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Research Paper

Intestinal inflammation and seizure susceptibility: understanding the role of tumour necrosis factor-α in a rat model

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Abstract

Objectives The aim of the study was to evaluate the correlation between colitis and susceptibility to seizures.

Methods Colitis was induced in Wistar rats by a single intracolonic administration of trinitrobenzene sulfonic acid (TNBS; 20 mg in 35% ethanol). The control group were given intracolonic vehicle. One group of rats with colitis were treated with thalidomide (150 mg/kg p.o.) daily for 14 days. The other colitis group received vehicle only. On day 15, seizure susceptibility was tested by administration of pentylenetetrazole (40 mg/kg i.p.). Colonic tissue was collected for estimation of morphological score, and malondialdehyde, superoxide dismutase, catalase and glutathione peroxidase. Tumour necrosis factor (TNF)-α levels were measured in serum and brain samples.

Key findings The colitis group showed a significant increase in seizure score and reduction in onset time compared with the control group. Thalidomide was protective against seizures, resulting in decreased seizure score and significantly delaying the onset of seizures. Thalidomide also provided significant protection against TNBS-induced colonic damage in terms of morphological and histological score and levels of lipid peroxidation, superoxide dismutase, catalase and glutathione peroxidase in colonic tissue. The level of TNF-α in serum was also reduced significantly whereas brain TNF-α level was reduced but not significantly.

Conclusions TNBS-induced colitis increased seizure susceptibility to a subconvulsive dose of pentylenetetrazole; the immunomodulator thalidomide was protective.

Keywords colitis; seizure; thalidomide; TNBS

Introduction

Epilepsy affects more than 50 million people worldwide, 5 million of whom have seizures more than once a month. Recent reports suggest higher incidence of seizures among patients with chronic inflammatory problems compared with the normal population.[1,2]

Various studies indicate that cytokines and their receptors are distributed widely in the peripheral and central nervous systems, and their expression is influenced by changes in tissue homeostasis. These observations, together with numerous and varied demonstrations of the actions of cytokines on the nervous system or neurons *in vivo* and *in vitro*, indicate that cytokines have important roles in neurobiology.[3] Recent evidence has shown that inflammatory cytokines and their receptors are present in various forebrain areas, synthesised locally in both neurons and glia.[4]

The involvement of cytokines in the pathogenesis of epilepsy has recently been suggested by evidence that limbic seizures increase the mRNA of inflammatory cytokines in rodent forebrain.[5] The additional release of tumour necrosis factor (TNF)-α and interleukin (IL)-1β from rat hippocampal slices is enhanced by seizures, and increased IL-1 immunoreactivity has been found in tissues from human brains with epilepsy.[6]

Colitis is a chronic inflammatory condition of the colon in which there are increased levels of inflammatory cytokines such as TNF-α, IL-1β and IL-6. A recent study by Rao *et al.*[7] has shown that various inflammatory cytokines have roles in the propagation of seizures. Reports suggest that seizure susceptibility was increased by a subconvulsive dose

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of pentylenetetrazole (PTZ; 40 mg/kg, i.p) in rats with colitis, and this was reduced by treatment with thalidomide. Several other studies have shown that cytokines are involved in determining neural excitability. IL-1β and TNF-α content have been shown to be increased in whole brain tissue after rats were kindled by electrical stimulation of the amygdala.[8,9] In addition, intracerebral TNF-α administration increased the susceptibility of the amygdala to kindling, while both TNF-α and IL-1β intensified spike wave discharges in wag/rij rats.[10,11] Moreover, an increased level of IL-1β in brain occurs in other experimental epilepsy models, and blockers of the IL-1β receptor possess potent antiepileptic activity.

The present study was designed to evaluate the correlation between cytokines induced by intestinal inflammation and the induction of seizures, and the role of the cytokine antagonist thalidomide in reducing the pathological process. We have studied thalidomide in a previous study at doses of 50, 100 and 150 mg/kg i.p.[12]

The primary aim of the study was to determine a relationship between gut inflammation and seizure susceptibility to PTZ in a rat model. The secondary aim was to scrutinise the role of cytokines and the antagonist thalidomide in explaining such a relationship.

Materials and Methods

Experimental animals

Eighteen Wistar rats of either sex weighing 150–200 g were divided into three groups of six. The animals were housed in standard laboratory conditions at 23 ± 2°C and a 12 h light–dark cycle. Animals had free access to rat chow (M/s Ashirwad Industries; Punjab, India) and water. Before conducting experiments animals were acclimatised to laboratory conditions for 7 days.

The study was approved the by the Institute Animal Ethics Committee of the Postgraduate Institute of Medical Education and Research.

Drugs and chemicals

Glutathione reductase, NADPH and thiobarbituric acid (TBA) were purchased from Sigma Chemical (St Louis, MO, USA). Phosphate-buffered saline (PBS), Tris-HCl buffer and EDTA were purchased from Central Drug House (P) Ltd (New Delhi, India). Reduced glutathione, hydroxylamine hydrochloride, trichloroacetic acid and nitroblue tetrazolium (NBT) were from M/s Sisco Research Laboratories Pvt. Ltd (Mumbai, India).

Induction of colitis

Rats were placed under light ether anesthesia and a rubber catheter (outer diameter 2 mm) lubricated with lidocaine jelly inserted rectally into the colon so that the tip was 8 cm inside the anus, approximately at the splenic flexure. Trinitrobenzene sulfonic acid (TNBS; Sigma) dissolved in 35% ethanol (v/v) was then instilled into the lumen of the colon via the rubber catheter. The total volume (0.25 ml) was expelled with additional air and the catheter removed. The TNBS enema was kept at 37°C. Control rats were given intracolonic vehicle only (0.25 ml 35% ethanol).

Study design

The thalidomide group had colitis induced as above and were then given thalidomide (150 mg/kg p.o.) daily for 14 days. This dose of thalidomide, determined in a pilot study, protected against PTZ-induced seziures in 50% of animals (i.e. seizure score < 3). The colitis group had colitis induced as described above and were given the thalidomide vehicle daily. The vehicle control group were given intra-colonic vehicle on day 0 and were treated thereafter with normal saline (1 ml p.o.) for 14 days.

PTZ-induced seizure susceptibility was tested as described below on day 15 (i.e. 24 h after the last treatment). Blood was then collected by intra-cardiac puncture under ether anaesthesia. Rats were killed by cervical dislocation[3] and the colon removed for histopathological examination to confirm inflammation. Mucosal scrapings were taken from the distal part of the colon for measurement of antioxidant and lipid peroxidation parameters, as described below.

Induction of seizures

PTZ (40 mg/kg) was administered i.p. Rats were then placed individually in Perplex glass chambers and observed for 90 min for recording of seizure score, as follows: 0 = no response; 1 = ear and facial twitching; 2 = one or two myoclonic jerks; 3 = more than 20 body jerks in 10 min; 4 = clonic forelimb convulsions; 5 = generalised tonic convulsion with episodes of rearing and falling down; 6 = generalised convulsion with tonic extension episode and status epilepticus.[13] A score ≥ 3 was taken as a positive response.

Estimation of TNF-α

TNF-α in serum was estimated using an ELISA kit, following the manufacturer's protocol (Diaclone Pvt. Ltd, Besancon Cedex, France).

Measurement of superoxide dismutase

Superoxide dismutase (SOD) was estimated by the method of Kono *et al*.[14] This method is based on the principle of the inhibitory effect of SOD on reduction of NBT dye by superoxide anions, which are generated by photo-oxidation of hydroxylamine hydrochloride.

Catalase

The activity of catalase was measured by the method of Luck.[15] Briefly, the reaction mixture consisted of Tris (50 mmol/l)–EDTA (5 mol/l) buffer (pH 7.0) and 10 mmol/l H_2O_2 (in 0.1 mol/l KH_2PO_4 buffer, pH 7.0) in a test cuvette. The reference cuvette contained Tris–EDTA solution and distilled water only. The cuvettes were incubated at 37°C for 10 min and the reaction started by the addition of diluted post-mitochondrial supernatant (PMS; 0.05 ml 10%) to both cuvettes. The rate of elimination of H_2O_2 by catalase was measured by recording change of absorbance per min at 240 nm for 4 min. Catalase activity was expressed as μmol H_2O_2 consumed/min per mg protein, using a molar extinction coefficient of 43.6 mmol/l per cm.

Measurement of glutathione peroxidase

Glutathione peroxidase (GPx) was estimated by the method of Paglia and Valentine[16] using PMS of colonic mucosa as the enzyme source. Briefly, the reaction mixture in both reference and test cuvette contained 50 mol/l phosphate buffer (pH 7.0), 0.1 mmol/l EDTA, 0.37 mol/l sodium azide, 0.1 mol/l glutathione (GSH), glutathione reductase (2.4 units (10 μl) per assay) and 2 mmol/l NADPH. PMS was added to the test cuvette only. The cuvettes were incubated at 37°C for 10 min and the reaction started by adding 35 μl 2.2 mol/l H_2O_2. Enzyme activity was expressed as the amount of NADPH oxidised to $NADP^+$, using the extinction coefficient of 6.2×10^3 mol/l per cm at 340 nm.

Measurement of lipid peroxidation

Lipid peroxidation in tissue homogenates was estimated by the method of Ohkawa *et al.*[17] Briefly, the reaction mixture contained Tris-HCl buffer (50 mol/l, pH 7.4). Butyl hydroperoxide (500 μmol/l in ethanol) and 1 mol/l $FeSO_4$. Reference samples contained an equal volume of ethanol. Samples were then incubated at 37°C and the reaction stopped after 90 min by the addition of 2 ml 8.1% sodium dodecyl sulfate followed by 1.5 ml 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed was estimated by adding 1.5 ml 0.8% TBA and heating at 95°C for 45 min. After cooling, the samples were centrifuged and the level of thiobarbituric acid-reactive species (TBARS) in supernatants measured at 532 nm using an extinction coefficient of 1.53×10^5 mol/l per cm.

Assessment of intestinal inflammation

Inflammation was determined from the histopathological examination score as follows:[18] 0 = no inflammatory site along the entire 10 cm length; 1 = slight inflammation evident as slight redness and villi visible under 15-fold magnification; 2 = intermediate inflammation, discontinuous hyperaemia and intermediate redness of villi; 3 = intensive inflammation indicated by hyperaemia and intensive redness of the villi.

Statistical analysis

Data are presented as means ± SD. Data were analysed by one-way analysis of variance followed by Tukey's HSD test, performed using SPSS statistical software. The Kruskal–Wallis test was used for non-parametric data. $P < 0.05$ was considered statistically significant.

Results

Two animals (one in the control group and one in the colitis group) died during the experiment of unknown cause and were replaced. Weight loss was observed in the colitis group but not in the thalidomide or control group. Two rats in the control group lost weight but mean weight loss was not significantly altered. There were significant changes in intestine-to-body weight ratio in the colitis group (9.8%) but not in the control or thalidomide groups.

Effect on seizures

The colitis group showed a significant increase in seizure severity score compared with the control group (3.5 ± 0.55 vs 5.17 ± 0.75; $P < 0.001$) and this score was reduced significantly by thalidomide treatment (3.0 ± 0.9; $P < 0.05$ vs colitis group), as shown in Figure 1. Time to onset of seizure was also reduced by thalidomide (data not shown).

Effects on antioxidant enzymes in colonic mucosa

Increased oxidative stress was seen in the colitis group compared with the control group (2.53 ± 0.48 vs 7.088 ± 0.62 nmol TBARS formed/min per mg protein) and this was reduced significantly by thalidomide treatment. (5.35 ± 0.67 nmol TBARS formed/min per mg protein; $P < 0.05$), as shown in Figure 2a.

Intestinal catalase, superoxide dismutase and glutathione peroxidase

Catalase activity was significantly lower in the colitis group than the control group (1.02 ± 0.26 vs 2.12 ± 0.34 μmol H_2O_2 consumed/min per mg protein; $P < 0.001$). Thalidomide treatment significantly increased catalase compared with the control group (1.52 ± 0.69 μmol H_2O_2 consumed/min per mg protein; $P < 0.05$) (Figure 2b).

SOD level was significantly higher in the thalidomide group than the colitis group (3.1 ± 0.84 vs 4.9 ± 0.96 IU/mg protein); $P < 0.05$) but was lower than in the control group (6.3 ± 0.74 IU/mg protein; $P < 0.05$) (Figure 2c).

GPx was higher in the control group than the colitis group (0.53 ± 0.11 vs 0.23 ± 0.07 μg/mg protein). Thalidomide treatment increased GPx level about 1.5 fold to 0.34 ± 0.07 μg/mg protein; $P < 0.05$) (Figure 2d).

We showed in a preliminary study that thalidomide had no effect on different biochemical parameters assessed in control animals (data not shown).

Effect of thalidomide on TNF-α

Pro-inflammatory TNF-α levels were significantly increased in the serum (3-fold) and brain (2-fold) of the rats with colitis compared with control rats ($P < 0.05$; Figure 3). The serum

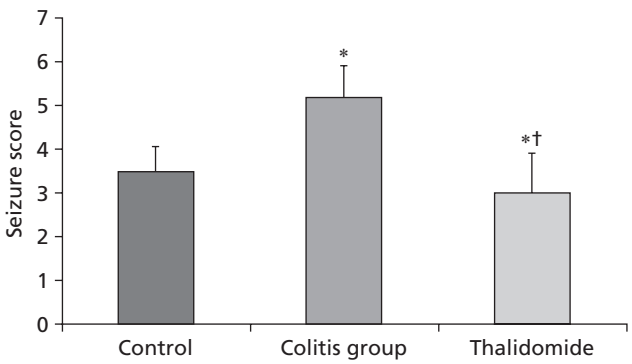

Figure 1 Effect of thalidomide on seizure susceptibility in rats with experimentally induced colitis. *$P < 0.001$ vs control; †$P < 0.05$ vs colitis group.

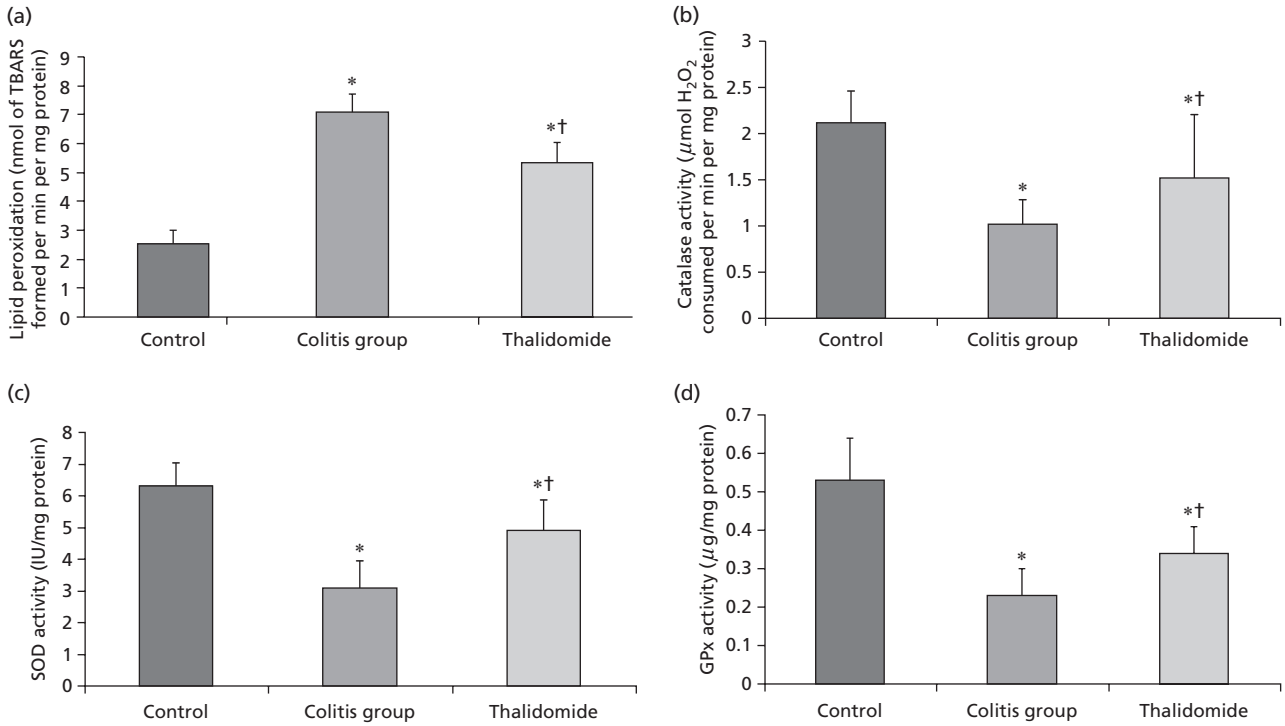

Figure 2 Effect of thalidomide on (a) lipid peroxidation (b) catalase (c) superoxide dismutase and (d) glutathione peroxidase in rats with experimentally induced colitis. GPx, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive species. *$P < 0.001$ vs control; †$P < 0.05$ vs colitis group.

TNF-α level was significantly decreased by thalidomide treatment compared with the colitis group ($P < 0.001$) but no statistically significant reduction was seen in brain TNF-α.

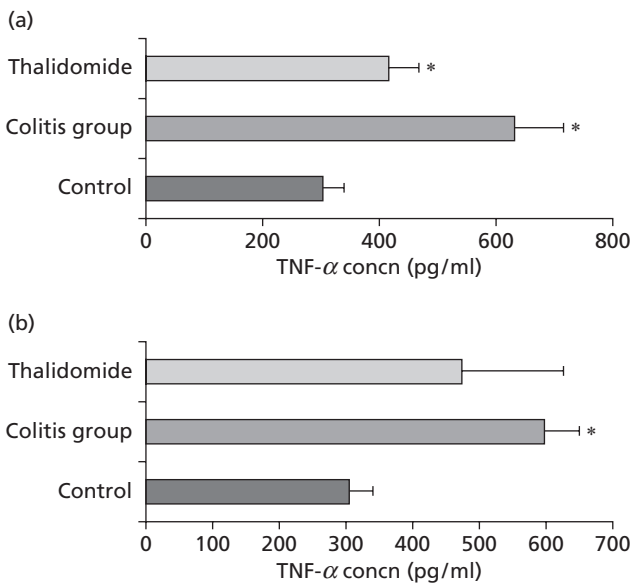

Figure 3 Effect of thalidomide on serum (a) and (b) brain levels of tumour necrosis factor-α in rats with experimentally induced colitis. TNF-α, tumour necrosis factor-α. *$P < 0.001$ vs control; †$P < 0.05$ vs colitis group.

Histopathological findings

Microscopic examination of the colon tissues revealed inflammation in the mucosa. The control group showed no changes at the cell level ($P < 0.001$). The tissue showed intact mucosa, and widened lamina propria and submucosa due to oedema (Figure 4a). The colitis group showed moderate-to-severe inflammation, indicated by plentiful neutrophils and eosinophils in the submucosal region and evidence of lymphocytic infiltration into crypts and focal crypt loss (Figure 4b). The thalidomide-treated group showed mild inflammation, with less infiltration by neutrophils and mild oedema (Figure 4c).

Discussion

The present study showed a link between the progression of colitis and seizure susceptibility. Levels of TNF-α were increased significantly by induction of colitis, and this was reduced by thalidomide treatment.

TNF-α activates pro-inflammatory signal transduction pathways in the rat hypothalamus. These signalling events lead to the transcriptional activation of an early responsive gene and induces expression of cytokines and cytokine responsive proteins such as IL-1β, IL-6, IL-10 and the suppressor of cytokine signalling-3.[13] In another study, a high concentration of mouse recombinant TNF-α (10 ng/ml) enhanced excitotoxicity when hippocampal slice cultures were simultaneously exposed to α-amino 3-hydroxy 5-methylisoxazole 4-propionic acid (AMPA) and TNF-α.

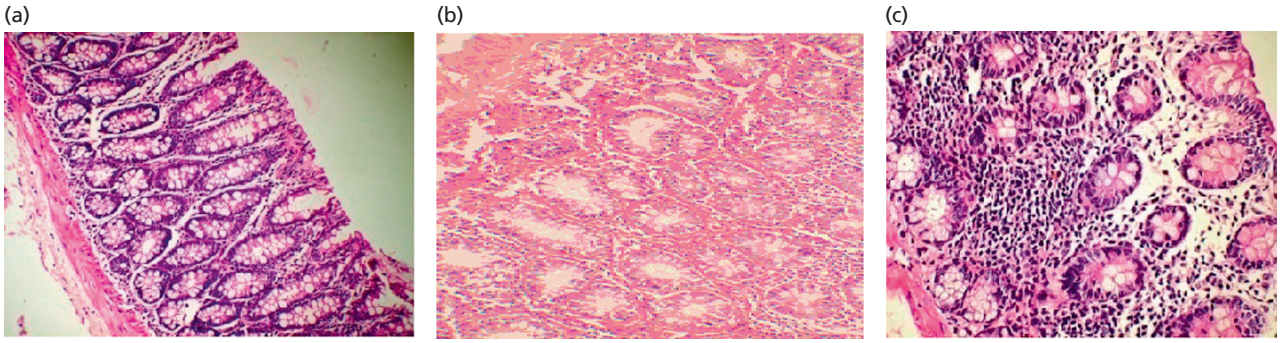

Figure 4 Histopathology of the colon tissue. Haematoxylin and eosin-stained microphotographs (×280) of (a) the vehicle-treated control group, showing normal intact mucosa with minimal inflammation in the lamina propria; (b) rats with experimentally induced colitis, showing an intact mucosal lining with moderate-to-severe inflammation, with neutrophils and eosinophils in the submucosal region; (c) thalidomide-treated rat, showing an intact mucosal lining with mild-to-moderate inflammation and a lamina rich in neutrophils.

Decreasing the concentration of TNF-α to 1 ng/ml resulted in neuroprotection against AMPA-induced neuronal death independently of the application protocol.[19] Increased brain levels of TNF-α resulted in significant inhibition of seizures in mice, and this action was mediated by neuronal p75 receptors. In the present study, the TNF-α level was significantly increased in blood and brain of the colitis group and was reduced by thalidomide treatment. Seizure scores were significantly higher in the colitis group than the control or thalidomide groups. Yuhas *et al.*[20] reported the involvement of IL-1 and TNF-α in the enhancement of PTZ-induced seizures caused by *Shigella dysenteriae*. TNF-α and IL-1β produced locally in the brain are also involved in sensitisation to PTZ-induced seizures. It has been reported that TNF-α and IL-1β mediate neurotoxicity synergistically through induction of nitric oxide (NO). NO also acts as a neurotransmitter, and its overproduction has been linked to induction of seizures.[21]

Several studies have reported relationships between brain and gut. Important findings suggest that colitis may increase permeability of the blood–brain barrier (BBB) and thus allow cytokines such as circulating TNF-α to cross the BBB by a receptor-mediated transport system.[22,23] Mayer[24] demonstrated that the central nervous system communicates with the intestine via the spinal cord, the dorsal root nuclei and intestinal neurons on the one hand and via neurohumoral or neuroendocrine systems on the other. Additional pathways of communication of signals of environmental stress are via the hypothalamic–pituitary axis or the sympathetic–adrenal medullary system.[25–28]

In addition, intracerebral administration of TNF-α was followed by increased susceptibility of the amygdala to kindling. Increases in levels of IL-1β have been shown in other experimental epilepsy models, and blockers of the IL-1β receptor have potent antiepileptic activity.[29,30] There are time-dependent and cell- and region-specific changes in the expression of IL-1 receptor type I during status epilepticus. IL-1 receptor type I in neurons mediates interleukin-1β-induced fast changes in hippocampal excitability while IL-1 receptor type I in astrocytes mediates IL-1β effects on neuronal survival in hostile conditions.[31]

In the present study, thalidomide reduced the level of TNF-α significantly in the serum and non-significantly in brain. Furthermore, biochemical parameters such as MDA level were increased in the colonic tissue of rats with experimentally induced colitis. Thalidomide significantly reduced MDA levels and hence ameliorated the tissue damage. This inhibition of MDA generation and lipid peroxidation may help to decrease tissue damage and thus the signs of inflammation.[13,31–33]

The main pathological feature of inflammatory bowel disease is an infiltration of polymorphonuclear neutrophils and mononuclear cells into the colonic tissue. Neutrophil and monocyte migration is triggered by chemotactic bacterial cell wall products and locally produced cytokines.[34] In addition, free radical chain reactions are aggravated by oxidative stress and subsequent lipid peroxidation, which may disrupt the integrity of the colonic mucosal barrier and activate inflammatory mediators. Evidence has shown that colonic MDA content is increased and colonic SOD levels decreased in both human and experimental animal studies.[33,35] In the present study, we showed that thalidomide administration significantly reduced the MDA level and increased levels of SOD, catalase and GPx, which ameliorated colon inflammation.

Conclusions

We have shown increased levels of the cytokine TNF-α following TNBS-induced colitis and an increase in seizure susceptibility, both of which were reduced by treatment with thalidomide.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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