Journal of Visualized Experiments

A TNBS-Induced Rodent Model to Study the Pathogenic Role of Mechanical Stress in Crohn's Disease

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63499R2
Full Title:	A TNBS-Induced Rodent Model to Study the Pathogenic Role of Mechanical Stress in Crohn's Disease
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Additional Information:	
Question	**Response**
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TITLE:

A TNBS-Induced Rodent Model to Study the Pathogenic Role of Mechanical Stress in Crohn's Disease

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SUMMARY:

The present protocol describes the development of a Crohn's-like colitis model in rodents. Transmural inflammation leads to stenosis at the TNBS instillation site, and mechanical enlargement is observed in the segment proximal to the stenosis. These changes allow studying mechanical stress in colitis.

ABSTRACT

Inflammatory bowel diseases (IBD) such as Crohn's disease (CD) are chronic inflammatory disorders of the gastrointestinal tract affecting approximately 20 per 1,00,000 in Europe and USA. CD is characterized by transmural inflammation, intestinal fibrosis, and luminal stenosis. Although anti-inflammatory therapies may help control inflammation, they have no efficacy on fibrosis and stenosis in CD. The pathogenesis of CD is not well understood. Current studies focus mainly on delineating dysregulated gut immune response mechanisms. While CD-associated transmural inflammation, intestinal fibrosis, and luminal stenosis all represent mechanical stress to the gut wall, the role of mechanical stress in CD is not well defined. To determine if mechanical stress plays an independent pathogenic role in CD, a protocol of TNBS-induced CD-like colitis model in rodents has been developed. This TNBS-induced transmural inflammation and fibrosis model resembles pathological hallmarks of CD in the colon. It is induced by intracolonic instillation of TNBS into the distal colon of adult Sprague-Dawley rats. In this model, transmural inflammation leads to stenosis at the TNBS instillation site (Site I). Mechanical distention is observed in the portion proximal to the instillation site (Site P), representing mechanical stress but not visible inflammation. Colonic portion distal to inflammation (Site D) presents neither

inflammation nor mechanical stress. Distinctive changes of gene expression, immune response, fibrosis, and smooth muscle growth at different sites (P, I, and D) were observed, highlighting a profound impact of mechanical stress. Therefore, this model of CD-like colitis will help us better understand CD's pathogenic mechanisms, particularly the role of mechanical stress and mechanical stress-induced gene expression in immune dysregulation, intestinal fibrosis, and tissue remodeling in CD.

INTRODUCTION:

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is characterized by chronic inflammation in the gastrointestinal (GI) tract. It affects ~1-2 million Americans[1]. The estimated annual costs for IBD care in the US are $11.8 billion. Unlike UC, the CD is characterized by transmural inflammation and stricture formation[2,3]. Stricture formation (stenosis) occurs in up to 70% of CD patients[3] and may be caused by transmural inflammation (inflammatory stenosis) or intestinal fibrosis (fibrotic stenosis)[4,5]. Intestinal fibrosis is characterized by excessive collagen deposition and other extracellular matrices (ECM) with smooth muscle cells (SMC) as one of the main mesenchymal cell types involved in the process[3,4]. Smooth muscle hyperplasia associated with hypertrophy is another significant histological change in fibrotic stenosis in CD[6]. Although stricture formation in CD is associated with chronic inflammation, no anti-inflammatory treatment is effective, except surgical treatment[2,6]. However, post-surgery recurrences are almost 100%, given sufficient time[2,7]. As an inflammatory response, fibrosis and SMC hyperplasia may also develop in non-inflammatory conditions (i.e., bowel obstruction) in the gut[8,9]; it is believed that both inflammation-dependent and independent mechanisms are involved in stricture formation[3,4]. Given that extensive research into the inflammation-dependent mechanisms has not translated into any effective therapy for stricture formation, studies into the possible role of inflammation-independent mechanisms in intestinal fibrosis are needed.

As a non-inflammatory factor, mechanical stress (MS) associated with edema, inflammatory cell infiltration, tissue deformation, fibrosis, and stenosis[10-13] is commonly encountered in IBD, especially CD, which is characterized by transmural inflammation. Mechanical stress is most remarkable in stenotic CD, where stenosis (inflammatory or fibrotic) in the inflammation site presents mechanical stress in the local tissue and leads to lumen distention in the segment proximal to the obstruction site[10,14]. Previous *in vitro* studies have demonstrated that mechanical stress alters gene expression of specific inflammatory mediators (i.e., COX-2, IL-6)[8,14,15] and growth factors (i.e., TGF-β) in the gastrointestinal tissues, especially gut smooth muscle cells (SMC)[16]. Recent studies also found that the expression of specific pro-fibrotic mediators such as connective tissue growth factor (CTGF) is highly sensitive to mechanical stress[17,18]. It was hypothesized that mechanical stress might play an independent pathogenic role in CD-associated inflammation, fibrosis, and tissue remodeling. However, the pathogenic significance of mechanical stress in gut inflammation, fibrosis, and smooth muscle hyperplasia in CD remains largely unexplored. This may be partly because inflammation is a more visible and better-studied process than mechanical stress. More importantly, there has been no well-defined animal model of IBD to distinguish the effect of mechanical stress from that of inflammation.

The current work describes a rodent model of Crohn's-like colitis induced by intracolonic injection of hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS)[19,20], which may serve the purpose to study the role of mechanical stress in CD. It was found that TNBS instillation induced a localized (~2 cm in length) transmural inflammation with lumen narrowing (stenosis) in the distal colon. The stenosis leads to marked bowel distention (mechanical stress)[14,15] but not visible inflammation in the colonic segment proximal to the instillation site. On the contrary, the colon segment distal to the stenosis site presents neither inflammation nor mechanical stress. Significant site-specific changes in gene expression, inflammation, fibrosis, and SMC hyperplasia were observed in the three different sites. The results suggest that mechanical stress, particularly mechanical stress-induced gene expression, may play a critical role in developing fibrosis and hyperplasia in Crohn's colitis.

PROTOCOL:

All animal experiments were conducted according to the institutional animal care and use committee of the University of Texas Medical Branch (#0907051C). Male or female Sprague-Dawley rats, ~8-9 weeks old, were used for the study.

1. Animal preparation

1.1. Fast rats for 24 h and treat them with laxative (bowel cleanser, see **Table of Materials**) overnight.

1.2. The next day, anesthetize rats using an anesthesia system (see **Table of Materials**) by exposing them to 2% isoflurane along with 1 L/min of oxygen during TNBS administration. Check for reflexes or pinch toes to confirm anesthetization.

1.3. Prepare fresh TNBS solution according to body weights.

NOTE: TNBS - 65 mg/kg of body weight in 250 µL of 40% ethanol was used.

1.4. Put rats in a supine position on the anesthesia table. To induce colitis, insert through the anus a medical-grade open-end polyurethane catheter for ~7-8 cm from the anal verge and gently instill TNBS (prepared in step 1.3) into the colon[19]. Administer the sham control rats with 250 µL of saline only.

1.5. After instilling TNBS or saline, keep rats in supine and slightly head-down position (~30°), with the anus closed for 2 min to help TNBS distribution and avoid spills.

1.6. Provide rats with food and water ad libitum for 7 days and observe the rats daily for body weight, food uptakes, feces, and general health condition.

2. Tissue preparations

2.1. On the day of euthanasia, euthanize the rats using CO_2 inhalation and confirm euthanasia with cervical dislocation.

2.2. Open the rat abdomen using surgical-grade scissors and forceps.

2.3. Carefully remove the entire colon (above the anal canal) and transfer the colon immediately to ice-cold 1x HBSS buffer.

2.4. Straighten the colon in the buffer and measure the colon length using a ruler. Take nylon thread and circle around the colon to measure the external circumference of the colon segments in control and TNBS-treated rats. Take full-thickness tissues for histology.

2.5. Cut open the colon along the mesenteric board and clean the colon well with HBSS buffer. Assess the colon for macroscopic inflammation score based on the criteria as previously described[19] with minimal modifications.

NOTE: 0 = normal mucosa; 1 = localized hyperemia but no erosions or ulcers; 2 = ulcer and stenosis (affected area < 5 mm); 3 = severe ulcer, scar, and stenosis (affected area > 5 mm).

2.6. Collect colonic tissue samples from site P (portion 2-3 cm before the oral margin of inflammation site), site I (inflammation site, typically 4-6 cm from the end of the colon, where TNBS is instilled to), and site D (portion 1-2 cm distal to the aboral margin of inflammation site), respectively from TNBS-treated rats.

NOTE: Colon tissue of ~1-2 cm-long was taken from each segment. In addition, the colon tissues of 2 cm long (~4-6 cm from the end of the colon) of the saline-treated rats were taken as sham control (S) (**Figure 1**).

2.7. Take tissue samples from each site for full-thickness preparation, and if desired, mucosa/submucosa and muscularis externa layers, respectively, as well[21,22].

2.8. Freeze tissue samples in liquid nitrogen first before storing them at -80 °C for storage up to one year and for future purposes (i.e., RNA preparations).

3. Histopathologic assessment of gut inflammation and fibrosis

3.1. Fix the full-thickness colon tissues in 10% formalin for 48 h, then transfer to 70% ethanol for 24-48 h.

3.2. Use a microtome to cut paraffin sections of 5 µm thickness for Hematoxylin and eosin (H&E) and Masson's Trichrome stains[6,19,23] (see **Table of Materials**), respectively.

3.3. Acquire and view images with an upright microscope equipped with a high-resolution camera with compatible software (see **Table of Materials**).

3.4. Grade inflammation and fibrosis indexes by two independent investigators, including a gastrointestinal surgical pathologist according to criteria described previously[6,23] with modifications. See **Supplementary File 1** for the scores.

3.5. Measure the thickness and cell numbers of the circular and longitudinal muscle layers per cross-section in four views of each H&E stained specimen and take the mean of the four measurements for each specimen.

4. RNA extraction and quantitative RT-PCR

4.1. Homogenize excised colon tissues obtained from the sham control and three sites (P, I, D) of TNBS colitis rats in the extraction reagent of an RNA extraction kit (see **Table of Materials**).

4.2. Isolate RNA from each sample utilizing the kit. Elute the RNA pellet in 30 µL of RNase-free water.

4.3. Quantify RNA concentration and check for purity using a microvolume UV-Vis spectrophotometer (see **Table of Materials**).

4.4. Use 1 µg of total RNA to synthesize cDNA[21,22] using the RNA synthesis kit (see **Table of Materials)**.

4.5. Analyze and quantify gene expression levels by performing real-time PCR with 50 ng of cDNA as a template, probes of IL-6, and CTGF using a commercial PCR kit for real-time PCR system (see **Table of Materials)**.

4.6. Use control gene 18S rRNA to normalize the samples and quantify relative gene expression utilizing the Cq values obtained.

5. Statistical analysis

5.1. Utilize statistical analysis software (see **Table of Materials**) to compare sham control and TNBS colitis rats.

5.2. Consider p value < 0.05 to be statistically significant[15,19].

5.3. To test the differences between two groups, use Student's t-test analysis and perform an ANOVA test if comparisons are more than two groups[15,19].

REPRESENTATIVE RESULTS:

Macroscopic view of Crohn's-like colitis induced by intra-colonic instillation of TNBS

As shown in **Figure 1**, intracolonic instillation of TNBS in rats induced a localized transmural inflammation (~2 cm in length) with thickened bowel wall and narrowed lumen (stenosis) in the site of instillation in the distal colon (**Figure 1A**). The site of TNBS instillation is referred to as site I. As a result of transmural inflammation and stenosis, both inflammation and mechanical stress are present in site I. The stenosis in site I led to marked lumen distention in the segment proximal to the site of TNBS instillation (site P) (**Figure 1**). The colon circumference was significantly increased in sites P and I, compared to sham control colon ($p < 0.05$ *vs*. control) (**Figure 1B**). While mechanically distended, site P did not show visible inflammation. On the contrary, the colonic segment distal to the TNBS instillation site is site D and presents neither inflammation nor mechanical distention (**Figure 1A,B**).

To help distinguish the effect of mechanical stress from inflammation, we followed a unique design in collecting tissue samples from the colon in the model described in step 2.6. Site P is the primary focus of the study, as this part is mechanically distended in the CD model. Site D is self-control, as it does not present mechanical stress. Sites P and D do not demonstrate any visible inflammation (**Figure 1**). However, the site I experience inflammation and mechanical stress (**Figure 1**).

Site-specific changes of inflammation score in sites P, I, and D in colitis rats

Criteria were developed in grading inflammation based on the macroscopic score of live tissue (0-3)[19] and the microscopic score of H&E stained specimens (0-3)[23] as described with modifications. The results showed that the macroscopic score of inflammation in site I was 2.70 ± 0.20 in the TNBS treated rats (7 days after induction of inflammation), dramatically increased compared to that of sham controls (0.30 ± 0.22, $p < 0.05$) and that of sites P (0.80 ± 0.26) and D (0.50 ± 0.22) of colitis rats (**Figure 1C**). The inflammation scores in sites P and D are not significantly increased compared to sham (**Figure 1C**). Microscopic imaging showed TNBS treatment-induced transmural inflammation in rats (**Figure 2A**). The microscopic score of inflammation in site I was 2.80 ± 0.27 in the TNBS treated rats, again significantly ($p < 0.05$, n = 5 each group) increased compared to that in sham controls (0.3 ± 0.2) and in sites P (1.0 ± 0.31) and D (0.80 ± 0.38) of colitis rats. The inflammation scores in sites P and D were not significantly increased compared to sham (**Figure 2C**).

Site-specific changes of fibrosis and smooth muscle hyperplasia and hypertrophy in sites P, I, and D in colitis rats

The fibrosis score was determined based on Mason's trichrome stain (**Figure 2B**) in different sites (P, I, D) (**Figure 2A**). The grading system for fibrosis is described in **Supplementary File 1**. It was found that fibrosis score is significantly increased not only in site I (2.60 ± 0.25) but also in site P (1.60 ± 0.24) of the colitis rats, compared to sham control (0.40 ± 0.25. $p < 0.05$) (**Figure 2D**). The thickness and cell numbers of circular and longitudinal smooth muscle layers were measured in different sites in H&E stained specimens (4 views per specimen). The thickness and cell numbers of both circular and longitudinal smooth muscle layers were significantly increased in sites I and P (**Figure 2E,F**). The site D in the colitis rats does not show any significant increase in fibrosis score, smooth muscle cell numbers, or muscle thickness (**Figure 2**).

Site-specific expression of mechano-sensitive genes in sites P, I, and D in colitis rats

IL-6 plays a critical role in gut inflammation, as it promotes T cell differentiation, damages barrier function, and affects neuromuscular function[8,24]. CTGF is a well-recognized pro-fibrotic mediator, as its inhibition can reverse the process of fibrosis[17]. More importantly, recent studies found that gene expression of IL-6 and CTGF is highly responsive to mechanical stress[8,14,18]. The site-specific expression of IL-6 and CTGF mRNAs were determined in the full thickness tissue of sham control and TNBS colitis rats. The mRNA expression levels of IL-6 and CTGF were significantly increased in the inflammation site (site I) compared to sham controls. In site P, where there is mechanical distention but no visible inflammation, the mRNA expression of IL-6 and CTGF was also dramatically increased compared to control rats (**Figure 3A,B**). However, IL-6 and CTGF mRNA levels in site D of colitis rats were not significantly different from that in sham controls (**Figure 3**).

FIGURE LEGENDS:

Figure 1: Rodent model of TNBS-induced CD-like colitis (7 day). (**A**) Outlook view of sham control and TNBS-treated colon (top) and macroscopic view of the mucosal surface of the distal colon (bottom). The yellow boxes indicate different sites of colon tissue. S, sham control; I, inflammation site; P, distended colon site proximal to inflammation site; D, non-distended site distal to inflammation. (**B**) Colon circumference in sham control and different sites (P, I, D) of TNBS-treated colon. (**C**) Macroscopic inflammation score of sham control colon and different sites of TNBS-treated colon. n = 5, *$p < 0.05$ *vs.* sham rats of the group. Bars represent SEM.

Figure 2: Histopathologic assessment. Microscopic views in H&E (**A**) and Masson's trichrome stains (**B**) showing collagen distribution in sham and different sites of colitis rats. Quantitative analysis shows increased microscopic inflammation index (**C**), fibrosis (**D**) and muscular thickness (hypertrophy) (**E**), and smooth muscle cell number (hyperplasia) (**F**) in sites I and P, but not D, in TNBS treated rats. In (**E**) and (**F**), the open bars are for the circular smooth muscle layer, and streaked bars are for the longitudinal smooth muscle layer. n = 5 in each group, *$p < 0.05$ *vs*. sham rats of the group. Bars in graphs represent SEM. Bars in (**A**) and (**B**) = 100 µm.

Figure 3: Site-specific expression of mechanosensitive genes (IL-6 and CTGF) in CD-like colitis. (**A**) Expression of IL-6 mRNA in sham control colon and different sites (P, I, and D) of TNBS-treated colitis colon. (**B**) Expression of CTGF mRNA in sham control colon and different sites (P, I, and D) of TNBS-treated colitis colon. n = 4 or 5, *$p < 0.05$ *vs*. S (sham control). Bars represent SEM.

Supplementary File 1: Score of inflammation and fibrosis.

DISCUSSION:

TNBS-induced colitis was introduced in 1989, and it is being used as an experimental model of Crohn's disease since then[19,20,23]. Significant features of this model in rodents include the development of a transmural inflammation that closely resembles the histopathological lesions developed in human Crohn's disease[19,20]. Previous studies on the model have focused mainly on aberrant immune response in the mucosa layer in the site of visible inflammation (the site I)[19,20,23]. Little attention has been paid to the bowel portions proximal and distal to inflammation.

The present study on the inflammation site, as well as the distended proximal segment and the non-distended distal segment, reveals apparent site-specific changes in gene expression, inflammatory response, and histopathological features. The model has been revisited to address the potential pathogenic role of mechanical stress in fibrosis and tissue remodeling in Crohn's disease.

It was found that acute inflammation is immediately developed after mucosal exposure of TNBS in ethanol and is peaked at day 3[19]. Transmural inflammation and inflammatory stenosis are present, which is associated with lumen distention in site P in every rat treated with TNBS. By day 7, chronic transmural inflammation is well developed in site I, as found in the current study and reported elsewhere[20]. Meanwhile, fibrotic changes are apparent in the site, characterized by excessive collagen deposition, as seen in the present study and elsewhere[20]. TNBS instillation in ethanol in the distal colon injures the local mucosa tissue[23] and leads to transmural inflammation in a localized area in the colon. The transmural inflammation with inflammatory infiltration, edema, and tissue deformation[10,12,13] in the instillation site present inflammation and mechanical stress[14] to the localized area (Site I). Moreover, transmural inflammation also causes luminal stenosis in site I[10]. It was found that stenosis, a partial bowel obstruction, causes mechanical distention in the proximal segment (site P). However, the part distal to the instillation site is not distended (site D). As found in the macroscopic and histological scoring systems, while site I presents both inflammation and mechanical stress, sites P and D do not present inflammation. Furthermore, site P shows significantly increased circumference (and thus mechanical stress, according to the law of Laplace[14]), but site D does not. Therefore, a study on the site-specific changes, especially the site P, will explore the pathogenic importance of mechanical stress in the inflammation model in the gut.

It was observed that the expression of mechano-sensitive genes IL-6 and CTGF was increased in site I and in mechanically stretched site P, but not in neighboring site D, where there is no mechanical distention. To examine the hypothesis that mechanical stress may contribute to the development of fibrosis and smooth muscle hyperplasia, fibrosis scores and measured smooth muscle cell numbers and muscle thickness in sites P, I, and D were determined separately. It was found that fibrosis and SMC hyperplasia are present in sites I and P. However, fibrosis and smooth muscle hyperplasia are not detected in site D. These findings indicate that mechanical stress may play an independent pathogenic role in collagen synthesis and cell proliferation in gut inflammation. Further studies are warranted to determine if this effect may be mediated by mechanical stress-induced expression of pro-fibrotic and growth factors such as CTGF in the sites P and I.

Although the described animal model itself can be used to address mechanical stress in stenotic CD-like colitis, it has limitations in the long-term goal to fully define the pathogenic role of mechanical stress in inflammation. For example, the effects of mechanical stress and inflammation in site I of the described model cannot be differentiated. Although it is assumed that mechanical stress is present in site I because of inflammatory infiltration, tissue deformation, stenosis, and distention, how much mechanical stress contributes to the pathological changes there cannot be determined. Further comprehensive studies using *in vitro*, *in vivo*, and *ex vivo*

approaches may be needed. For example, preventing mechanical distention by feeding the colitis animals exclusively with a clear liquid diet[25] may help create a loss-of-mechanical distention status in the colitis model. On the other hand, induction of pure mechanical distention by obstruction band[15] may help create a gain-of-mechanical stress model. Moreover, the *in vitro* mechanical stretch model in cultured cells[14,15] assists in the quantitative determination of the effects of mechanical stress on gene expression and function, as the mode and extent of mechanical stress can be finely controlled in the *in vitro* setting.

To prepare a reproducible model of transmural and stenotic inflammation in CD-like colitis as described in the study, TNBS at 65 mg/kg in 250 µL of 40% ethanol must be used. The model was tested mainly in rats, male or female, 8-9 weeks old. After instillation of TNBS at the dose, transmural inflammation is consistently developed at the local instillation site. The inflammation is associated with inflammatory cells infiltration, edema, and bowel wall thickening, leading to lumen narrowing at the instillation site, resembling pathological characteristics of Crohn's disease[20]. Pilot studies in the lab showed that TNBS at doses lower than 50 mg/kg in the same volume of 40% ethanol might cause gut inflammation but not reliable stenosis in site I. Thus, there would be no apparent mechanical distention in site P. On the other hand, TNBS at doses greater than 80 mg/kg may cause severe inflammation and fatalities. The current protocol using TNBS at 65 mg/kg in 250 µL of 40% ethanol hardly causes fatalities (1 in 16 rats of TNBS colitis).

It was found that bowel cleansing the day before instillation of TNBS is an important step to ensure a relatively clean colon for a reliable model of stenotic colitis. For that, rats need to fast for 24 h and give bowel cleanser overnight before TNBS treatment. It is also important to keep rats in a supine and slightly head-down position with the anus closed for 2 min after instillation of TNBS. This helps to ensure a good distribution of TNBS inside the distal colon.

In summary, it was found that intracolonic instillation of TNBS at 65 mg/kg in 250 µL of 40% ethanol consistently induced CD-like colitis in rats. Transmural inflammation in the model is associated with stenosis at the TNBS instillation site. Mechanical distention, but not inflammation, is observed in the segment proximal to the stenosis. Neither inflammation nor mechanical stress is present in the segment distal to the stenosis. With these changes in different colon sites in a colitis rat, mechanical stress from inflammation in CD-like colitis can be distinguished.

ACKNOWLEDGMENTS:
This work is supported in part by grants from NIH (R01 DK124611 to XZS) and the US Department of Defense (W81XWH-20-1-0681 to XZS). This histology work was done with the help of the UTMB Surgical Pathology Lab.

DISCLOSURES:
The authors report no conflict of interest and have nothing to disclose.

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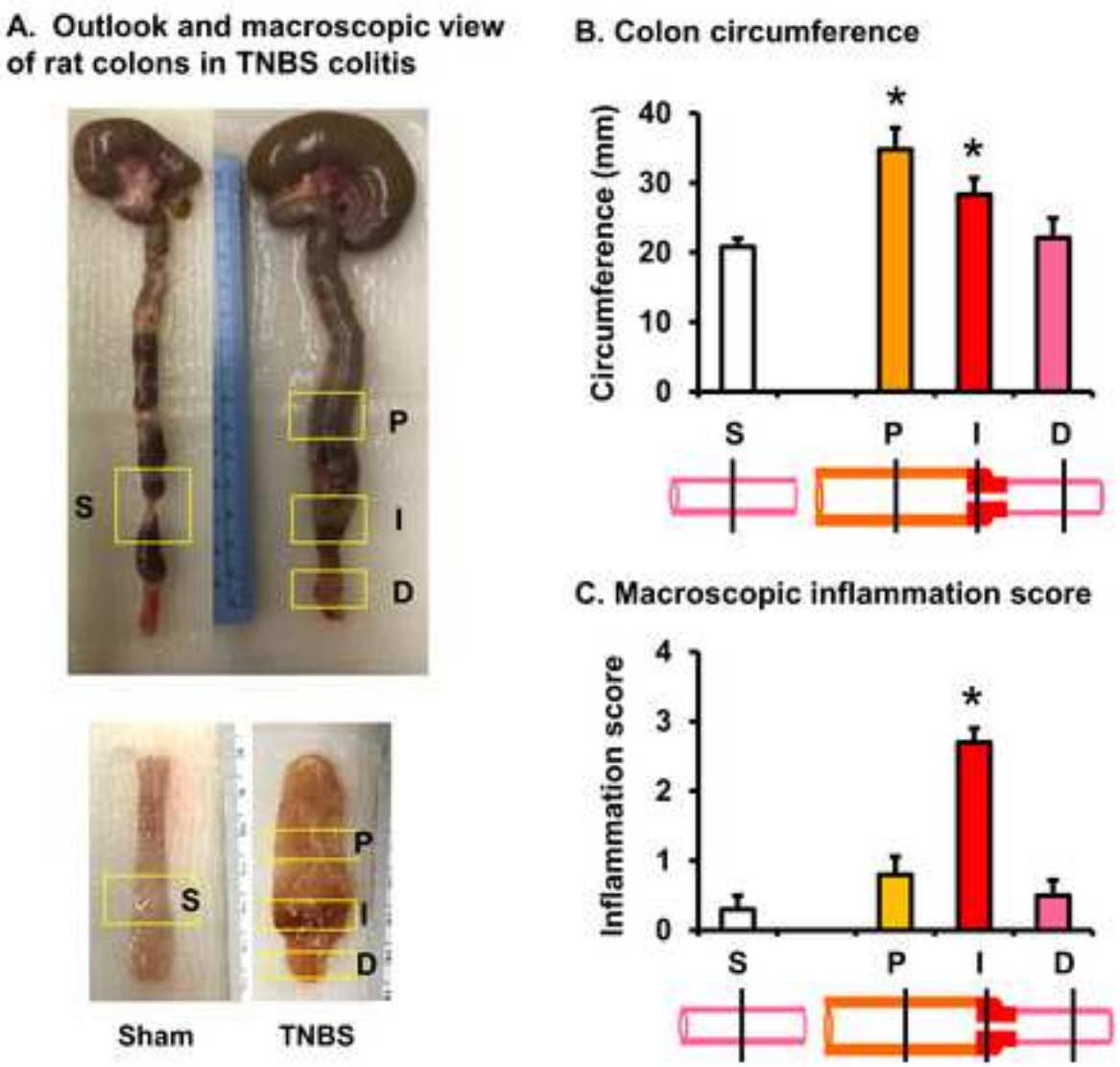
Figure 1



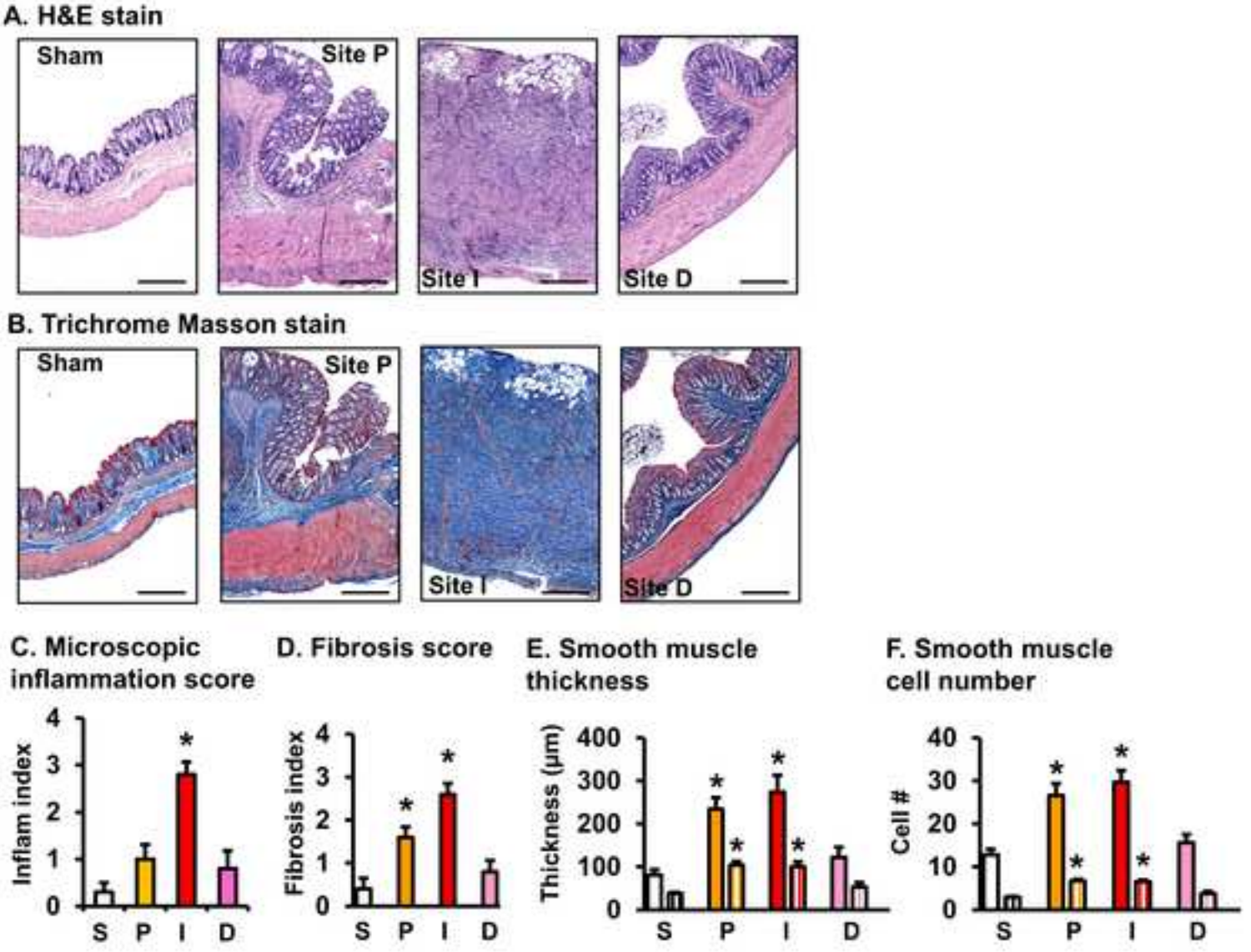
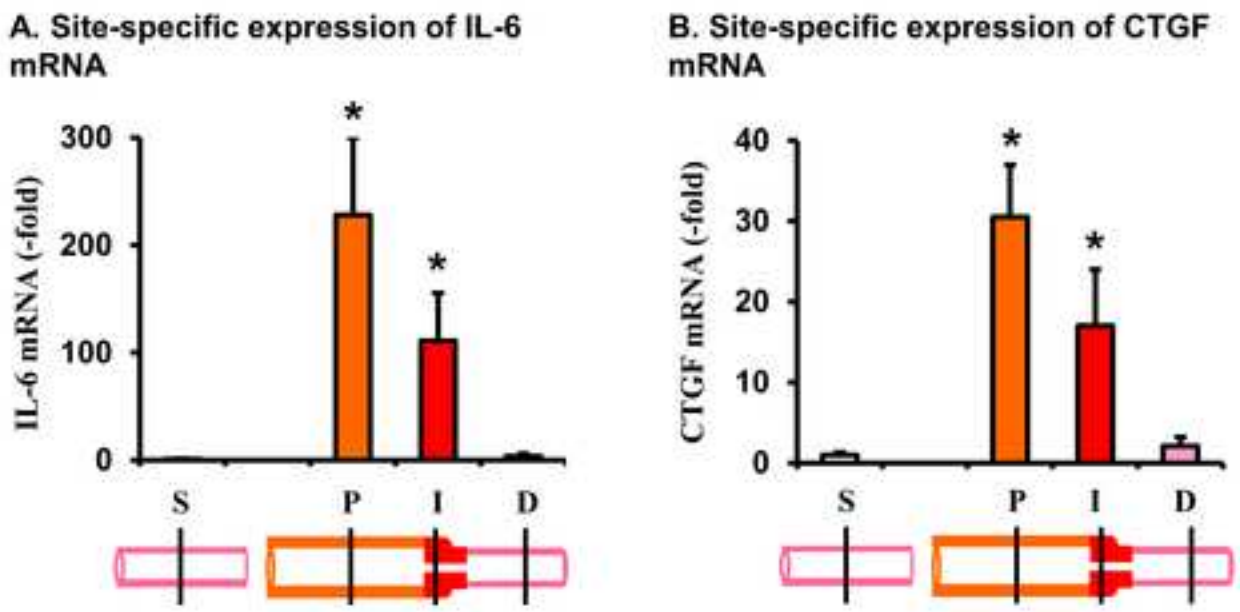


Figure 3



Jan. 5th, 2022

Dear editor and reviewers,

We thank you all for an extensive review of our submission. We believe that we have significantly improved the paper with your helpful comments and suggestions. We are now submitting the revised paper, figures (Fig. 2), Table of Materials for your consideration for publication. Per reviewers' suggestion, we have modified the title slightly. The revised title is "TNBS-induced Crohn's-like Colitis: a rodent model to study the pathogenic role of mechanical stress in Crohn's disease". The point-by-point response to the comments is attached as below. Thank you again.

Sincerely,

Xuan-Zheng Shi, MB, MS, AGAF

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We are thankful for the extensive review and valuable suggestions to improve the quality of our manuscript. Manuscript has been checked for any spelling or grammar issues multiple times.

2. Please reword the following lines to avoid previously published work: 189-194, 267-270.

Response: The sentences were rephrased as suggested by the editor.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Done as suggested.

4. Please ensure that all the abbreviations are defined at the first instance.

Response: Done as suggested.

5. Please format in-text journal references to appear as numbered superscripts after the appropriate statement(s).

Response: Yes, all in-text journal references were modified as they appear as superscripts.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Envigo, Alice, TX, E-Z Anesthesia, Covidien, Tissue-TEK Prisma, Agilent, Nikon, SuperScript™, etc.

Response: Thank you for letting us know. We have changed all the commercial names to generic names and made sure that they were referenced in table of materials.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Response: The numbering of the protocol has been adjusted according to JoVE instruction.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: Thank you for your valuable suggestions. We understand the relevance of detailed description of protocol and manuscript has been revised accordingly.

9. Please add more details to your protocol steps:

Line 115: For the medication, please mention the dosage. Also please include the details in the Table of Materials.

Line 135: How the entire colon length is measured?

Line 146: How long the samples can be stored?

Line 152: "sent to UTMB Histopathological Lab for paraffin sectioning", please include this in the acknowledgement section instead of the protocol step.

Line 154-156: Please remove the reagents from the protocol steps. Instead, please add the functions of the reagents in the Comments column of the Table of Materials.

Line 158: Please provide the details of the ACT-1 software in the Table of Materials. Is this freely available?

Line 172: How the thickness of circular smooth muscle layer and cell numbers per cross section were determined? Please provide details. Citations from published work can also be added.

Line 190: Please include the details of Graph pad prism in the Table of materials.

Line 192: consider p value of < 0.05 to be statistically significant – please include citations to support this.

Response: We appreciate the critical observations and suggestions. All the modifications are done accordingly in the revision.

10. Please include one line space between the protocol steps and highlight that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: The key steps involved in our protocol were highlighted and one line space is included.

11. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and also is in-line with the Title of the manscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of

the step includes at least one action that is written in imperative tense. However, the NOTEs cannot be filmed, so please do not highlight.

Response: Thank you, whole sentences were highlighted.

12. Figure 1/2/3: What do the error bars denote: standard error or standard mean? Please specify in the legend.

Response: Figure legends were added with “bars represent standard error” in the revised manuscript.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., Last Name, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references.

Response: All the references were formatted according to the JoVE journal instructions.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript is well written, and the protocol is clear.

Major Concerns:
None

Minor Concerns:
1. Line 142, please give the definitions of site P, I and D in the methods, instead of those in the results (line 201, 204, 207).

Response: Thank you for your comments and suggestions.

This has been revised accordingly.

2. The resolution of figure 2A and 2B is too low. The morphology is not clear. Please provide high-resolution images.

Response: In order to show the full thickness of the colon specimens, 4x magnification images were taken. We have provided high-resolution images (600 dpi). It is possible that resolution goes low in the combined PDF format. Sorry about it.

3. Did you see any granulomas in rats?

Response: We saw some areas of clustered inflammatory cells, similar to granulomas, in the colon (site I) of TNBS treated rats.

Reviewer #2:

Manuscript Summary:

The manuscript by Geesala et al. described a refined protocol of TNBS-induced CD-like colitis animal model that has distended region proximal to the inflammation site and non-distended region distal to the inflammation site. This animal protocol will be suitable for the study of mechanical stress-induced changes in the gut in inflammatory bowel disease that are independent of inflammation.

Major Concerns:

No major concerns.

Minor Concerns:

1. How long is the distended region extended proximal to the site of TNBS instillation? It looks like the cecum is also distended in TNBS-treated rat in Fig. 1A. Does it happen to all TNBS-treated rats?

Response: Thank you for the comments and questions.

TNBS at the dose specified in our protocol consistently leads to inflammatory stenosis and proximal distention in all rats. The distended region is usually of 4~5 cm proximal to the inflammation site. In severe cases, it may extend to the cecum as shown in Fig. 1.A.

2. Page 7, line 1. Is it possible to count the smooth muscle cells on tissue slides stained by Masson's Trichrome method?

Response: We used slides of H&E stain for measurements of smooth muscle cell number and thickness. This is now specified in the revision. Sorry about the confusion.

3. Fig. 2B, site I. The staining mostly looks blue. It is hard to tell the border of the circular muscle layer. thus, It is unclear how the muscle thickness and smooth muscle numbers were measured at site I.

Response: In the slides of H&E stain, it is not difficult to find the borders. Also, we took 4 views and measurements for each specimen. The image in Fig 2 for site I is of severe inflammation and fibrosis. By adjusting focus and taking different views (4 views/sample) of the specimen, we can have good measurements.

Reviewer #3:

Manuscript Summary:

The authors aim to detect the role of mechanical stress in fibrosis (and inflammation) in CD and present an animal model (rat) in which they us TNBS induced colitis through intracolonic instillation of TNBS (65 mg/kg) in 250µL of 40% ethanol into the distal colon. They then describe histological as well as qPCR based analysis of their outcomes.

Overall it is a quite interesting model as they used a TNBS colitis model to make mechanical stress visible by distend proximal lumen. And fibrosis was observed in the colon proximal to the TNBS instillation site. They owe the fibrogenesis to the mechanical stress as little inflammation was found, which is in line with the clinical observation, since anti-inflammatory therapy doesn't work well on stenosis patients and fibrosis can still progress without inflammation. There are major revisions needed prior to acceptance.

Major Concerns:

1. The authors empathize the model can be used to detect the role of mechanical stress in inflammation, fibrosis, and tissue remodeling in Crohn's disease in the whole article. Although inflammation is usually associated with mechanical stress, the relation between mechanical stress and inflammation is not revealed in this model. And they seem to interpret that fibrosis is induced without inflammation, and solely caused by mechanical stress. It might be better to adjust the title as well as the content and delete the word "inflammation". Also the abstract states that barrier function was changed, which was not investigated in the model at all. Please complete the analysis or delete the paragraph.

Response: Thank you for the comments and questions.

Per reviewer's suggestion, the word "inflammation" is deleted from the title, and the context is adjusted accordingly. The words "barrier function" are deleted in the abstract.

2.Why do the authors skip the presensitazition step like other TNBS model? Is fibrosis can be induced without repeated cycles?

Response: We tested single dose and multiple doses of TNBS in pilot study and found that single dose of TNBS as described in our protocol consistently induced transmural colitis and fibrotic changes in Sprague-Dawley rats (not in mice). Fibrosis induced by repeated cycles of TNBS was no more severe than single dose did in rats. Moreover, multiple doses made it difficult to identify sites P, I, and D.

3. When they measure the muscle layer, they only measured circular muscle layer, is the longitudinal muscle layer affected or not?

Response: Yes, the longitudinal muscle layer is affected as well. We now included the data of longitudinal layer (Fig. 2 E and F).

4. The image of site P and D in Fig.2A and 2B doesn't look like distal colon. They look a little bit more like proximal colon.

Response: We only took the distal part of the colon for study in this work (i.e. for sites P, I, D), as shown in Fig. 1. The proximal colon was not taken for this study.

5. How do they explain the Site P was detected with higher IL-6 and CTGF mRNA expression with lower inflammation and fibrosis grade compared with Site I?

Response: As shown in Fig. 3, the IL-6 and CTGF mRNA expression in site P was not statistically higher than in site I. However, in some individual colitis rats, the expression levels of IL-6 or CTGF in site P might be higher than in site I, while inflammation was not visible there. In a separate study using mechanical obstruction model, we also saw similar extent of increase of IL-6 and CTGF expression in the distended colon segment proximal to obstruction, compared to non-distended distal segment (data not shown in this paper). This indicates that mechanical stress certainly induces up-regulation of pro-inflammatory and profibrotic mediators. This is further elaborated in Discussion.

6. The analysis is quite superficial. What markers for mechanical stress can be used to complete the analysis? Also, what changes to the extra cellular matrix did the authors investigate? TNBS colitis for induction of inflammation and fibrosis is a very common and old model, the authors truly need to add additional analysis of their mechanical stress theory to prove their point.

Response: Yes, TNBS-induced colitis is a widely used model mimicking Crohn's-like colitis. While almost all studies focus on the inflammation site (site I) of the model, we focus on the segment prior to inflammation (site P) in this study and take site I and side D as a reference only. This is because site P does not have visible inflammation, but marked distention as shown in Fig. 1. We have measured external circumference of the colon segments as a marker of distention (Fig. 1). According to the law of Laplace, mechanical stress in the tissue is directly proportional to the size of lumen radius (circumference/2π).

As the reviewer stated earlier, the inflammation site (with inflammatory infiltrations, edema and tissue deformation) is supposedly associated with mechanical stress. But our focus in this study is not the inflammation site, as we do not have other good markers to differentiate the effects of

inflammation and mechanical stress in vivo in the site I of the model. However, we do plan to take comprehensive approaches (i.e. in vitro, in vivo, and ex vivo tools, prevention of lumen distention in the colitis model, and comparison of the colitis model with pure mechanical obstruction model) in the future to further differentiate the effects of mechanical stress from inflammatory process. This is further elaborated in Discussion.

This article is to introduce the model and design for the study of mechanical stress in CD-like colitis. We believe that we have shown representative data to demonstrate the usefulness of the model. Our histology studies have shown increased collagen deposition and fibrosis in sites I and P (Fig. 2).

Minor Concerns:

1. Why they took Site I sample 4-6cm away from the anal verge while they insert 7-8cm during instillation?

Response: As stated in the Methods and Results, site I tissue was taken 4~6 cm from the end of the colon (**not from the anal verge**). The colon was isolated above the anal canal, and the anal canal is ~ 2 cm long in rats. However, when we instilled TNBS, the catheter was inserted 7~8 cm from the anal verge.

Score of inflammation and fibrosis:

Score of inflammation: 0 = normal view; 1 = minimal inflammation (involving less than 1/3 area of the specimen) with infiltration largely in the mucosa and submucosa; 2 = moderate inflammation (involving 1/3-2/3 area of the specimen), ulceration, and tissue thickening (transmural infiltration involving muscle layer); 3 = severe transmural inflammation (involving more than 2/3 area of the specimen), ulcer, and tissue deformation.

Scores of fibrosis: 0 = normal view; 1 = mild increase of extracellular matrix components in mucosa and submucosa layers (involving less than 1/3 area of the specimen); 2 = moderate increase of extracellular matrix components in mucosa and submucosa layers, and mild increase in muscularis externa (involving 1/3~2/3 area of the specimen); 3 = severe increase of extracellular matrix components in mucosa, submucosa layers, and muscularis externa, with tissue deformation (involving more than 2/3 area of the specimen).