An Improved and Reliable Method for the Induction of Colitis in Rats Using 2,4,6-Trinitrobenzene Sulfonic Acid

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Abstract. We performed this study to develop a reliable method for inducing colitis in rats using 2,4,6-trinitrobenzene sulfonic acid (TNBS) to reduce the variation in ulcer size. A pair of ring forceps was used to clamp the colon and 0.1 M TNBS in ethanol was injected into the luminal side of the clamped portion. This method resulted in a small coefficient of variation of the ulcer index. A significant linearity was observed by plotting ulcer size against days after ulcer induction in both logarithm scales. These findings show that this technique is simple and reliable and that ulcers heal linearly.

Keywords: inflammatory bowel disease, colitis model, 2,4,6-trinitrobenzene sulfonic acid (TNBS)

A simple and reproducible rat model of chronic colonic inflammation and ulceration has previously been developed by the intraluminal instillation of a solution containing a ‘barrier breaker’ and a hapten, 2,4,6-trinitrobenzene sulfonic acid (TNBS) (1). Many recent articles have reported the mechanism of ulceration and the efficacy of drug treatments using this method of colonic inflammation (2 – 6). However, when using this technique, we found that the size of the ulcer was affected by the luminal conditions of the colon, such as the number and wetness of the feces.

A relatively new rat model, in which gastric ulcers are induced by the luminal application of acetic acid to an area clamped with ring forceps (kissing gastric ulcers), has been used for the screening of anti-ulcer drugs (7). Using a similar technique, the present study aimed to develop a simple and reliable method for inducing colonic ulcers with TNBS in rats in order to reduce the variation in ulcer size.

Our previous study in a gastric ulcer model, which was induced by the submucosal injection of acetic acid, showed that an approximately linear relationship existed between the logarithm of the days after ulcer induction (x) and the logarithm of the ulcer index (y), resulting in the following equation: \( \log y = a \log x + b \) (8). The slope (a) indicates the ulcer-reducing rate. The present study evaluated whether this formula fitted the new colitis model.

All animal experiments were performed according to the “Guiding Principles for the Care and Use of Laboratory Animals” approved by The Japanese Pharmacological Society.

Male Sprague-Dawley rats (200 – 250 g) were purchased from SLC (Shizuoka) and housed for 1 week prior to the commencement of the experiments under a constant temperature of 21 ± 2°C, humidity of 55 ± 15%, and a 12-h light/dark cycle.

The rats were anesthetized with an intraperitoneal administration of sodium pentobarbital (Nembutal; Dinabot, Tokyo) at a dose of 35 mg/kg. Following lower abdominal laparotomy, the colon was exposed. The middle part of the colon was pinched with ring forceps (inside diameter: 8 mm), and 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS (Fluka, Tokyo) was injected into the luminal site of the clamped portion of the colon. The injection tube with the needle (26 gauge) attached is shown in Fig. 1. After 2 min, the colon was returned to the abdominal cavity and the incision was sutured. In order to compare this technique with the conventional method, 0.2 ml of 35% or 50% ethanol solution containing a final concentration of 0.1 M TNBS was instilled from the anus using a catheter at an approximate length of 8 cm.

Following cervical dislocation, the colon was excised.
from all animals and cut along the mesentery to allow for ulcer evaluation. The size of the ulcer was measured, and the ulcer index was calculated from the length and width measurements (mm²).

The time course of ulcer healing was observed by sacrificing the rats at appropriate intervals from 3 to 31 days after ulcer induction. Following macroscopic observations, the colonic specimens were fixed in 10% neutral buffered formalin and histological examinations were performed. Tissue sections were prepared and

Fig. 1. Schematic drawing showing the induction of colitis in rats by the intraluminal application of an ethanol solution with TNBS. The colon was clamped with a pair of ring forceps, and 0.2 ml of ethanol solution with TNBS was injected into the luminal side of the clamped portion using an injection tube with an attached needle. Clamping was kept in place for 2 min following the injection.

Fig. 2. Macroscopic (A) and microscopic (B) findings of colitis in rats 3 days after ulcer induction with 0.2 ml of 35% ethanol solution containing a final concentration of 0.1 M TNBS at the middle position of the colon. Colitis with a marked margin different from the normal mucosa and with round lesions of the colon was induced evenly in each rat (A). The lesions penetrated the muscularis mucosae (B). Hematoxylin and eosin staining; magnification ×40.
stained with hematoxylin and eosin, and histological observations were performed under a light microscope.

Ulcer indexes were plotted against days after ulcer induction using logarithm scales for both, and a regression curve was obtained. The results were expressed as the mean ± the standard deviation (S.D.).

On day 3, colitis with a marked margin that differed from the normal mucosa had successfully been induced by the application of 35% ethanol solution with TNBS (Fig. 2). The modified area of the colon was clearly ulcerated, and the lesions were characterized by edema, epithelial exfoliation, and infiltration of leukocytes. The colitis was also seen to penetrate the muscularis mucosae (Fig. 2).

The ulcer index calculated on day 3 was 129 ± 7.7 (n = 4), and the coefficient of variation was 6.0. In the case of 35% ethanol only, the ulcer index on day 3 was 30.8 ± 5.6 (n = 4), which significantly differed from that of the 35% ethanol solution with TNBS (P < 0.01, by Student’s t-test). These findings show that TNBS plays an important role in the genesis of colitis.

Interestingly, ulcers were not induced by the conventional method of instilling 0.2 ml of 35% ethanol solution with TNBS into the colon from the anus (n = 4). However, when the conventional method was used with a 50% ethanol solution and TNBS, the ulcer index was 188 ± 121 (n = 4) and the coefficient of variation was 64; this presented a stark contrast to the novel method. An outlier value of 10 mm² for one of the rats produced this variation in the ulcer index.

The results showed that the ulcer index decreased over time. By plotting both parameters on logarithm scales, a significant negative linearity was observed following the equation: \[ \log y = -1.43 \log x + 2.74 \ (r = 0.987, P < 0.05; \text{Fig. 3}) \].

It is important to reduce the variation in the ulcer index, especially when evaluating the efficacy of test samples on TNBS-induced colitis. Previously, we found that the size of the ulcer varied depending on the luminal conditions of the colon, such as the number or wetness of the feces. In the present study, we developed a simple and reliable method for inducing colonic ulcers in rats by injecting ethanol solution with TNBS into the luminal site of the clamped colon. This method produced a small coefficient of variation compared with the conventional method of injecting ethanol solution with TNBS into the colon from the anus.

Moreover, we showed that 35% ethanol solution with TNBS did not induce colitis when the conventional method was used. Many previous reports have used 50% ethanol for inducing colitis. The original paper describing the conventional method, published by Morris and colleagues (1), also used 50% ethanol. The difference in the ethanol concentration might explain why, in the present study, colitis was not initially induced by the conventional method. Indeed, in the case of 50% ethanol solution with TNBS, colitis was induced; however, its coefficient of variation value was extremely large.

The findings from the present study show that the precise induction of colitis using ring forceps is a reliable method that induces colitis evenly with small coefficients of variation. Moreover, even in cases where the efficacy of the sample is moderate or weak, this approach might make it possible to detect a useful activity by changing the concentration of ethanol. Indeed, colitis could be induced even at a low concentration of 10% ethanol, although the ulcer index using 10% ethanol solution with TNBS was smaller than that using 35% ethanol solution with TNBS (data not shown).

Histological observations characterized the lesions by the presence of edema, epithelial exfoliation, and infiltration of leukocytes and showed that the ulcer had penetrated the muscularis mucosae. Similarly, Morris and colleagues (1) observed lesions that were characterized by edema, hemorrhage, epithelial exfoliation, and infiltration of polymorphonuclear leukocytes, although the muscularis mucosae remained intact. This difference was probably caused by the different experimental conditions, although the concentration of ethanol in the present study was lower. We previously reported, in acetic acid-induced gastric ulcers, that the lesion penetrated the muscularis mucosae, and relapse was observed by endoscopic observation (9). Therefore, in the present model, the colitis might relapse, similar to the findings in
acetic acid-induced gastric ulcers. However, further studies will be necessary to clarify this point.

In the present study, by plotting both parameters using logarithm scales, a significant negative linearity was observed following the equation: \( \log y = -1.43 \log x + 2.74 \). This correlated with our previous findings in the acetic acid-induced gastric ulcer model, in which an approximately linear relationship existed between the logarithm of the days after ulcer induction (x) and the logarithm of the ulcer index (y), resulting in the following equation: \( \log y = a \log x + b \) (9). These results suggest that the ulcers heal according to a regular formula, although the lesion models differed from one another. Further studies will be needed to clarify the mechanism of this interesting phenomenon.

By comparing the slopes (a), we previously found that the efficacy of the testing sample could be evaluated in the acetic acid-induced gastric ulcer model (9). Therefore, in the present colitis model, the efficacy of the testing sample might be evaluated by comparing the slopes (a) between the control and test groups.

In conclusion, we found that this method of precise colitis induction resulted in a smaller coefficient of variation of the ulcer index. This technique might therefore be a useful tool for screening effective samples for colitis and for studying the mechanism of colitis.

References