ORIGINAL ARTICLE

Key factors in developing the trinitrobenzene sulfonic acid-induced post-inflammatory irritable bowel syndrome model in rats

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Abstract

AIM: To investigate the key factors in developing the trinitrobenzene sulfonic acid (TNBS)-induced post-inflammatory irritable bowel syndrome (PI-IBS) model in rats.

METHODS: TNBS was administered to rats at the following conditions: (1) with different doses (20, 10, 5 mg/0.8 mL per rat); (2) with same dose in different concentrations (20 mg/rat, 25, 50 mg/mL); (3) in different ethanol percentage (25%, 50%); and (4) at depth either 4 cm or 8 cm from anus. At 5 d and 4 wk after TNBS administration, inflammation severity and inflammation resolution were evaluated. At 4 and 8 wk after TNBS application, visceral hyperalgesia and enterochromaffin (EC) cell hyperplasia were assayed by abdominal withdrawal reflex test, silver staining and capillary electrophoresis.

RESULTS: Our results showed that: (1) TNBS induced dose-dependent acute inflammation and inflammation resolution. At 5 d post TNBS, the pathological score and myeloperoxidase (MPO) activity in all TNBS treated rats were significantly elevated compared to that of the control (9.48 ± 1.86, 8.18 ± 0.67, 5.78 ± 0.77 *vs* 0, and 3.55 ± 1.11, 1.80 ± 0.82, 0.97 ± 0.08 unit/mg *vs* 0.14 ± 0.01 unit/mg, $P < 0.05$). At 4 wk post TNBS, the pathological score in high and median dose TNBS-treated rats were still significantly higher than that of the control (1.52 ± 0.38 and 0.80 ± 0.35 *vs* 0, $P < 0.05$); (2) Intracolonic TNBS administration position affected the persistence of visceral hyperalgesia. At 4 wk post TNBS, abdominal withdrawal reflex (AWR) threshold pressure in all TNBS-treated groups were decreased compared to that of the control (21.52 ± 1.73 and 27.10 ± 1.94 mmHg *vs* 34.44 ± 1.89 mmHg, $P < 0.05$). At 8 wk post TNBS, AWR threshold pressure in 8 cm administration group was still significantly decreased (23.33 ± 1.33 mmHg *vs* 36.79 ± 2.29 mmHg, $P < 0.05$); (3) Ethanol percentage affected the TNBS-induced inflammation severity and visceral hyperalgesia. In TNBS-25% ethanol-treated group, the pathological score and MPO activity were significantly lowered compared to that of the TNBS-50% ethanol-treated group, while AWR threshold pressure were significantly elevated (36.33 ± 0.61 mmHg *vs* 23.33 ± 1.33 mmHg, $P < 0.05$); and (4) TNBS (5 mg/0.8 mL per rat, in 50% ethanol, 8 cm from anus)-treated rats recovered completely from the inflammation with acquired visceral hyperalgesia and EC cell hyperplasia at 4 wk after TNBS administration.

CONCLUSION: TNBS dosage, concentration, intraco-

Ionic administration position, and ethanol percentage play important roles in developing visceral hyperalgesia and EC cell hyperplasia of TNBS-induced PI-IBS rats.

INTRODUCTION

Post-infectious irritable bowel syndrome (PI-IBS) is a subgroup of IBS in which the IBS patients developed their symptoms after recovery from an acute gastrointestinal infection[1,2]. The features of PI-IBS, such as urgency, loose stool, and abdominal pain, are very similar to those of the diarrhea-predominant IBS[3,4]. Prospective studies indicate that 3%-36% of enteric infections may lead to the generation of new IBS symptoms; the precise incidence mainly depends on the infecting organism and the host responses[1]. Though the term "PI-IBS" was first coined by Chaudhary and Truelove in 1962[5], little attention has been paid to it until recently[1]. The causes and/or underlying mechanisms of PI-IBS are still not fully understood, although it is believed that altered gut flora, changed intestinal permeability, activated gut immunity, and functional or structural changes in enteric nervous system are important factors[1,6].

Validated animal models, which aim to mimic one or more features of human disease, play important roles in the studies of mechanisms and potential therapeutic agents for diseases. Based on the current understanding of the clinical features and underlying mechanisms of PI-IBS, the ideal model for PI-IBS should bear the character with one or more features of IBS, such as visceral hypersensitivity, motility dysfunction, alterations in permeability or secretion, and enterochromaffin (EC) cell hyperplasia, and complete recovery from initial infection/inflammation[7]. The chemical agent-induced post-inflammatory IBS animal models have been widely used in mechanistic studies of PI-IBS because the chemical agent-induced inflammatory response and host immune activation can, to some extent, mimic the characteristics of PI-IBS[8,9], and these alterations are consistent with the findings from clinical studies[10,11]. Our previous systematic review showed that the trinitrobenzene sulfonic

acid (TNBS)-induced model is the most commonly used PI-IBS model[7], and the major features of IBS (i.e., visceral hypersensitivity, motility dysfunction, and alteration in permeability or secretion), can be developed in this model. However, there are large variations in the protocol of model development, such as TNBS dosage and concentration, position of intracolonic TNBS administration, ethanol percentage, and the optimal time after intracolonic TNBS administration for model application, *etc.*[7]. These variations make comparison between studies to be very difficult, if not impossible.

As a chemical hapten, TNBS is capable of binding to tissue proteins and stimulating T helper 1 cell mediated immunity; thus, it has been widely used to induce acute colitis by intracolonic administration[12]. It has been reported that TNBS-induced acute inflammation and damage becomes maximal from 3 d to 1 wk after instillation[9]. Previous studies have shown that TNBS had dose-dependent effects on mucosal inflammation[13,14], suggesting dosage is an important factor in TNBS-induced inflammation severity and colonic recovery from such inflammation. However, in previous studies on the TNBS-induced PI-IBS model, the dosage of TNBS varied from 20 mg per rat to 30 mg per rat[7]. Further, there is no consensus on how long it will take for TNBS-induced inflammation recovery after TNBS administration. Some studies, using TNBS (30 mg/rat), took 4 or 6 wk for inflammation recovery after intracolonic TNBS administration[15,16], while another study took at least 8 wk[17]. In addition, TNBS is generally administered into the colon lumen at either 4 cm or 8 cm from the anus[7]. Given the anatomical and physiological differences between these two sites (descending colon *vs* transverse colon), it is possible - but not known - whether the position of TNBS administration influences the results. Furthermore, ethanol is routinely used as a breaker to the mucosal barrier in TNBS-induced PI-IBS studies, and previous studies have shown that 30%-50% ethanol alone induced acute inflammation and hyperemia[18,19]. The commonly used ethanol percentage in developing TNBS-induced PI-IBS model are 25% and 50%[14,20]; and whether the ethanol percentage influences the development of TNBS-induced PI-IBS rat model is still not clear.

Concerning the above variations in developing protocol of the TNBS-induced PI-IBS rat model, the present study aimed to investigate the effects of some impact factors (i.e., TNBS dosage and concentration, position of TNBS administration, and ethanol percentage) on TNBS-induced acute inflammation, inflammation resolution, and later acquired features of IBS (i.e., visceral hyperalgesia and EC cell hyperplasia).

MATERIALS AND METHODS

Materials

TNBS (2,4,6-trinitrobenzenesulfonic acid solution), hexadecyltrimethylammonium bromide, o-dianisidine dihydrochloride, sodium hyposulfite, and silver nitrate were

Table 1 Study design and the treatments

Groups	Treatment (mg/mL per rat, i.col.)	Ethanol (%)	Position (cm to anus)	Time points for evaluation		
				5 d	4 wk	8 wk
1	TNBS, 20/0.4 per rat	50	8	IE	IE	-
2	TNBS, 20/0.8 per rat	50	8	IE	IE	-
3	TNBS, 10/0.8 per rat	50	8	IE	IE	-
4	TNBS, 5/0.8 per rat	50	8	IE	IE/PE	PE/EE
5	TNBS, 5/0.8 per rat	50	4	IE	IE/PE	PE
6	TNBS, 5/0.8 per rat	25	8	IE	IE/PE	PE
7	Saline, 0.8/rat	-	8	IE	IE/PE	PE/EE

TNBS: Trinitrobenzene sulfonic acid; IE: Inflammation evaluation; PE: Pain evaluation; EE: Enterochromaffin cell evaluation.

all purchased from Sigma-Aldrich (St. Louis, MO, United States). Chloral hydrate was purchased from Kou Hing Hong Scientific Supplies (Hong Kong, China).

Animals
Male Sprague-Dawley rats (aged 6 wk with body weight around 220 g) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. Rats were housed 5 per cage and maintained at 25 ℃ under 12 h-12 h alternating light-dark cycle with free access to food and water. Rats were maintained in laboratory conditions for 1 wk to adapt to the environment before each experiment. All animal studies were carried out in accordance with the guidelines of the Committee on Use of Human and Animal Subjects in Teaching and Research, Hong Kong Baptist University.

Experimental design
The rats were randomly divided into 7 groups. TNBS (20 mg/0.4 mL per rat) was given to rats in group 1 (n = 10). The rats in group 2 (n = 10), group 3 (n = 10), and group 4 (n = 14) were intracolonically administered with TNBS at a dose of 20, 10 and 5 mg per rat in 0.8 mL ethanol, respectively. In the above 4 groups, TNBS in 50% ethanol saline solution was administered at a depth of 8 cm from the anus. Rats in group 5 (n = 14) were given TNBS (5 mg/0.8 mL per rat in 50% ethanol) at a depth of 4 cm from the anus, while rats in group 6 (n = 14) were given TNBS (5 mg/0.8 mL per rat in 25% ethanol) at 8 cm from the anus. Group 7 (n = 14) was set as a control; rats in this group were given saline 0.8 mL at a depth of 8 cm from the anus (Table 1).

Five days after TNBS or saline administration, 4 rats in each group were selected randomly, and the colon tissues from these rats were harvested for inflammation evaluation. The remaining rats were allowed to recover until the pain threshold pressure was measured. At 4 wk after TNBS administration, five rats in each group were selected randomly for pain threshold pressure evaluation; then the colon tissues were collected for inflammation recovery examination. At 8 wk after TNBS administration, the rest of the rats in each group went through pain threshold pressure evaluation to test the stability of visceral hyperalgesia. In order to further investigate if the TNBS-induced PI-IBS model had EC cell hyperplasia in the colon tissue, a part of proximal and distal colon tissues were also collected for EC cell number counting and serotonin [5-hydroxytryptamine (5-HT)] content determination.

Induction of colitis
Colitis was induced according to previous reports, with little modification[16]. Briefly, rats were fasted for 24 h before experiments, and then deeply anesthetized with chloral hydrate (350 mg/kg, i.p.). A fine plastic catheter (external diameter = 0.96 mm) was gently inserted into the descending colon at a depth of 4 cm or 8 cm from anus. The rats were kept in a head-down vertical position, and then TNBS was instilled slowly into the colon lumen within 1 min. After TNBS instillation, the catheter was left in place for 1 min and then slowly removed. The TNBS-treated rats were left on a warm mound of bedding in head-down position to prevent drug leakage until they regained consciousness. The control rats were similarly administered with 0.8 mL saline instead of TNBS.

Tissue preparation
Rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.); then an approximately 6 cm long piece of colon tissue with drug administration position in the middle was removed. After the photos were taken, 3 cm of the distal part (proximal to the anus) was fixed in 4% paraformaldehyde for histological evaluation., the remaining 3 cm (distal to the anus) was placed in liquid nitrogen and stored in a freezer at -80 ℃ for myeloperoxidase activity assay. In addition, approximate 3 cm of proximal colon (1-2 cm from cecum) and 3 cm of distal colon (1-2 cm from anus) tissue were also harvested from the PI-IBS model rats; the proximal part (about 1 cm) was fixed in 4% paraformaldehyde for EC cell number counting; the rest was placed in liquid nitrogen, and stored at -80 ℃ for 5-HT content determination.

Histological evaluation
The colon sections (5 μm thick) collected at 5 d and 4 wk after TNBS administration were all stained with hematoxylin and eosin (H and E). Masson trichrome staining[21] was performed on sections collected at 4 wk after TNBS administration for fibrosis evaluation. All sections were examined under a Nikon light microscope (Nikon Inc., Japan). The severity of the acute inflammation and the degree of inflammation resolution was graded using the macroscopic and histological scoring criteria (Tables 2 and 3), which were modified based on previous reports[22,23] according to the pathologist's suggestion. Five random fields were selected in each slide; images were captured with 100 × magnifications and analyzed using Image J NIH software. For fibroplasia evaluation, the area stained blue by Masson trichrome staining was measured and adjusted to reflect the total area of the colon tissue[21].

Myeloperoxidase activity assay
Myeloperoxidase (MPO) is an enzyme released by neu-

Table 2 Criteria for macroscopic scoring of colonic ulceration and inflammation

Score	Appearance
0	Normal appearance
1	Ulceration with inflammation at 1 or 2 sites
2	More sites of ulceration and inflammation
3	Major sites of damage extending > 1.5 cm along length of colon
4	Major sites of damage extending > 3 cm along length of colon

trophils in tissue under inflammatory conditions, and the level of MPO activity correlates directly with severity of inflammation[17]. In this study, MPO activity was measured by the modified method described by Krawisz *et al*[24] and Diop *et al*[18]. Briefly, the colon tissues were cut into small pieces and homogenized in 0.5% hexadecyltrimethylammonium bromide 1 mL per 100 mg of colon tissue. The homogenates were centrifuged at 19 000 *g* at 4 ℃ for 15 min. Aliquots of 80 mL supernatant were mixed with 120 μL potassium phosphate buffer (50 mmol, pH 6.0) with 0.0005% o-dianisidine dihydrochloride and 0.1% hydrogen peroxide. MPO activity was calculated from the rate of absorbance change during 1 min at 460 nm; one unit of MPO activity is equal to 1.13×10^{-2} changes in absorbance at 25 ℃. The results were normalized to the wet weight of colon tissue and expressed as MPO units/mg tissue.

Abdominal withdrawal reflex
Abdominal withdrawal reflex (AWR) test was performed as previously described to detect the pain threshold pressure[25]. Briefly, rats were lightly anesthetized with ether in order to place a 6 cm long flexible latex balloon into the descending colon and rectum through the anus. The end of the balloon was secured at least 1 cm proximal to the anal verge. Rats were then allowed to recover for at least 30 min. The tube of the balloon was connected *via* a Y-connector to a sphygmomanometer and colorectal distension was applied in increments of 5 mmHg until a visible contraction of the abdominal wall was observed by an investigator blinded to the treatment. The pain threshold pressure was defined as the intensity of colorectal distension that elicited an observable AWR, i.e., a sudden and persistent abdominal muscle contraction with abdomen lift off the platform (Score 3). The pain threshold pressure of all groups was recorded and repeated five times with intervals of at least 5 min for recovery.

Enterochromaffin cell counting
Tissue sections (5 μm thick) were deparaffinized in xylene, and rehydrated with graded ethanol for silver staining according to a method previously described, with little modification[26]. Sections were incubated with 5% ammoniacal silver solution for 4 h at room temperature, then 2 h in 56 ℃ and subsequently 12 h at room temperature in a dark humidified chamber. After rinsing

with water, 5% sodium hyposulfite was added and sections were incubated for 5 min at room temperature. The brown to black silver precipitate in the cytoplasm of EC cells was considered as a positive reaction. Five random fields at 200× magnifications for each section were captured and saved in the same size and resolution by a researcher blinded to treatment. After calibration, the mucosal fields were circled, and the areas of mucosa were calculated using Image J NIH software. EC cell density was calculated and expressed as the number of EC cells per mm^2 of mucosal area.

Serotonin content assessment
5-HT content in the colonic tissue was assayed following the previously reported procedure[27]. Briefly, the colon segment was homogenized in 15% iced trichloroacetic acid; the supernatant of each sample was filtered using 0.22 μm filters and extracted with diethyl ether, then the prepared samples were added to derivatization solution and analyzed by capillary electrophoresis with laser-induced fluorescence detection.

Statistical analysis
Data are presented as mean ± SE. Differences between two groups were analyzed by Student *t* test. When multiple groups were compared, data were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

RESULTS

Effect of trinitrobenzene sulfonic acid concentration on body weight and mortality rate
Based on the findings from our previous systematic review, TNBS (20 mg/rat, in 50% ethanol) was selected and given to rats at 8 cm from anus in 0.4 or 0.8 mL volume. As shown in Table 4, compared to the control group, the body weight of TNBS-treated rats all decreased markedly with loose and bloody stools at 5 d post-TNBS ($P < 0.01$). Moreover, the body weight in high-concentration TNBS (50 mg/mL)-treated rats was significantly lowered when compared to that treated with low-concentration TNBS (25 mg/mL) ($P < 0.05$), and 3 rats in the high-concentration TNBS (50 mg/mL)-treated group were found dead. At 4 wk after TNBS administration, the average body weight of high-concentration TNBS-treated rats was still significantly decreased compared to that of rats treated with low-concentration TNBS and saline ($P < 0.05$); no significant difference was found in the body weight between low-concentration TNBS-treated rats and the saline-treated ones.

Effect of trinitrobenzene sulfonic acid dosage on the severity of acute inflammation and inflammation resolution
Five days after TNBS administration, the results from MPO activity assay and histopathological evaluation showed that, TNBS, when administered at the doses of

Table 3 Criteria of histological scoring in colon tissue

Variables	Severity and scoring			
	0	1	2	3
Ulceration	No ulcer	Ulcerations not exceeding lamina muscularis mucosae	Ulcerations not exceeding submucosa	Ulcerations exceeding submucosa
Edema	Normal thickness	Submucosal expansion < 30%	Submucosal expansion 30%-100%	Submucosal expansion > 100%
Inflammatory cells	No infiltration	Few scattered cells	Distributed but not dense	Dense
Fibroplasia	Normal collagen	Increase < 30%	Increase 30%-50%	Increase > 50%

Table 4 Effects of trinitrobenzene sulfonic acid concentration on body weight and mortality rate

Treatment	TNBS concentration	Body weight (g)		Mortality rate (%)
		5 d	4 wk	
Saline	-	249.6 ± 26.7	343.0 ± 28.9	0
TNBS 20 mg/0.8 mL per rat	25 mg/mL	214.5 ± 16.8[b]	324.5 ± 24.8	0
TNBS 20 mg/0.4 mL per rat	50 mg/mL	179.2 ± 21.4[d]	291.8 ± 42.6[a]	33

[a]$P < 0.05$, [b]$P < 0.01$ *vs* saline-treated group; [d]$P < 0.01$ *vs* TNBS (20 mg/0.8 mL)-treated group. TNBS: Trinitrobenzene sulfonic acid.

20, 10, and 5 mg/0.8 mL per rat in 50% ethanol, dose-dependently induced acute inflammation and damage in the colon tissue of rats (Figure 1). The results indicate that the higher the dose TNBS used, the more severe the inflammation and damage induced. As shown in macroscopic appearance and H and E sections (Figure 1A and B), high dose TNBS (20 mg/rat) induced multiple larger and deeper ulcerations, and dense inflammatory cell infiltration when compared to that treated with low dose TNBS.

As shown in Figure 2, four weeks after TNBS (5-20 mg/rat) administration, there was no significant difference in MPO activity between TNBS-treated rats and the control groups, suggesting no acute inflammation was left at this period. However, results from pathological evaluation showed that the histological scores in high dose (20 mg/rat) and median dose (10 mg/rat) TNBS-treated groups were still significantly higher compared to that of the control ($P < 0.05$), but no significant difference was found between low dose TNBS-treated rats and the control, suggesting low dose TNBS-treated rats had completely recovered from the initial inflammation at 4 wk post TNBS. As shown in Figure 2A and B, the colons from high and medium dose TNBS treated rats lost their normal appearance, and more collagen was found in the submucosa and smooth muscle in Masson staining sections, suggesting the occurrence of fibroplasia.

Effect of trinitrobenzene sulfonic acid administration position, and ethanol percentage on the severity of acute inflammation and inflammation resolution

Knowing that low dose TNBS (5 mg/0.8 mL per rat in 50% ethanol)-treated rats can completely recover from initial inflammation at 4 wk after TNBS administration, further studies were based on this dosage selection. As shown in Figure 3, compared to the control, MPO activ-

ity assay and histopathological evaluation all demonstrated that low dose TNBS (5 mg/0.8 mL per rat, in 50% ethanol), when given to rats at 8 cm or 4 cm from anus, induced marked acute inflammation and ulcers at 5 d post TNBS administration ($P < 0.01$), no difference was found between these two groups. However, the same dose TNBS (5 mg/0.8 mL per rat, 8 cm depth) in 25% ethanol induced significant mild non-ulcer inflammation compared to that treated with TNBS in 50% ethanol ($P < 0.01$). Four weeks after TNBS administration, there were no significant differences in MPO activity and histopathological score among all TNBS-treated groups and the control, suggesting that the rats treated with low dose TNBS (5 mg/0.8 mL per rat) had all completely recovered at 4 wk after TNBS administration.

Effect of trinitrobenzene sulfonic acid administration position, and ethanol percentage on the visceral hyperalgesia

To investigate whether the low dose TNBS (5 mg/0.8 mL per rat)-treated rats acquired long-lasting visceral hyperalgesia after inflammation resolution, AWR test was applied to all these TNBS-treated rats at 4 and 8 wk after TNBS administration. As shown in Figure 4, at 4 wk after TNBS administration, the pain threshold pressure in all TNBS-treated rats was decreased significantly compared to the control ($P < 0.05$), but no significant difference was found among these TNBS-treated rats. At 8 wk after TNBS administration, the pain threshold pressure in rats treated with TNBS at the depth of 8 cm was still significantly decreased compared to that of the controls ($P < 0.05$), but no significant difference was found in rats treated with TNBS at 4 cm from anus or in 25% ethanol.

Enterochromaffin cell hyperplasia in trinitrobenzene sulfonic acid-induced post-infectious irritable bowel syndrome rat model

To investigate whether the rats treated with low dose TNBS (5 mg/0.8 mL per rat, in 50% ethanol) at 8 cm from anus also acquired other features of PI-IBS, the EC cell number and 5-HT content in the proximal and distal colon tissues were further tested at 4 and 8 wk after TNBS administration. As shown in Figure 5, compared to the control, 5-HT content in the proximal colon, but not the distal colon, were significantly increased, with 32% ($P < 0.01$) and 23% ($P < 0.05$) increased at 4 and 8 wk after TNBS administration, respectively. A
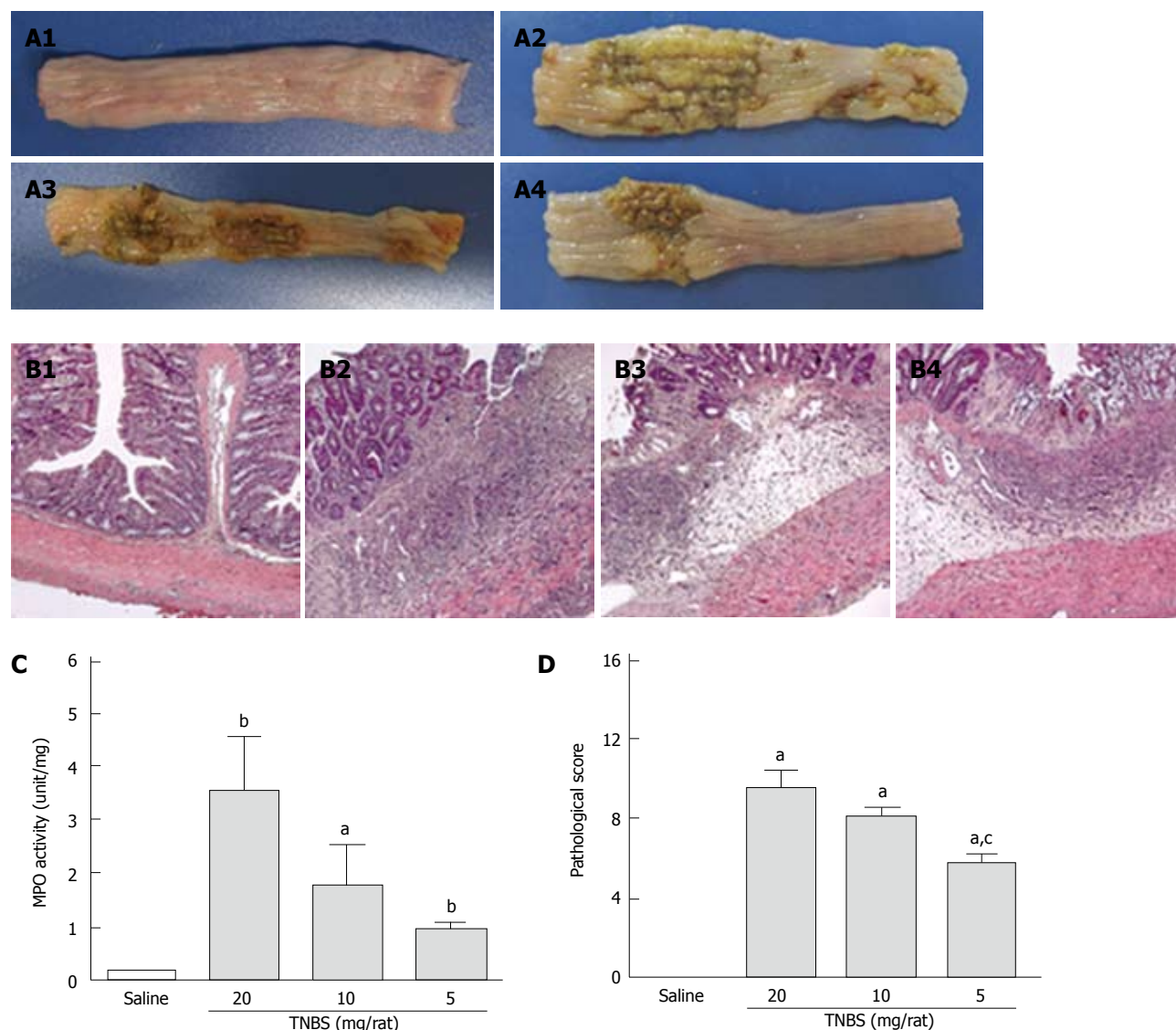
Figure 1 Acute colonic inflammation and damage induced by different doses of trinitrobenzene sulfonic acid at 5 d after trinitrobenzene sulfonic acid administration. Panel A depicts the appearance of colon tissue in saline (A1), high dose trinitrobenzene sulfonic acid (TNBS) (20 mg/rat, A2), medium dose TNBS (10 mg/rat, A3), and low dose TNBS (5 mg/rat, A4)-treated groups; Panel B depicts the representative histological changes of colon tissue in saline (B1), high dose TNBS (B2), median dose TNBS (B3), and low dose TNBS (B4)-treated groups (hematoxylin and eosin staining, 100×); Statistical analysis of myeloperoxidase (MPO) activity is shown in panel (C), and pathological score in panel (D). Data are shown as mean ± SE, n = 4 per group. [a]$P < 0.05$, [b]$P < 0.01$ *vs* saline-treated group; [c]$P < 0.05$ *vs* high dose TNBS-treated group (one-way analysis of variance, Student-Newman-Keuls).

similar trend can also be found in the EC cell number; i.e., there were 68% and 60% increases at 4 and 8 wk after TNBS administration, respectively ($P < 0.01$), suggesting this TNBS-induced PI-IBS model also presents EC cell hyperplasia in the colon tissue.

DISCUSSION

TNBS is an agent commonly used in inducing post-inflammatory IBS model currently. Concerning the variations in developing a protocol of the TNBS-induced PI-IBS rat model and the three necessary features of PI-IBS animal model (i.e., initial inflammation/infection, inflammation/infection resolution, and acquired symptoms of IBS), the present study investigated the effects of TNBS dosage and concentration, intracolonic TNBS administration position, and ethanol percentage on the development of the TNBS-induced PI-IBS rat model. Our results showed that: (1) TNBS induced dose- and concentration-dependent acute inflammation and inflammation resolution at the dose range of 5-20 mg/rat; (2) Intracolonic TNBS administration position affected the persistence of later acquired visceral hyperalgesia, but not the initial inflammation severity; (3) Ethanol percentage affected the TNBS-induced inflammation severity and later acquired visceral hyperalgesia, and low ethanol percentage reduced the degree of TNBS-induced acute inflammation and shortened the persistence of acquired visceral hyperalgesia; and (4) The protocol with TNBS at 5 mg/0.8 mL per rat, dissolved in 50% ethanol, intracolonically administered 8 cm from anus may be a proper protocol in which rats can recover completely from the
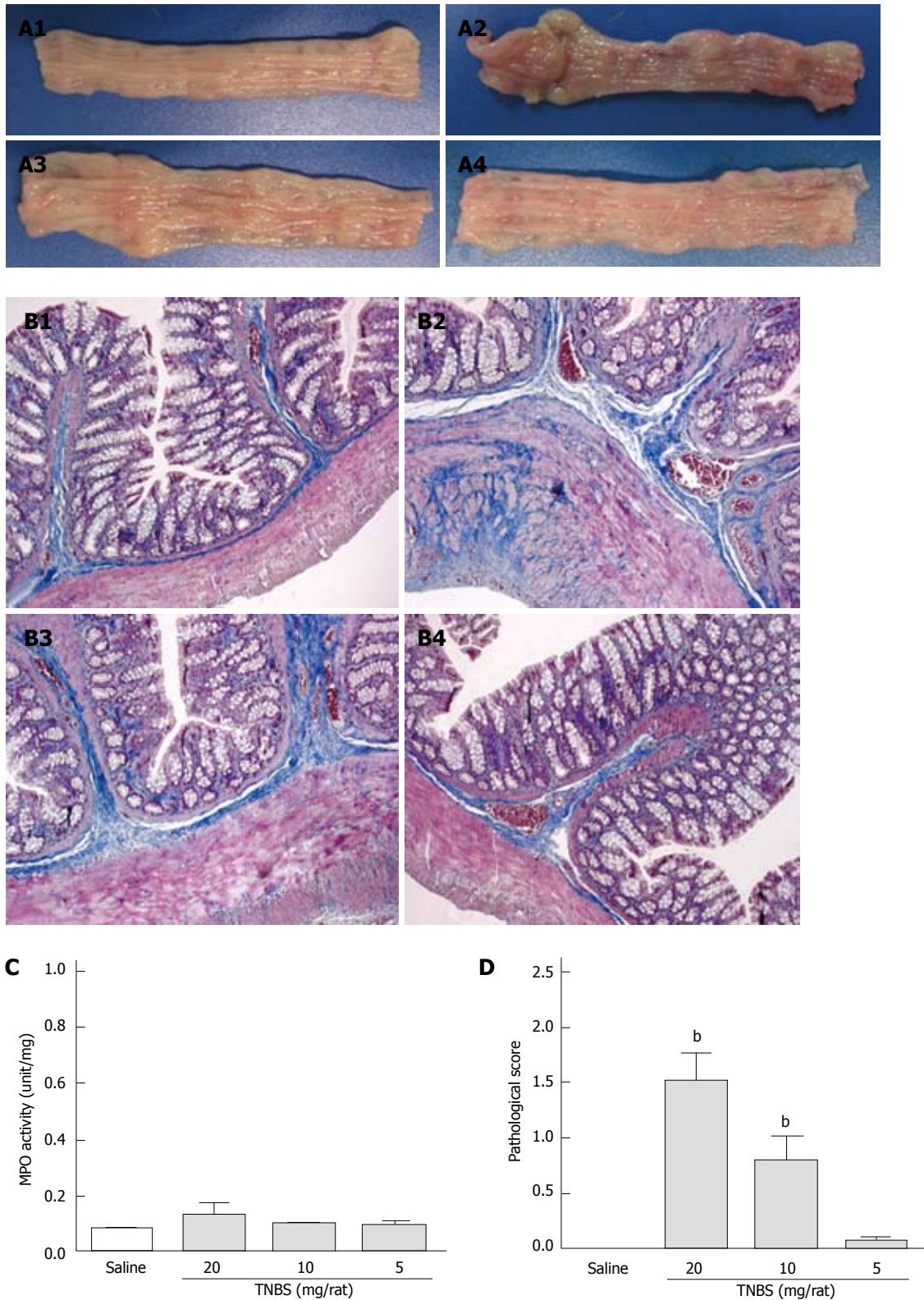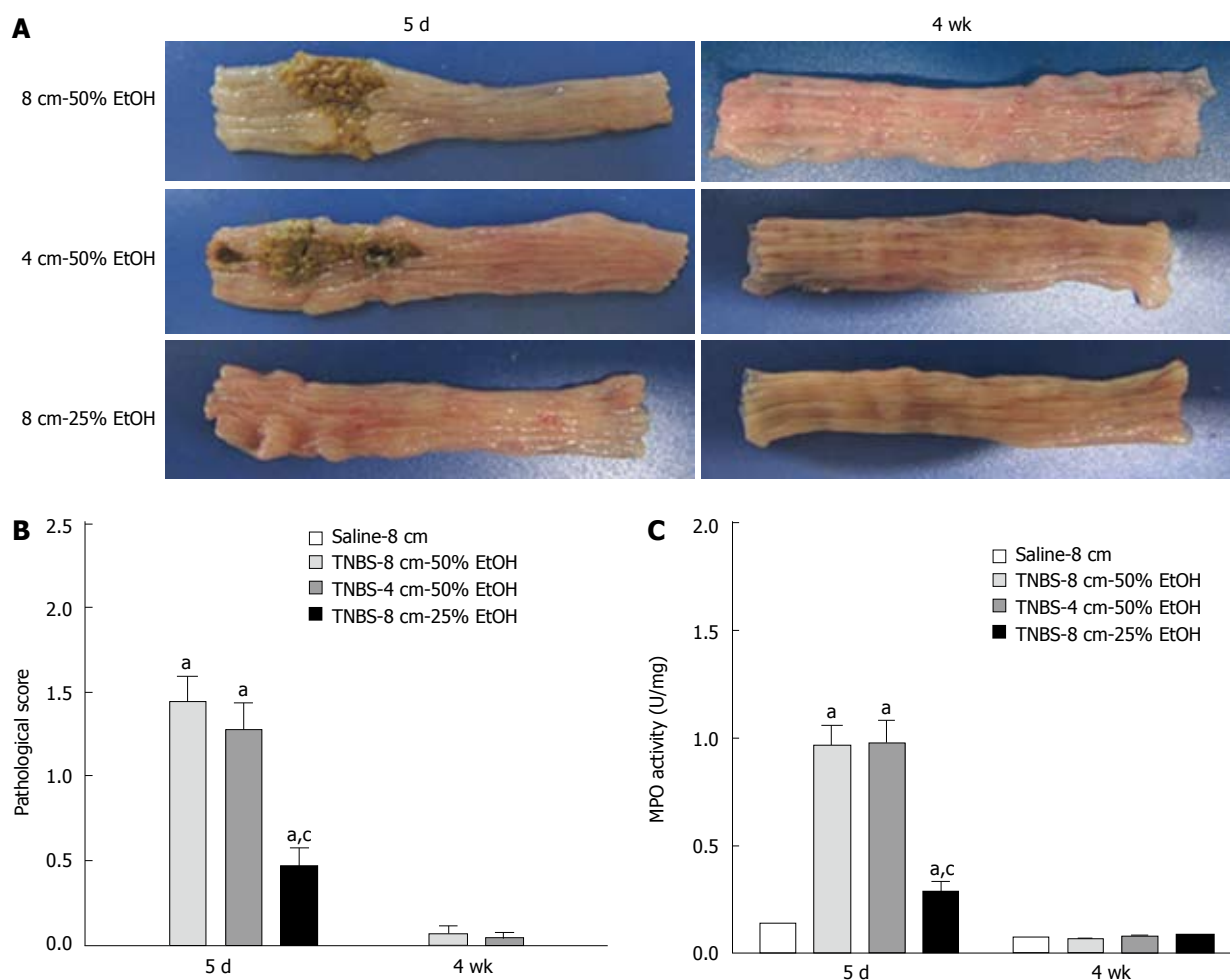
Figure 2 Effect of trinitrobenzene sulfonic acid dosage on severity of acute inflammation and inflammation resolution. The colon tissue was collected at 4 wk after trinitrobenzene sulfonic acid (TNBS) administration. Panel A depicts the appearance of colon tissue in saline (A1), high dose TNBS (20 mg/rat, A2), medium dose TNBS (10 mg/rat, A3), and low dose TNBS (5 mg/rat, A4)-treated rats; Panel B depicts the representative histological changes of colon tissue in saline (B1), high dose TNBS (B2), medium dose TNBS (B3), and low dose TNBS (B4)-treated rats (Masson trichrome staining, 100×); Statistical analysis of myeloperoxidase (MPO) activity is shown in panel (C), and pathological score in panel (D). Data are shown as mean ± SE, $n = 4$ per group. [b]$P < 0.01$ *vs* saline-treated group (one-way analysis of variance, Student-Newman-Keuls).

initial inflammation but acquire persistent visceral hyperalgesia and EC cell hyperplasia at 4 wk after TNBS

administration.

As shown in our results, in the dose range of 5-20

A

B

C

Figure 3 Effects of trinitrobenzene sulfonic acid administration position and ethanol percentage on severity of acute inflammation and inflammation resolution. Panel A depicts the appearance of colon tissue in rats treated with trinitrobenzene sulfonic acid (TNBS) at 4 cm or 8 cm from anus or in 25% ethanol. The colon tissues were collected at 5 d or 4 wk after TNBS administration, respectively; Statistical analysis of pathological score is shown in panel (B), and myeloperoxidase (MPO) activity in panel (C). Data are shown as mean ± SE, n = 4-5 per group. [a]P < 0.05 *vs* saline-treated rats, [c]P < 0.05 *vs* rats treated with TNBS at 8 cm from the anus in 50% ethanol (one-way analysis of variance, Student-Newman-Keuls).

A

B
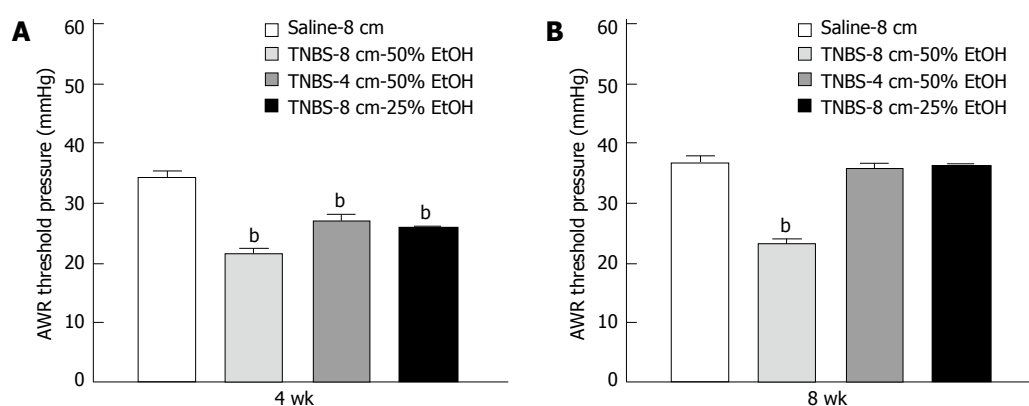
Figure 4 Effects of low dose trinitrobenzene sulfonic acid administration position and ethanol percentage on later acquired visceral hyperalgesia. Statistical analysis of pain threshold pressure at 4 wk and 8 wk after trinitrobenzene sulfonic acid (TNBS) administration are shown in panel (A) and (B), respectively. Data are shown as mean ± SE, n = 5 per group. [b]P < 0.01 *vs* saline-treated rats (one-way analysis of variance, Student-Newman-Keuls). AWR: Abdominal withdrawal reflex.

mg per rat, the higher the dose of TNBS, the more severe the acute inflammation and mucosa damage. This finding is consistent with previous reports that the severity and duration in TNBS-induced inflammation is dose-dependent[13,14,17]. It is notable that the same dose of TNBS (20 mg/rat) when given to rats in different vehicle volumes induced different degrees of damage; a higher TNBS concentration (50 mg/mL) induced
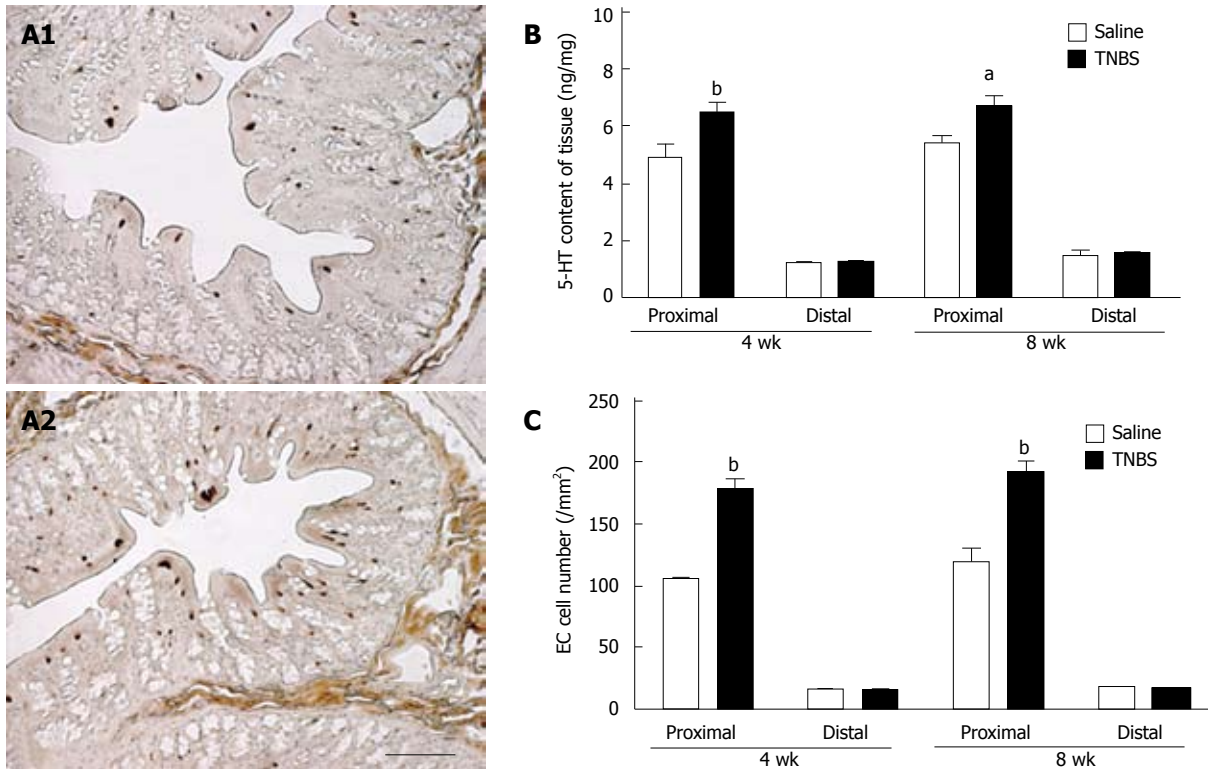
Figure 5 Persistent increases of enterochromaffin cell number and serotonin content in the colon tissue of trinitrobenzene sulfonic acid-induced post-infectious irritable bowel syndrome rat model. The colon tissue was collected at 4 wk and 8 wk after trinitrobenzene sulfonic acid (TNBS) administration. Panel A depicts the representative enterochromaffin (EC) cell staining in colon mucosa, the samples were from rats treated with saline (A1), or TNBS (A2) at 4 wk post TNBS (Scale bar, 100 μm). Statistical analysis of serotonin content is shown in panel (B), and EC cell number in panel (C). Data are shown as mean ± SE, *n* = 5 per group. [a]*P* < 0.05, [b]*P* < 0.01 *vs* saline-treated rats. 5-HT: 5-hydroxytryptamine.

more severe damage, even causing animal death in the acute phase. Our pathological results also showed that high-concentration TNBS-treated rats presented localized more severe inflammation and deeper ulcerations around the position of TNBS administration, which may be responsible for the occurrence of colitis complications, i.e., intestinal obstruction, intra-abdominal infection, and animal death. Concerning the fact that inflammation severity and duration have close correlation with TNBS dosage, the effects of TNBS dosage on inflammation resolution was assessed at 4 wk after TNBS administration. According to the pathologist's suggestion, fibroplasia evaluation index was added into the pathological evaluation criteria for the first time, so as to exactly evaluate inflammation resolution. Our results showed that the animals receiving low dose TNBS (5 mg/rat) recovered completely at 4 wk after TNBS administration, but those receiving high (20 mg/rat) and medium (10 mg/rat) dose were not recovered. This result seems to somewhat conflict with the finding from a previous study in which the rats receiving TNBS (5-20 mg/rat, in 25% ethanol) all recovered after 4 wk[14]. We speculate that this discrepancy may come from the protocol differences between these two studies, such as ethanol percentage, animal strain, or evaluation criteria. Consistent with our findings, the previous study showed that colonic inflammation induced by TNBS (30 mg/rat, in 50% ethanol) lasted for at least 8 wk[17], suggesting the

inflammation resolution is also dose-dependent. Based on previous findings and our results, TNBS dosage plays a critical role in TNBS-induced inflammation severity and resolution, and low dose TNBS (5 mg/rat)-induced inflammation can recover completely at 4 wk after TNBS administration.

Currently, 25% and 50% ethanol are both used as the carrier of TNBS in establishing TNBS-induced PI-IBS model and TNBS is commonly given to rats at the depth of either 4 cm or 8 cm from the anus. Based on low dose TNBS (5 mg/0.8 mL per rat) selection, we further investigated the effects of ethanol percentage and intracolonic administration position on the severity and resolution of TNBS-induced inflammation. Our results showed that rats treated with low dose TNBS at a depth of 4 cm or 8 cm presented similar inflammation severity and resolution, while the rats treated with TNBS in 25% ethanol developed milder non-ulcer inflammation compared to those treated with TNBS in 50% ethanol. As the carrier of TNBS, ethanol is known as a breaker to the mucosal barrier, and 30%-50% ethanol alone has been proved to induce acute inflammation and hyperemia[18,28]. Moreover, previous study have shown that 30% ethanol alone induced small areas of hyperemia, while TNBS combined with 30% ethanol induced larger area of necrosis and hyperemia in the colon[18]. These findings indicate that ethanol alone can induce concentration-dependent inflammation, and can be considered

Table 5 Detailed protocols in developing trinitrobenzene sulfonic acid induced post-inflammatory irritable bowel syndrome model in Sprague-Dawley rats

Procedures	Cautions
1. 7-wk-old male Sprague-Dawley rats, fast 24 h before TNBS administration	
2. Mix 1 volume of 12.5 mg/mL TNBS-saline solution with 1 volume of absolute ethanol	Fresh preparation
3. Weight and anesthetized rats with chloral hydrate (350 mg/kg, i.p.)	Anesthetization may last for 3-4 h
4. Insert a catheter into the colon at 8 cm from anus	Proceed carefully to avoid damage the colon wall
5. Keep the rat with head-down vertical position, instill 0.8 mL TNBS solution slowly into the colon lumen within 1 min	Handle slowly to avoid TNBS leakage
6. Keep the rat in head-down vertical position for 1 min before gently removing the catheter	
7. Put the rats in a mound of bedding in head-down position until consciousness recovery	Ensure TNBS solution remains completely in the colon; Keep the rats warm
8. On day 3, 7, 14, 21 and 28, observe and weight the rats	On day 3, body weight decreased by 10%-20% with unformed bloody stool; On day 7, body weight regained and reached the original level, the stool become formed but still soft; From day 7 to day 28, body weight increased smoothly, and finally reached the control level

TNBS: Trinitrobenzene sulfonic acid.

as an invasive helper in enhancing the effects of TNBS. Therefore, it seems reasonable that milder inflammation was induced in rats treated with equal dose of TNBS (5 mg/rat) but in lower ethanol percentage (25%). These results can also explain why high dose or high concentration TNBS have also been used by some researchers in establishing a PI-IBS model: low concentration ethanol may ameliorate the effects of TNBS[13,14].

Visceral hyperalgesia, the major feature of IBS, was further tested in low dose TNBS (5 mg/0.8 mL per rat) treated rats. From 4 wk to 8 wk post TNBS intracolonic administration at a depth of 8 cm, significant and persistent decrease in visceral pain threshold pressure was found, while the intracolonic TNBS administration at a depth of 4 cm or in 25% ethanol presented short-term acquired visceral hyperalgesia. Nowadays, the duration and severity of inflammation are considered as important risk factors in the development of PI-IBS[29], and severe inflammation which results in deep impairment of the underlining nerve fibers has been proposed to play an important role in the pathogenesis of IBS[30]. Based on the above evidence, we believe the unstable visceral hyperalgesia (i.e., lasting for less than 4 wk) in rats treated with TNBS in 25% ethanol may have close correlation with the mild non-ulcer inflammation observed at the acute phase of inflammation. These results also provide us with the information that mild inflammation, even without ulceration, can induce later short-term visceral hyperalgesia. However, it seems interesting that the same dose of TNBS when given at the different positions of colon (transverse vs descending) induced different features of acquired visceral hyperalgesia (persistent vs short-term), even though the TNBS-induced acute inflammation and damage was similar at first. Considering the unclear and complex pathogenesis of visceral hypersensitivity in PI-IBS, it is not easy to provide a clear explanation for the current findings. However,

during the acute phase of TNBS-induced inflammation, obvious colon dilation and a large amount of retained feces were found in the inflamed transverse colon, but not in the inflamed distal colon. We speculate that the persistent colonic inflammation and dilation may play important roles in enteric nerve system plasticity, and thus influence the feature of acquired visceral hypersensitivity. Currently, peripheral and central neuroplastic changes are considered to be associated with post-inflammatory persistent visceral hypersensitivity[1,31]. It is also well known that transient colorectal distension in the neonatal period can result in chronic visceral hypersensitivity and motility dysfunction in adulthood[25,32], and the underlying mechanism is thought to be associated with alterations in peripheral and central nerve systems[33]. Moreover, considering the different structure and functions of the transverse and descending colons, the regional differences in gut flora[34], mast cells[35] and EC cells[36] may also contribute to the different feature of acquired visceral hypersensitivity observed in our study. Further studies are needed to clarify these issues.

EC cell hyperplasia has been found in the colonic specimens of IBS and PI-IBS patients[37-39], and EC cell hyperplasia has been reported to play important role in the development of visceral hypersensitivity in IBS patients[40,41]. To identify whether visceral hyperalgesia is also accompanied with EC cell hyperplasia in the TNBS-induced PI-IBS rat model, EC cell number and 5-HT content were further investigated in the proximal and distal colon of PI-IBS rats. As shown in our study, the EC cell number and 5-HT content in the proximal colon, but not the distal colon, were significantly and persistently increased in this TNBS model, suggesting the occurrence of EC cell hyperplasia. However, this result differed from the finding from IBS patients, as EC cell hyperplasia in rectal mucosa was commonly reported[3]. This may be explained by the different distribution of

EC cells in the gut mucosa between human beings and rats: the vast majority of EC cells in humans mainly resides in the small intestine and rectum[42] while the major source of EC cells in rats largely locates in the cecum with a decline trend from the proximal to the distal colon[36]. Consistent with our findings, previous studies also showed that 5-HT content in the rat proximal colon tissue was significantly higher (about 5 fold) than that in the distal colon[43,44]. Therefore, it seems that EC cell hyperplasia in the proximal colon can be regarded as one of the features of the TNBS-induced PI-IBS rat model.

This present study, for the first time, showed that TNBS dosage and concentration, position of intracolonic TNBS administration, and ethanol percentage play important roles in developing the TNBS-induced PI-IBS model in Sprague-Dawley rats, suggesting more attention should be paid to these factors when developing a PI-IBS model with TNBS. Concerning the gene differences among animal strains, the protocol set up here may need some modulation when other rat strains except Sprague-Dawley rats, are used. Though this TNBS model presented persistent visceral hyperalgesia and EC cell hyperplasia for as long as 4 wk, more studies are still needed to observe the long-lasting visceral hyperalgesia and identify other features of PI-IBS in this model.

In the present study, we found out that the protocol with TNBS at 5 mg/0.8 mL per rat, dissolved in 50% ethanol, intracolonically administered at 8 cm from the anus resulted in persistent visceral hyperalgesia and colonic EC cell hyperplasia after complete recovery from the initial inflammation in rats. With this protocol, low-dose TNBS-induced mild mucosa damage and colonic inflammation is reproducible without any animal loss; visceral hyperalgesia and EC cell hyperplasia which last for at least 4 wk occur as early as 4 wk after TNBS administration. The details of this protocol are presented in Table 5. Ensuring the results from different laboratories would be comparable, we believe that widespread adoption of a recommended protocol will save a great quantity of time and resources.

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COMMENTS

Background
Post-infectious irritable bowel syndrome (PI-IBS) is a subgroup of IBS in which the patients developed their symptoms after recovery from an acute gastrointestinal infection. Previous systematic review showed that the trinitrobenzene sulfonic acid (TNBS)-induced model is the most commonly used PI-IBS model, but there are large variations in the protocol of model development. These variations make comparison between studies to be very difficult, if not impossible.

Research frontiers
This study aimed to investigate the effects of TNBS dosage, concentration, intracolonic administration position, and ethanol percentage on TNBS-induced acute inflammation, inflammation resolution, and later acquired features of IBS

(i.e., visceral hyperalgesia and enterochromaffin cell hyperplasia).

Innovations and breakthroughs
Recent studies have highlighted the important status of PI-IBS in functional diseases, as the clear onset and well defined pathophysiological changes of PI-IBS will help people understand not only the PI-IBS but also other subtypes of IBS. Moreover, the validated animal models, which aim to mimic one or more features of human disease, play important roles in the studies of mechanisms and potential therapeutic agents for diseases. This is the first study aimed to investigate the effects of key factors on developing TNBS-induced PI-IBS model in rats. The results and recommended protocol presented in this study will save a great deal of time and resources and ensure the results from different laboratories be comparable.

Applications
This study provides direct information about the effects of key factors on TNBS-induced acute inflammation, inflammation resolution, and later acquired features of IBS. More attention should be paid to these key factors when developing PI-IBS rat model with TNBS.

Peer review
This is a good descriptive study in which authors investigate the effects of TNBS dosage and concentration, intracolonic administration position, and ethanol percentage on the developing TNBS-induced PI-IBS rat model. The topic is interesting and useful for the study of pathophysiology of PI-IBS and the development of therapeutic agents.

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