**ABSTRACT**

The 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced acute inflammatory bowel disease (IBD) model in the rat is discussed, focusing on the details of the TNBS instillation and highlighting the advantages and limitations of this model. For determination of the time-dependent action of 50% ethanol and different doses of TNBS, male Wistar rats were treated with 50% ethanol or 10 mg or 30 mg of TNBS dissolved in 50% ethanol. The TNBS-induced inflammation peaked 48-72 h after installation and the colitis caused by 30 mg of TNBS was more severe than that caused by 10 mg of TNBS. To test the effectiveness of sulfasalazine (SASP), male rats were treated with 10 mg of TNBS or with 10 mg of TNBS and SASP, and 72 h later the extent of mucosal damage was determined. Orally administered 50 mg/kg/day SASP proved to reduce the TNBS-induced colonic inflammation in rats significantly. The TNBS-induced colitis model facilitates a better understanding of the immunopathological mechanisms of IBD. Optimization of the dose of TNBS and oral SASP as positive control in TNBS-induced colitis in rats furnishes an appropriate test system for new anti-IBD drugs.

**KEY WORDS**

colitis rat IBD model TNBS

Inflammatory bowel disease (IBD) is a chronic disease of the gastrointestinal tract, primarily manifested as Crohn’s disease (CD) and ulcerative colitis (UC). Environmental, genetic and immunological factors all contribute significantly to the pathophysiology of IBD, but the precise mechanisms remain unclear (Kaser et al. 2010). Epidemiological studies have revealed an increasing incidence of IBD in northwestern Europe, the USA and eastern Canada, suggesting that environmental and lifestyle factors play a major role in the development and progression of this disease (Vatn 2008; Kappelman et al. 2013; Ng et al. 2013).

Several animal models have been developed in attempts to understand the pathogenesis of IBD and to test pharmacological molecules and therapeutic targets. However, CD and UC are chronic diseases, while animal models are necessarily primarily acute, in order to limit the discomfort and the pain caused to the animals and to reduce the costs associated with the use of reagents and supplies. Invertebrate models of IBD, involving *Caenorhabditis elegans* (Chinnadurai et al. 2008; Kang et al. 2010) and *Drosophila melanogaster* (Liu et al. 2010; Apidianakis et al. 2011), have been used to investigate the genetic factors and signalling pathways involved in the pathogenesis of these diseases and to identify novel therapeutic agents (Lin et al. 2011). However, the various vertebrate models (most commonly mice and rats) more closely resemble the complexity of the human physiology than those with invertebrate animals, and may reflect the different subtypes of patients with IBD, and they are important in preclinical studies (Jurjus et al. 2004).

Rodent models of chemically induced IBD are widely used because of their simplicity and the possibility of the control of the degree of inflammation (Table 1). However, the differences in protocols lead to difficulties in reproducing reported experiments and in comparing measured parameters between studies. The inconsistent variables generally include the rodent strains used, the amounts and concentrations of chemical agents and the time of sacrifice in relation to the treatment.

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) model was originally described by Morris et al. (Morris et al. 1989). TNBS (0.5 to 4.0 mg for mice and 10 to 30 mg for rats) is dissolved in 45% or 50% ethanol, which can lead to the destruction of the mucosal barrier. As a hapten, TNBS can bind to the endogenous proteins, giving rise to hapten-protein formation, which induces the interleukin-12 (IL-12) and T helper 1-mediated local immunological response. The activated macrophages produce inflammatory mediators,
such as tumor necrosis factor-α, IL-6 and IL-1α (Ishiguro et al. 2010; Strober et al. 1998), which in turn result in transmural inflammation with weight loss and diarrhoea. The TNBS model has many advantages, such as the simple process and the short duration of the experiment. Moreover, it is widely applicable to vertebrates, including mice (it is important to note, however, that the susceptibility to TNBS-induced colitis varies between different mouse strains: SJL and BALB/c mice are susceptible), rats, guinea pigs and rabbits, and can be used either acutely with a single TNBS treatment or chronically with the repeated administration of TNBS (Terai et al. 2014). On the other hand, this model has the major limitation that it cannot mimic the relapsing phase of CD (Table 1).

Among the reference drugs with anti-IBD effects, hydrocortisone acetate (20 mg/kg, i.p.), Asacol (100 mg/kg, p.o.) (Motavallian-Naeini et al. 2012), 5-aminosalicylic acid (5-ASA; 8, 25 or 75 mg/kg, intracolonal) (Horvath et al. 2008) and the 5-ASA-releasing sulfasalazine (SASP; 360 mg/kg, p.o.) (Byrav et al. 2013) have been found effective in ameliorating inflammation.

The primary aim of the present study was to investigate a TNBS-induced acute IBD model in the rat, focusing on the details of the TNBS instillation and highlighting the advantages and limitations of this model, in which anti-colitis drugs can be tested. A further aim was to describe a newly developed method for treatment with SASP as a positive control in a TNBS-induced acute colitis model.

### Materials and Methods

#### Animals

Male Wistar rats (200-250 g, Toxi-Coop Zrt., Hungary) were housed in groups (4 or 5 rats in each cage). Food was withdrawn overnight before the induction of colitis; otherwise, the animals had access to food and drinking water *ad libitum* throughout the experiments. The animal care and research protocols were in full accordance with the guidelines of the University of Szeged.

#### Experimental design for determination of the time-dependent action of 50% ethanol or TNBS (10 or 30 mg) dissolved in 50% ethanol

The animals were randomly divided into three groups, to which 50% ethanol (n=36), 10 mg of TNBS (n=27) or 30 mg of TNBS (n=90) was administered. 12 h before the induction of colitis, the rats were fasted.

TNBS (also called picrylsulfonic acid, from Sigma-Aldrich) as received from the manufacturer was aliquoted and stored at -20 °C in order to prevent its effectiveness. The TNBS solution for administration was prepared immediately before treatment: 10 mg or 30 mg of TNBS was dissolved in 50% ethanol for instillation in a final volume of 250 μl per rat. Following dissolution, the TNBS solutions were protected from light, because they are light-sensitive and unstable at room temperature.

The intracolonic administration of the ethanol or the TNBS (dissolved in ethanol) was performed under transient ether anaesthesia with the aid of an 8 cm long plastic catheter (800/100/260PO, ReplantMed) attached to a 1 ml syringe. After the instillation, the rats were kept on their back for about 30 s before being returned to their cages.

The body weight was measured immediately before and 24, 48 and 72 h after ethanol or TNBS treatment.

Both before and 24, 48 and 72 h after treatment with 10 mg of TNBS, and 1.5, 3, 6, 12, 24, 48 and 72 h and 6 and 10 days after treatment with 30 mg of TNBS and 6, 12, 24 and 48 h after treatment with ethanol, rats (n=9 at each timepoint) were sacrificed by cervical dislocation under ether anaesthesia, and the 8 cm portion of the colon distal from the rectum was removed for histological examination.

### Table 1. Advantages and disadvantages of the most common chemically-induced animal models of IBD.

<table>
<thead>
<tr>
<th>Models</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>The general health of the animals is similar to that of the controls.</td>
<td>The treatment is more complex.</td>
<td>Yamada et al. 1992 Fabia et al. 1992</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Induces inflammation not in the colon, but in the ileum and jejunum. Simple administration (subcutaneous) without anaesthesia.</td>
<td>The inflammation is not localized in the colon.</td>
<td>Yamada et al. 1993 Piepoli et al. 2005</td>
</tr>
</tbody>
</table>

TNBS = 2,4,6-trinitrobenzenesulfonic acid, DSS = dextran sulfate sodium

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The TNBS rat model of colitis was dissected, longitudinally opened, gently rinsed with ice-cold physiological saline, and photographed (Panasonic Lumix DMC-TZ6 digital camera) for determination of the extent of macroscopic colonic inflammatory damage.

**Experimental design for tests of the effectiveness of a SASP in the TNBS model (Table 2)**

The animals were randomly divided into 2 groups, for the administration of 10 mg of TNBS (n=9) or 10 mg of TNBS + SASP 50 mg/kg/day (positive control group, n=9). For 12 h before the induction of colitis, all of the rats were fasted. The animals were treated with TNBS (10 mg dissolved in 50% ethanol for a final volume of 250 μl per rat). The detailed steps of the TNBS treatment were described above.

The animals in the positive control group were treated with TNBS and SASP as follows: 25 mg/kg SASP was dissolved in 1% carboxymethylcellulose (CMC) for a final volume of 250 μl per rat. The animals were treated orally with SASP solution twice a day for 3 days, the first treatment taking place 2 h before the instillation of TNBS.

72 h after the treatment with TNBS, the 8 cm distal segment of the colon was collected as described above.

**Parameters utilized to analyse the severity of the induced inflammation**

Body weight change: the weight of each animal was measured every day, starting on the day before TNBS treatment, and the change in weight was calculated as a percentage, the baseline (100%) being taken as the weight on the day of the TNBS challenge.

Percentage of damaged mucosa: the extent of macroscopically apparent inflammation, ulceration and tissue disruption was determined in a randomized manner from colour images via computerized planimetry (proprietary computerized planimetry software, developed in our laboratory was used: Stat_2_1_1). The surface area of macroscopically visible mucosal involvement was calculated and expressed as a percentage of the surface area of the total 8 cm colonic segment under study.

**Statistical analysis**

Results are shown as means ± S.E.M; statistical comparisons were performed with the two-tailed Student *t*-test.

**Results**

**Time-dependent changes in colonic inflammation induced by 50% ethanol, or by 10 mg or 30 mg of TNBS**

Our findings revealed the role and time-dependent effects of ethanol (the vehicle of TNBS) and the different doses of TNBS in the development of acute colonic inflammation. 50% ethanol give rise to damage to the mucosal barrier 6-12 h after treatment, and the macroscopic changes were reversed after 2 days. The TNBS-induced inflammation peaked 48-72 h after instillation, and this was followed by a slow recovery process. The maximum extent of the lesion caused by 30 mg of TNBS (62.8 ± 5.5% of the 8 cm segment of the colon) was slightly greater than that caused by 10 mg of TNBS (58.0 ± 3.0% of the 8 cm segment of the colon) (Fig. 1A). Moreover, in the 30 mg TNBS group 5 rats died during the study, whereas in the 10 mg TNBS group there were no deaths.

**Changes in body weight induced by treatment with 10 mg or 30 mg of TNBS**

The rats treated with 30 mg of TNBS exhibited a significantly greater weight loss as compared with the animals challenged with 10 mg of TNBS on the third day (Fig. 1B).

**SASP, a positive control for the TNBS colitis model**

SASP administered orally twice a day in a dose of 25 mg/kg/day in 1% CMC significantly reduced the colonic inflamma-

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**Table 2. Treatment groups.**

<table>
<thead>
<tr>
<th>Group (n=9)</th>
<th>Treatment</th>
<th>Route options</th>
<th>Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>EtOH</td>
<td>Intracolonic</td>
<td>Single dose of 250 μl of 50% EtOH</td>
</tr>
<tr>
<td>TNBS-treated</td>
<td>TNBS in 50% EtOH</td>
<td>Intracolonic</td>
<td>Single dose of 10 mg/250 μl of 50% EtOH</td>
</tr>
<tr>
<td>Positive control</td>
<td>TNBS in 50% EtOH + SASP in 1% CMC</td>
<td>Intracolonic</td>
<td>Single dose of 10 mg/250 μl of 50% EtOH</td>
</tr>
<tr>
<td>(anti-IBD drug)</td>
<td></td>
<td>Oral</td>
<td>Daily 2 x 25 mg/250 μl 1% CMC</td>
</tr>
<tr>
<td>Test item</td>
<td>TNBS in 50% EtOH + test item</td>
<td>Intracolonic</td>
<td>Single dose of 10 mg/250 μl of 50% EtOH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral</td>
<td>Twice daily</td>
</tr>
</tbody>
</table>

EtOH = ethanol, TNBS = 2,4,6-trinitrobenzensulfonic acid solution, SASP = sulfasalazine, CMC = carboxymethylcellulose
tion in the TNBS (10 mg)-treated rats, from 51.2 ± 3.7% to 28 ± 2.1% 72 h after the TNBS challenge (Fig. 2A).

The representative images demonstrate that the TNBS (10 mg) treatment caused macroscopic damage in the 8 cm segment of the colon, such as ulceration and wall thickening (Fig. 2B), while the SASP treatment exerted an anti-inflammatory effect on the TNBS (10 mg)-induced colitis (Fig. 2C).

**Discussion**

The use of animal models is important for the attainment of a better understanding of the pathological mechanisms involved in IBD, and such models can contribute to the development of new modes of treatments for humans. The present study related to a detailed protocol of TNBS treatment in the rat, with methods designed to monitor colonic inflammation, including the effects of different doses of TNBS, ethanol and SASP on the development of colonic inflammation.

TNBS-induced colitis can mimic many of the features of CD. The body weight loss is an indicator of the severity of the colitis, because TNBS/ethanol treatment causes diarrhoea, and the discomfort decreases the appetite (Ibrahim et al. 2011). In accordance with previous findings, we found that there was a greater decrease in body weight in the rats treated with 30 mg of TNBS at 72 h than in those treated with 10 mg

Figure 1. Time-dependent changes in the percentage of mucosal damage induced by 10 mg of TNBS, 30 mg of TNBS or 50% ethanol (A). (The intermittent x axis shows the time-scale: hours and days.) Results are given as means ± S.E.M., n=7-9. Time-dependent body weight changes caused by challenge with 10 mg or 30 mg of TNBS (B). Results are given as means ± S.E.M., *P<0.05 as compared with the 30 mg TNBS group on the 3rd day, n=7-9.
The TNBS rat model of colitis

Moreover, in the 10 mg TBNS group, no rat died as a result of the TNBS treatment.

The dose of TNBS and the percentage of ethanol applied vary among the reported investigations. In agreement with previous studies, we found that the severity of TNBS-induced colonic inflammation is dose-dependent (Motavallian-Naeini et al. 2012, Brenna et al. 2013). Menozzi et al. observed that 30 mg of TNBS resulted in the need for a longer regeneration of the histological changes as compared with 10 mg of TNBS (Menozzi et al. 2006). The findings relating to the body weight changes, the mucosal damage and the survival rate following the treatments with the two different doses of TNBS suggest that 10 mg of TNBS is a more appropriate dose with which to induce colonic inflammation.

As the time-related changes in ethanol-induced mucosal damage indicated, ethanol plays an important role in the initial phase (12 h after treatment) of the development of inflammation. Hong-Yan Qin et al. demonstrated that the same dose of TNBS in different vehicle volumes caused different degrees of damage, TNBS in 25% ethanol gave rise to a lower pathological score and to a lower level of myeloperoxidase activity relative to those after TNBS in 50% ethanol (Qin et al. 2012).

We used SASP (brand names Azulfidine in the U.S., and Salazopyrin and Sulazine in Europe) as a positive control. SASP is a sulfa drug and a widely used anti-colitic agent in human IBD therapy. Earlier findings revealed that SASP and 5-ASA are poorly absorbed in the gut (Yen et al. 2012), while 5-ASA, the pharmacologically active metabolite of SASP, ingested directly into the colon is effective in decreasing colitis (Horvath et al. 2008). The oral administration of SASP is better than local ingestion of the drug into the inflamed colon, as there is then less risk of aggravation of the injury of the inflamed colon; moreover, the oral administration of SASP can serve as a positive control (Ghatule et al. 2014, Zhang et al. 2014). The present study has demonstrated that SASP given orally at a dose of 50 mg/kg/day twice a day resulted in an improvement in the colonic inflammation induced by 10 mg of TNBS.

In conclusion, this report has described a chemically-induced rodent model of intestinal inflammation focusing on TNBS-induced acute colitis in rats, a widely-used simple animal model characterized by transmural inflammation with weight loss. It emerged that 10 mg of TNBS is a more suitable dose than 30 mg of TNBS, and, as a reference drug, orally administered SASP is preferable to an enema (Horvath et al. 2008) for attenuation of acute TNBS inflammation in rats.

This model may promote a better understanding of the immunopathological mechanisms of IBD via the testing of new compounds in the preclinical phase. It is hoped that this

Figure 2. Effect of 50 mg/kg/day sulfasalazine (SASP) on the severity of colonic inflammation induced by 10 mg of TNBS (A). Results are given as means ± S.E.M., ***P<0.001 as compared with the TNBS group, n=9. Representative images of the 8 cm colonic segment from each group: TNBS 10 mg (B), TNBS 10 mg + SASP 50 mg/kg/day (C).
detailed description of the TNBS colitis protocol used in our laboratory will be helpful to other research teams.

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