Acta Farmacêutica Portuguesa
2013, vol. **2,** n. **2,** pp. **89-95**

Development of TNBS-induced colitis: animal model to test new pharmacological approaches

Mateus, V.[1,2], Faisca, P.[3], Mota-Filipe, H.[2], Sepodes, B.[2], Pinto, R.[2]

ARTIGO ORIGINAL | ORIGINAL ARTICLE

ABSTRACT

IBD is a gastro-intestinal disorder marked with chronic inflammation of intestinal epithelium, damaging mucosal tissue and manifests into several intestinal and extra-intestinal symptoms. Currently used medical therapy is able to induce and maintain the patient in remission, however no modifies or reverses the underlying pathogenic mechanism. The research of other medical approaches is crucial to the treatment of IBD and, for this, it´s important to use animal models to mimic the characteristics of disease in real life. The aim of the study is to develop an animal model of TNBS-induced colitis to test new pharmacological approaches. TNBS was instilled intracolonic single dose as described by Morris et al. It was administered 2,5% TNBS in 50% ethanol through a catheter carefully inserted into the colon. Mice were kept in a Tredelenburg position to avoid reflux. On day 4 and 7, the animals were sacrificed by cervical dislocation. The induction was confirmed based on clinical symptoms/signs, ALP determination and histopathological analysis. At day 4, TNBS group presented a decreased body weight and an alteration of intestinal motility characterized by diarrhea, severe edema of the anus and moderate morbidity, while in the two control groups weren't identified any alteration on the clinical symptoms/signs with an increase of the body weight. TNBS group presented the highest concentrations of ALP comparing with control groups. The histopathology analysis revealed severe necrosis of the mucosa with widespread necrosis of the intestinal glands. Severe hemorrhagic and purulent exsudates were observed in the submucosa, muscular and serosa. TNBS group presented clinical symptoms/ signs and histopathological features compatible with a correct induction of UC. The peak of manifestations became maximal at day 4 after induction. This study allows concluding that it's possible to develop a TNBS- induced colitis 4 days after instillation.

Keywords: IBD, TNBS-induced colitis, Inflammation, Metabolic pathways, Pharmacological targets

[1]Escola Superior de Tecnologia da Saúde de Lisboa - IPL
[2]Pharmacology and Translational Research Group, iMED – Faculdade de Farmácia, Universidade de Lisboa
[3]Departamento de Anatomia Patologica, Faculdade de Medicina Veterinária - ULHT

Endereço para correspondência: Rui Pinto *E-mail*: rapinto@ff.ul.pt

Submetido/ Submitted: 23 de outubro de 2013| Aceite/Accepted: 20 de novembro de 2013

 ISSN: 2182-3340

RESUMO

DII é um distúrbio gastro-intestinal caracterizado por inflamação crónica do epitélio intestinal com dano associado da mucosa, manifestando-se a partir de sintomas intestinais e extra-intestinais. A terapia médica utilizada é capaz de induzir e manter o doente em remissão, mas não modifica ou inverte o mecanismo patogénico subjacente. A procura de outras abordagens terapêuticas é crucial para o tratamento de DII e, para tal, é importante o uso de modelos animais para mimetizar as características da doença. O objetivo do estudo é desenvolver um modelo animal de colite induzida por TNBS de modo a testar novas abordagens farmacológicas. O TNBS foi instilado por via intra-colónica em dose única como descrito por Morris et al. Foi administrado 2,5% de TNBS em 50% de etanol através de um cateter inserido no cólon. Os animais foram mantidos em posição Tredelenburg para evitar o refluxo. Nos dias 4 e 7, os animais foram sacrificados por deslocamento cervical. A indução de colite foi caracterizada com base nos sintomas/sinais clínicos, determinação de ALP e análise histopatológica. No dia 4, o grupo TNBS apresentou uma diminuição do peso corporal e uma alteração da motilidade intestinal caracterizada por diarreia, edema severo do ânus e morbilidade moderada, enquanto nos dois grupos controlo não foram identificados quaisquer alterações nos sintomas/ sinais clínicos com um aumento do peso corporal. O grupo TNBS apresentou as maiores concentrações de ALP, comparando com os grupos controlo. A análise histopatológica demonstrou necrose grave da mucosa com necrose generalizada das glândulas intestinais. Foi observado exsudato hemorrágico e purulento ao nível da submucosa, muscular e serosa. O grupo TNBS apresentou sintomas/sinais clínicos e características histopatológicas compatíveis com uma correta indução de colite. O pico das manifestações tornou-se máximo ao 4º dia após a indução. Este estudo permite concluir que é possível desenvolver colite induzida por TNBS 4 dias após a instilação.
Palavras-Chaves: DII, Colite induzida por TNBS, Inflamação, Vias metabólicas, Alvos farmacológicos.

INTRODUTION

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases of the gastrointestinal tract, characterized by chronic recur- rent ulceration of the bowels1. IBD affects between 7–10% of people worldwide, mainly of Caucasian descent[2,3], promoting significant gastrointestinal symptoms, like bloody diarrhea, abdominal pain, anemia, weight loss and other extra-intestinal manifestations[1]. Interplay between several factors, like genetic predisposition, environmental trigger and aberrant immune reaction seem to contribute to initiation and progression of IBD[4,5].

Pathogenesis of IBD is not fully understood, but two broad hypotheses have arisen regarding its fundamental nature. The first contends that primary dysregulation of the mucosal immune system leads to excessive immunologic responses to normal microflora. The second suggests that changes in the composition of gut microflora and/or deranged epithelial barrier function elicit pathologic responses from the normal mucosal immune system. Currently, it's well accepted that IBD is indeed characterized by an abnormal mucosal immune response but that microbial factors and epithelial cell abnormalities can facilitate this response[6].

Currently used medical therapy of IBD consists of salicylates, corticosteroids, immunesuppressants and immunomodulators. These drug treatments aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism[1,7]. Second-generation agents have been developed with improved drug delivery, increased efficacy and decreased side effects frequency[1]. However, their use may result in severe side effects and complications, such as an increased rate of malignancies or infectious diseases[7]. The research of other medical approaches is crucial to the treatment of IBD and, for this, it is important to use animal models to mimic the characteristics of disease in real life[8].

Animal models are widely used to study pathogenesis of human diseases and to test new therapeutics[9]. Most of these models are based either on chemical induction, immune cell transfer or gene targeting[4]. Trinitrobenzene sulfonic acid (TNBS) promotes a chemical induction of colitis by intrarectal instillation of the haptenating substances TNBS dissolved in ethanol resulting in acute inflammation with ulcers in rat and mouse[10]. The acute transmural damage became maximal from 3 days to 1 week after instillation, and resolved within 2 weeks[8,10-12]. The protocols of the TNBS-induced IBD model are not standardized, such as the dosage of TNBS, the depth of TNBS administration, and the time point for model evaluation. Therefore, it knows that the effects of TNBS are dose dependent[8]. Thus, the aim of the study is to develop an animal model of TNBS-induced colitis to test new pharmacological approaches.

MATERIAL AND METHODS

Materials

TNBS 5% aqueous solution was purchased from Sigma Chemical Co. Ketamine (Imalgene® 1000) was purchased from Merial. Xilazine (Rompun® 2%) was purchased from Bayer. ADVIA® kit was purchased from Siemens Healthcare Diagnostics.

Animals

Male CD-1 mice, 30-40 g in weight and 5-6 weeks of age, were housed in standard polypropylene cages with ad libitum access to food and water in the Faculty of Pharmacy Central Animal Facility in the University of Lisbon.

Experimental groups

Three groups of mice were used in the study, which received an intracolonic administration of different preparations. The first group (n = 6) received 100 µl of 2.5% of TNBS in 50% ethanol (TNBS group) for induction of TNBS-colitis. The second group (n=3) received 100µl of 50% ethanol (ethanol group). The third group (n=2) received 100µl of saline solution (sham group). It was used two control animals groups, namely ethanol and sham group.

Induction of TNBS-colitis

TNBS was instilled intracolonic single dose as described by Morris et al (1989). Briefly, mice were left unfed during 24h. In the induction day, mice were anesthetized with Ketamine 100mg/Kg + Xilazine 10mg/Kg IP and a catheter was carefully inserted into the colon until the tip was 4 cm proximal to the anus. Then, 2,5% TNBS in 50% ethanol was administered and mice were kept for 1 min in a Tredelenburg position to

avoid reflux[13,14]. On day 4 and 7, the animals were sacrificed by cervical dislocation, however a cardiac puncture was made immediately before in order to obtain samples for determination of serum alkaline phosphatase (ALP). The abdomen was opened by a midline incision. The small intestine and colon were removed, freed from surrounding tissues and washed with phosphate buffered saline. The results between day 4 and 7 were subsequently compared.

Clinical symptoms/signs

The animals were observed daily, monitoring body weight, morbidity, stool consistency and anus appearance.

Biochemical Marker

ALP in the sample catalyzes the hydrolysis of colorless p-nitrophenyl phosphate to give p--nitrophenol and inorganic phosphate. At the pH of the assay (10.3 e 10.4), the p-nitrophenol is in the yellow phenoxide form. The rate of absorbance increase at 410/478 nm is directly proportional to the ALP activity in the sample. Optimized concentrations of zinc and magnesium ions are present to activate the ALP in the sample. The measurement was made by an automatic analyzer: ADVIA 1200.

Histopathological analysis

Histopathology was carried out by an independent histopathologist of the Faculty of Veterinary Medicine of Universidade Lusófona de Humanidades e Tecnologias. The intestine samples were fixed in 10% neutral buffered formalin, processed routinely for paraffin embedding, sectioned at 5µm, and stained with hematoxylin and eosin. The morphological features of small intestine and colon were evaluated in the same conditions for all studied groups (TNBS group, ethanol group and sham group), according the number of days after induction and the localization of sections. It was evaluated at day 4 and 7, with several sections of small intestine due to its length and with three sections of colon (proximal to the cecum, in the middle of the colon and distal to the rectum).

Microscopic assessment of colitis severity

The assessment of colitis severity was based on previously described parameters15:

a)epithelial damage (0 = none, 1 = minimal loss of goblet cells, 2 = extensive loss of goblet cells, 3 = minimal loss of crypts and extensive loss of gob-let cells, and 4 = extensive loss of crypts);

b)infiltration (0 = none, 1 = infiltrate around crypt bases, 2 = infiltrate in muscularis mucosa, 3 = extensive infiltrate in muscularis mucosa with edema, and 4 = infiltration of submucosa).

The histological activity index (HAI) was calculated as the sum of the epithelium and infiltration score, resulting in the total HAI score ranging from 0 (unaffected) to 8 (severe colitis).

RESULTS

Monitoring of clinical symptoms/signs

Animals were observed daily for morbidity, stool consistency and anus appearance. At day 1, TNBS group presented an alteration of intestinal motility characterized by diarrhea or soft stools, severe edema of the anus and moderate morbidity. Ethanol group showed the same clinical signs, but lightly. At day 4 and 7, TNBS group kept the observed clinical signs, while in the ethanol group wasn't identified any alteration. Sham group remained without any alterations during the study.

Regarding body weight, ethanol and sham groups increased the weight of mice during the study, but TNBS group showed a decreased body weight at day 4 (TABLE 1).

Table 1. Average of body weight during the study

	AVERAGE OF BODY WEIGHT ± SD (g)		
	DAY 0	DAY 4	DAY 7
TNBS GROUP (n=6)	34.3 ± 4.7	29 ± 0	43.3 ± 4.6
ETHANOL GROUP (n=3)	33.6 ± 1.6	44 ± 0	40.5 ± 2.5
SHAM GROUP (n=2)	37 ± 2	43 ± 0	47 ± 0

At day 2, there were 2 deaths in the TNBS group. The macroscopic analysis showed a severe obstruction to the colon filled with large fecal pellets. Severe swelling of the intestinal wall, inflammation with presence of generalized strokes. The lesions were apparently consistent with toxic megacolon.

Biochemical Marker

ALP was identified in all experimental groups, but in different concentrations depending of the group was evaluated. TNBS group

presented the highest values around 32.9 ± 5.5 U/L of serum concentration comparing with the other two groups. Ethanol and sham groups presented decreased values but quite similar around 10.8±1.5 U/L and 8.5±0 U/L, respectively.

Assessment of small intestine lesions

The small intestine was analyzed and it showed similar histological results among evaluated groups and the number of days after induction. Apparently, no macroscopic lesions were observed, but microscopically, a slight lymphoplasmacytic infiltrate in the lamina propria was identified and it was similar to all studied groups independently of the day after induction (Figure 1).

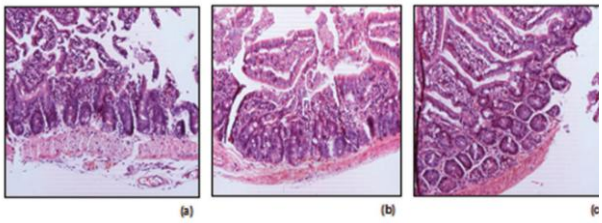

Figure 1. Histopathological features of small intestine sections (100x) from (a) TNBS group, (b) Ethanol group, (c)Sham group.

Assessment of colitis severity

The colon was analyzed and it showed different histological results depending of which group was evaluated and the number of days after induction. Regarding macroscopic evaluation of colon, it was observed hemorrhagic focus and edema. Microscopically, the TNBS group presented severe lesions at day 2 and day 4, whereas no substantial morphological changes were detected at day 7 (Figure 2). The histopathology analysis revealed severe necrosis of the mucosa with widespread necrosis of the intestinal glands. The remaining intestinal glands were ectasic with squamous metaplasia. Severe hemorrhagic and purulent exsudates were observed in the submucosa, muscular and serosa.

The assessment of colitis severity was based on two main parameters namely (a) epithelial damage and (b) infiltration. At day 2 and 4, it was observed the higher score (HAI of 8 – severe colitis) for all samples evaluated on the TNBS group comparatively to the samples of ethanol and sham group, which it was observed no lesions (HAI of 0-unaffected).

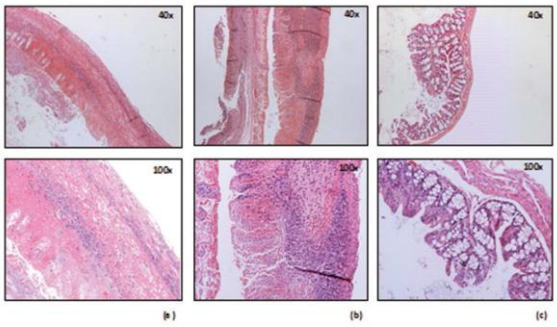

Figure 2. Morphologic changes of colon from TNBS group in day (a) 2, (b) 4 and (c) 7. In the ethanol and sham group, no histological alterations were observed in the colon (HAI of 0–unaffected), independently of the day after induction (Figure 3).

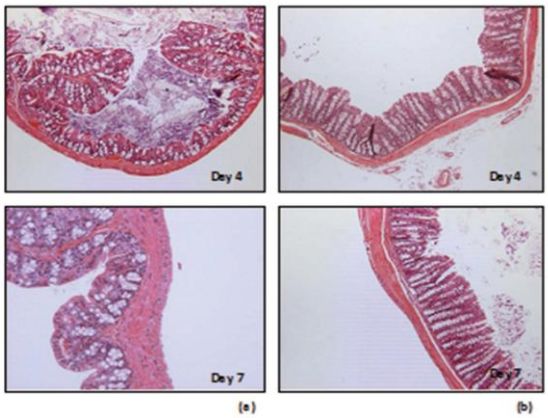

Figure 3. Histopathological features of colon (40x) from Ethanol group and (b) Sham group, in day 4 and 7.

DISCUSSION

The pathogenesis of IBD is similar between human disease and TNBS-induced colitis[16], and this is the reason why so many research groups are now using this model to investigate novel approaches for the treatment of IBD.

Regarding monitoring of clinical symptoms/ signs, TNBS group presented an alteration of intestinal motility characterized by diarrhea or soft stools, severe edema of the anus and moderate morbidity, while ethanol and sham groups remained without any alterations. These clinical manifestations in the TNBS group were expected and compatible with a correct induction of UC[15,16]. The manifestations became maximal at day 4 after induction and resolved at day 7. The literature refers that manifestations became maximal from 3 days to 1 week after instillation, and resolved within 2 weeks[8,10-12], depending of the dosage of TNBS, the depth of TNBS administration, and the time point for model evaluation8. The peak of clinical symptoms/signs is also confirmed by the decreased

body weight of TNBS group at day 4, comparing with ethanol and sham groups that increased its body weight during the study.

ALP is regularly measured in clinical practice and its changes in serum levels are observed in a number of clinical conditions of organs where it can be found like bone, liver, bowel and kidney[17]. Therefore, ALP was measured in all experimental groups and TNBS group presented the highest values around 32.9 ± 5.5 U/L of serum concentration comparing with ethanol and sham groups (10.8±1.5 U/L and 8.5±0 U/L, respectively). The low concentrations of APL in both control groups (ethanol and sham group) suggest that the origin of increased APL in the TNBS group is due to intestinal lesion inducted in this study. These our results are consistent with other studies, which observed a higher AP activity in the colon from colitic animals compared to the non-colitic animals from acute intestinal inflammation model induced by TNBS[18,19]. Intestinal ALP has been considered a phenotypic marker of differentiation, which is up-regulated in experimental chronic diarrhea and IBD[19,20]. It's a small intestinal brush-border enzyme that functions as a gut mucosal defense factor, providing resistance to bacterial invasion when the intestine is subject to a certain lesion like local or distant ischemic injury[21]. Based on these results, administration of exogenous intestinal ALP enzyme to patients with the active form of IBD may be a therapeutic option[22].

The histopathological analysis showed a slight lymphocytic inflammatory infiltrate in the lamina propria similar with all experimental groups. These lesions are consistent with a sub-acute to chronic process, suggesting no relationship with this study. Perhaps, the reason is that mice are not Specific Pathogen Free, even because there are similar for all experimental groups. These should therefore be devalued.

The morphological features of colon in the TNBS group were evaluated at day 2, 4 and 7. The results revealed severe lesions of tissue between day 2 and day 4, whereas it was detected no substantial morphological changes at day 7. The peak of clinical symptoms/signs is also confirmed by the severe lesions at day 4. No histological alterations were observed in the colon from ethanol group and sham group. The histopathology analysis revealed severe necrosis of the mucosa with widespread necrosis of the intestinal glands. The remaining intestinal glands were ectasic with squamous metaplasia. Severe hemorrhagic and purulent exsudates were observed in the submucosa, muscular and serosa. These lesions are consistent with a correct induction of UC by TNBS[15,16].

CONCLUSION

This study allows concluding that it's possible to develop a TNBS-induced colitis 4 days after instillation. This model will be interesting to clarify the inflammatory mechanisms associated with IBD in order to propose other pharmacological modulation of the inflammatory response than the currently known, with the main objective to facilitate a more effective and selective treatment for this disease.

REFERÊNCIAS BIBLIOGRÁFICAS

1.Pithadia A, Jain S. Treatment of inflammatory bowel disease (IBD). Pharmacological Reports. 2011; 63:629-42

2.Hanauer S. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. Inflamm Bowel Dis. 2006; 12(1): 3–9

3.Spiegel B. The burden of IBS: looking at metrics. Curr Gastroenterol Rep. 2009; 11:265–9

4.Wirtz S, Neurath M. Mouse models of inflamma- tory bowel disease. Adv Drug Del Rev. 2007; 59:1073–83

5.Mayer L. Evolving paradigms in the pathogenesis of IBD. J Gastroenterol. 2010; 45:9–16

6.Strober W. Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. J Clin Invest. 2007; 117:514–21 in doi: 10.1172/ JCI30587

7.Engel M, Neurath M. New pathophysiological in-sights and modern treatment of IBD. J Gastroenterol. 2010; 45:571-83

8.Qin H, Wu J, Tong X, Sung J, Xu H, Bian Z. Systematic review of animal models of post-infectious/ post-inflammatory irritable bowel syndrome. J Gastroenterol. 2011; 46:164–74

9.Szczepanik M, Góralska M, Marcińska K, Wiacek M, Strzepa A, Dorozynska I, Szczepanik M. Epicutaneous immunization with protein antigen TNP-Ig alleviates TNBS-induced colitis in mice. Pharmacological Reports. 2012; 64: 1497-504

10.Morris G, Beck P, Herridge M, Depew W, Szewczuk M, Wallace J. Hapten-induced model of chronic inflammation and ulceration in the

rat colon. Gastroenterology. 1989; 96:795–803
11.Linden D, Foley K, McQuoid C, Simpson J, Shar- key K, Mawe G. Serotonin transporter function and expression are reduced in mice with TNBS-induced colitis. Neurogastroenterol Motility. 2005; 17:565–74
12.Lamb K, Zhong F, Gebhart G, Bielefeldt K. Exper- imental colitis in mice and sensitization of con- verging visceral and somatic afferent pathways. Am J Physiol Gastrointest Liver Physiol. 2006; 290:G451–7
13.Wirtz S, Neufert C, Weigmann B, Neurath
14.M. Chemically induced mouse models of intestinal inflammation. Nat Protocols. 2007; 2: 541–6
15.Mazzon E, Muià C, Paola D, Genovese T, Menegazzi M, De Sarro A, Suzuki H, Cuzzocrea
16.S. Green tea polyphenol extract attenuates colon injury induced by experimental colitis. Free Radic Res. 2005; 39(9):1017-25
17.Alex P, Zachos N, Nguen T, Gonzales L, Chen T-E, Conklin L, Centola M, Li X. Distinct cytokine patterns identified converging visceral and somatic afferent pathways. American Journal of Physiology - Gastrointestinal and Liver Physiology from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm Bowel Dis. 2009; 15:341–52
18.Pawar P, Gilda S, Sharma S, Jagtap S, Paradkar A, Mahadik K, Ranjekar P, Harsulkar A. Rectal gel ap- plication of Withania somnifera root extract ex- pounds anti inflammatory and mucorestorative activity in TNBS-induced Inflammatory Bowel Disease. BMC Complement Altern Med. 2011; 11(34):1-9
19.Tinnion R, Embleton N. How to use.alkaline phosphatase in neonatology. Archives of Disease in Childhood–Education and Practice.2012; 97(4):157-63 in doi:10.1136/archdischild-2012-301633
20.Cruz T, Gálvez J, Crespo E, Ocete M, Zarzuelo
21.Effects of silymarin on the acute stage of the trinitrobenzene sulphonic acid model of rat colitis. Planta Medica. 2001; 67:94-6
22.Luchini A, Rodrigues-orsi P, Cestari S, Seito L, Witaicenis A, Pellizzon C, Stasi L. Intestinal Anti-inflammatory Activity of Coumarin and 4-Hydroxycoumarin in the Trinitrobenzenesulphonic Acid Model of Rat Colitis. Biol Pharm Bull. 2008; 31(7):1343-50
23.Galvéz J, de la Cruz J, Zarzuelo A, Sánchez de la Cuesta F. Flavonoid inhibition of enzymic and nonenzymic lipid peroxidation in rat liver differs from its influence on the glutathione-related enzymes. Pharmacology. 1995; 51:127-33
24.Ramasamy S, Nguyen D, Eston M, Alam S, Moss A, Ebrahimi F, Biswas B, Mostafa G, Chen K, Kaliannan K, Yammine H, Narisawa S, Millán J, Warren H, Hohmann E, Mizoguchi E, Reinecker H, Bhan A, Snapper S, Malo M, Hodin R. Intestinal alkaline phosphatase has beneficial effects in mouse models of chronic colitis. Inflamm Bowel Dis. 2011; 17(2):532–42 in doi: 10.1002/ibd.21377
25.Molnár K, Vannay A, Szebeni B, Bánki N, Sziksz E, Cseh A, Györffy H, Lakatos P, Papp M, Arató A, Veres G. Intestinal alkaline phosphatase in the colonic mucosa of children with inflammatory bowel disease. World J Gastroenterol. 2012; 18(25):3254-9