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THE ROLE OF THE DELTA OPIOID RECEPTOR IN TWO MURINE MODELS OF COLITIS

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ABSTRACT

Crohn's disease (CD) and ulcerative colitis (UC) are classified as chronic inflammatory disorders of the gastrointestinal tract, and are classically termed inflammatory bowel disease. Currently, there is no known cause or medical cure of this often times debilitating disease and the reported estimated annual cost for medical treatment within the United States is $2 billion. In this study, we investigated the effects of a naltrexone (NTX), a nonselective opioid receptor antagonist, in two murine models of colitis. We compared the anti-inflammatory effects of high dose NTX (1.0 mg/kg) to that of the low dose NTX (0.1 mg/kg) therapy administered subcutaneously (SQ). We also compared the specific opioid delta receptor antagonists, naltrindole (NTI), a delta-1 receptor antagonist, and 7-benzylidenenaltrexone (BNTX), a delta 2-receptor antagonist, in order to study how selective blockade of the delta receptor affects colonic inflammation. A small subset of mice were used to examine whether inflammation was influenced by agonist effects of the delta receptor, and this was achieved by administering [D-Pen2, 5] - Enkephalin hydrate (DPDPE) SQ. For this purpose, male C57BL/6NCrl mice (15 to 20 g; age 6 to 8 wk.; n=110) and female Balb/cAnNCrl mice (15 to 20 g; 6-8 wk.; n=115) were used for the Dextran Sodium Sulfate (DSS) model (that mimics ulcerative colitis) as well as the 2, 4, 6-Trinitrobenzenesulfonic acid (TNBS) model (that mimics Crohn's disease) respectively. Disease activity indices (DAIs) were used to quantitatively measure colonic symptoms and overall illness. We found that administration with NTX (0.1mg/kg, 1.0 mg/kg) significantly improved overall scores in the DSS model of colitis on days 4 and 5 of therapy. The volume percentage of red blood cells within the blood, also known as hematocrit (HCT), is a good indicator when determining whether anemia or dehydration is present. BNTX and NTX therapy (0.1mg/kg, 1.0 mg/kg) were shown to return HCT values closer to normal values. Therapy with NTX (0.1mg/kg) demonstrated improved colon length, which in active cases of colitis would otherwise be shortened. Microscopic

evaluation is a good diagnostic tool to accurately assess active or chronic inflammatory conditions. Treatment with NTX (1.0 mg/kg) significantly reduced histologic indices. In the TNBS model, low dose NTX significantly improved HCT whereas therapy with high dose NTX significantly reduced macroscopic ulcers. Based on the current findings, therapy with NTX exhibited some therapeutic effects in two murine models of colitis, whereas delta agonists and antagonists had limited efficacy.

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ABBREVIATIONS

µL	Microliter
BNTX	7-Benzylidenenaltrexone
CD	Crohn's disease
DAI	Disease activity index
DPDPE	[D-Pen2, 5] - Enkephalin hydrate
DSS	Dextran Sodium Sulfate
gm	Grams
HCT	Hematocrit
H&E	Hematoxylin & eosin
IBD	Inflammatory bowel disease
kg	Kilograms
mL	Milliliter
MPO	Myeloperoxidase
NTI	Naltrindole
NTX	Naltrexone
SQ	Subcutaneous
TNBS	2, 4, 6-Trinitrobenzenesulfonic acid
UC	Ulcerative colitis
WBC	White blood cell

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Philippians 3:14 states “I press on toward the goal to win the prize for which God has called me heavenward in Christ Jesus” and Philippians 4:13 states “I can do all things through Christ who strengthens me”. These passages have been recited numerous times throughout my journey here at Penn State Hershey College of Medicine. I truly believe that this experience would not have been possible without the help from my Lord and Savior Jesus Christ.

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Chapter 1

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) together are classically referred to as inflammatory bowel disease (IBD), a condition that can lead to long term and sometimes irreversible impairment of gastrointestinal function and structure (Bouma & Strober, 2003). Although the etiology of IBD is unknown, research suggests environment, genetics, immune, non- immune, and microbial factors play a role in the onset of disease (Leone, Chang, & Devkota, 2013). While there are many similar characteristics between CD and UC, there are also marked differences in the pathologic process and features as well (Beaugerie & Sokol, 2012). The terminal ileum, cecum, peri-anal, and colon are sites often affected in CD, whereas, the inflammatory process of UC involves the rectum and extends proximally in a continuous fashion, while remaining restricted to the colon. Histologically, trans-mural inflammation with the presence of granulomas, fissuring ulceration, and fibrosis are seen in CD (Bouma & Strober, 2003). On the other hand, UC tends to affect the superficial mucosal layers with infiltration of mononuclear and polymorph nuclear leukocytes. Ulcerations and crypt abscesses are also identified on microscopic evaluation. Lastly, the clinical features and complications of these disease processes also vary. Individuals with CD often experience diarrhea, abdominal pain, strictures and bowel obstruction, abscess formation, and fistula formation to the skin and internal organs. UC patients develop severe diarrhea, anemia due to blood loss, mega-colon, and perforation of the colon. Patients with chronic UC and CD have an increased risk of developing colorectal cancer and patients with small intestinal CD are at increased risk of small bowel adenocarcinoma (Triantafillidis, Nasioulas, & Kosmidis, 2009).

Both CD and UC have a prevalence range of 100 - 200 cases per 100,000 individuals in Europe and North America (Economou & Pappas, 2008). Kaplan *et al.* reported in 2005 that the mean annual costs for CD and UC patients were $8265 and $5066 respectively. In 2007, IBD nearly accounted for $2 billion in annual medical costs in the United States (Loftus & Sandborn, 2002). Despite advances in treatments, there still remains a need for safe, well-tolerated therapeutics that have a rapid onset and function in maintaining long term remission (Perše & Cerar, 2012). Current therapies include compounds that reduce the inflammatory response, such as 5-aminosalicylate compounds, corticosteroids, immune modulators (i.e. azathioprine, methotrexate, cyclosporine), and immunotherapies (i.e. anti-TNF-α antibodies) (Bandzar, Gupta, & Platt, 2013; Baumgart & Sandborn, 2012). In recent years, accumulating evidence has pointed to the roles of endogenous opioids and opioid receptors in modulating immune responses (Matters et al., 2008; Smith et al., 2007, 2011; Zagon, 2011). Three classic and distinct opioid receptors exist (i.e. δ, κ, and μ) of variable densities in various tissues within the body and have differences in the selectivity and affinity of opioid drugs (Institute for Laboratory Animal Research, 2009; McDonald, 2005; Koneru *et al.*, 2009). A nonclassical opioid receptor has also been described (Donahue, McLaughlin, & Zagon, 2011) that is nuclear membrane bound and termed the opioid growth factor receptor (OGFr). An oral non-selective opioid receptor antagonist that blocks all four opioid receptors, Naltrexone (NTX), has been shown to significantly improve inflammatory scores in the Dextran Sodium Sulfate (DSS) animal model of IBD (Matters et al., 2008) and in human subjects with Crohn's disease (Smith et al., 2007). NTX therapy also lowered plasma inflammation markers and promoted mucosal healing in adult patients with active Crohn's disease (Smith et al., 2011).

Since delta opioid receptors are the predominant receptor subtype associated with inflammatory cells (van Rijn, Defriel, & Whistler, 2013), in the current study it was hypothesized that the mechanism by which NTX reduced colonic inflammation in IBD was mediated via

blockade at the delta receptor. In order to test this hypothesis, two delta receptor selective antagonists [naltrindole (NTI), a delta-1 receptor antagonist and 7-benzylidenenaltrexone (BNTX), a delta-2 receptor antagonist], were therapeutically administered to animals with chemically-induced inflammatory bowel disease. Control animals received no therapy or diluent in the absence of experimentally-induced colitis. A small subset of mice were treated with a delta receptor agonist, [D-Pen2, 5] - Enkephalin hydrate (DPDPE) to evaluate if the inflammatory response would be abolished. Murine animal models of both UC (DSS induced colitis) and CD (TNBS induced colitis) were utilized. The primary outcome of this investigation was to assess improvement of intestinal inflammation by histology. Secondary outcomes included reduction in intestinal inflammation as assessed by myeloperoxidase (MPO) activity in colonic tissue, disease activity index score, hematological analyses, and gross examination of the large intestine.

The results from these studies should help elucidate whether NTX reverses intestinal inflammation through the delta opioid receptor or another mechanism, as well as determining the utility of drugs selectively interacting with the delta opioid receptor for the treatment of IBD. Furthermore, these studies will examine for the first time, the role of opioid agents in an animal model of CD.

Chapter 2

Materials and Methods

Animals

Male C57BL/6NCrl mice (15 to 20 g; age 6 to 8 wk.; *n*=110) and female Balb/cAnNCrl mice (15 to 20 g; 6-8 wk.; *n*=115) were used in this study and were obtained from Charles River Laboratories (Wilmington, MA). The male mice were individually housed in polycarbonate cages on corncob bedding (7092, Harlan Teklad, Madison, WI) with *ad lib* access to irradiated rodent chow (2918, Harlan Teklad, Madison, WI) and tap water. The female mice were socially housed (5 mice/cage) under the same conditions as stated above. The animal facility was programmed with a 12:12-h light: dark cycle. Room temperature was maintained at 20 ± 2ºC and air humidity was within the range of 30 to 60%. Cages were either placed on shelves under static conditions with microfilter lids or on a ventilated rack at 12 air changes hourly (Allentown Caging Equipment, Allentown, NJ). Environmental enrichment was provided in the form of nesting material or colored plastic tubes. Mice were allowed 1 week acclimation prior to any experimental manipulations. According to vendor health reports, mice were free of mouse hepatitis virus, mouse minute virus, mouse parvovirus, mouse rotavirus, encephalomyelitis virus, pneumonia virus of mice, Sendai virus, lymphocytic choriomeningitis virus, murine norovirus, ectromelia virus, Hantaan virus, mouse adenovirus types 1 and 2, mouse cytomegalovirus, respiratory enteric virus III, K virus, lactate dehydrogenase elevating virus, polyoma virus, thymic virus, β-hemolytic *Streptococcus* spp., *Bordetella bronchiseptica, Citrobacter rodentium*, C*lostridium piliforme*, *Corynebacterium kutscheri, Klebsiella oxytoca*, *Klebsiella pneumonia*, *Mycoplasma* spp., *Pasteruella pneumotropica*, other *Pasteruella* spp., *Pseudomonas aeruginosa*,

Salmonella spp., *Staphylococcus aureus*, *Streptococcus pneumonia*, cilia-associated respiratory bacillus, *Helicobacter hepaticus*, *H. bilis*, *Pneumocystis carinii*, endo-and ectoparasites, enteric protozoa, and *Encephalitozoan cuniculi*. All experiments were conducted in accordance with institutional guidelines, the *Guide* For The Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 2011) and approved by the Penn State College of Medicine Institutional Animal Care and Uses Committee.

Chemicals

DSS (molecular weight 36,000-50,000) was obtained from MP Biomedicals (Solon, OH). TNBS was obtained from Sigma-Aldrich (St. Louis, MO). Key reagents for the MPO assay were (3, 3', 5, 5' tetramethylbenzidine [TMB], hexadecyltrimethylammonium bromide [HTAB], N, N dimethylformamide, and hydrogen peroxide) were obtained from Sigma-Aldrich (St. Louis, MO). NTX, NTI, BNTX, and DPDPE were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in filtered tap water using a 0.2 µm PES Bottle Top Filtration system from VWR (Radnor, PA). TNBS was dissolved in ethanol (50% w/vol).

DSS Induced Colitis

Part 1. C57BL/6NCrl mice were randomized by weight to receive either DSS in filtered drinking water (n=30) or filtered tap water (n=30). The acute model of DSS induced colitis was modified from Okayasu *et al*. In brief, DSS was prepared fresh daily and added to the drinking water at a final concentration of 2%. The DSS treatment was continued for 7 days and water intake measured daily for both treatment groups. Mice were subdivided into three treatment groups (n=10) and given a subcutaneously (SQ) injection (27g x ½", Cardinal Health, McGraw

Park, IL) of NTX [0.1 mg/kg (low-dose) or 1.0 mg/kg(high-dose)] or sterile saline in order to assess dose dependent effects. The control groups received DSS only (colitis control) or filtered water only (no colitis control). The dose volumes for saline and NTX treatments were 5 ml/kg. The injections were started on the first day of DSS administration and were administered once daily (by the same person and between the hours or 08:00 to 09:00) until study endpoint. Table 1 outlines the treatment and doses administered for each group. A disease activity index (DAI) score was calculated for each mouse based on the criteria outlined by Murthy et al., 1993 (Table 1) using percent weight change, stool occult blood, and stool consistency. The presence of occult blood was determined by a hemoccult fecal blood kit (Beckman-Coulter, Fullerton, CA). The animals were monitored daily for decreased body weight and evidence of morbidity or mortality. Mice were euthanized on day 7 by carbon dioxide inhalation. Cardiocentesis was performed and blood samples were submitted for a complete blood count (CBC) analysis. Reference ranges were used according Fox *et al*, 2002.

Part II. C57BL/6NCrl mice were randomized by weight to receive either DSS in filtered drinking water (n=40) or filtered tap water (n=10) as described above. Mice (n=10/group) were given a once daily SQ injection (27g x ½", Cardinal Health, McGraw Park, IL) of NTX, NTI, and BNTX (0.1 mg/kg) or sterile saline for 7 days. The control groups were similar to that as stated above. The dose volumes for saline and NTX, NTI, and BNTX treatments were 5 ml/kg. DAIs were calculated and assessed as described above. Mice were euthanized on day 7 by carbon dioxide inhalation. Cardiocentesis was performed and blood samples were submitted for CBC analysis. Reference ranges were used according to Fox *et al*, 2002.

Table 1. Treatments and doses administered for DSS colitis

Group	Therapy (mg/kg)	Water	N
No colitis control	Saline	Untreated	20
Opioid receptor antagonist	NTX 1.0	Untreated	10
Opioid receptor antagonist	NTX 0.1	Untreated	10
Colitis control	Saline	2% DSS	20
Opioid receptor antagonist	NTX 1.0	2% DSS	10
Opioid receptor antagonist	NTX 0.1	2% DSS	20
δ -2 antagonist	BNTX 0.1	2% DSS	10
δ -1 antagonist	NTI 0.1	2% DSS	10

δ=delta

Table 2. Criteria for scoring disease activity index

Score	Weight loss (%)	Stool consistency	Occult blood or gross bleeding
0	None	Normal	Negative
1	1-5	Loose stool	Negative
2	5-10	Loose stool	Hemoccult positive
3	10-15	Diarrhea	Hemoccult positive
4	>15	Diarrhea	Gross bleeding

DAI was calculated by combining score of weight loss (from baseline), stool consistency, and occult or gross bleeding/3

TNBS Induced Colitis

Part I. Balb/cAnNCrl mice (*n*=60) were randomized by weight into groups receiving intracolonic administration of TNBS (*n*=30), PBS (*n*=25), or 50% ethanol (*n*=5). Balb/c mice were chosen due to their susceptibility to TNBS induced colitis as opposed to C57BL/6 mice, which are resistant (Scheiffele, 2002). Mice were subdivided into three treatment groups and given a SQ injection (27g x ½", Cardinal Health, McGraw Park, IL) of NTX (0.1 mg/kg or 1.0 mg/kg; *n*=20/ group) or sterile saline (*n*=20) one hour prior to enema administration. The dose volumes for saline and NTX were 5 ml/kg. Table 3 outlines the treatment and doses administered for each group. The acute model of TNBS induced colitis was modified from Scheiffele, 2002 and Fitzpatrick *et al.*, 2010 . In brief, mice were fasted overnight (total of 10 hours) and the following day, received a single 40 µL dose per rectum of TNBS (20 mg/kg in 50% ethanol/PBS) under isoflurane anesthesia (3-4%) for 5 minutes. The enema was administered 4cm into the colon using a 100 µL Hamilton syringe (Fischer Scientific; Waltham, MA) attached to a 20g x 1.5" gavage needle (Fischer Scientific; Waltham, MA). Mice were allowed access to *ad lib* feed and water upon recovery from anesthesia. The animals were monitored daily for decreased body weight and evidence of morbidity or mortality. Mice were euthanized on day 6 by carbon dioxide inhalation. Cardiocentesis was performed and blood samples were submitted for CBC analysis. Reference ranges were used according to Fox *et al*, 2002.

Part II. Balb/cAnNCrl mice (*n*=55) were randomized by weight into treatment groups receiving intracolonic administration of TNBS (*n*=33), PBS (*n*=17), or 50% ethanol (*n*=5). Mice (n=5/group) were given a once daily SQ injection of NTX, NTI, and BNTX (0.1 mg/kg) or sterile saline (*n*=16). To further examine the role of the delta receptor, a delta agonist, DPDPE, was used. Mice were given either 0.1 mg/kg or 1 mg/kg SQ injection (*n*=24) in the colitis and no colitis groups. Dose volumes for saline and all administered drugs was 5 ml/kg. Enema procedure

was performed as stated above. Mice were allowed access to *ad lib* feed and water upon recovery from anesthesia. Mice were euthanized on day 6 by carbon dioxide inhalation. Cardiocentesis was performed and blood samples were submitted for CBC analysis. Reference ranges were used according to Fox *et al*, 2002.

Table 3. Treatments and doses administered for TNBS colitis

Group	Therapy (mg/kg)	Enema	N
Vehicle	Saline	EtOH	10
No colitis control	Saline	PBS	10
Opioid receptor antagonist	NTX 1.0	PBS	10
δ agonist	DPDPE 1.0	PBS	6
Opioid receptor antagonist	NTX 0.1	PBS	10
δ agonist	DPDPE 0.1	PBS	6
Colitis control	Saline	TNBS	16
Opioid receptor antagonist	NTX 1.0	TNBS	10
δ agonist	DPDPE 1.0	TNBS	6
Opioid receptor antagonist	NTX 0.1	TNBS	15
δ agonist	DPDPE 0.1	TNBS	6
δ -2 antagonist	BNTX 0.1	TNBS	5
δ -1 antagonist	NTI 0.1	TNBS	5

δ=delta

Macroscopic Colonic Evaluation

On the day mice from the TNBS groups were euthanized, the colons were transected from the rectum, and tissues rinsed in PBS. Proximal and distal sections were evaluated for macroscopic changes according to the method of (Reuter, Asfaha, Buret, Sharkey, & Wallace, 1996). In brief, the scoring system evaluated a combination of various features, such as, ulcers, adhesions, evidence of diarrhea, and colonic thickness. Table 4 provides the subjective criteria for this scoring method.

Table 4. Criteria for the assessment of macroscopic colonic damage

Feature	**Score**
Ulceration:	
Normal appearance	0
Focal hyperemia, no ulcers	1
Ulceration without hyperemia or bowel wall thickening	2
Ulceration with inflammation at one site	3
Ulceration/inflammation at two or more sites	4
Major sites of damage extending >1cm along length of colon	5
When damage extend >2 cm along colon, score increased by 1 for each additional cm involved	6-10
Adhesions:	
None	0
Minor (colon can be separated from other tissue with litle effort)	1
Major	2
Diarrhea:	
No	0
Yes	1
Thickness:	
Maximal bowel wall thickness (x), in millimters	

Total score = (ulceration + adhesion + diarrhea) (thickness)

Colonic Histopathologic Evaluation

At necropsy, the entire colon was excised from the cecocolic junction to the anus and the colon length was measured as in indirect marker of inflammation. Only 5cm of measured colon was weighed and sections were then longitudinally bisected into proximal and distal portions for the TNBS study. The remaining section was placed in liquid nitrogen and stored in a -80ºC freezer for myeloperoxidase (MPO) assay. For the DSS study, a 2.5cm representative section of colon was examine histologically. The remaining section was used for the MPO assay. For both colitis models, colons were swiss rolled according to the technique by Moolenbeek & Ruitenberg, 1981. Histologic specimens were fixed in 10% neutral buffered formalin, paraffin embedded, and sectioned for hematoxylin and eosin (H&E) staining. Colon sections were microscopically evaluated and blindly scored by a board certified veterinary pathologist. Severity of inflammation, ulceration, percent area involved, and hyperplasia/dysplasia were determined on a 11 point scale as described by Ju et al., 2009b (Table 5).

Table 5. Microscopic evaluation for DSS and TNBS induced colitis

Feature	Description	Score
Inflammation Severity	Normal colonic mucosa	0
	Mild: either focal or widely separated multifocal inflammation limited to basal 1/3 of mucosa with loss of crypts	1
	Moderate: either multifocal of locally extensive and/or fibrosis up to 2/3 of the crypts	2
	Severe: mucosal ulcers with monocytes and polymorphonuclear leukocytes infiltrated into the mucosa, submucosa, muscularis propria and/or subserosa	3
Ulceration	Absent	0
	Present	1
Inflammation Area	0%	0
	1-25%	1
	26-50%	2
	51-75%	3
	76-100%	4
Hyperplasia/Dysplasia	Normal	0
	Mild: epithelial cells lined normally, but crypts 2 to 4 times thicker than normal crypts	1
	Low-grade: 2 to 4 times thicker epithelium, hyperchromatic cells, few goblet cells, and scattered crypts developing an arborizing pattern	2
	High-grade: >4 times thicker epithelium, hyperchromasia, few to no goblet cells, high mitotic index in the crypts with arborizing pattern, and crypts extended to muscularis mucosa or submucosa	3

The inflammation index was the sum of scores of four individual inflammatory parameters: inflammation severity, ulceration, inflammation area involved, and hyperplasia and dysplasia

MPO Assay

In order to assess granulocytic infiltration and to quantify MPO activity, a 2.5cm segment of colon was processed as previously described and modified from Fitzpatrick, Wang, & Le, 2000. Briefly, tissues were homogenized in HTAB buffer and the homogenate centrifuged at 10,000 rpm for 15 minutes at 4ºC. The pellet was retained for MPO measurement using the TMB substrate method. Absorbance was measured at 655 nm on a spectrophotometer. All measurements were performed in triplicate. One unit of MPO activity was defined as the amount that caused a change in absorbance of 1.0/min at 37°C (Fitzpatrick et al., 2000). MPO activity is expressed as units per gram of tissue.

Statistical Analysis

Statistical analysis of the colon lengths and weights, MPO, DAIs, hematology values, macroscopic damage, and histology of colon sections were analyzed by using a two sample Wilcox rank sum test (SAS; version 9.3; Cary, NC). DAIs for both colitis studies and weight change across each time point in the TNBS colitis groups were analyzed using the generalized estimating equation model. Statistical significance was defined as a p value of less than or equal 0.05. For each group of mice, data is presented as mean ± SD.

Chapter 3

Results

Colon Length

In active cases of IBD, the length of the colon will be shortened. In the current study, the length of colon significantly differed between the colitis control and no colitis control treatment groups in the DSS model of colitis ($p<0.05$). Administration of NTX (0.1 mg/kg) for 7 days significantly increased colon length towards the normal length ($p=0.02$) (Figures 1A and 1B). In the TNBS colitis model, no differences were observed between treatment or control groups (Figures 2A and 2B).

Colon Weight

Due to the severity of inflammation and associated submucosal edema and/or fibrosis, the weight of the colon may be markedly increased in active cases of colitis. We found that the control groups differed significantly from each other in the DSS model ($p<0.05$) (Figures 3A and 3B). Although there was a decrease in weight between the low and high dose NTX groups, this did not reach significance (Figure 3A). The low dose NTX treatment group differed significantly from the colitis control group ($p=0.05$) in the TNBS colitis model (Figures 4A and 4B), but actually increased the colonic weight suggesting worsening of edema, ulceration and/or fibrosis.

Hematologic Analysis

A CBC was collected via cardiocentesis at time of necropsy. In response to inflammation or stress, a leukocytosis may be present on the hemogram. In cases of dehydration or anemia from blood loss, HCT values can be altered. There were no differences in white blood cell (WBC)

counts in the DSS model, however, there was significance in HCT values amongst control groups ($p<0.05$), probably related to rectal bleeding in the DSS control group. Values returned closer to the normal reference range in both NTX treatment groups ($p=0.01$) as well as the BNTX colitis group ($p=0.01$) (Table 6). In the TNBS model, HCT was significantly lower in the low dose NTX group compared to the colitis control group ($p=0.01$). Although outside of the normal reference range, treatment with low dose NTX and DPDPE significantly differed (by lower values) from the colitis control ($p = 0.01$ and $p = 0.03$). WBC counts were significantly higher and closer to normal in mice that received NTI compared to the colitis control group ($p=0.04$). No differences were seen in the other treatment groups (Table 7).

Myeloperoxidase (MPO) Activity

The myeloperoxidase enzyme activity was measured to indirectly assess neutrophil influx into the colon, as an indicator of the amount of local inflammation. Control groups differed significantly ($p<0.05$) in the acute DSS model, whereas no significance was reached between treatment groups (Figures 5A and 5B). In the TNBS model, control groups of PBS/EtOH differed from low dose NTX control ($p=0.01$) and high dose NTX control ($p=0.02$) (Figure 6A). High dose NTX treatment groups had lower levels of MPO; however, this did not reach significance. Treatment with DPDPE (1.0mg/kg) and BNTX (0.1 mg/kg) did not reduce MPO activity compared to the colitis control group ($p=0.02$ and $p=0.05$) (Figure 6B)

Disease Activity Index (DAI)

Measurements of weight, stool consistency, and occult blood were calculated daily to evaluate progression of disease in the DSS models of colitis. DAI scores were the greatest in the DSS-control animals compared to non-DSS controls indicating the model for induction of colitis was valid. Treatment with low dose and high dose NTX significantly reduced DAI scores on days

4 and 5 compared to the colitis control group (Figure 7A). Administration of BNTX and NTI also improved DAI scores on days 5 and 6, and 4, 5, and 6; however, significance was not reached with these therapies (Figure 7B). The reduction in DAI scores was most likely attributed to the therapeutic effects of the drugs. The potential mechanistic basis for NTX therapy is, a decrease in mucosal ulcers would result in the decrease amount of hematochezia, increased food consumption occurs when illness is not active and therefore results in increase or maintenance of weight, and the consistency of stool changes from loose to firm when vascular permeability and intestinal motility and reduced.

Macroscopic Evaluation of TNBS Induced Colitis Mice

The macroscopic evaluation of colon samples was determined by the sum of ulcers, adhesions, and diarrhea multiplied by total thickness of the sample for each mouse. Ideally, lower gross observation scores would be obtained with effective therapy. The colitis control group to that of no colitis almost reached significance. Treatment with high dose NTX significantly lowered the ulcer score compared to mice in colitis control groups (p=0.03) (Figure 8). Therapy with low dose NTX, DPDPE (both doses), and NTI reduced ulcer scores but did not reach significance. BNTX treatment did not alter ulcer scores (data not shown). The overall macroscopic damage induced by TNBS enema administration did not differ between mice receiving therapy compared to no treatment, due to substantial variability in the TNBS treatment group.

Progression of Body Weight in TNBS Colitis

Control non-colitis mice appropriately gained weight throughout the duration of the study. Mice receiving the lower dose of NTX lost weight one day after enema administration and did not gain a significant amount of weight throughout the course of therapy. Alternatively, mice

in the high dose NTX group gained weight compared to the colitis control group (Figure 9A). Treatment with DPDPE at the high dose allowed mice to maintain their weight post colitis induction. In comparison to the low dose of DPDPE, mice lost weight, but eventually were able to recover close to baseline (Figure 9B).

Effects of Opioid Receptor Therapy at the Histologic Level

Examining colon tissues microscopically for evidence of colitis is an accurate assessment when measuring therapeutic effects of various types of drugs (Cooper, et al., 1993), and in this case, anti-inflammatory effects of opioid receptor agents. Pathologic changes ranged from, but were not limited to, focal to multifocal thromboemboli in the mucosa and submucosa, myocyte degeneration in the muscularis propria, focally extensive fibrous replacement in the muscularis propria, and focally extensive serosal fibrosis with granulomatous inflammation and multinucleated foreign body type giant cells. The proximal colon was largely unaffected in the TNBS model as expected. The overall index score, that was obtained by the summation of inflammation severity, ulceration, percentage of inflamed area, and hyperplasia/dysplasia, was used as the criteria for comparison between groups. Significant differences were observed between the treatment controls to that of no colitis control. Differences were also observed between mice receiving filtered water to DSS control groups, thereby indicating that the model was effective. High dose NTX significantly reduced overall inflammation within the colon. Select opioid receptor-antagonists (i.e. BNTX, NTI) did not demonstrate an inflammatory effect as scores compared to the colitis group as indicated by the increased values (Table 8). Within the TNBS model, no significant differences were observed between the treatment or control groups. The delta receptor agonist DPDPE (0.1 mg/kg and 1.0 mg/kg) and antagonists NTX (1.0 mg/kg) and NTI were able to reduce inflammation indices; however this change did not reach significance. This seems to be due to variability in the TNBS control group.

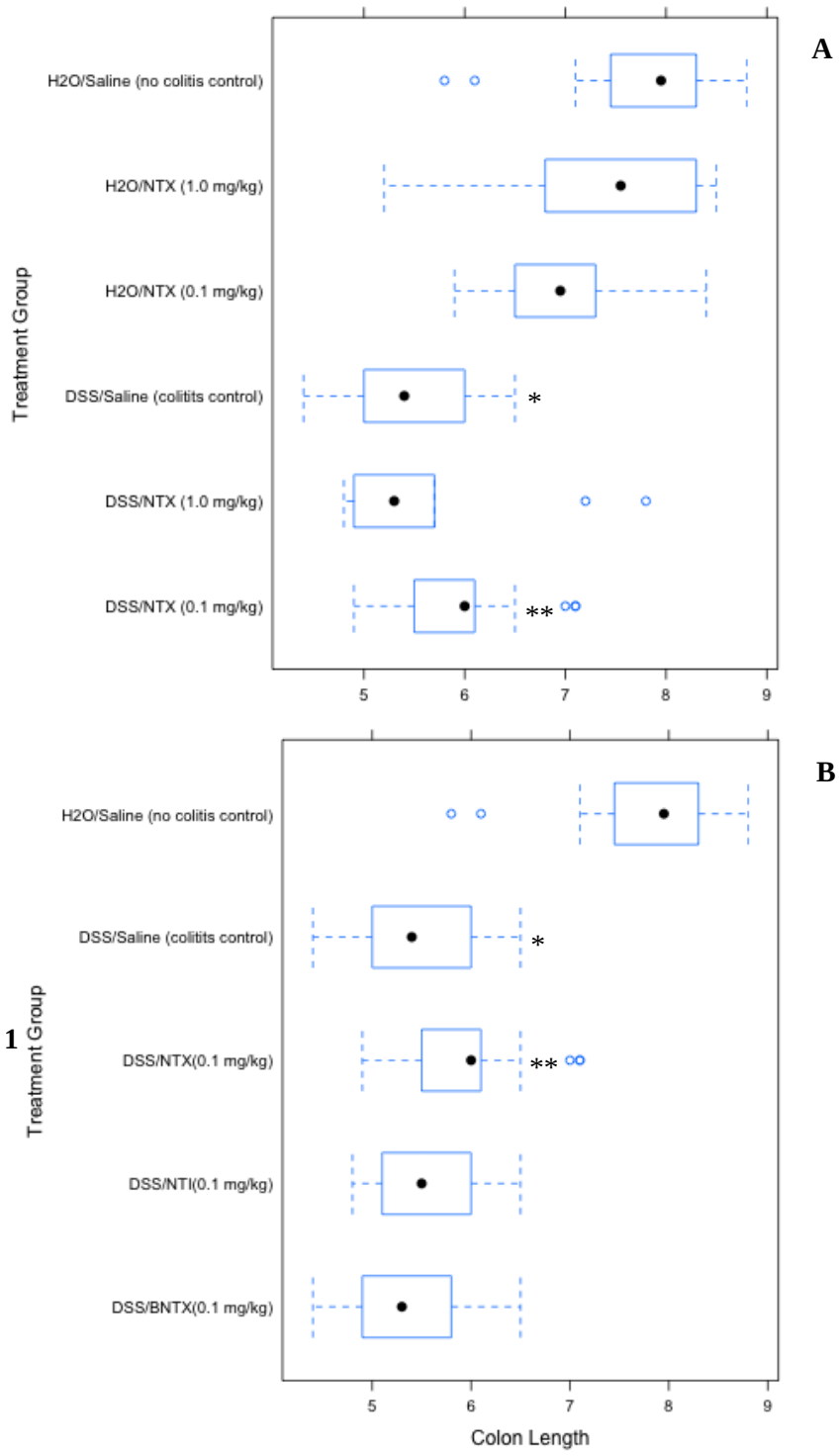


Figure 1. Colon length measured in cm for DSS colitis. A) Results from DSS-1 study showed the control groups significantly differed from each other (* p<0.05). NTX (0.1 mg/kg) significantly improved colon length (** p<0.02). Mean values ± SD for no colitis

control, high dose NTX, low dose NTX, colitis control, high dose NTX, low dose NTX were 7.75 ± 0.77, 6.98 ± 0.70, 7.39 ± 1.02, 5.48 ± 0.62, 5.99 ± 0.64, 5.64 ± 1.03 respectively. B). In the second DSS study, treatment with BNTX and NTI did not change colon length. Mean values ± SD for no colitis control, colitis control, and therapy with BNTX, NTI and NTX were 7.75 ± 0.77, 5.49 ± 0.62, 5.33 ± 0.62, 5.57 ± 0.57, 5.99 ± 0.64 respectively.

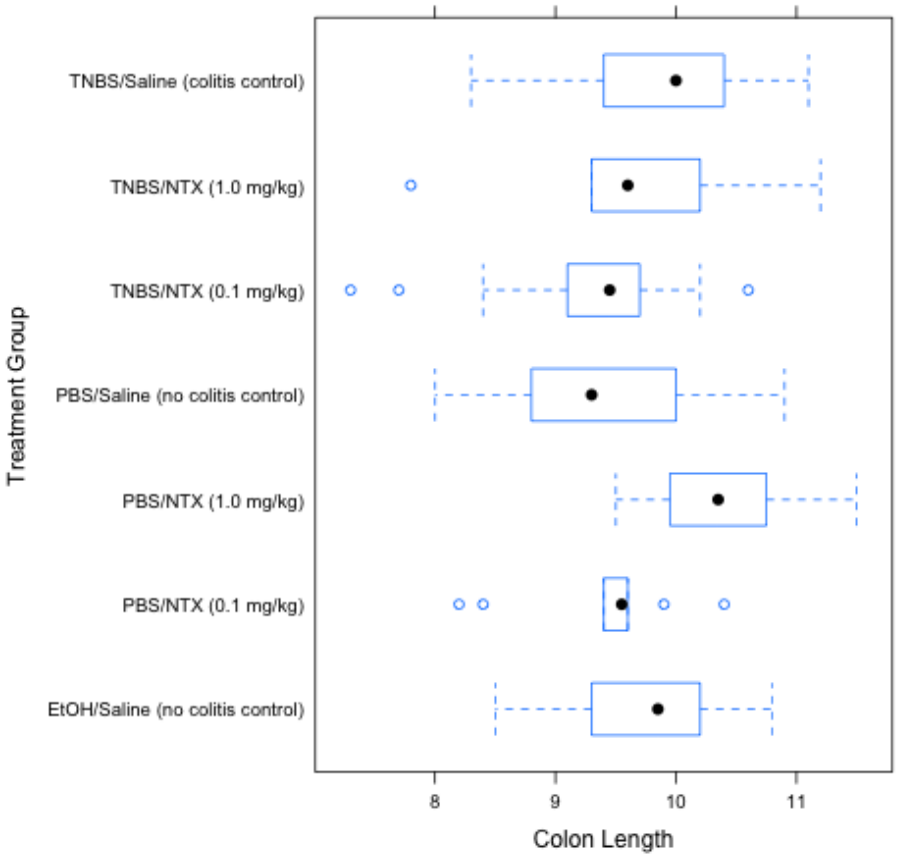


Figure 2A. Colon length measured in cm for TNBS colitis. No differences were observed between control or treatment groups. Mean values ± SD for no colitis control, vehicle, colitis control, low dose and high dose control, low dose and high dose treatment were

9.38 ± 0.88, 9.74 ± 0.65, 9.80 ±0.85, 9.41 ± 0.65, 10.39 ± 0.64, 9.25 ± 0.92, and 9.61± 1.12, respectively.

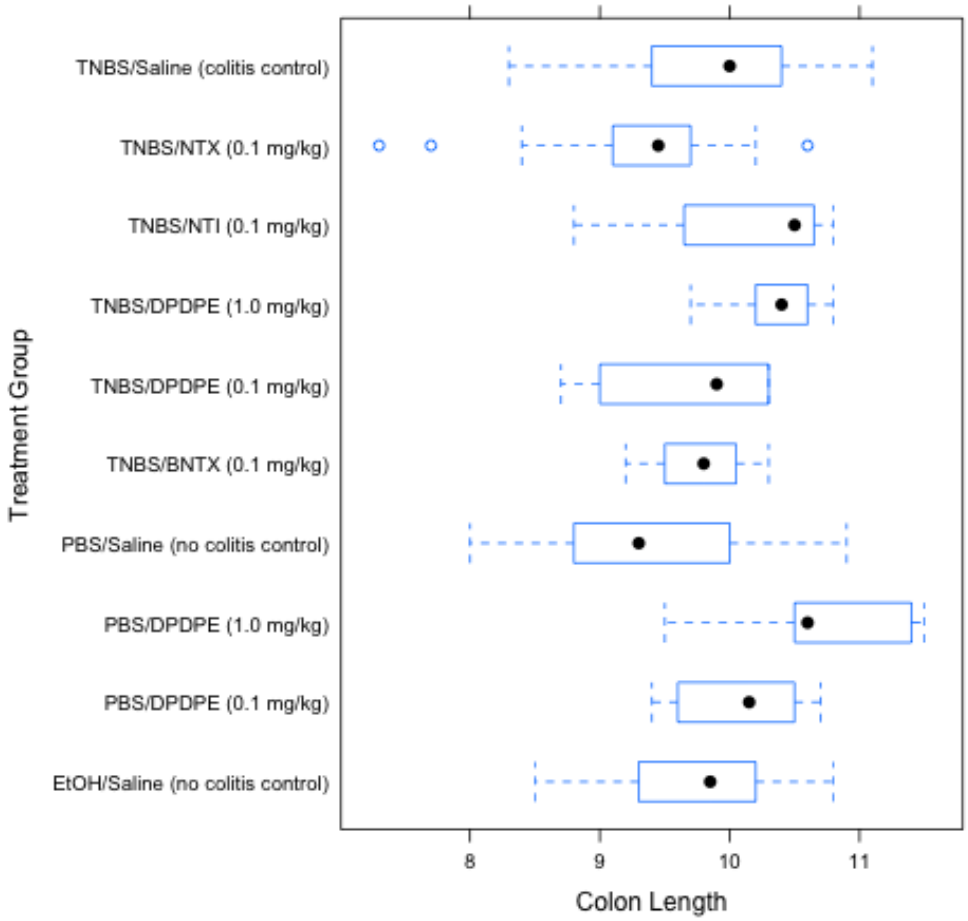


Figure 2B. Colon length measured in cm for TNBS colitis. No differences were observed between control or treatment groups. Mean values ± SD for no colitis control, vehicle, colitis control, low dose NTX, low dose and high dose DPDPE, NTI, BNTX, low dose and high dose DPDPE control were 9.38 ± 0.88, 9.74 ± 0.65, 9.80 ±0.85, 9.25 ± 0.91, 9.68 ± 0.70, 10.34 ± 0.42, 10.15 ± 0.91, 9.76 ± 0.55, 10.08 ± 0.50 and 10.7± 0.8, respectively.

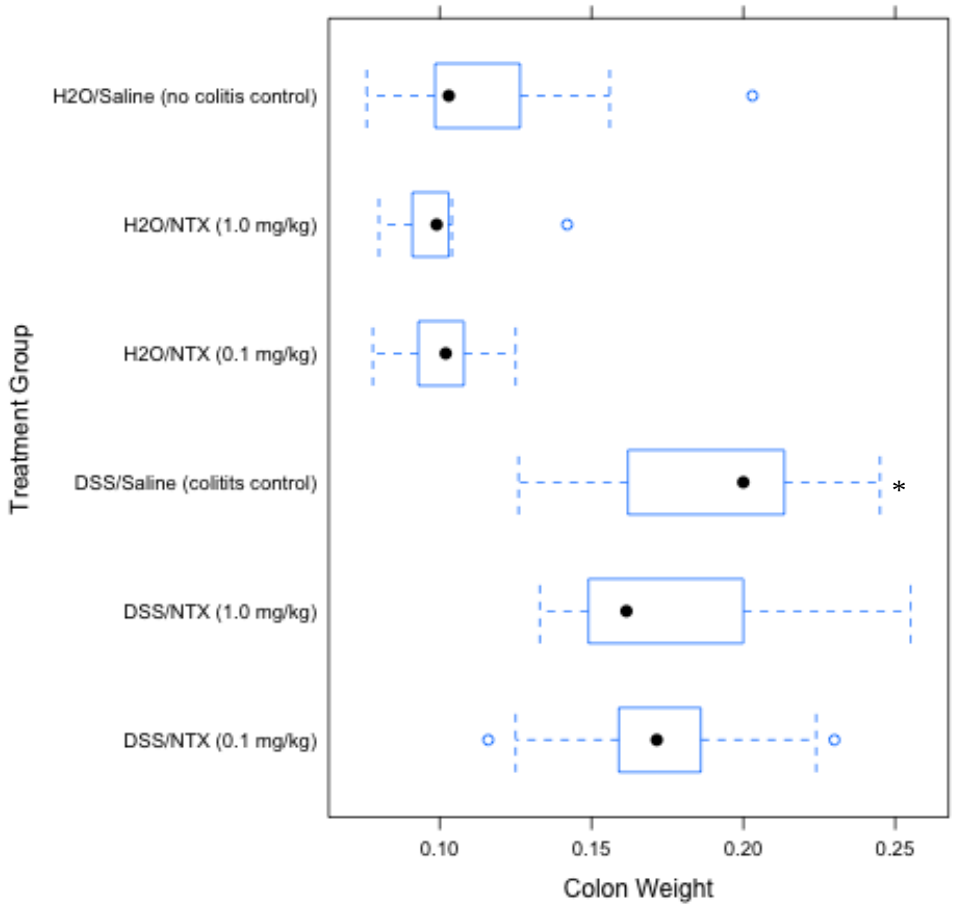


Figure 3A. Colon weight (gm) in DSS induced colitis. The no colitis control and colitis control groups significantly differed (* $p<0.05$). Treatment with NTX improved colon weight, although this was not significant. Mean values ± SD for no colitis control, high dose NTX, low dose NTX, colitis control, high dose NTX, low dose NTX were 0.11 ± 0.03, 0.10 ± 0.01, 0.10 ± 0.01, 0.19 ± 0.03, 0.17 ± 0.03, 0.17 ±0.04, respectively.

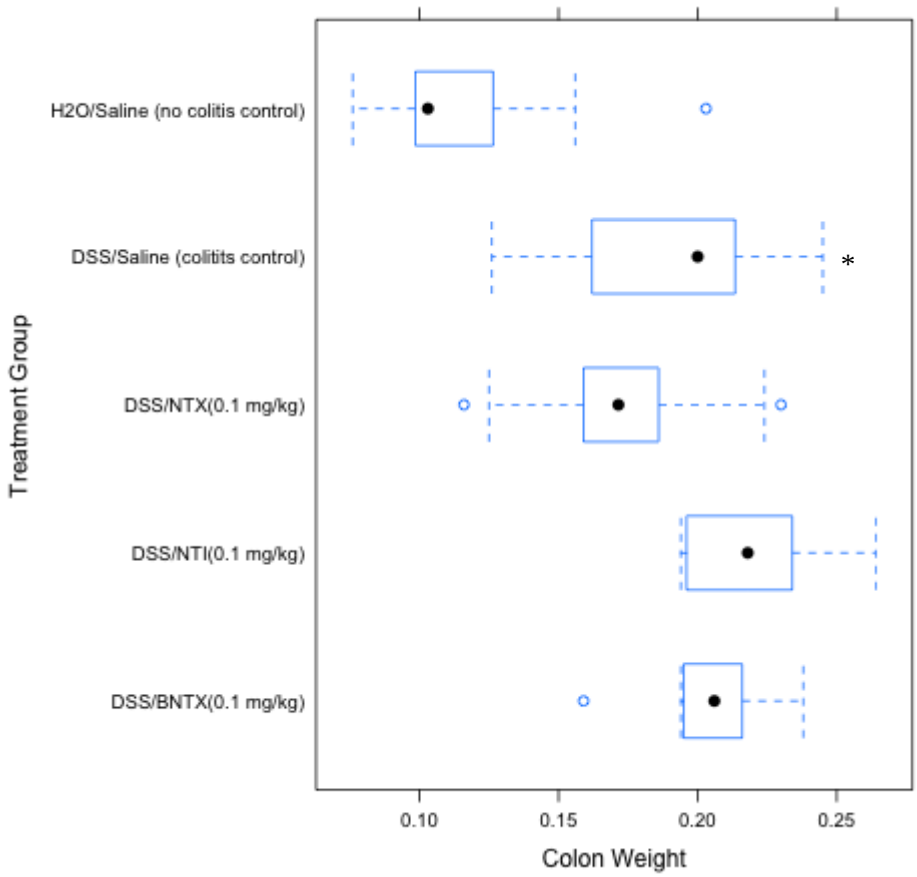


Figure 3B. Colon weight (gm) in DSS-induced colitis. No colitis control and colitis control groups significantly differed (* $p<0.05$). Treatment with either BNTX or NTI resulted in the return of colon weight toward normal levels. Mean values ± SD for no colitis control, colitis control, and therapy with BNTX, NTI and NTX were 0.11 ± 0.03, 0.19 ± 0.03, 0.20 ± 0.02, 0.22 ± 0.02, and 0.17 ± 0.03, respectively.

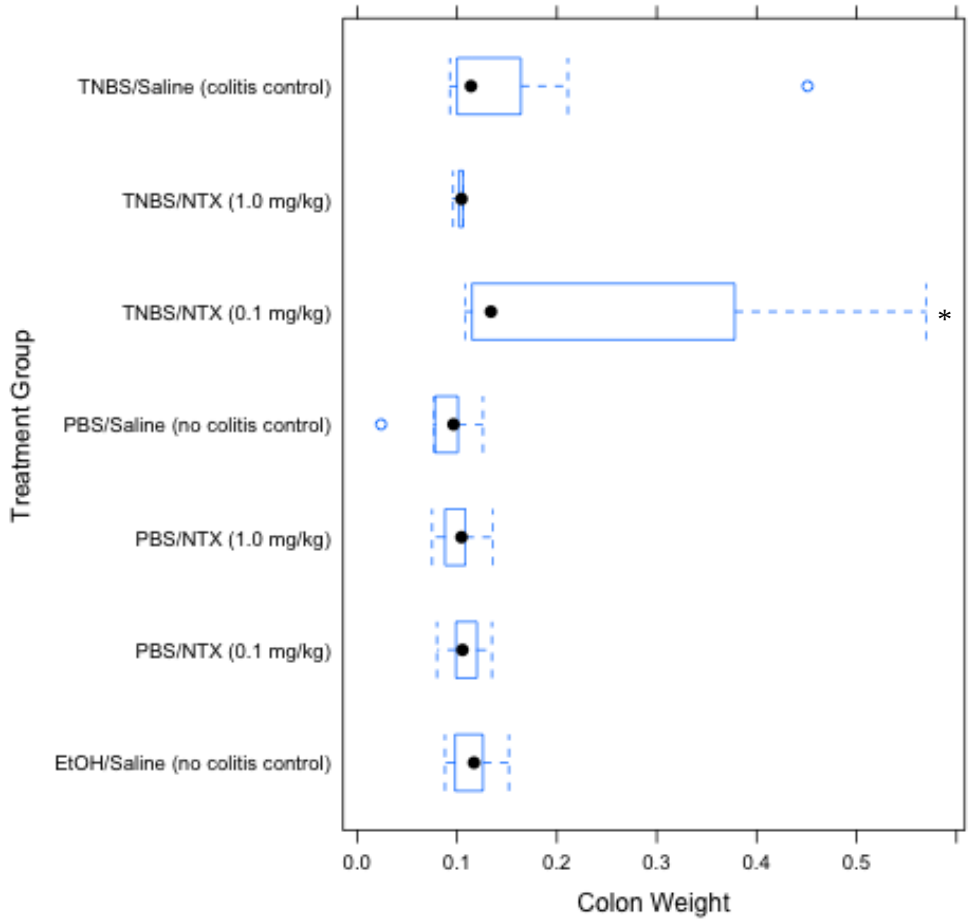


Figure 4A. Colon weight (gm) in TNBS induced colitis. Low dose NTX yielded a significant increase in colon weight compared to the colitis control group (p=0.05). No other significant differences in colon weight were found in the TNBS model. Mean values ± SD for no colitis control, vehicle, colitis control, low dose and high dose control, low dose and high dose treatment were 0.08 ± 0.02, 0.11 ± 0.01, 0.15 ±0.09, 0.11 ± 0.01, 0.10 ± 0.01, 0.24 ± 0.17, and 0.10 ± 0.003 respectively.

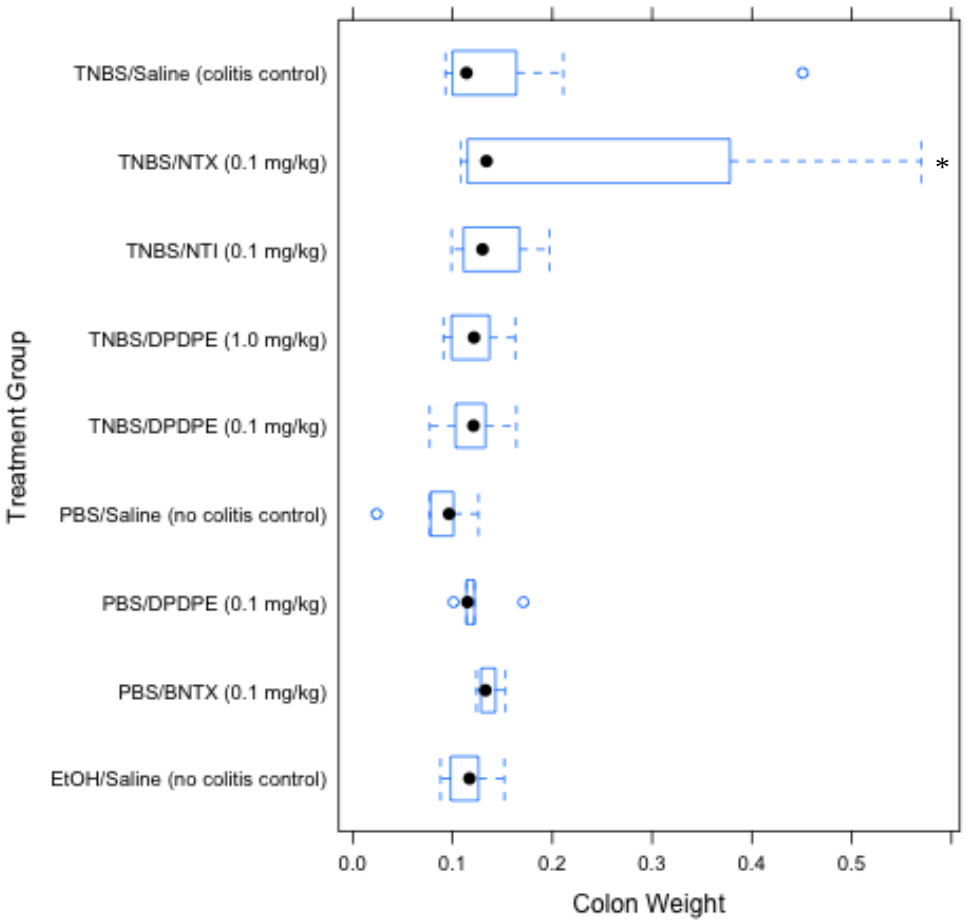


Figure 4B. Colon weight (gm) in TNBS induced colitis. Low dose also yielded a significant increase in colon weight compared to the colitis control group (p=0.05) in the second TNBS experimental model. Likewise, no other changes in colon weight were observed between the other treatment groups. Mean values ± SD for no colitis control, vehicle, colitis control, low dose NTX, low dose and high dose DPDPE, NTI, BNTX, low dose and high dose DPDPE control were 0.09 ± 0.02, 0.12 ± 0.02, 0.15 ±0.01, 0.24 ± 0.17, 0.12 ± 0.02, 0.13 ± 0.02, 0.13 ± 0.04, 0.14 ± 0.01, 0.12 ± 0.02 and 0.11± 0.01, respectively.

Table 6. Hematologic analysis of mice with DSS-induced colitis. A) DSS experimental group 1 and B) DSS experimental group 2. No differences were noted in WBC values in any treatment group. HCT values in colitis control groups were significantly decreased compared to mice receiving no DSS (*p<0.05). NTX treatment improved HCT values; however, this did not reach significance.

A

Treatment Group	WBC (range 5.1-11.26 x 10^3/µl)	HCT (range 42-44%)
H_20/Saline	5.05 ± 0.49	47.15 ± 0.56
H_20/NTX (0.1 mg/kg)	4.17 ± 0.34	47.91 ± 1.01
H_20/ (1.0 mg/kg)	4.40 ± 0.51	47.04 ± 1.54
DSS/Saline	4.25 ± 0.39	33.31 ± 2.20*
DSS/NTX (0.1 mg/kg)	5.02 ± 0.36	40.05 ± 2.26
DSS/NTX (1.0 mg/kg)	4.67 ± 0.54	42.14 ± 2.03

Data are presented as mean values ± SEM

B

Treatment Group	WBC (range 5.1-11.26 x 10^3/µl)	HCT (range 42-44%)
H_20/Saline	5.05 ± 0.49	47.15 ± 0.56
DSS/Saline	4.25 ± 0.39	33.31 ± 2.20*
DSS/BNTX (0.1 mg/kg)	4.59 ± 0.25	33.19 ± 3.03
DSS/NTI (0.1 mg/kg)	5.54 ± 0.72	37.09 ± 2.06
DSS/NTX (0.1 mg/kg)	5.02 ± 0.36	40.05 ± 2.26

Data are presented as mean values ± SEM

Table 7. Hematologic analysis of mice with TNBS induced colitis. A) Experimental group 1 and B) Experimental group 2. WBC was close to normal reference range in mice receiving NTI therapy and significantly differed compared to colitis control (* p=0.04). No differences were noted in WBC values in any other treatment group. HCT values in low dose NTX and DPDPE groups were significantly lower compared to mice receiving no therapy (** p=0.01; ***p = 0.03), however values were outside of normal reference range. Although samples were obtained for the DPDPE treatment group, samples were not of diagnostic quality and were therefore not included in the analysis.

A

Treatment Group	**WBC (range 5.1-11.26 x 10^3/µl)**	**HCT (range 42-44%)**
PBS/Saline	6.08 ± 1.29	60.25 ± 1.46
EtOH/Saline	5.25 ± 0.57	57.13 ± 1.88
TNBS/Saline	4.56 ± 0.39	62.0 ± 1.21
PBS/NTX (0.1 mg/kg)	4.03 ± 0.60	58.71 ± 1.19
PBS/NTX (1.0 mg/kg)	4.30 ± 0.48	62.08 ± 1.28
TNBS/NTX (0.1 mg/kg)	5.16 ± 0.40	50.64 ± 2.81**
TNBS/NTX (1.0 mg/kg)	4.60 ± 0.90	58.50 ± 2.50

Data are presented as mean values ± SEM

Treatment Group	WBC (range 5.1-11.26 x 10^3/µl)	HCT (range 42-44%)
PBS/Saline	6.08 ± 1.29	60.25 ± 1.46
EtOH/Saline	5.25 ± 0.57	57.13 ± 1.88
TNBS/Saline	4.56 ± 0.39	62.0 ± 1.21
TNBS/NTX (0.1 mg/kg)	5.16 ± 0.40	50.64 ± 2.81 **
TNBS/DPDPE (0.1 mg/kg)	6.58 ± 1.34	54.0 ± 2.16***
TNBS/DPDPE (1.0 mg/kg)	NS	NS
TNBS/NTI (0.1 mg/kg)	7.20 ± 0.50 *	56.0 ± 3.00
TNBS/BNTX (0.1 mg/kg)	5.15 ± 2.35	61.25 ± 2.25
PBS/DPDPE (0.1 mg/kg)	6.50 ± 1.10	60.0 ± 1.00
PBS/DPDPE (1.0 mg/kg)	4.20 ± NA	66.0 ± NA

Data are presented as mean values ± SEM

NS= sample not available for diagnostic evaluation

B

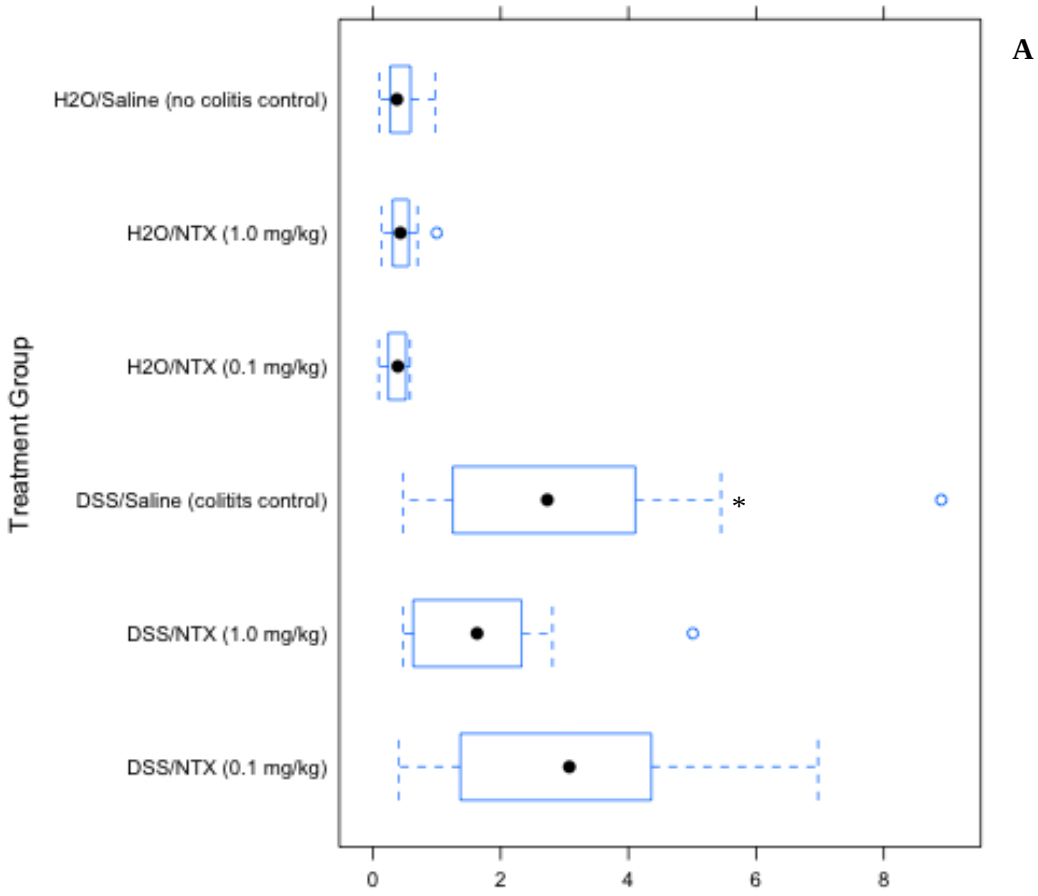


Figure 5A. MPO activity in the DSS colitis model. Colitis control groups were significantly different from mice receiving untreated water (* $p<0.05$). High dose NTX reduced the MPO levels; however, this did not reach significance. Mean values ± SD for no colitis control, high dose NTX, low dose NTX, colitis control, high dose NTX, low dose NTX were 0.44 ± 0.25, 0.37 ± 0.18, 0.46 ± 0.25, 2.96 ± 2.10, 2.96 ± 1.98 and 1.83 ± 1.35, respectively.

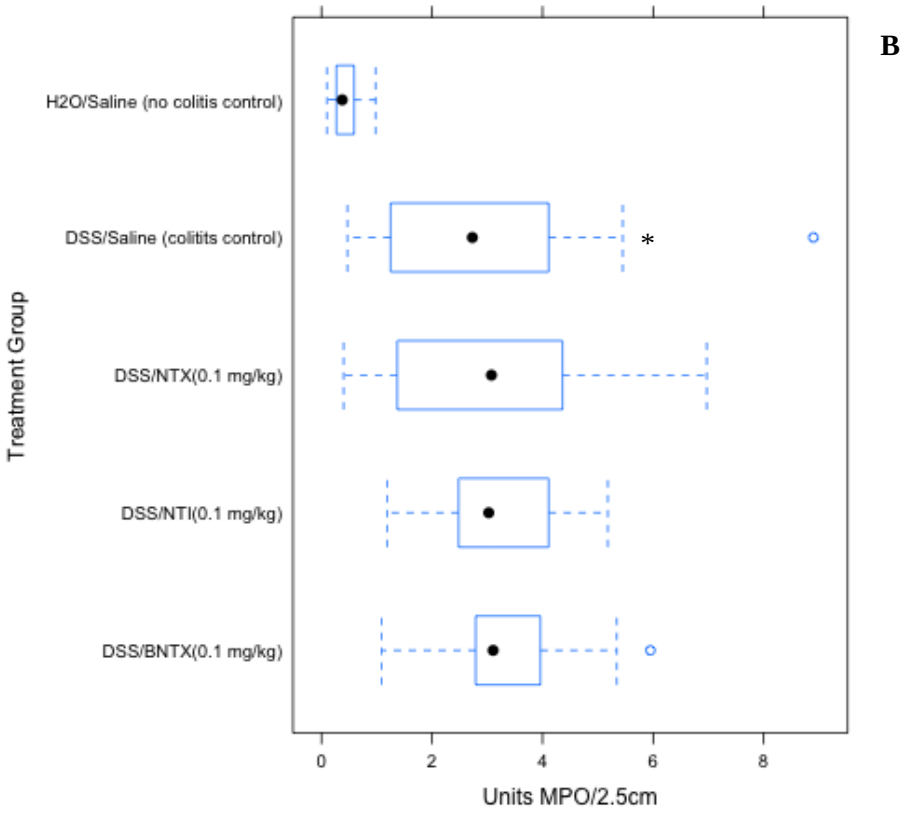


Figure 5B. MPO activity in the DSS colitis model. Colitis control groups were significantly different from mice receiving untreated water (* $p<0.05$). Mean values ± SD for no colitis control, colitis control, and therapy with BNTX, NTI and NTX were 0.44 ± 0.25, 2.96 ± 2.10, 3.44 ± 1.39, 3.21 ± 1.43 and 2.96 ± 1.98, respectively.

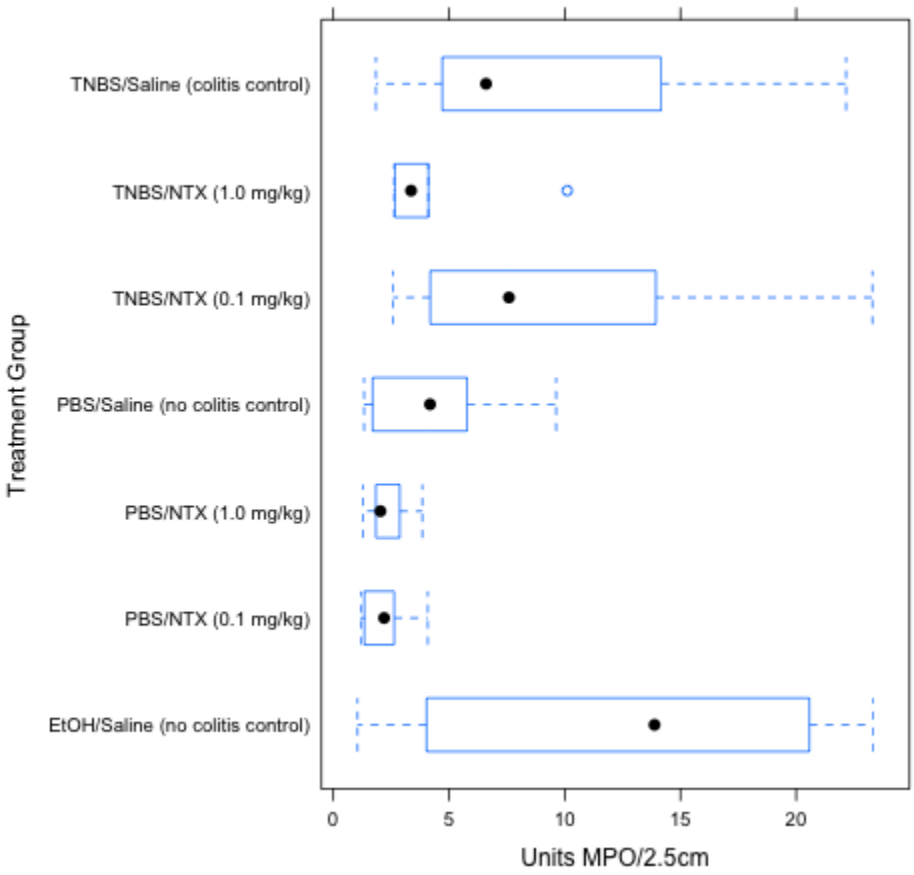


Figure 6A. MPO activity in the TNBS colitis model. High dose NTX reduced the MPO levels; however, this did not reach significance. Mean values ± SD for no colitis control, vehicle, colitis control, low dose and high dose control, low dose and high dose treatment were 4.57 ± 3.01, 12.52 ± 8.64, 9.29 ± 6.80, 2.21 ± 0.91, 2.33 ± 0.88, 9.63± 6.85, and 4.37 ± 2.87, respectively.

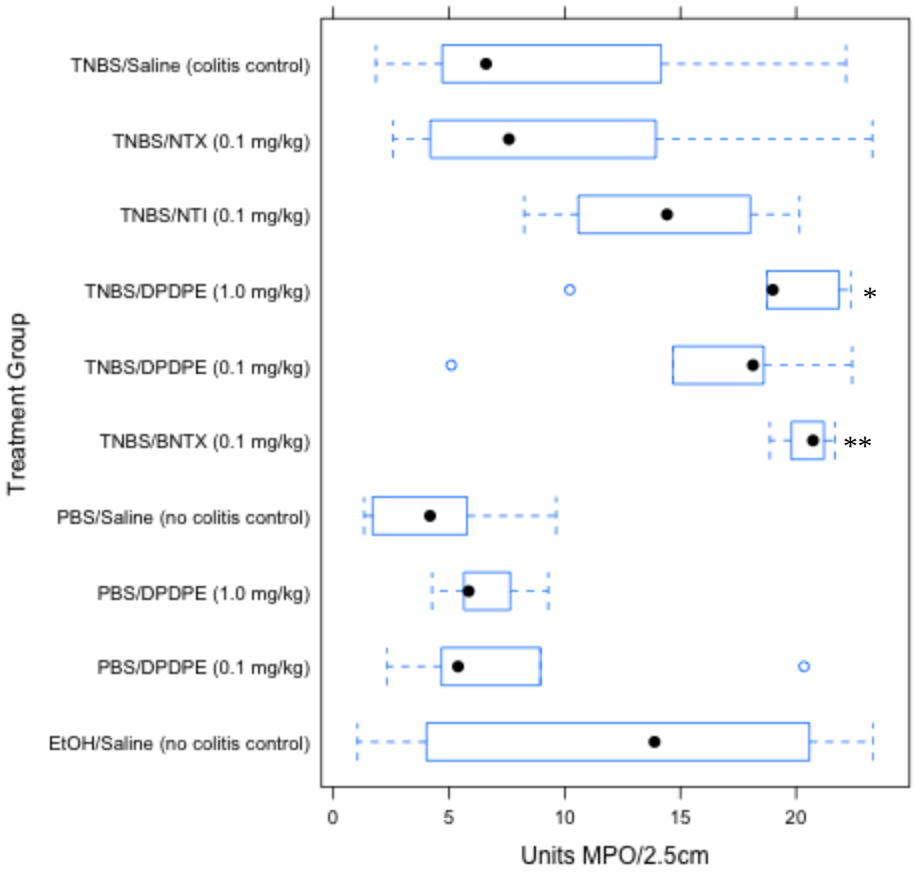


Figure 6B. MPO activity in the TNBS colitis model. Treatment with DPDPE and BNTX failed to reduce MPO activity and was significantly higher than the colitis control group (* p=0.02 and ** p =0.05) indicating that treatment was ineffective. Mean values ± SD for no colitis control, vehicle, colitis control, low dose NTX, low dose and high dose DPDPE, NTI, BNTX, low dose and high dose DPDPE control were 4.57 ± 3.01, 12.52 ± 8.64, 9.29 ± 6.80, 9.63 ± 6.84, 16.18 ± 5.96, 18.43 ± 4.88, 14.31 ± 5.01, 20.42 ± 1.43, 7.84 ± 6.49 and 6.54 ± 1.95, respectively.

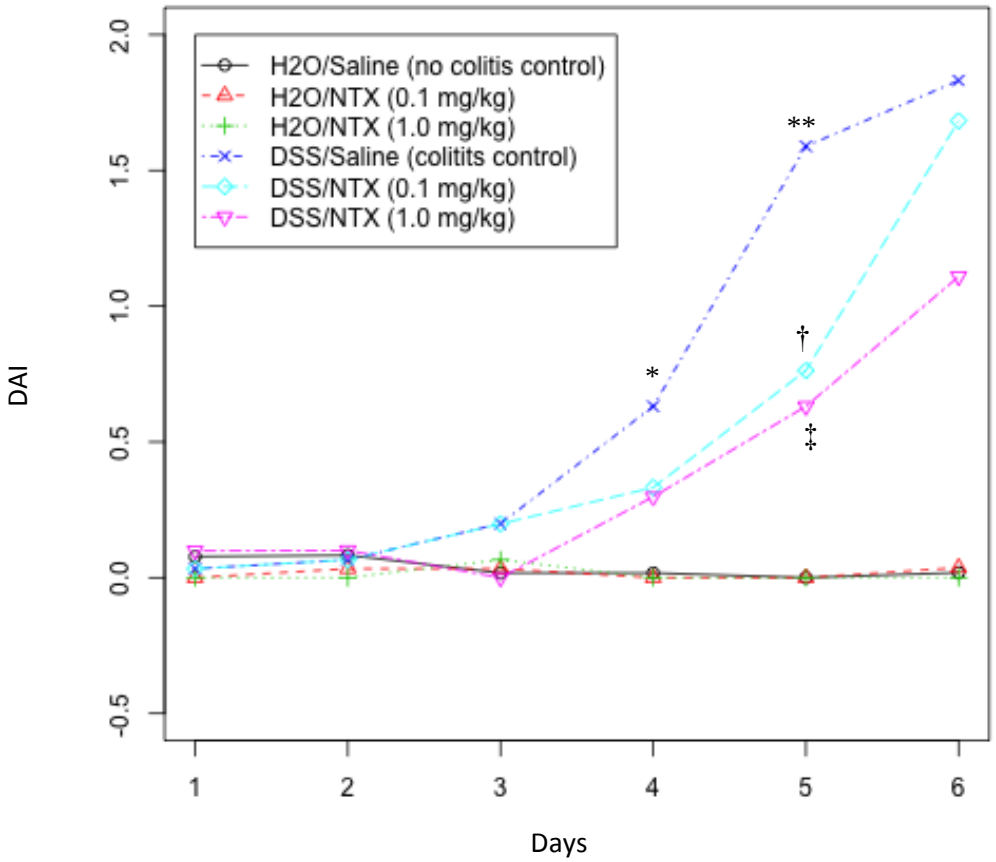


Figure 7A. Disease activity index in DSS treated mice. The control groups significantly differed from each other on days 4 (*p = 0.003) and 5 (**p <0.05). Both low and high dose NTX therapy significantly reduced DAI scores on days 4 and 5compared to the colitis control group († p = 0.003 and ‡ p = 0.001)**.**

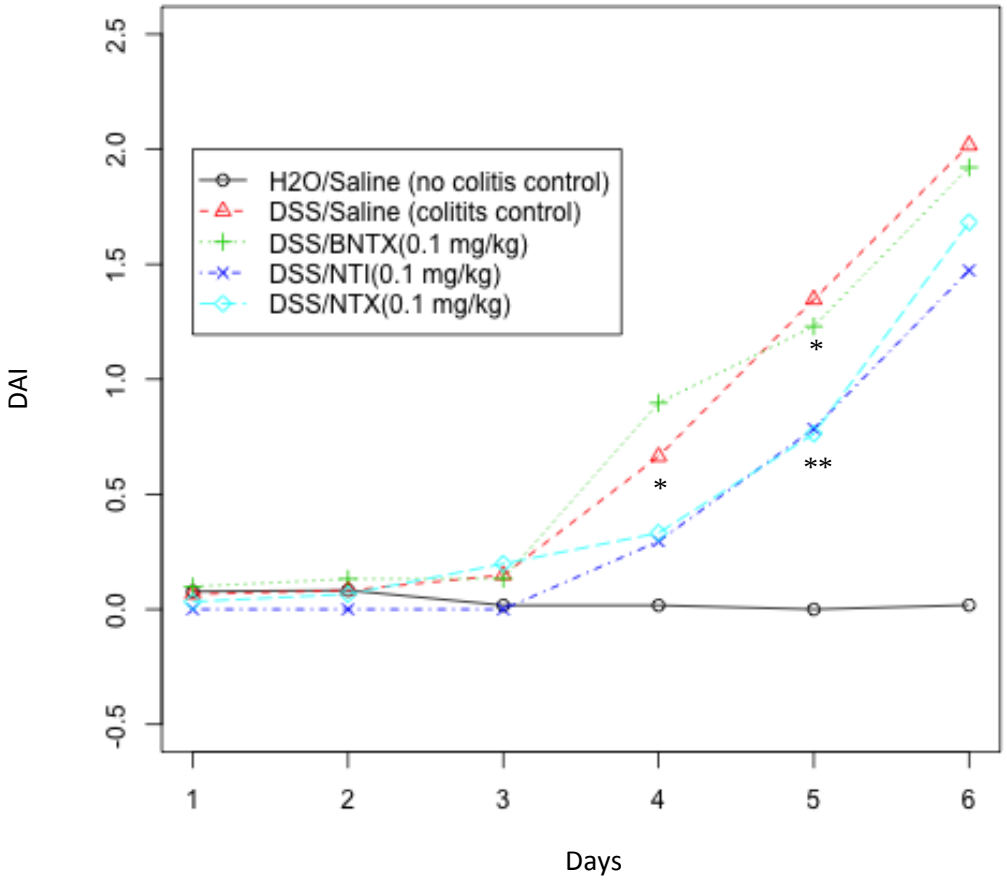


Figure 7B. Disease activity index for DSS induced colitis. NTI and NTX significantly reduced DAI scores on day 5 of therapy compared to the colitis control (**p =0.01). Control groups significantly differed on day 4 and 5 of treatment (*p <0.05).

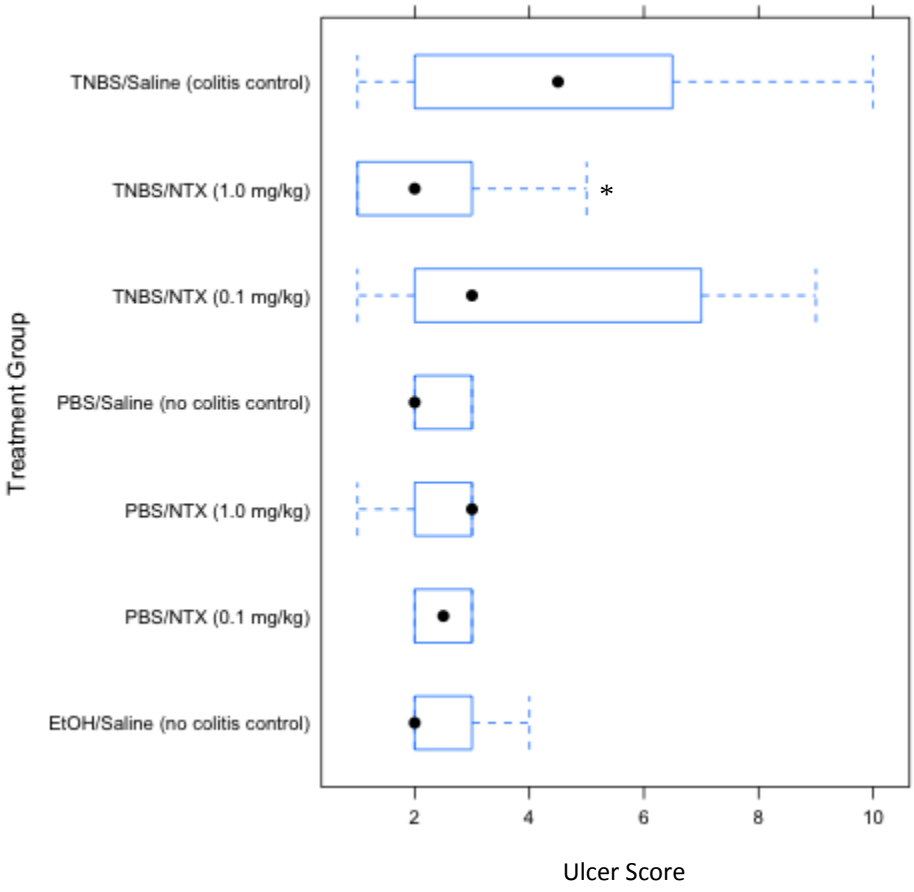


Figure 8. Ulcer score in the TNBS colitis model. High dose NTX significantly lowered the macroscopic ulcer score in mice with colitis compare the colitis control group (*p =0.03). Mean values ± SD for no colitis control, vehicle, colitis control, low dose and high dose control, low dose and high dose treatment were 2.30 ± 0.48, 2.50 ± 0.71, 4.31 ± 2.75, 2.50 ± 0.53, 2.40 ± 0.84, 4.40 ± 2.61 and 2.10 ± 1.23, respectively.

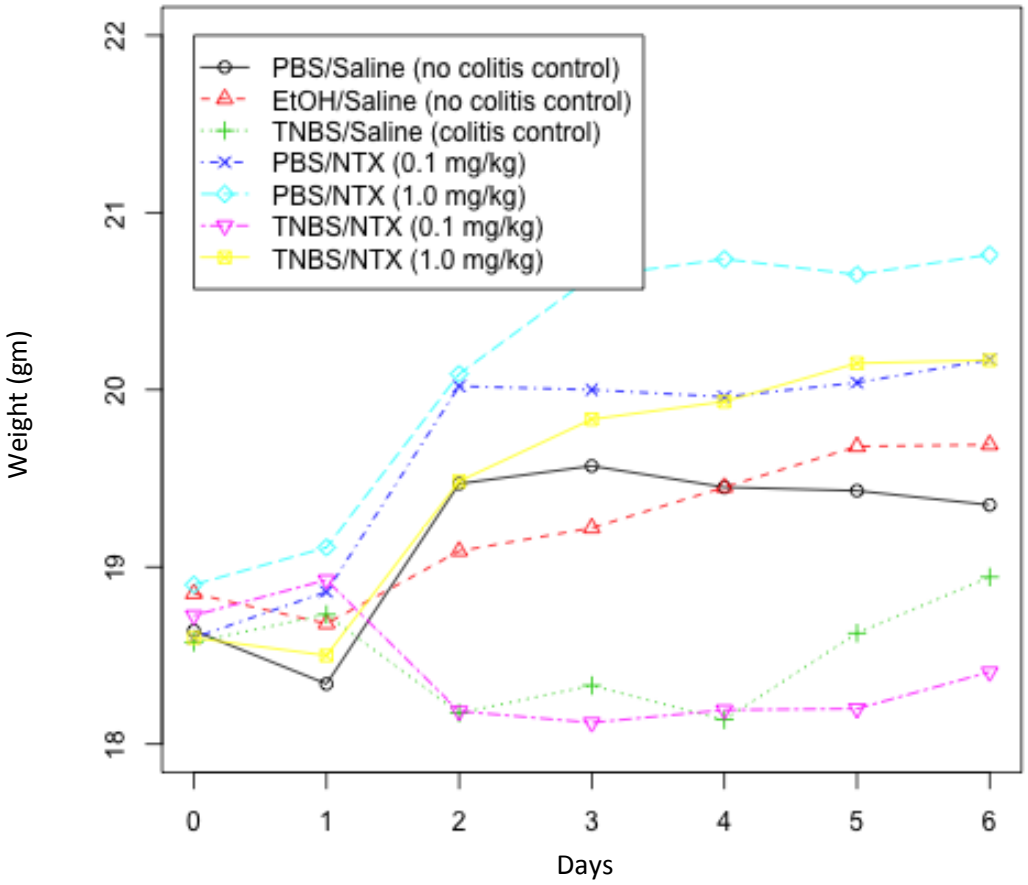


Figure 9A. Progression of bodyweight in the TNBS colitis model. Mice in the high dose NTX group gained weight compared to the colitis control group whereas mice receiving low dose NTX were exhibited a decrease in bodyweight.

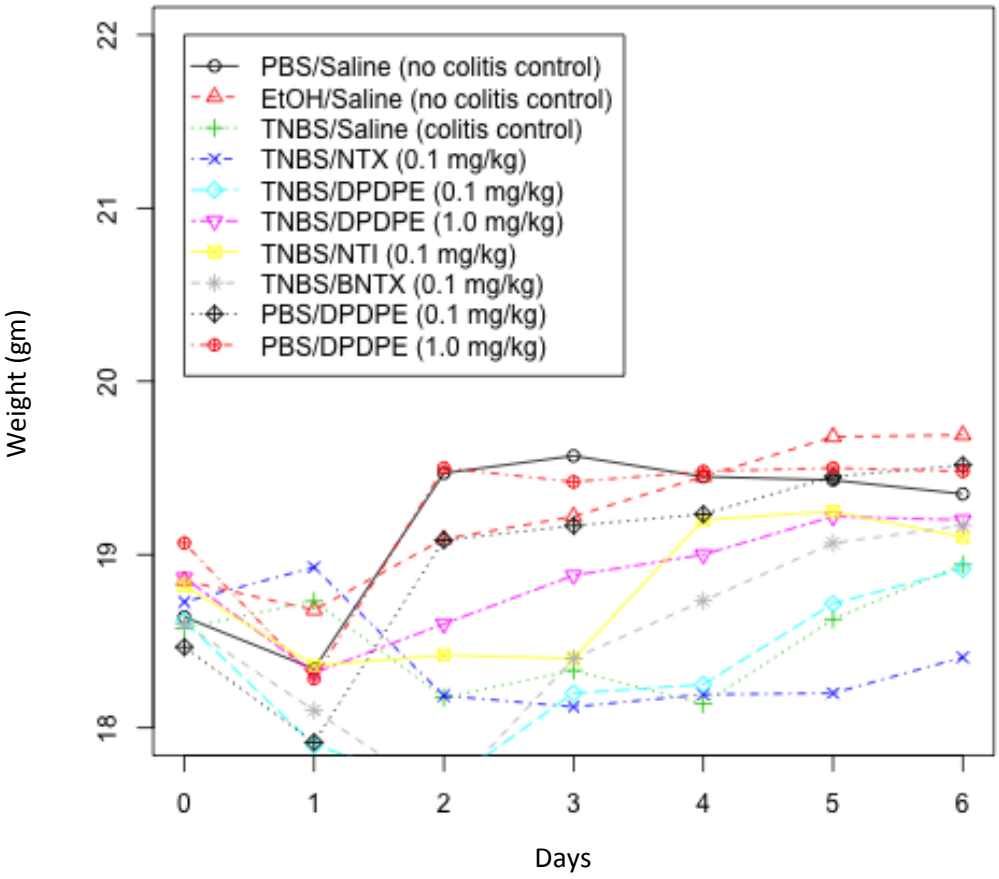


Figure 9B. Progression of body weight in mice in the TNBS colitis model .Treatment with DPDPE at the high dose allowed mice to maintain their weight post colitis induction. In comparison to the low dose DPDPE, mice lost weight, but were eventually able to recover close to baseline. Low dose NTX did not improve body weights compared to the no colitis control.

Table 8. Inflammation indices in the DSS/TNBS colitis models. A) Significant differences were observed between the treatment controls to that of no colitis control (*p =0.04 and **p =0.008). Differences were also observed between mice receiving filtered water to DSS control groups (†p =0.001). High dose NTX was shown to reduce overall inflammation within the colon (‡ p =0.039). B) Control groups were significantly different from each other (* p = 0.003).

A

Treatment Group	Index Score
H_20/Saline	1.63 ± 3.06
H_20/NTX (0.1 mg/kg)	4.33 ± 4.53 *
H_20/ (1.0 mg/kg)	5.56 ± 3.84 **
DSS/Saline	5.79 ± 3.84 †
DSS/NTX (0.1 mg/kg)	4.95 ± 3.61
DSS/NTX (1.0 mg/kg)	2.30 ± 3.27‡

Data are presented as mean values ± SD

B

Treatment Group	Index Score
H_20/Saline	1.72 ± 3.12
DSS/Saline	5.50 ± 3.95*
DSS/BNTX (0.1 mg/kg)	7.80 ± 1.03
DSS/NTI (0.1 mg/kg)	6.22 ± 3.15
DSS/NTX (0.1 mg/kg)	5.17 ± 3.61

Data are presented as mean values ± SD

C) No significant differences were observed between the treatment groups however differences were noted between the control and vehicle group (* and ** p <0.005). D) Treatment with various opioid receptor compounds did not reduce inflammation scores.

C

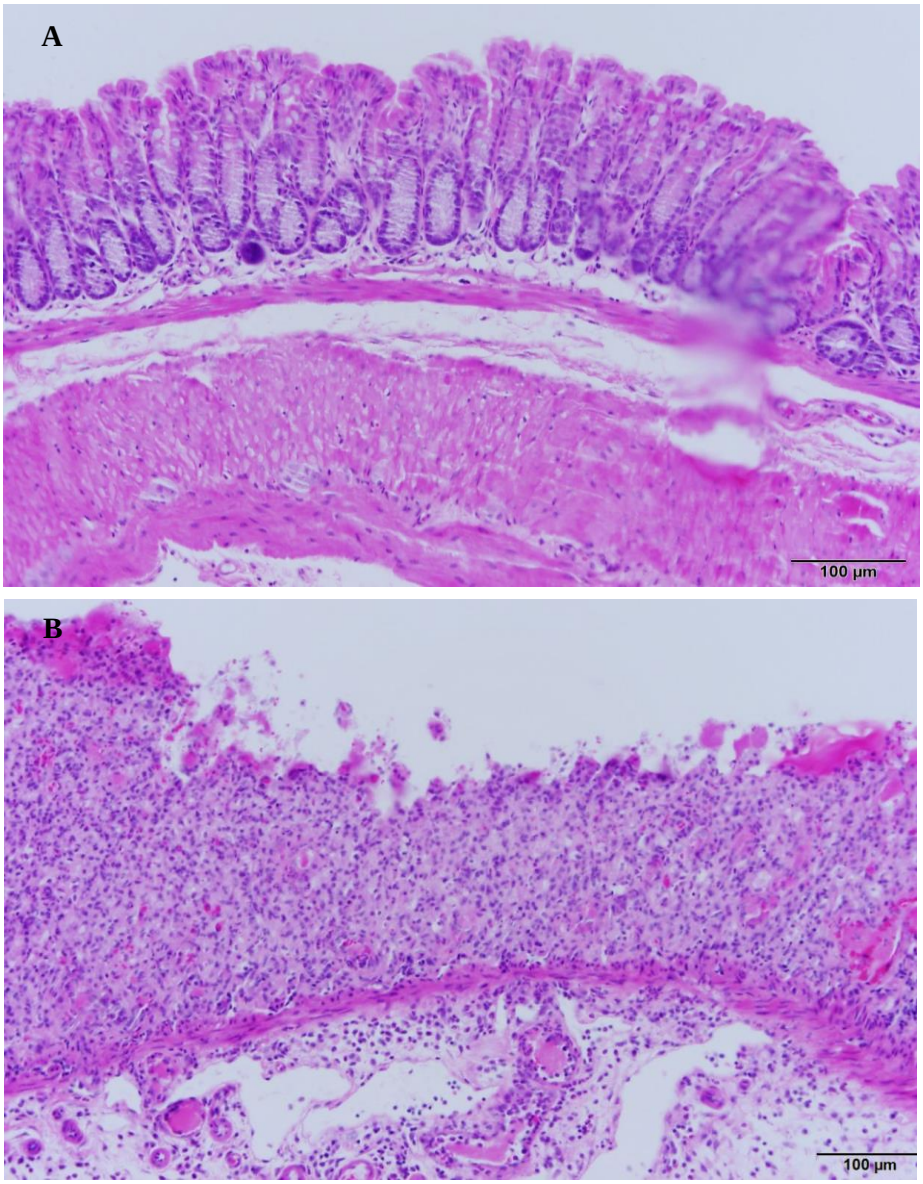
Treatment Group	Index Score
PBS/Saline	1.90 ± 1.45
EtOH/Saline	4.60 ± 2.07 **
TNBS/Saline	4.85 ± 2.48 *
PBS/NTX (0.1 mg/kg)	3.20 ± 1.32
PBS/NTX (1.0 mg/kg)	1.75 ± 1.16
TNBS/NTX (0.1 mg/kg)	5.86 ± 2.66
TNBS/NTX (1.0 mg/kg)	3.83 ± 3.06

Data are presented as mean values ± SD

D

Treatment Group	Index Score
PBS/Saline	1.90 ± 1.45
EtOH/Saline	4.60 ± 2.07 **
TNBS/Saline	4.85 ± 2.48 *
TNBS/NTX (0.1 mg/kg)	5.85 ± 2.66
TNBS/DPDPE (0.1 mg/kg)	4.16 ± 2.79
TNBS/DPDPE (1.0 mg/kg)	3.80 ± 2.86
TNBS/NTI (0.1 mg/kg)	3.25 ± 2.75
TNBS/BNTX (0.1 mg/kg)	5.00 ± 2.65
PBS/DPDPE (0.1 mg/kg)	2.33 ± 3.50
PBS/DPDPE (1.0 mg/kg)	1.00 ± 1.41

Data are presented as mean values ± SD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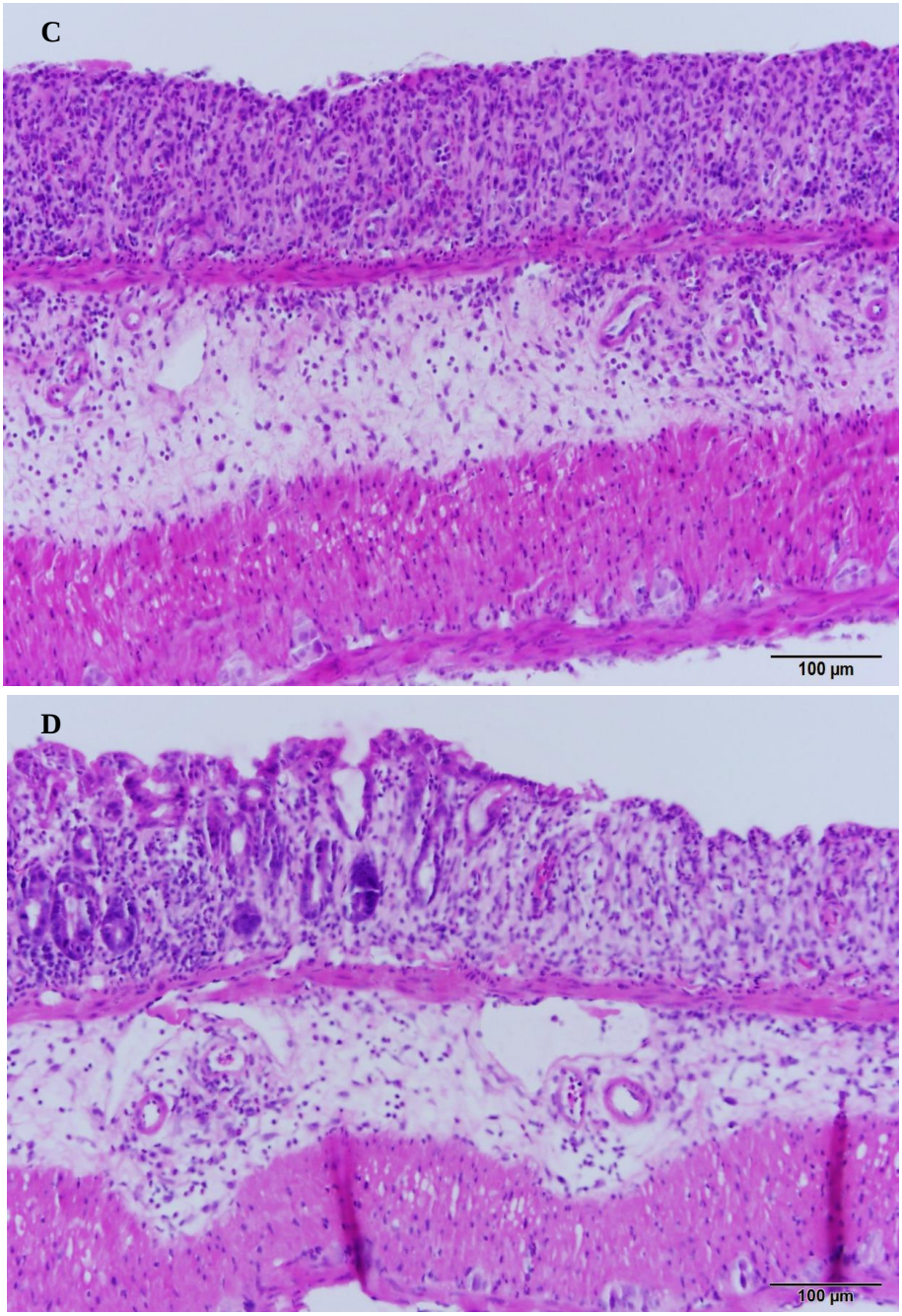
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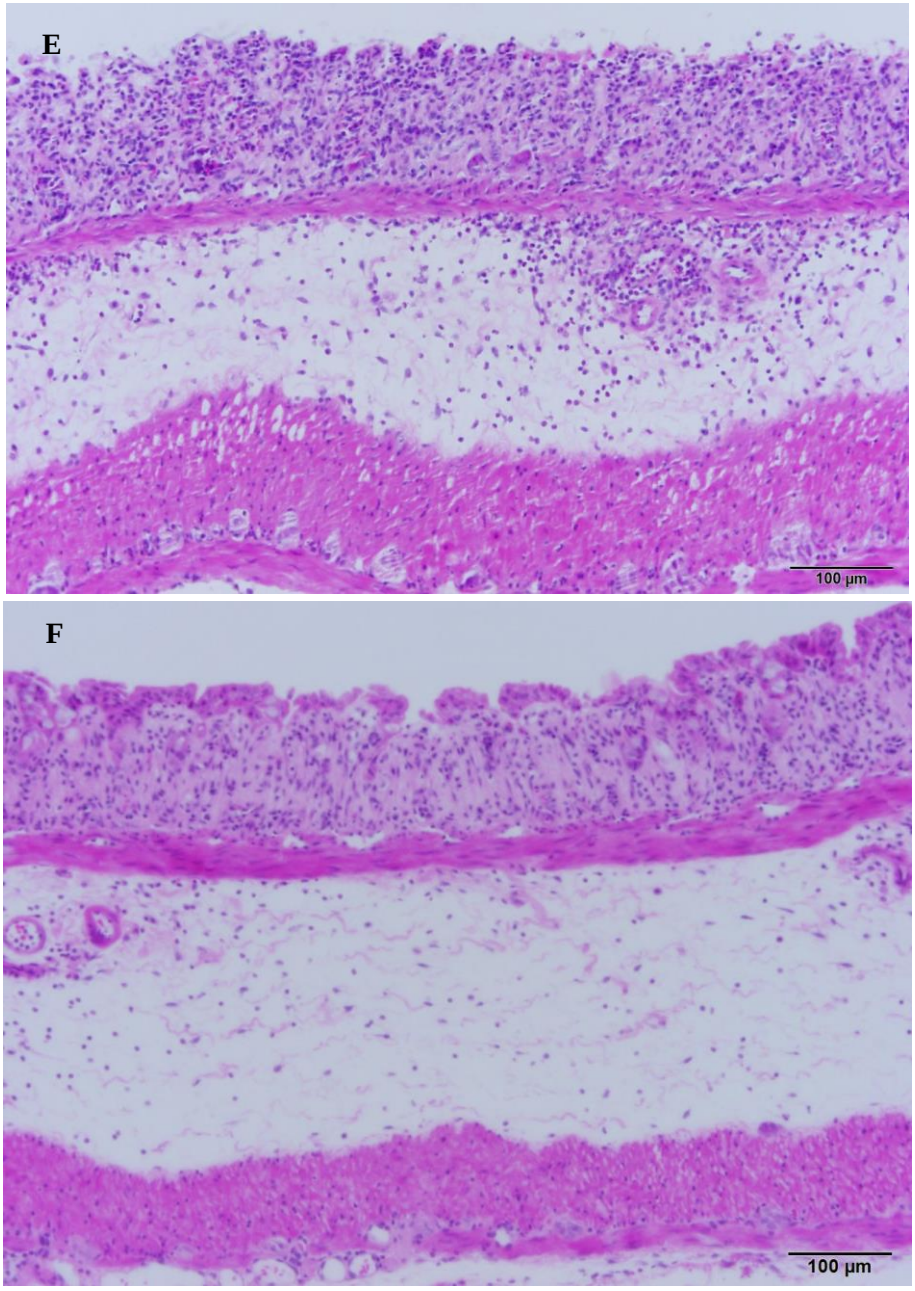
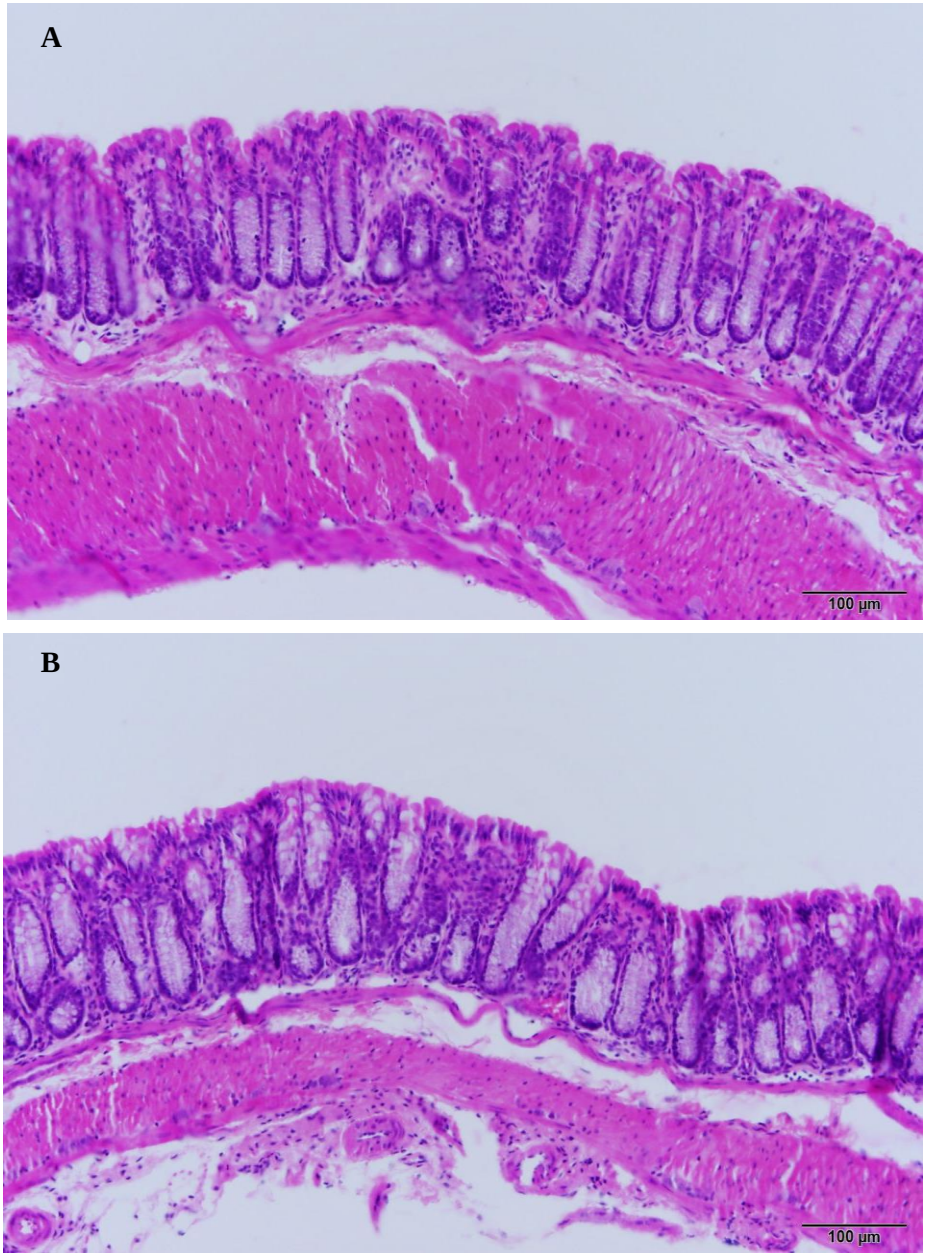


Figure 10. Histologic representation of the colon in DSS model of colitis (H&E; scale bar 100 µm). A) No colitis control – normal section of colon. B) Colitis control – severe ulceration with loss of crypts and mononuclear and polymorphonuclear inflammatory infiltrates. C) Treatment with BNTX – submucosal edema as well as inflammatory infiltrates expand the lamina propia and infiltrate the submucosa. D) Treatment with NTI – loss of crypts with submucosal edema and inflammation. E) Treatment with NTX (0.1 mg/kg) – similar pathologic lesions are present to that of the other therapy groups. F) Treatment with NTX (1.0 mg/kg) – mononuclear inflammatory cells expand the lamina propria as well as submucosal edema is present.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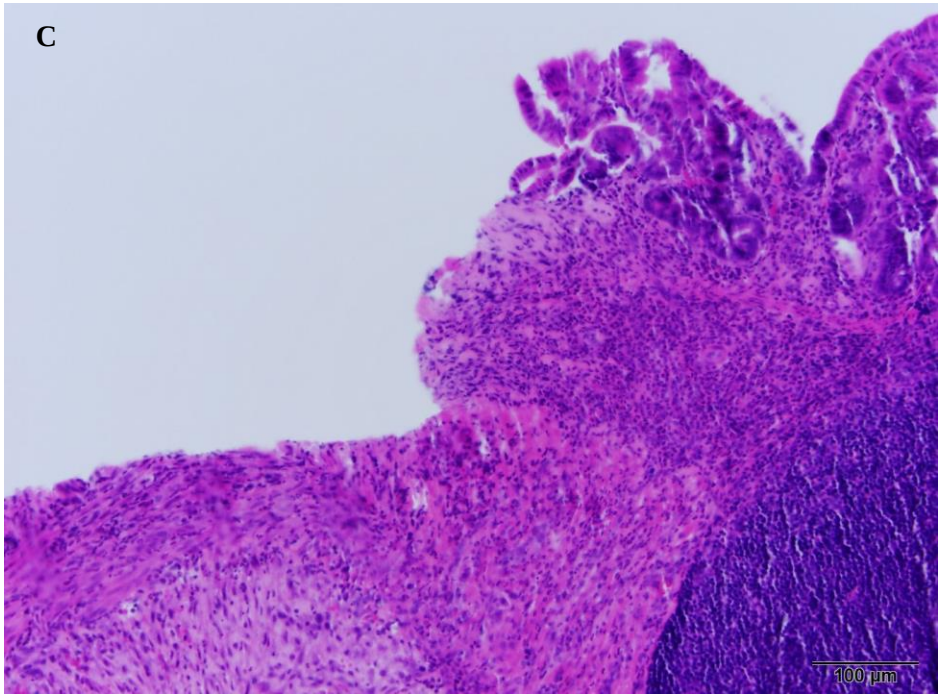
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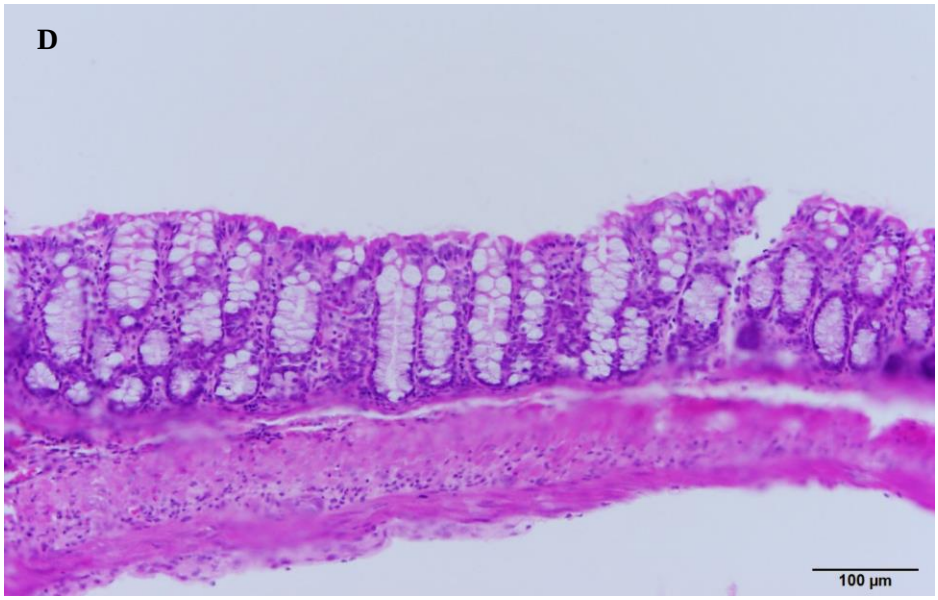
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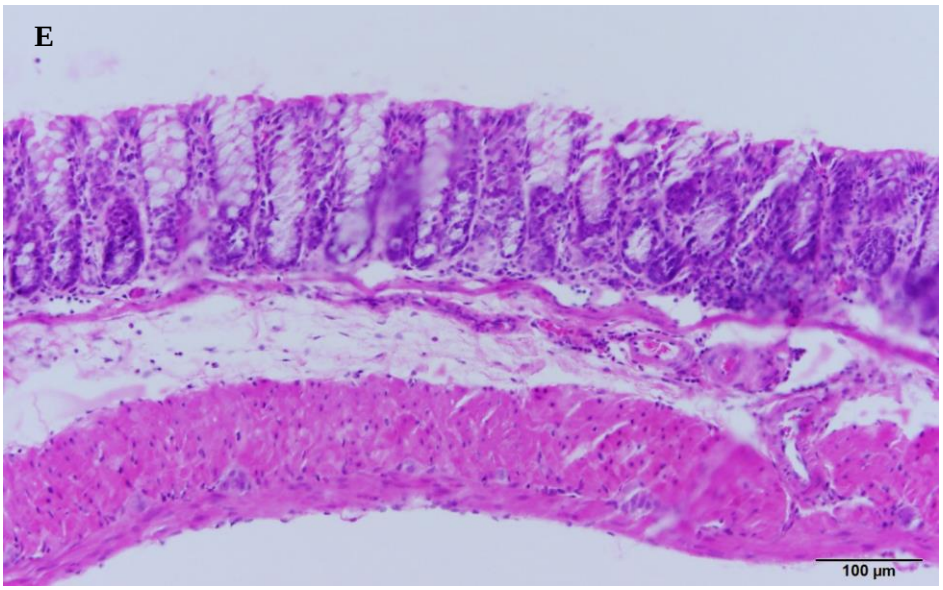
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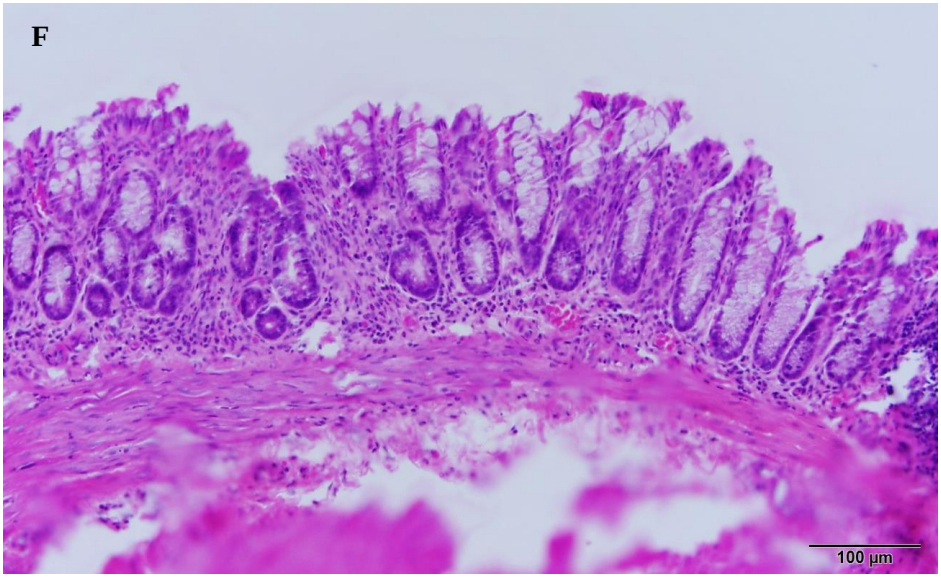
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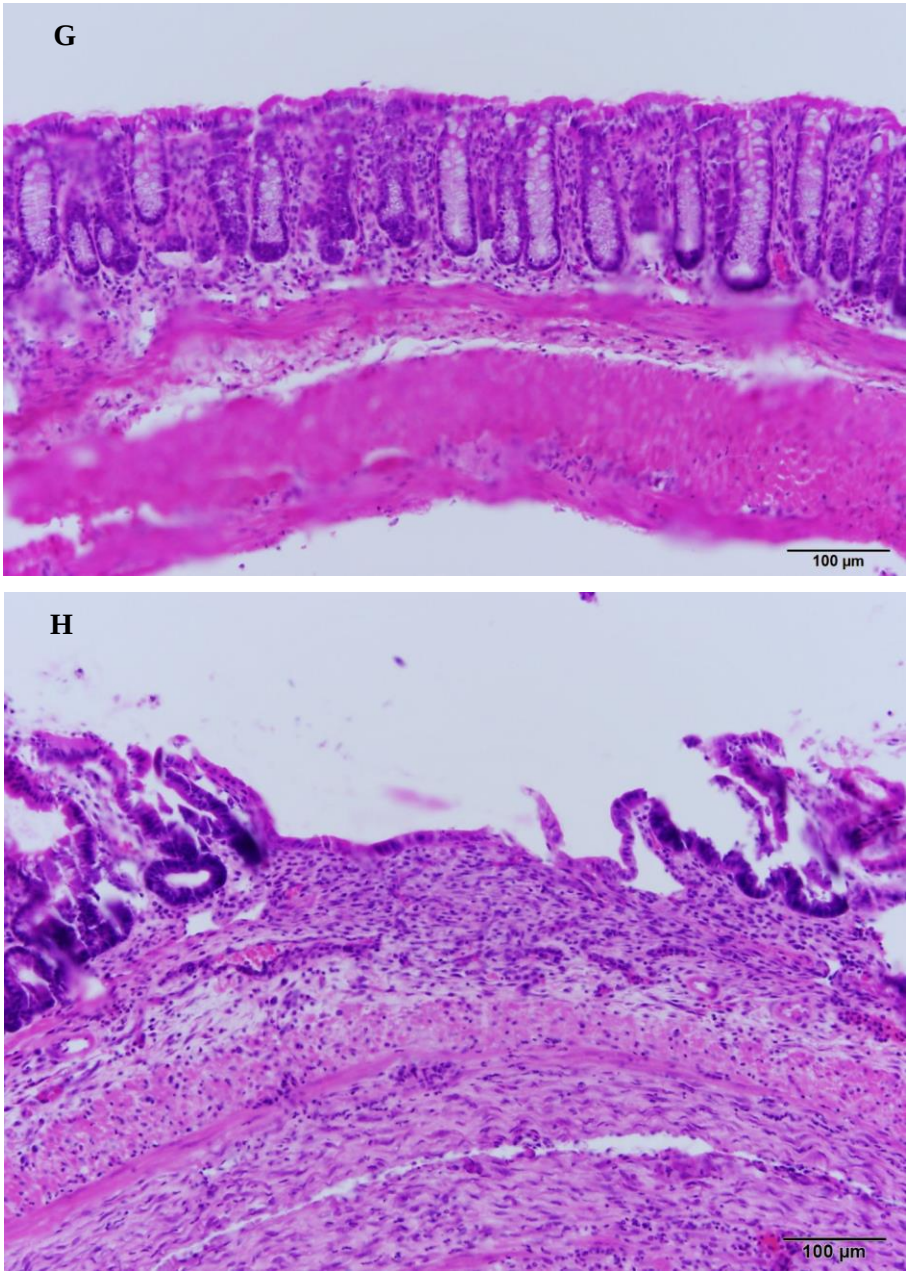

Figure 11. Histologic representation of the colon in TNBS model of colitis (H&E; scale bar 100 µm). A) No colitis control – normal section of colon. B) Vehicle – multifocal areas of mononuclear inflammation expanding the lamina propria. C) Colitis control – focally extensive ulceration with fibrosis. D) Treatment with DPDPE (0.1 mg/kg) – mild inflammation. E) Treatment with DPDPE (1.0 mg/kg) – mild submucosal edema. F) Treatment with NTI – moderate fibrosis and inflammation within the lamina propria. G) BNTX – mild submucosal inflammation. H) NTX (0.1 mg/kg) – focally extensive ulceration of the mucosal epithelium.

Chapter 4

Discussion

In the present study, we were able to successfully induce colonic inflammation in two established murine models of experimental colitis. Achievement of these experimental models was confirmed with histology by the presence of acute ulcerations, submucosal edema, and polymorphonuclear as well as mononuclear inflammatory infiltrates. These IBD models were also confirmed by the elevation in MPO activity, increased DAIs, and greater colon lengths and weights within the colitis control groups. Therapy with NTX (0.1 mg/kg) significantly increased the colon length towards the normal values. Both doses improved DAI scores on days 4 and 5, and NTX (1.0 mg/kg) significantly lowered inflammation indices in the DSS induced model of colitis. These findings are consistent with and provide validation of a previous report regarding NTX therapy in DSS-treated mice with moderately induced colitis (Matters et al., 2008). At present, there are no studies reported in the literature to our knowledge, evaluating therapeutic effects of NTX in TNBS-induced murine colitis. In this model, we also demonstrated improvement of colitis with NTX therapy. NTX (1.0 mg/kg) significantly reduced gross ulcer scores as well as decreased MPO activity in the tissues (however this did not reach significance). Low dose NTX also improved HCT levels on CBC analysis.

Expression of the mu, delta, and kappa opioid receptors is present in the brain, spinal cord, and the myenteric and submucosal plexuses of the gastrointestinal tract (Bagnol, Mansour, Akil, & Watson, 1997) and in lower concentrations in enterocytes (Lang, Davison, Bates, & Meddings, 1996). Opioid receptors in the gastrointestinal tract participate in the inhibitory modulation of gut motility and secretion (Pol, Alameda, & Puig, 2001). Although opioid

receptors and their ligands play a critical role in the physiology and pathophysiology of the gastrointestinal tract, the anti-inflammatory actions of opioids are largely unrecognized.

The mu opioid receptor has been shown to exert an anti-inflammatory effect in the colon through regulation of cytokine production and T cell proliferation (Philippe et al., 2003). The use of Salvinorin A has been reported to have anti-inflammatory as well as analgesic effects in mice with colitis which is mediated through the kappa receptor (Fichna et al., 2012). Few studies have investigated the role of the delta receptor as it pertains to IBD. Wade *et al.* suggested that increases in the delta opioid receptor activity and expression in intestinal tissue were the result of inflammatory stress (Wade et al., 2012). Another study found that DPDPE, a delta agonist, had no effect on transit time in naïve mice, however, reduced castor oil induced diarrhea (Shook, Lemcke, Gehrig, Hruby, & Burks, 1989), and thereby suggested that castor oil enhanced the activity of the delta receptor. Similar findings were described by Pol *et al.* whereby diarrhea caused by croton oil induced intestinal inflammation and potentiated the delta opioid receptor agonist inhibition of gastrointestinal transit (Pol, Ferrer, & Puig, 1994). Essentially, the nature and duration of stress can affect expression levels of the delta receptor and therefore may influence efficacy to the specific ligand.

In the current study it was hypothesized that reduction in colonic inflammation may occur through blockade of the delta receptor, since delta opioid receptors are the predominant receptor subtype associated with inflammatory cells (van Rijn et al., 2013). The results revealed that treatment with NTI and BNTX had varying levels of efficacy. For instance, therapy with BNTX and NTI did not improve colon length or weight in mice receiving TNBS or DSS induced colitis. Similar negative results were also evident when assessing MPO activity in the colon tissue with these compounds. Conversely, DAI scores were slightly improved throughout the course of the study, although significance was not obtained. It is possible that the dose was not optimal to

achieve therapeutic effects, or, use of these specific delta receptor antagonists does not play a role in gastrointestinal inflammation induced via DSS or TNBS.

A small subset of mice was used in the TNBS study to evaluate if the inflammatory response would be abolished by administering the delta agonist DPDPE. We found that DPDPE had minimal effects on colon length and weight as well as reduced HCT levels when administered at the lower dose (however this was not significant). DPDPE also failed to reduce MPO activity within the colon of mice given an enema containing TNBS. The higher dose of DPDPE allowed mice to maintain body weight throughout the study compared to the colitis controls as well as lowered inflammation indices (although not significant). Jiménez *et al.* reported that use of delta opioid receptor DPDPE reduced acute and chronic intestinal inflammation (Jimenez, Puig, & Pol, 2006). In this study, similar pharmacologic effects were not observed, which may have been attributed to the lower dose (0.1 mg/kg compared to 5 mg/kg). Also, DPDPE may have more effect on the small intestine opioid receptors as opposed to the large intestine.

The effects of NTX varied between the two colitis models. Low dose NTX improved colon length and weight, DAI scores, and HCT values in the DSS model, whereas, high dose NTX decreased colon weight, lowered MPO activity, reduced ulcer scores, and maintained body weight in the TNBS colitis mice. Because the etiology and pathogenesis of CD and UC are different, these findings may therefore result in the differing therapeutic effects of NTX as evidenced in this study.

Individuals with active colitis exhibit clinical signs of weight loss, dehydration, occult rectal bleeding and abdominal pain. Although these observations are part of the expected disease involvement in animal models, allowance of these characteristics would not be compatible with the best care from an animal welfare perspective according to the *Guide* (Institute for Laboratory Animal Research, 2011) and were therefore considered limitations of this study. Mice

experiencing >15% body weight loss or gross bleeding were euthanized prior to study endpoint according to humane care as outlined in the protocol. In some cases, mice expired prior to the study endpoint, thereby reducing the number of mice in various treatment groups. This etiology of death most likely was attributed to their advanced disease state. In other instances use of inhalation anesthesia also contributes to unexpected mortalities (Fish, Brown, Danneman, & Zaras, 2008). Another limitation of this study involved blood collection by cardiocentesis. Due to dehydration and/or blood loss, hematologic samples were often difficult to obtain or were not of diagnostic quality. Intrarectal administration of TNBS caused varying degrees of colitis in control and treated mice, so despite efforts to retain the enema via the operator, it is possible that the haptenating agent did not remain in the colon long enough to induce inflammation and was expelled from the anus of the mice during recovery from anesthesia.

In conclusion, we were able to successfully show that non-selective opioid receptor blockade exerted some preventive and therapeutic intestinal anti-inflammatory effects in mice with colitis. Since improvement was demonstrated in both murine experimental models of IBD, our work suggested that opioid receptor blockade could potentially be beneficial in both CD and UC. The use of opioid receptor antagonists for the treatment of IBD may be advantageous in that these compounds do not display adverse side effects such as bone marrow suppression, a common toxicity reported with administration of the current therapeutic agents (i.e. corticosteroids, immunomodulators, and biologics). Consistent with this speculation is that the white blood cell indices were not suppressed and HCT values were improved in mice treated with NTX.

Since there were not any significant changes with the use of delta opioid receptor antagonists or agonists, our study would suggest that the beneficial effects of NTX are not mediated through the delta receptor in IBD. Additional studies are needed to determine efficacy for the delta receptor as well as the other opioid receptors (mu, kappa, and OGF) in IBD. One of

the key features of intestinal inflammation is upregulation of opioid receptors in the gastrointestinal tissues, both at the mRNA and protein level (Jimenez, Puig, & Pol, 2006; Pol et al., 2001; Pol, Palacio, & Puig, 2003). Future studies may include assessment of proinflammatory cytokines, dose optimization for NTX, dose optimization for selective delta opioid receptor antagonists, use of different selective antagonists, and the role of opioid receptor blockade in chronic animal models of IBD.

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