Indian Journal of Experimental Biology
Vol. 49, September 2011, pp. 672-678

Effect of combination of thalidomide and sulfasalazine in experimentally induced inflammatory bowel disease in rats

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Received 26 November 2010; revised 12 May 2011

Thalidomide provided significant protection against tri nitro benzene sulfonic acid induced colitis. Combination therapy also reduced colonic inflammation and all the biochemical parameters (myeloperoxidase assay, malondialdehyde assay and tumor necrosis factor-α, estimation) were significant as compared to control as well as thalidomide alone treated group. Combination therapy showed additive effect of thalidomide which restored lipid peroxidation as well as reduced myeloperoxidase and TNF-α towards the normal levels. Morphological and histological scores were significantly reduced in combination groups. In experimental model of colitis, oral administration of thalidomide (150 mg/kg) alone as well as its combination with sulfasalazine (360 mg/kg) significantly reduced the colonic inflammation. The results indicate the additive effect of thalidomide with sulfasalazine in rat colitis model which requires further confirmation in human studies.

Keywords: Inflammatory bowel disease, Malondialdehyde, Myeloperoxidase, Sulfasalazine, Thalidomide, Tumor necrosis factor- α

Inflammatory bowel disease (IBD) is a gamut of chronic idiopathic inflammatory intestinal conditions and involves a group of inflammatory disorders of the colon and small intestine. Two major manifestations of IBD are: ulcerative colitis (UC) and Crohn's disease (CD). IBD is more common in European and North American countries than tropical African, South American and Asian countries. In Western countries the incidence is 11 cases per 1,00,000 of ulcerative colitis and 7 cases per 1,00,000 of Crohn's disease, and currently the incidence is estimated to be about equal. However, incidence in Indian population is less as compared to Western population, though at is on increasing in Asia[1-4].

IBD affecting mainly the gastrointestinal tract is of unknown etiology. Studies on etiopathogenesis of IBD and screening of potential therapeutic agents have been hampered by the paucity of reproducible and histopathologically relevant animal model of chronic inflammation. Although a variety of genetic, infectious, immunological, and psychological factors have been implicated in its causation one mechanism which is considered to play a crucial role is mediated by inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-8 (IL-8) which are secreted from macrophages[5]. IL-1 and TNF-α upregulate the adhesion molecules (B-selectin and ICAM-1). This causes the adherence of neutrophils to endothelium, helps in neutrophil recruitment at the site of inflammation and activates CD4 lymphocytes, which secrete IL-3 and IL-4 further activating the mast and plasma cells. These mast cells secrete platelet activating factor (PAF) and leukotrienes, which are further responsible for inflammation. The plasma cells secrete Immunoglobulins (Ig) G and E. IL-8 attracts the activated neutrophils thereby causing degranulation of the toxic proteases and other reactive oxygen species (ROS), which are cytotoxic and cause ulceration of intestine. Increased lipid peroxidation and neutrophil degranulation leading to release of malondialdehyde and myeloperoxidase are the other most common characteristics involved in the pathophysiology of IBD[5].

There are several options available for the symptomatic therapy of IBD[6], mainly including 5-aminosalicyclic acid (sulfasalazine), immunosuppressives (azathioprine, mercaptopurine, methotrexate), corticosteroids, antibiotics (metronidazole, ciprofloxacin), and IgG anti TNF-α

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antibody (infliximab). The current therapy that exerts its action mainly by dampening the generalized inflammatory response, is very limited and unpredictable[7-10].

Thalidomide was introduced in 1956 as a hypnotic drug and approved for treatment of erythema nodosum leprosum (ENL) in 1998. Thalidomide has anti-inflammatory and immunomodulatory properties[10], and suppresses the secretion of certain cytokines. It causes stimulation of cytotoxic T-cells with overall increase in the number of T- lymphocytes[10]. It downregulates the expression of surface adhesion molecules and major histocompatibility antigens on endothelial and epidermal cells and thereby causes reduction of leukocyte adherence[10-12]. Thalidomide also reduces the levels of TNF-α by accelerating the degradation of its mRNA. It indirectly influences the production of other lipopolysaccharide induced cytokines like IL-1, IL-6 or granulocyte monocytes colony stimulating factor[13]. Thalidomide is also beneficial in conditions where it is necessary to suppress TNF-α production. These findings project thalidomide as a promising drug for the treatment of IBD. So far no study has been conducted to evaluate the efficacy of combination of thalidomide with sulfasalazine in treatment of inflammatory bowel disease. Hence, the present study has been undertaken to evaluate the combined effect of thalidomide along with sulfasalazine in experimentally induced inflammatory bowel disease in rats.

Materials and Methods

Experimental animals—Adult Wistar rats of either sex weighing between 150-250 g were procured from the Institute's Central Animal House. The animals were housed in standard laboratory conditions at 25°C and 12:12h L:D cycle. Animals were kept in polycarbonate cage and had free access to rat chow diet and water *ad libitum.* Before conducting experiments, animals were acclimatized to laboratory conditions for 7 days.

Drugs and chemicals—Thalidomide (Dabur, Pharmaceuticals, Sahibabad, UP, India), 2,4,6-trinitrobenzene sulphonic acid (Sigma Chemicals, St. Louis, MO, USA), ethanol and sulfasalazine (Wallace Pharmaceuticals, Mumbai, India) were used. Rat TNF-α ELISA kit was purchased from (Diaclone, Besancon, France).

The study was conducted after obtaining approval from Institute's Animals Ethics Committee (IAEC), PGIMER, Chandigarh, India.

Induction of colitis—Single intra-colonic administration of 20 mg TNBS [dissolved in 35% ethanol; (v/v)] in the descending colon was used to induce colitis. Rats were subjected to light ether anesthesia and a rubber catheter lubricated with lignocaine jelly (OD, 2 mm) was inserted rectally into the colon through anus such that tip is 8 cm inside from anus, approximately at the splenic flexure. TNBS was instilled into the lumen of the colon through rubber catheter. The total volume was expelled with additional air and the catheter was removed. After completion of study period of two weeks rats were sacrificed by cervical dislocation under ether anaesthesia for assessment of various parameters[14]. The animals (30) were divided into following 5 groups of beach:

Group I: (vehicle i.e. ethanol treated group) in this group, 0.25 ml of 35% ethanol was given as enema to the rats after ether anesthesia as per the method described earlier.

Group II: (TNBS + ethanol), solution of TNBS (20 mg) sodium salt in 35% ethanol was delivered as enema (0.25 ml) to the rats after anaesthetizing with ether to induce colitis.

Group III: (TNBS + thalidomide), TNBS was delivered as per group II. Thalidomide (150 mg/kg)[30] was given, orally daily by intra-gastric tube for 2 weeks.

Group IV: (TNBS + sulfasalazine), TNBS was delivered as per group II. Sulphasalazine (360 mg/kg[30]) was given, orally daily by intra-gastric tube for 2 weeks.

Group V: (TNBS + thalidomide + sulfasalazine) TNBS was delivered as per group II. Combination of thalidomide (150 mg/kg) and sulfasalazine (360 mg/kg) was given orally daily by intra-gastric tube for 2 weeks.

Assessment of inflammation—The distal 10 cm of the colon was quickly excised, freed of adherent adipose tissue and incubated in Tris buffer for 30 min at 37°C in shaking water bath (1ml/100mg tissue). The colon was dissected longitudinally into three pieces for histological analysis, myeloperoxidase[16], malondialdehyde[17] assay and TNF-α estimation[18]. A gross inflammatory index was visually assessed for inflammation according to the following scores:

Enteritis gross morphology score[14]: 0: no inflammatory sign in the whole of 10 cm of intestine; 1: slight inflammation, slight redness, villi visible under 15-fold magnification; 2: intermediate

inflammation, discontinuous hyperaemia intermediate redness of mucosa; and 3: intensive inflammation, hyperemia, intensive redness of mucosa.

Histological analysis: The histopathological examination was done by using hematoxylin and eosin staining in a blinded fashion as described by Levine *et al*[15]. The tissue obtained were fixed in 10% buffer formalin. Histopathological analysis was done by following scores: 0: normal; 1: mild mixed infiltrates in the lamina propria; 2: focal superficial ulceration of the mucosa only, moderate cryptitis and crypt abscess; 3: deep ulceration penetrating colonic wall through mucosa till muscularis mucosa and severe inflammation; and 4: necrosis through large bowel wall.

Statistical analysis—The data are expressed as mean $\pm$ SD and median with interquartile range. One-way analysis of variance (ANOVA) followed by appropriate post hoc test (Bonferonni's test) for parametric value. Non-parametric values were compared using Kruskal Wallis test followed by Dunn's multiple comparison test, P <0.05 was considered as statistically significant.

Results

Effect of combination of thalidomide and sulphasalazine on myeloperoxidase, malondialdehyde activities and TNF-α levels are given in Table 1.

Histological examination—The histological examination of the colon tissue showed presence of inflammation in the mucosa. In Group I, there was no change at the cell level. Scanner view of the tissue showed intact mucosa, widened lamina propria and submucosa due to edema (Fig. 1). There were significant changes in group II (TNBS +35% ethanol). Plentiful neutrophils and eosinophils were visible in the submucosal region. There was also indication of lymphocytic infiltration into crypts and focal crypt loss. These findings indicated the moderate to severe inflammation of the colon, and so the histological score was very high compared to vehicle treated group (Fig. 2). In thalidomide treated group, microscopy revealed lesser infiltration of the neutrophils along with mild inflammation and edema (Fig. 3). The histological score was highly reduced in this group. The histological findings in group IV showed mild infiltration of neutrophils and edema of the submucosal region (Fig. 4). In this case, score was lower than group II. In group V, the score was reduced, however it was greater than group IV (Table 1, Fig. 5).

Discussion

TNBS induced colitis model in rats has been described by Morris and colleagues[19]. This experimental method mimics similar disease condition

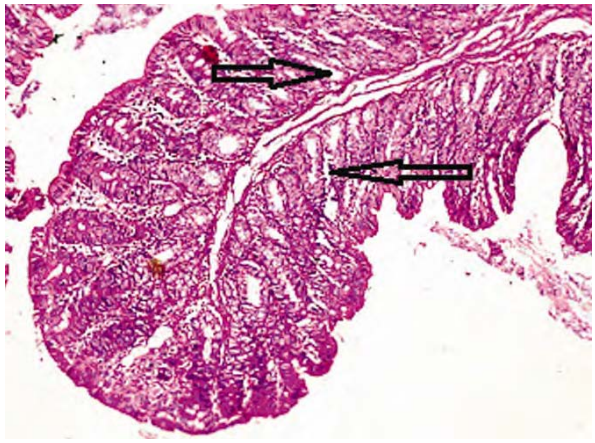


Fig. 1—Normal intact mucosa with minimal inflammation in lamina propria in vehicle treated group (Group I), (H & E ×140).

Table 1—Effect of combination of thalidomide and sulfasalazine on various parameters measured in rats. [Values are mean ± SE (parametric values) and median with interquartile range (non-parametric values)]. P values: [a]<0.05; [b]<0.01; [c]<0.001

Biochemical parameters	Vehicle (ethanol) Group I	TNBS Group II	TM (150 mg/kg) Group III	SSZ (360 mg/kg) Group V	TM (150 mg/kg) + SSZ (360 mg/kg) Group IV
MDA (nM/90min/mg protein)	0.90 ± 0.29	3.22 ± 0.559[c]	1.44 ±0.107 [c]	1.06 ± 0.091 [b]	0.89± 0.084[c]
MPO(µmol H_2O_2 consumed/min/mg protein)	0.50±0.123	3.05± 0.429 [c]	1.29±0.445[c]	0.79±0.074 [c]	0.65 ±0.252[c]
TNF-α (pg/ml)	0.042 ±0.017	0.118 ±0.010 [c]	0.060±0.016[c]	0.0780 ±0 .014 [c]	0.04 ±0.006[c]
MI (0-3)	0.50±0.548	2.00±.632[a]	0.00±0.00[b]	1.17± 0.408[b]	0.50±0.548[c]
HI (0-4)	0.50±0.548	2.83±.408 [a]	0.667±0.817[b]	1.500±0.837	1.00±0.894[b]

Values are compared between TNBS+ Ethanol group and the rest.
MI= Morphological index, HI= Histological index, TNBS= Trinitrobenzene sulphonic acid, TM= Thalidomide, SSZ= Sulphasalazine, F-One way ANOVA value, KW-Kruskal Wallis test value.
[c]Comparison of TNBS with vehicle control

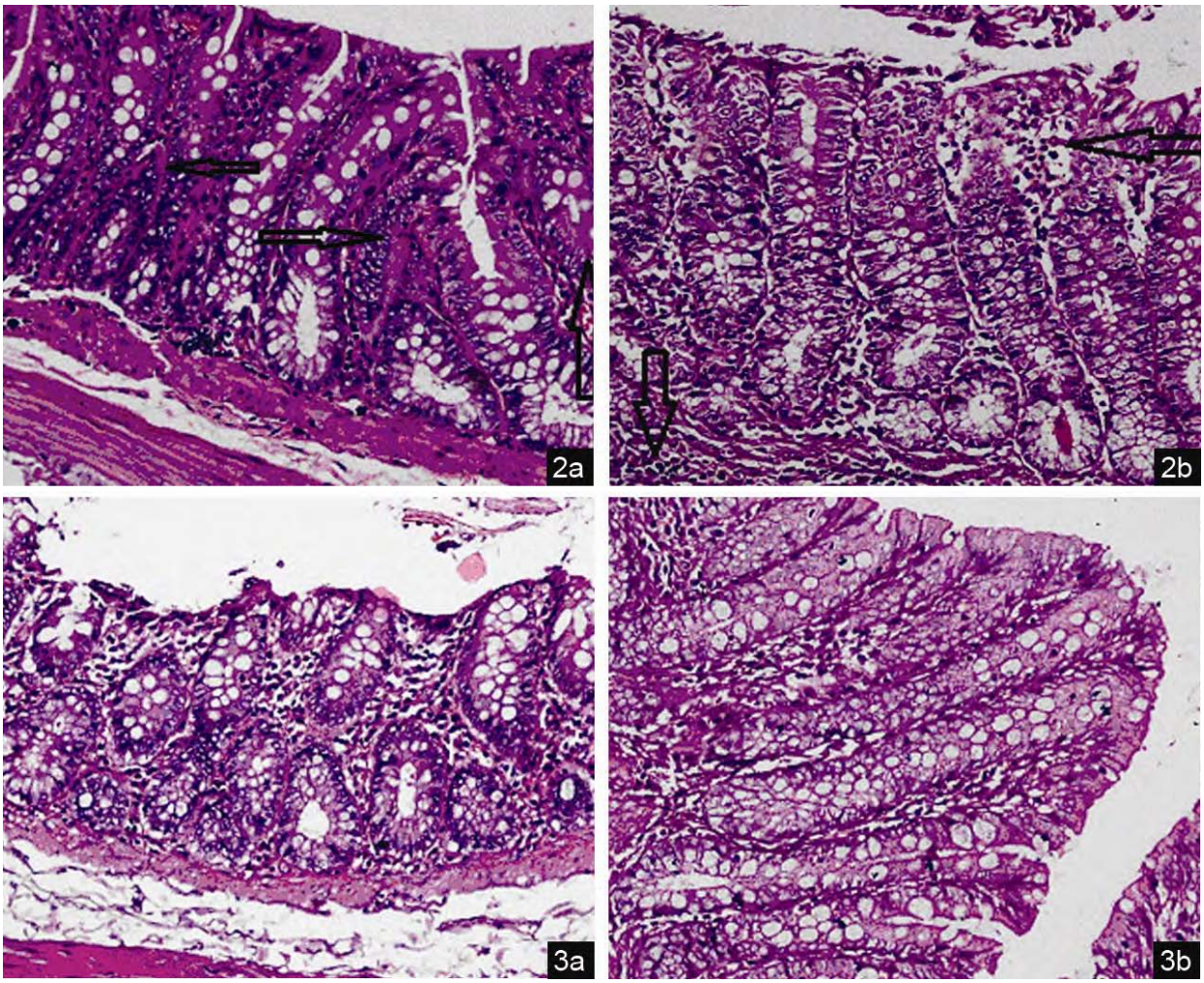
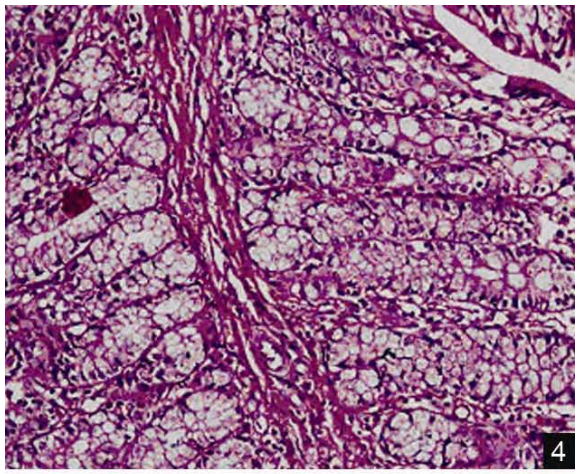


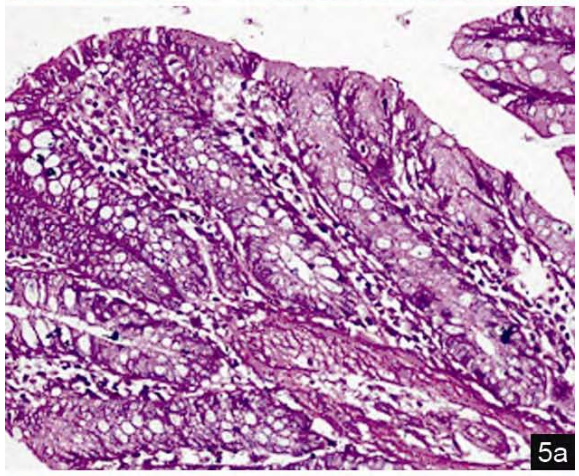
Fig. 2 (a)—Intact mucosal lining with moderately dense inflammation in Group II (TNBS), (H & E × 280), (b)—Lymphoplasmocytic infiltrates and mixed with neutrophils (H & E × 550).

Fig. 3 (a)—Intact mucosal lining epithelium with mild inflammation in lamina propria in Group III (TM, 150 mg/kg), (H & E × 280); (b)—Intact mucosal lining epithelium with mild inflammation in lamina propria in Group III (TM 150 mg /kg), (H & E × 550).

as evidenced in human IBD and thus is considered a well established model. Intracolonic administration of the TNBS results in long lasting chronic ulceration and inflammation of the rat colon. This damage was characterized by marked thickening of colonic wall, infiltration of polymorphonuclear leukocytes, granuloma formation. Ward[20] and Shorter *et al.*[21], proposed the hypothesis that chronic inflammation of intestine may occur as a consequence of increased permeability of mucosa to a luminal antigen which can be cleared by immune system. Following features of this model make it attractive for study of inflammatory bowel disease: (i) inflammation is induced by a single intraluminal administration without any previous sensitization of the animal or the surgery and the severity and persistence of the damage is very reproducible; (ii) animal used is rat, so the model is relatively inexpensive. (iii) inflammation produced by TNBS and 35% ethanol is long lasting with significant thickening of the colonic wall associated with cellular infiltration and ulcer persisting for long period. Long duration of inflammation provides a suitable period in which potential treatment can be assessed. This model also allows for the study of events characterizing the progression from acute to chronic inflammation, which histopathologically resembles the features of human inflammatory bowel disease, particularly Crohn's disease.

In TNBS challenged group, the infiltration of polymorphonuclear and mononuclear cells at the site of inflammation has been documented. The





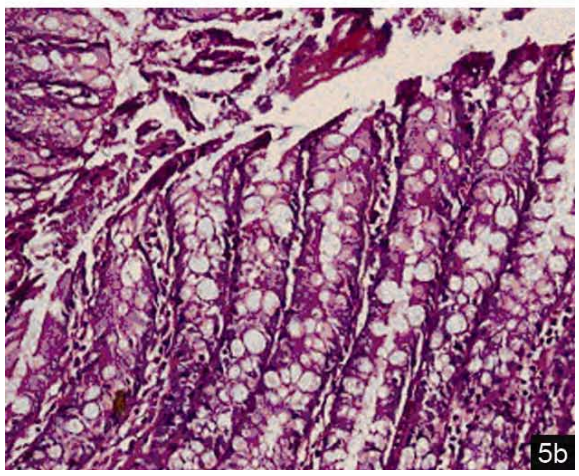


Fig. 4—Normal mucosal lining epithelium with mild edema and minimal inflammation in combination therapy in Group IV (TM 150+SSZ 360 mg/kg), (H & E × 280).

Fig. 5 (a)—Intact mucosal lining epithelium with mild to moderate inflammation in lamina rich in eosinophils in Group V (SSZ, 360 mg /kg), (H & E × 140); (b)—Intact mucosal lining epithelium with mild to moderate inflammation in lamina propria admixed with eosinophils in Group V (SSZ, 360mg/kg), (H & E × 280).

morphological changes included redness, edema, and distortion of mucosa and were similar to a previous study by Barbier *et al*.[22] and Joshua ka-Shun Ko *et al*[23]. Some animals also showed adhesions in TNBS challenged group than control group. The main pathological feature of IBD is infiltration of neutrophils and monocytes into the intestinal tissue. Neutrophils and monocytes migration is in turn triggered by chemo tactic bacterial cell wall product and locally produced cytokine[24,25]. The neutrophils generate reactive oxygen metabolites such as superoxide anions; hydrogen peroxide, N-chlorinated derivatives as well as the release of granule enzyme which are responsible for endothelial damage[26]. The histological findings also support the infiltration of neutrophils in the mucosal and submucosal layer. It was found that the morphological and histological scores were reduced by thalidomide and the combination therapy. This indicates that thalidomide acts through the effect on polymorphonuclear cell functioning as it reduces phagocytosis by inhibiting polymorphonuclear leukocytes and monocytes without causing any cytotoxicity[10, 27].

MPO is secreted by the neutrophils at the site of inflammation. Estimation of MPO activity is an important parameter which indicates neutrophil concentration and activity. Thus measurement of MPO activity correlates with the rate of neutrophils in the inflammation. Direct inhibitory effect of thalidomide on MPO activity and thus on the neutrophils recruitment at the site of inflammation has been reported[24]. However, the present results indicated more reduction in MPO level which shows decreased inflammation of intestine.

MDA level serves as a convenient index for determining the extent of the tissue damage via lipid peroxidation as its levels get increased at the site of tissue injury. In the present study, thalidomide significantly reduced the MDA levels in the intestinal tissue thereby decreasing the severity of the tissue damage which also directly correlated with the histopathological results and cytokine levels. However the MDA level was more reduced in the combination group.

TNF-α, a pro-inflammatory cytokine, is secreted by lymphocytes (T and B) and mast cells and plays a role in IBD[28]. During inflammation it gets over expressed. In the present study too, TNF-α was increased in TNBS treated group and was reduced in thalidomide and combination therapy groups, thereby confirming

the previous hypothesis regarding the role of TNF-α in IBD.

Experimental findings reveal that sulfasalazine also reduced these parameters but to a lesser extent as compared to the combination therapy, thus indicating the synergistic effect of sulphasalazine with thalidomide. However sulphasalazine reduces inflammation by different mechanism involving the inhibition of cyclooxygenase and 5-lipoxygenase pathway of arachidonic acid metabolism[29].

Conclusion

On the basis of present results, it can be concluded that thalidomide is significantly effective in reducing colonic inflammation. But the effects are much more pronounced when it is combined with sulfasalazine. However these findings require further confirmation through human trials before any clinical implementation.

Conflict of interest

The authors have no financial or proprietary interest in any of the products mentioned in this manuscript.

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