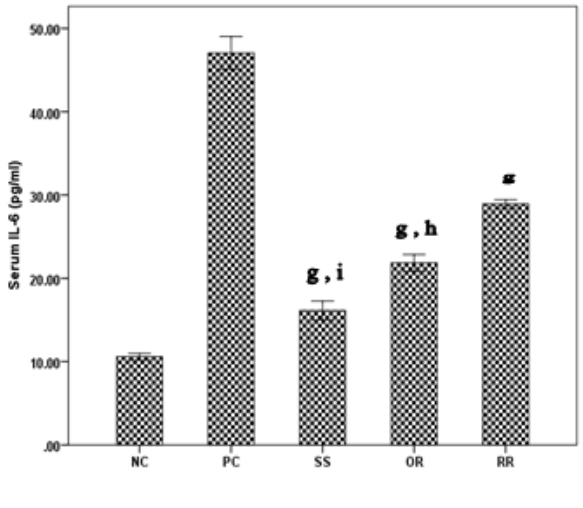

Figure 6. **The IL6 content in different rat's groups. The experiments were done in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at P < 0.05 according to Tukey test.**

Histopathological examination:

In the colonic sections stained with H&E, the TNBS-colitic rats revealed destroyed glands, marked inflammatory infiltration, focal necrosis of mucosa and submucosa, loss of lining epithelium, and diffuse submucosal edema (Figure 7). For Masson trichrome, the TNBS group showed numerous congested blood vessels in the propria submucosa with edema around the CT fibers and blood vessels. Accumulation of lymphocytes was located in the propria submucosa, and some large nodules were located in the mucosa-submucosa (Figure 8). For PAS, the TNBS group showed numerous goblet cells with positive PAS reaction prominent between the lining epithelium of the crypts. Some crypts of Lieberkühn showed ulceration and accumulation of leucocytes (Figure 9). For the three stains, all treatments reversed these TNBS-induced changes with varying degrees. Sulfasalazine exerted the greatest improvement and oral roflumilast caused more improvement than rectal roflumilast.

Immunohistochemical results:

In the colonic sections stained for Ki 67, the TNBS-colitic rats revealed very faint positive reaction (Figure 10) while for immunohistochemical staining for TNF-α, the TNBS group showed strong positive reaction (Figure 11). For the two stains, all treatments reversed these TNBS-induced changes with varying degrees. Sulfasalazine exerted the greatest improvement and oral roflumilast caused more improvement than rectal roflumilast.

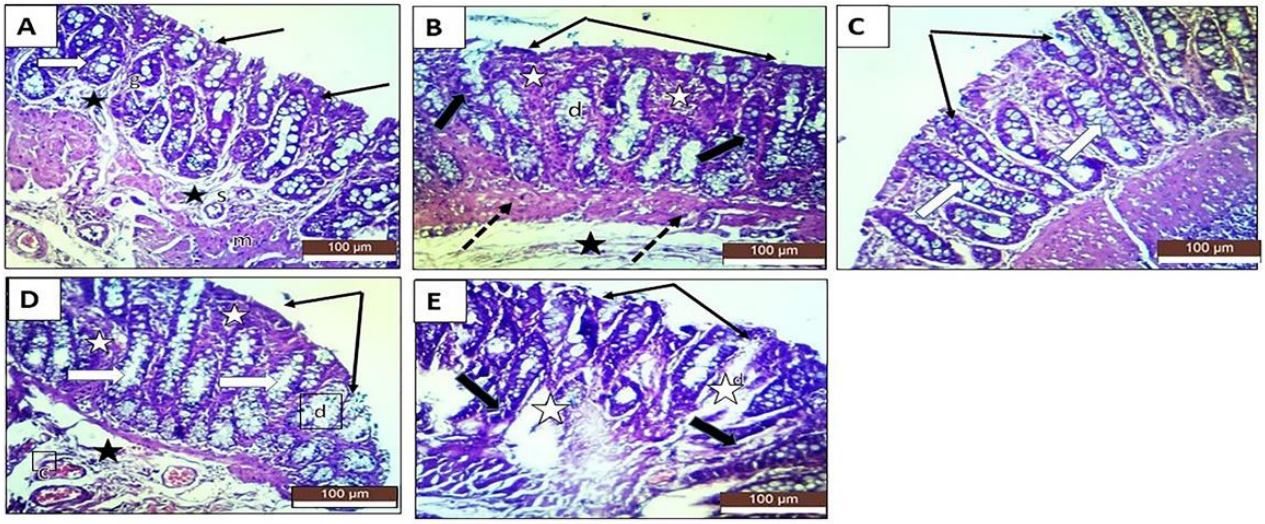

Figure 7. **Microphotographs of colon sections showing effects of oral and rectal roflumilast (H&E, X20). (A) Normal control group showing normal colonic structure with tunica submucosa consisting of loose CT rich in blood vessels and lymph vessels (s) and tunica musculosa consisting of inner circular and outer longitudinal smooth muscle fibers (m). (B) Positive control group showing destruction in the glands (d) and marked inflammatory infiltration. (C) Sulfasalazine group showing moderate to marked improvement with few desquamated cells especially the colonocytes (d) and minimal inflammatory infiltrate. (D) Oral roflumilast group showing moderate improvement with thickening of the colonic wall with patches of fibrosis (f) and epithelial desquamation (d). (E) Rectal roflumilast group showing mild improvement with desquamated colonic cells (d) especially the cells lining the crypts and with numerous congested blood vessels (c) in the propria submucosa.**

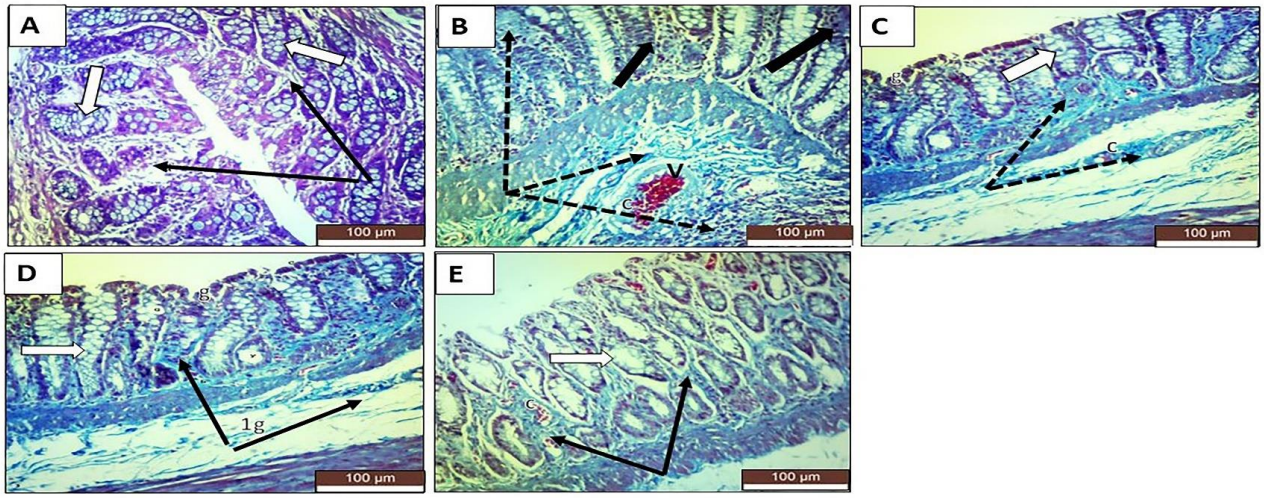

Figure 8. **Microphotographs of colon sections showing effects of oral and rectal roflumilast (Masson trichrome, X20). (A) Normal control group showing normal colonic collagen fibers (lg) and diffuse lymphocytes in the propria submucosa and numerous goblet cells (g). (B) Positive control group showing numerous congested blood vessels in the propria submucosa with edema around the CT fibers and blood vessels (c). Accumulation of lymphocytes was located in the propria submucosa, and some large nodules were located in the mucosa-submucosa (l). (C) Sulfasalazine group showing some congested blood vessels (c) in the propria submucosa and diffuse lymphocytes and numerous goblet cells (g). (D) Oral roflumilast group showing numerous congested blood vessels (c) between the crypts of Lieberkühn (intestinal glands). (E) Rectal roflumilast group showing some ulcerated crypts of Lieberkühn (u) with accumulation of leucocytes. 284x144mm (300 x 300 DPI)**

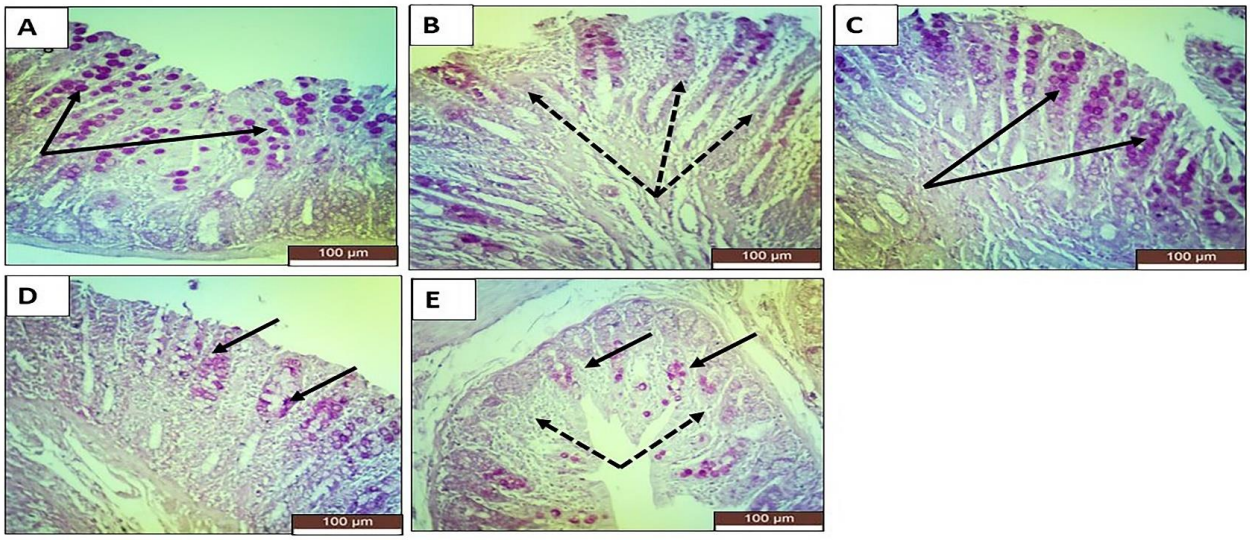

Figure 9. **Microphotographs of colon sections showing effects of oral and rectal roflumilast (PAS, X 20). (A) Normal control group showing numerous goblet cells with positive PAS reaction between the enterocytes (g). The propria submucosa CT showed very faint PAS positive fibers. (B) Positive control group showing numerous goblet cells with positive PAS reaction (g) prominent between the lining epithelium of the crypts. Some crypts of Lieberkühn showed ulceration and accumulation of leucocytes. (C) Sulfasalazine group showing PAS-positive goblet cells while the CT of the propria submucosa showed faint positive PAS reaction (g). (D) Oral roflumilast group showing goblet (g) cells with faint positive PAS reaction. (E) Rectal roflumilast group showing PAS-positive goblet cells of the crypt lining. 261x159mm (300 x 300 DPI)**

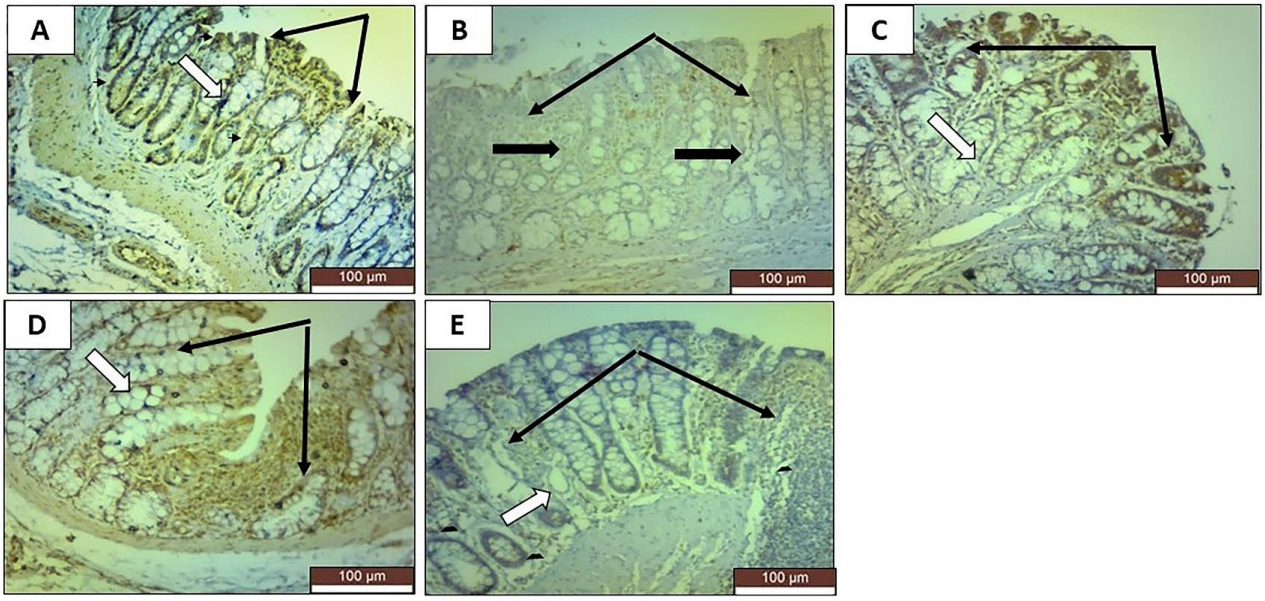

Figure 10. **Immunostaining for Ki-67 of colonocytes and crypt cells showing effects of oral and rectal roflumilast. (A) Normal control group showing positive reaction. (B) Positive control group showing very faint positive reaction. (C) Sulfasalazine group showing faint positive reaction. (D) Oral roflumilast group showing faint positive reaction. (E) Rectal roflumilast group showing very faint positive reaction (20X). 284x144mm (300 x 300 DPI)**

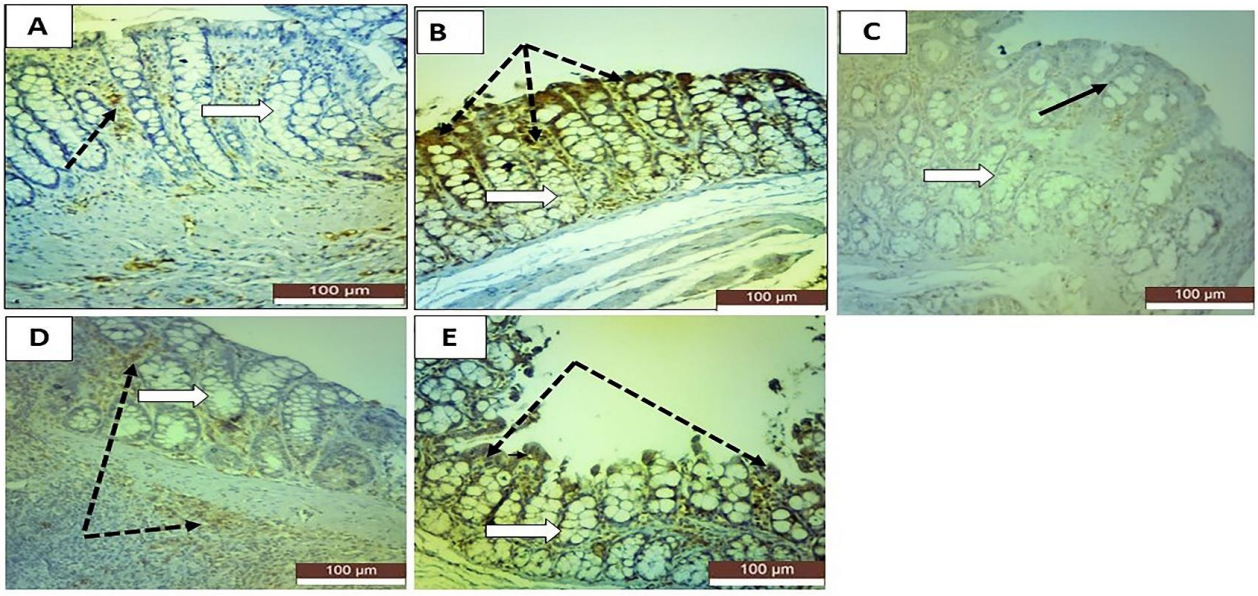

Figure 11. **Immunostaining for TNF-α of colonocytes and crypt cells showing effects of oral and rectal roflumilast. (A) Normal control group showing negative reaction. (B) Positive control group showing positive reaction. (C) Sulfasalazine group showing very faint positive reaction. (D) Oral roflumilast group showing moderate positive reaction. (E) Rectal roflumilast group showing strong positive reaction (20X). 270x161mm (300 x 300 DPI)**

The phosphodiesterase IV (PDE4) is an intracellular enzyme which increases production of the proinflammatory mediators and decreases production of the anti-inflammatory mediators, and thus it is implicated in pathogenesis of many inflammatory diseases. PDE4 inactivates cyclic adenosine monophosphate (cAMP) and is the main PDE isoenzyme in the mononuclear inflammatory cells, the main source of production of TNF-α. It was reported that elevation of TNF-α plays a crucial role in the pathogenesis of inflammatory bowel disease (IBD). IL-2 is another common cellular inflammatory factor. In inflammatory conditions, levels of TNF-α and IL-2 rapidly increase and thus activate WBCs, promote the migration of inflammatory cells, and expand the inflammatory response (Oh et al., 2017). Inhibition of PDE4 is associated with a broad anti-inflammatory activity including suppression of TNF-α. Thus, specific inhibition of PDE4 could be effective in treatment of several chronic inflammatory disorders (Banner and Trevethick, 2004, Li et al., 2018, Loher et al., 2003). Inhibition of PDE4 might provide a novel approach in the prevention and treatment of IBD (Videla et al., 2006). Unfortunately, clinical use of PDE4 inhibitors such as rolipram was limited by their adverse effects, but interestingly, roflumilast, the first-licensed member in this class, is a highly selective PDE4 inhibitor and is clinically effective at a relatively low dose compared with other PDE4 inhibitors. It improved episodic memory in subjects with minimal cognitive impairment at a non-emetic dose with plasma levels of about five times lower than the approved dose for COPD treatment. Use of these low doses minimizes the typical side effects of the PDE 4 inhibitors such as vomiting (Sugin et al., 2020). Roflumilast showed a more potent anti-inflammatory activity in both animals and humans and was more well-tolerated than the early PDE4 inhibitors like rolipram and cilomilast (Bundschuh et al., 2001).

The results of the current study agree with a previous study which revealed that in mice with dextran sulphate sodium (DSS)-induced colitis, oral roflumilast (1 or 5 mg/kg/d) dose-dependently improved the disease clinical score (weight loss, stool consistency and bleeding), colon length, and colonic TNF-α production. However, it did not improve the histologic score (Rieder et al., 2013). In addition, roflumilast showed potential anti-inflammatory effects in DSS-induced ulcerative colitis in male Wistar rats. Colitis was determined by assessing colon length, weight loss, histologic colon score, and measuring the concentrations of TNF-α, nitric oxide, cAMP, MPO activity and inducible nitric oxide synthase (iNOS) gene expression in colonic tissue. Roflumilast (5 mg/kg) attenuated the severity of colitis as evidenced by increased colon length, improved body weight loss, and improved colon histologic score compared to the DSS group. It also decreased colon concentrations of TNF-α, NO and MPO activity and down-regulated the iNOS gene expression. The results of roflumilast were comparable to those exerted by sulfasalazine (El-Ashmawy et al.,

2018). The colon length is an indirect measure for the severity of colonic inflammation (Loher et al., 2003). In agreement with our results, roflumilast was reported to partially reverse the TNBS-induced reduction in colon length at 1 and 5 mg/kg/day and to decrease the elevated colonic TNF-α concentration (Rieder et al., 2013). Moreover, in sepsis-induced liver damage in mice, roflumilast inhibited the expression of TNF-α, and IL-6 (Feng et al., 2017).

In the current study, we examined the effects of rectal roflumilast on TNBS-induced CD in a trial to prove that local roflumilast through rectal application could help in treatment of CD. Our results showed that rectal roflumilast reversed the TNBS-induced colitic changes indicating local anti-inflammatory effects. A previous study reported that oral roflumilast has local anti-inflammatory effects. In that study, colitis was induced in rats and the local effects of oral roflumilast at sites of inflammation were examined. It was found that treatment with oral roflumilast dose-dependently ameliorated the clinical score of colitis, led to a reduced shortening of the colon length, and decreased local expression of TNFα in colonic tissue, but this improvement was not associated with lowering the histologic score (Rieder et al., 2013). In addition, in a clinical trial, roflumilast cream applied topically once daily to affected areas of psoriasis was superior to vehicle cream in causing to an almost clear state at six weeks. Thus, it seems that topical roflumilast has the potential to help existing therapies in many inflammatory skin diseases (Lebwohl et al., 2020). Moreover, it is known that activation of adenylate cyclase by prostaglandin E2 or prostacyclin may exert a synergistic effect with PDE inhibition to enhance cAMP and reduce inflammatory cellular effects (Sinha et al., 1995). The inflamed mucosa in IBD patients has elevated levels of prostaglandin E2 and prostacyclin and therefore, administration of specific PDE inhibitors might lead to the strongest local effects in the gut. Consequently, although the extent of improvement with rectal roflumilast is less than that with oral roflumilast, but this improvement could suggest a potential role of rectal roflumilast as an add-on therapy for CD.

In the current study, roflumilast improved the histopathological and immunohistochemical changes induced by TNBS in rats. This agrees with a previous study which showed a close relation between crypt lesions and clinical activity of colitis (Cooper et al., 1993), but contradicts with another study which reported non-significant change on the histologic score with roflumilast (Rieder et al., 2013). The antigen KI-67 is a nuclear protein that is considered a marker of cellular proliferation. KI-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent in resting (quiescent) cells (G0) (Cuylen et al., 2016). In DSS-induced UC, epithelial apoptosis increased approximately 5-fold and the mitotic cells decreased about half-fold as compared to the controls. KI-67 immunohistochemistry showed that cells with cell cycle arrest at the G0 stage in the crypt increased approximately 2-fold as compared to the controls indicating decreased proliferation. This might lead to a breakdown of the epithelial barrier function, and thus facilitate the mucosal invasion of intraluminal microorganisms in DSS-induced colitis (Araki et al., 2010).

The colon of the second group (TNBS) showed desquamation of the colonocytes and destruction of some crypts, congested blood vessels were noticed with diffuse odema in the propria and submucosa, the goblet cells were increased and the crypts of liberkhum showed ulceration and accumulation of leucocytes.

These findings were supported by the results of (Cosnes, 2011, Danes, 2011 and De souza, 2016) which showed lesions of different size are simultaneously present. The mucosa may appear normal or may show multiple small (1–2 mm in size) punctiform, rounded nodules or superficial erosions known as ‘aphthoid lesions’. Over a period of time, the erosions become confluent and give rise to larger longitudinal ulcers, known as serpiginous ulcers.

the results of (Feller, 2007, Feng, 2017, Gajendran, 2018, Germia and Jewell, 2014 and Germia et al.,2012) showed ulcers at the base of crypts with neutrophils streaming into the bowel lumen, which leads in a later phase to mountain peak ulcers, villous abnormalities, and damage of small capillaries (including capillary thrombi) with subsequent loss of surface epithelial cells, these results were similar to our finding typically in group one (control positive group)

The lymphocytes were accumulated in the propria submucosa in the form of lymphoid nodules which obsecured the underlying structure and may lead to obstruction of the lymph vessels. These results were supported by the finding of (Van Kruiningen et al., 2014) which recorded a dense network of lymphocytes, histocytes, and macrophages within the lymphatic system results in the obstruction of regional lymphatics. This complex structure was observed in all layers of the intestinal wall in CD. Coincidently, transmural inflammation, multiple lymphoid aggregations in the submucosa, and beaded changes of the serosa occurred only where the lymphatics were located.

This suggests granulomatous lymphangitis as the underlying physiopathological mechanism of CD.

Our finding in third group showed (oral Roflumilast) aggregation of lymphocytes was located in the propria submucosa. Some colonocytes showed desquamation but were few compared to the control positive group.

Our finding in fourth group showed (rectal Roflumilast) congested blood vessels in the propria submucosa. Accumulation of lymphocytes was located in the propria submucosa and some large nodules were located in the mucosa-submucosa. Some crypts of liberkhum showed ulceration and accumulation of leucocytes.

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are aetiologically idiopathic, chronic, relapsing, and refractory inflammatory conditions that results from the interactions of gene susceptibility, environmental factors, disturbance of immune homeostasis, and microbiological anomaly in the gastrointestinal tract (Sartor, 2006).

TNBS instillation included focal necrosis of the mucosa, erosion, loss of goblet cells, and submucosal edema characterized by high level of inflammatory cell infiltration. The combination of A-PL and sulfasalazine afford protection against TNBS induced colonic damage (Yousefi et al., 2020).

Colonic inflammation involves the disruption of the apparatus of colonic mucosa and ulceration, resulting in the infiltration of inflammatory cells such as inflammatory monocytes and macrophages and thickening of the lamina propria (Buchheister et al.,2017).

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