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**Alleviated mucosal and neuronal damage in a rat model of Crohn's disease**

Talapka P *et al.* Rat model of Crohn's disease

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## **Abstract**

### ***BACKGROUND***

#### ***AIM***

To establish a rat model suitable to investigate the repetitive relapsing inflammations (RRI) characteristic to Crohn's disease.

#### ***METHODS***

Colitis was induced by 2, 4, 6-trinitrobenzenesulfonic acid (TNBS). RRI were mimicked by repeating administrations of TNBS. Tissue samples were taken from control, once, twice and three times treated rats from the inflamed and adjacent non-inflamed colonic segments at different timepoints during the acute intestinal inflammation. The means of the ulcerated area were measured to evaluate the macroscopic mucosal damage. The density of myenteric neurons was determined on wholemounts by HuC/HuD immunohistochemistry. Heme oxygenase-1 (HO-1) expression was evaluated by molecular biological techniques.

#### ***RESULTS***

TNBS-treated rats displayed severe colitis, but the mortality was negligible, and an increase of body weight was characteristic throughout the experimental period. The widespread loss of myenteric neurons, and marked but transient HO-1 up-regulation were demonstrated after the first TNBS administration. After repeated doses the length of the recovery time and extent of the ulcerous colonic segments were markedly decreased, and the neuronal loss was on a smaller scale and was limited to the inflamed area. HO-1 mRNA level was notably greater than after a single dose and overexpression was sustained throughout the timepoints examined. Nevertheless, the HO-1 protein up-regulation after the second TNBS treatment proved to be transient. Following the third treatment HO-1 protein expression could not be detected.

#### ***CONCLUSION***

Experimentally provoked RRI may exert a protective preconditioning effect against the

mucosal and neuronal damage. The persistent up-regulation of HO-1 mRNA expression may correlate with this.

**Key words:** Crohn's disease; Experimental rat model; Heme oxygenase-1; Myenteric neurons; Repetitive relapsing inflammation

**Core tip:** We report our first results derived from a newly developed rat model with chronic experimental colitis allowed us to modelling the recurring periods of recrudescence and remission in Crohn's disease. Colitis was induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). Repetitive recurrent inflammations (RRI) were mimicked by repeated administrations of TNBS. This study demonstrates for the first time that experimentally provoked RRI develop preconditioning effect by speeding up mucosal healing and restoring myenteric neuronal injury. Decreased severity of gut inflammation after repeated TNBS treatments might be associated with the persistent up-regulation of heme-oxygenase 1 messenger RNA expression.

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## INTRODUCTION

Inflammatory bowel diseases (IBDs) are a group of chronic intestinal inflammatory conditions. The major types of IBDs are Crohn's disease (CD) and ulcerative colitis. CD is characterized by relapsing transmural inflammation that can affect any part of the gastrointestinal tract. Although the pathogenesis of CD is still unclear, the most widely accepted hypothesis is that an impairment of the mucosal barrier function due to a dysregulated immune response to an environmental factor can generate prolonged inflammation<sup>[1,2]</sup>. The development of irreversible pathological alterations including stricturing and penetrating complications in response to the repetitive relapsing inflammations (RRI) characteristic of CD can indicate the transition from the early to the late disease which is dependent on the intensity rather than on the duration of the inflammation<sup>[3]</sup>.

The intestinal symptoms common among CD patients are often caused by intestinal motility abnormalities related to enteric neuropathy. The intestinal motor functions are regulated by the myenteric neurons. The evidence suggests that both the quantitative properties and function of the myenteric neurons are altered substantially by intestinal inflammation<sup>[4]</sup>. In a previous study 40% loss of myenteric neurons was demonstrated in the inflamed segment of the colon four days following the induction of colitis in rats<sup>[5]</sup>. Moreover, the complete loss of myenteric neurons was observed in the strictured region<sup>[6]</sup>. Persistent alterations in neuronal signalling were documented even after the resolution of the colitis. The AH neurons, one electrophysiological type of enteric neurons that function as intrinsic afferent neurons, remained hyperexcitable eight weeks after the induction of colitis. In the same experiment larger amplitudes of fast excitatory postsynaptic potentials were measured in the S neurons as compared with the controls<sup>[7]</sup>. Physiological disturbances are not restricted to the site of the inflammation. Suppression of noradrenaline release was observed in both the distal and the transverse colon and in the terminal ileum in rats with 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis<sup>[8]</sup>.

During the past two decades, experimental animal models of IBD have proved to

be important tools for the detection of potential therapeutic agents and for the investigation of the pathogenesis. One of the most widely used haptinizing agents TNBS is used to induce colitis, resulting in mucosal inflammation mediated by a Th1 response. Nevertheless, in most cases the induction of acute necrotizing enterocolitis with a single high dose of TNBS was not able to generate the pathological alterations characteristic of CD and was accompanied by a high mortality rate<sup>[9]</sup>. The repeated administration of TNBS in increasing doses resulted in chronic colitis and fibrosis in mice<sup>[10]</sup> and in rats<sup>[11]</sup> that most likely reflects the characteristics of the chronic phase of CD. Nevertheless, considerable levels of mortality, 25% and 18% respectively were still demonstrated.

Our aim was to establish a rat model of chronic colitis with the possible lowest mortality rate, suitable for the investigations of the long-term consequences of acute inflammation. In the present study the extent of mucosal and myenteric neuronal injuries were investigated in the acute phase of the experimentally mimicked RRI. Since increased expression of heme oxygenase-1 (HO-1) has been described in patients with CD<sup>[12,13]</sup>, it has been suggested that the activation of HO-1 may act as a natural defence to alleviate inflammation and tissue damage<sup>[14-16]</sup>. Therefore, the quantitative changes in HO-1 mRNA and protein expression were also evaluated here.

## **MATERIALS AND METHODS**

### *Animal model*

All experiments were approved by from the Local Ethics Committee for Animal Research Studies at the University of Szeged. Adult male Sprague-Dawley rats, weighing 200-220 g, kept on standard laboratory chow (Bioplan Kft., Hungary) and with free access to drinking water, were used throughout the experiments. They were housed in a restricted access room with controlled temperature (23 °C) and a light/dark (12 h:12 h) cycle. Colitis was induced locally with TNBS (Sigma-Aldrich, St. Louis, MO, United States; 10 mg) dissolved in 0.25 mL of 25% ethanol administered with a polyethylene cannula 8 cm proximal to the anus under pentobarbital anaesthesia (45 mg/kg i.p.). The animals were deprived of food at 24 h before the induction of inflammation. Relapsing inflammations were mimicked by repeating the administration of TNBS with a two week-lag. The animals were randomly divided into control ( $n = 18$ ), and once ( $n = 13$ ),

twice ( $n = 14$ ) or three times ( $n = 11$ ) TNBS-treated groups. The control animals received an enema of 0.25 mL of 0.9% saline. The rats were weighed weekly and monitored for activity, bloody diarrhoea and mortality.

### *Tissue handling*

The animals were killed by cervical dislocation under chloral hydrate anaesthesia (375 mg/kg i. p.) two ( $n = 11$ ), four ( $n = 13$ ) and eight ( $n = 14$ ) days following the TNBS treatments. The final 8 cm region of the descending colon from the anus was dissected. Tissue samples were taken from the inflamed segment and also proximally and distally to the inflamed segment of the colon. The gut segments were cut along the mesentery and pinched flat. Digital photographs were taken to evaluate the macroscopic mucosal damage. The extent of the ulceration was measured two, four and eight days after the first and repeated TNBS treatments. The means of the ulcerated area ( $\text{cm}^2$ ) per area of total colon segments ( $\text{cm}^2$ ) were determined by Image J 1.44 (National Institute of Health, Bethesda, MD, United States) and the the mean  $\pm$  SE was calculated with GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, United States). After cutting longitudinally, half of the colon samples were processed for immunohistochemistry. The other halves were further divided and processed either for qRT-PCR or western blotting analysis. Tissue samples for qRT-PCR were incubated overnight at 4 °C in RNA Later (Qiagen, Venlo, The Netherlands). Those for western blotting analysis were frozen immediately in liquid N<sub>2</sub> and stored at -80 °C until use.

### *Investigation of the quantitative properties of the myenteric neurons*

Wholemout preparations were immunostained with the pan-neuronal marker HuC/HuD as described earlier<sup>[17]</sup>. Briefly, wholemounts were incubated overnight with anti-human neuronal protein HuC/HuD developed in mouse (Sigma-Aldrich, St. Louis, MO, United States; final dilution 1:50). After washing in phosphate buffer (PB, 0.05 mol/L), tissue samples were incubated with biotinylated anti-mouse IgG (Amersham, Buckinghamshire, United Kingdom; final dilution 1:100) for 6 h, followed by overnight incubation in streptavidin-biotinylated horseradish peroxidase (Amersham, Buckinghamshire, United Kingdom; final dilution 1:100). Peroxidase activity was

revealed by using 3, 3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, United States) as substrate chromogen. Wholemounds were then mounted on gelatin-coated slides in glycerol-PB. Twenty digital photographs at 200x magnification were taken from each colonic segment from each experimental group with an Olympus BX51 light microscope equipped with an Olympus DP70 camera. The number of neurons was counted with Plexus Pattern Analysis software<sup>[18]</sup>. Statistical analysis was performed by using one-way ANOVA and the Newman-Keuls test. The results were evaluated with GraphPad Prism 4.0, and a probability  $P < 0.05$  was set as the level of significance. The results were expressed as mean  $\pm$  SE.

### ***Quantification of HO-1 mRNA expression by qRT-PCR***

Tissue samples were homogenized in AccuZol (Bioneer, Daejeon, South Korea) directly before qRT-PCR. Total RNA was prepared from tissue homogenates as suggested by the manufacturer (Bioneer, Daejeon, South Korea). The reverse transcription was achieved by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, United States). 2  $\mu$ g of total RNA from HO-1 was transcribed in 15  $\mu$ L of reaction mixture (1.2  $\mu$ L dNTPs, 1.5  $\mu$ L of MultiScribe Reverse Transcriptase (50 U/ $\mu$ L), 3  $\mu$ L of RT Buffer, 3  $\mu$ L of RT primer and 6.3  $\mu$ L of nuclease-free water). All reactions were carried out for 10 min at 25 °C and then for 2 h at 37 °C in MyGenie32 Thermal Block (Bioneer, Daejeon, South Korea). qRT-PCR was performed in an Exicycler 96 (Bioneer, Daejeon, South Korea) in a total volume of 20  $\mu$ L containing 10  $\mu$ L of FastStart SYBR Green PCR Master Mix, 1  $\mu$ L of specific primer (0.5 pmol/ $\mu$ L) and 50 ng of cDNA template. The PCR program began with a 15-min initial step at 95 °C to activate the Taq DNA polymerase. This was followed by 45 cycles of 15 s at 95 °C for denaturation, 45 s at 60 °C for annealing and 25 s at 72 °C for extension. The sequences of primers were derived from NCBI RefSeq Database entry NM\_012580.2 for HO-1 (forward: 5' gtcaagcacagggtgacaga 3' and reverse: 5' ctgcagctcctcaaacagc 3'). Every sample was measured three times and the comparative  $C_T$  ( $\Delta\Delta C_T$ ) method was applied with the Exicycler 96 Analysis Software (Bioneer, Daejeon, South Korea) for the relative quantification of transcription levels. Hypoxanthine guanine phosphoribosyltransferase (NBCI RefSeq Database entry: NM\_012583.2; forward: 5' gaccggttctgtcatgtcg 3' and



reverse 5' acctggttcatcatcactaatcac 3') was used as a housekeeping gene to normalize expression data. The results were expressed as means  $\pm$  SD.

#### ***Western blotting analysis of the expression of HO-1 protein***

Tissue samples were homogenized in TRIS-mannitol buffer and then total protein was denatured from each sample as described earlier<sup>[19]</sup>. 10  $\mu$ g of total cellular protein was separated by SDS-PAGE and was transferred to nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom). The membrane was probed with anti-HO-1 monoclonal antibody (Enzo Life Sciences, Farmingdale, NY, United States; final dilution 1:1000) and then was incubated with horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States; final dilution 1:2000) at room temperature. Immunoreaction was visualized with an enhanced chemiluminescence system Immobilon Western HRP Substrate (Millipore Corporation, Billerica, MA, United States) and scanned with LI-COR C-DiGit™ Blot Scanner (Li-Cor Corporate, Lincoln, NE, United States).

## **RESULTS**

### ***Macroscopic observations***

Consistent with the findings of previous studies, the TNBS-treated rats already displayed severe ulcerative intestinal inflammation associated with weakness and bloody diarrhoea on the first day after the induction of colitis with either single or repeated doses of TNBS. The mortality rate was negligible: only two animals died during the experiments. Independently of how many doses were administered, the symptoms always presented with the same severity, but the length of the recovery time decreased spectacularly after repeated treatments. Except for the first week of acute inflammation, when a gain in weight was not detected, a gradual increase in body weight was characteristic in all the rats throughout the experimental period (Table 1). The severe symptoms, like bloody diarrhoea accompanied by acute inflammation lasted for seven or eight days after the administration of a single dose of TNBS but were already resolved four or five days after repeated treatments. The accelerated mucosal healing after the repeated TNBS administrations was clearly demonstrated by the significant differences in the mean ulcerated areas on days two, four and eight after the

induction of colitis (Figure 1). The mean area of the ulcerated colonic segment decreased significantly in all TNBS-treated groups between days two and eight following induction. Whereas, the area was still noteworthy eight days after the single TNBS administration, it was significantly reduced and hardly detectable after the second (Figure 1) and even undetectable after the third treatment (not shown). However, long-range hyperaemia was always detected in all colonic samples even on day eight after colitis induction.

### *Quantitative changes of the myenteric neurons*

Whole mounts of colonic segments after HuC/HuD immunohistochemistry were used to evaluate the density of the myenteric neurons (Figure 2). Data were always compared to the age-matched controls (Figure 3). Irrespective of the number of TNBS treatments, the colitis in the acute phase was always associated with a rapid and significant loss of HuC/HuD-immunoreactive myenteric neurons. Significant decrease in the number of neurons was first demonstrated four days after the administration of a single dose of TNBS, when 43% of the neurons were lost (Figure 3). Further significant decrease in neuronal density was demonstrated until day eight, when the number of neurons was 58% less relative to the age-matched controls (Figure 3). After the second and third TNBS treatments significant decreases in myenteric neuronal number were also observed first on day four after the induction of colitis, but in these cases the neuronal loss was less extensive (36% and 23%, respectively) and did not accelerate between days four and eight after the induction of colitis (Figure 3). Moreover, the decrease in myenteric neuronal density in the acute phase of the inflammation was not limited to the apparent inflamed area. Proximally and distally to the inflamed colonic segment a significant neuronal (25% and 34%, respectively) was noticed four days after the first TNBS administration. However following the repeated doses of TNBS a significant loss of neurons in the colonic segments adjacent proximally and distally to the inflamed area was not detected (Figure 3).

### *Quantitative changes in HO-1 mRNA expression*

The HO-1 mRNA expression was evaluated by qRT-PCR in all three colonic segments after single and repeated TNBS treatments. The HO-1 gene expression was markedly

induced in the inflamed segment on day four after the first TNBS administration (Figure 4). However four days after the second treatment an approximately 50% higher HO-1 mRNA level was detected (Figure 4). The HO-1 mRNA expression then declined until day eight in the samples from the once TNBS-treated rats, and to a lesser extent also in the twice TNBS-treated rats, but it has never returned to the baseline level (Figure 4). Nevertheless, after the third treatment a nearly 45% increase in gene expression was measured between days four and eight and this high level of HO-1 mRNA expression was sustained even in the chronic phase of inflammation (not shown). Proximally to the inflamed colonic segment a mild increase of HO-1 mRNA expression was observed in the acute phase of the inflammation four days after the single and repeated TNBS doses, but in the distal segment the HO-1 mRNA remained at the baseline level at this timepoint. Eight days after the TNBS treatments expression of HO-1 mRNA in the non-inflamed sites adjacent to the inflamed colonic segments was down-regulated (Figure 4).

#### *Changes in HO-1 protein expression*

The changes in HO-1 protein expression were evaluated by western blotting analysis in the inflamed segments of the colon samples following single and repeated TNBS treatments. Four days after the first and second TNBS administration a notable up-regulation of HO-1 protein expression was demonstrated. Following treatment with a single dose, HO-1 protein expression declined fast to the control level, while after repeated TNBS doses it has not returned back to the baseline level until the eighth day. Nevertheless, after the third TNBS treatment, HO-1 protein expression could not be detected at all (Figure 5).

## **DISCUSSION**

To mimic RRI characteristic of that in CD patients<sup>[20]</sup>, a rat model of chronic colitis was established with repeated administration of TNBS. In contrast with literature data<sup>[11,21]</sup> the rats in this case were treated with a low dose of TNBS (10 mg) dissolved in 25% ethanol. Already on the first day post-induction, severe mucosal inflammation associated with bloody diarrhoea, significant myenteric neuronal loss and marked HO-1

up-regulation indicated the primary events of acute inflammation after the single and after each repeated TNBS treatment. Despite the severity of the initial symptoms the mortality rate was negligible, which makes this rat model more suitable for investigations of the long-term consequences of acute intestinal inflammation than other TNBS models published to<sup>[9,11,21-25]</sup>. Besides the low mortality rate, the alleviated macroscopic mucosal damage and accelerated mucosal healing were salient features in the acute phases of inflammation after repeated TNBS treatment. However, regardless of the number of treatments long-range hyperaemia was always detected several days after the period of acute inflammation. This observation indicates that the post-inflammation remodelling of the vascular pattern is delayed in these rats. The delay in returning to the normal vascular pattern well after the mucosal healing is otherwise a regular problem in clinical practice<sup>[26-28]</sup>. We therefore consider that the rat model developed here will be suitable in future studies to reveal the molecular events behind vascular remodelling in acute intestinal inflammation and thereby help to open up new therapeutic avenues for the treatment of CD patients.

Although there are an appreciable number of unexplainable differences in the findings regarding CD-related alterations in the numbers of enteric nerve cells in human patients and animal models<sup>[29-31]</sup>, the quantitative alterations of the ENS are now considered a hallmark of bowel inflammation. We therefore set out to establish whether the experimentally provoked RRI also alleviates the inflammatory damage in the ENS and reduces the rapid, significant and widespread loss of myenteric neurons demonstrated four and even more eight days after the administration of a single dose of TNBS. After the second and third TNBS treatments significant but less extensive decreases in the number of myenteric neurons were also observed first on day four post-induction, but further neuronal loss was never demonstrated. Examination of the intestinal segments adjacent proximally and distally to the inflamed area likewise significant decreases in neuronal density after the single dose of TNBS indicating the spreading of the neuronal injury outside the inflamed area. Literature data from surgical practice which indicate a neuronal pathway for the spreading of intestinal inflammation at the resection margins<sup>[32-34]</sup> are in accordance with these findings. However, after the administration of repeated doses of TNBS the neuronal loss was strictly limited to the

inflamed segments of the colon: no proximal or distal spread of the neuronal injury was noticed. Further long-range studies with the present model are now in progress to elucidate the pathogenetic role of the myenteric plexus in the spreading of CD.

These observations of alleviated mucosal and neuronal injury, accelerated mucosal healing and reduced and restricted neuronal cell loss clearly indicate the preconditioning effect of the experimentally provoked RRI. However, the accelerated recovery in the acute phase of inflammation might be misleading in early diagnosis of CD, and thus in avoiding the development of chronic complications. Therefore, studies on the long-term structural and functional consequences of this protective effect are currently in progress in our laboratory.

Since an increased expression of HO-1 was previously described in patients with CD as a crucial mediator of the mucosal defence<sup>[12,13]</sup>, we expected changes in HO-1 gene expression behind the mucosal and neuronal defence demonstrated here. The HO-1 mRNA expression was therefore evaluated by qRT-PCR after single and repeated TNBS treatments. Marked increase in HO-1 gene expression was already demonstrated on day four after administration of the single dose of TNBS and the expression was enhanced to a great extent after the second and even more so after the third treatment. There was a decline in HO-1 expression between days four and eight in samples from the once and twice TNBS-treated rats, although it never returned to the baseline level. However after the third treatment there was no decline at all, but rather a more enhanced expression of HO-1 mRNA was detected until the post-induction day eight. HO-1 mRNA down-regulation in adjacent non-inflamed sites at the same time led us to hypothesize that an early inactivation of the endogen antioxidant defence system at the borderline of inflammation might contribute to the recurrence of CD proximal to the inflamed gut segments observed regularly after surgical resection<sup>[27,33,34]</sup>.

In parallel with the sustained up-regulation of HO-1 mRNA expression the increase in HO-1 protein level proved to be transient here and after the third treatment HO-1 protein expression could not be detected at all. Therefore, we suggest a post-transcriptional control mechanism for HO-1 expression in RRI. Recent studies<sup>[35-37]</sup> indicate that a regulatory feedback network may exist between HO-1 and microRNAs (miRs) for controlling gene expression at the post-transcriptional level in response to

oxidative damage<sup>[38]</sup>. However, the details of this feedback mechanism needs to be explored, we hypothesize that HO-1 up-regulation under RRI resulted in elevated amount of miRs, which in turn could lead to the inhibition of HO-1 protein expression after the third TNBS treatment.

In conclusion, we assumed that the alleviated mucosal and neuronal damage in the acute phase of RRI may be associated with the posttranscriptional regulation of HO-1 mRNA expression. Thus a better understanding of the mechanisms and regulatory factors involved in these regulatory processes might be of therapeutic interest.

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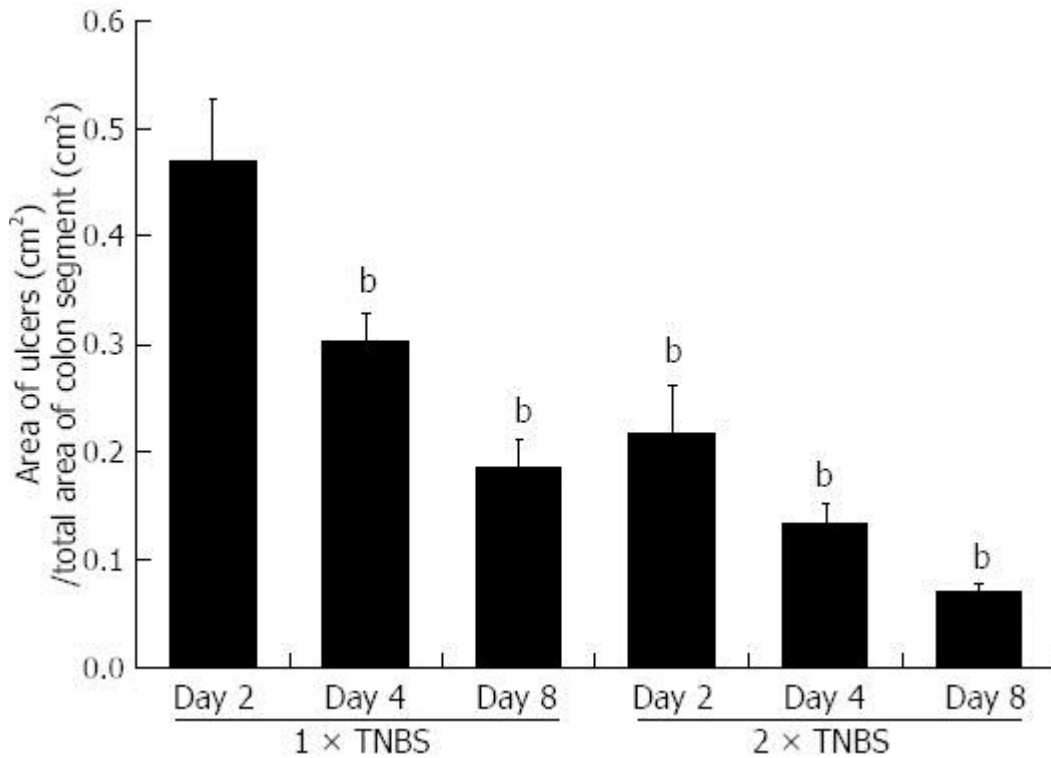
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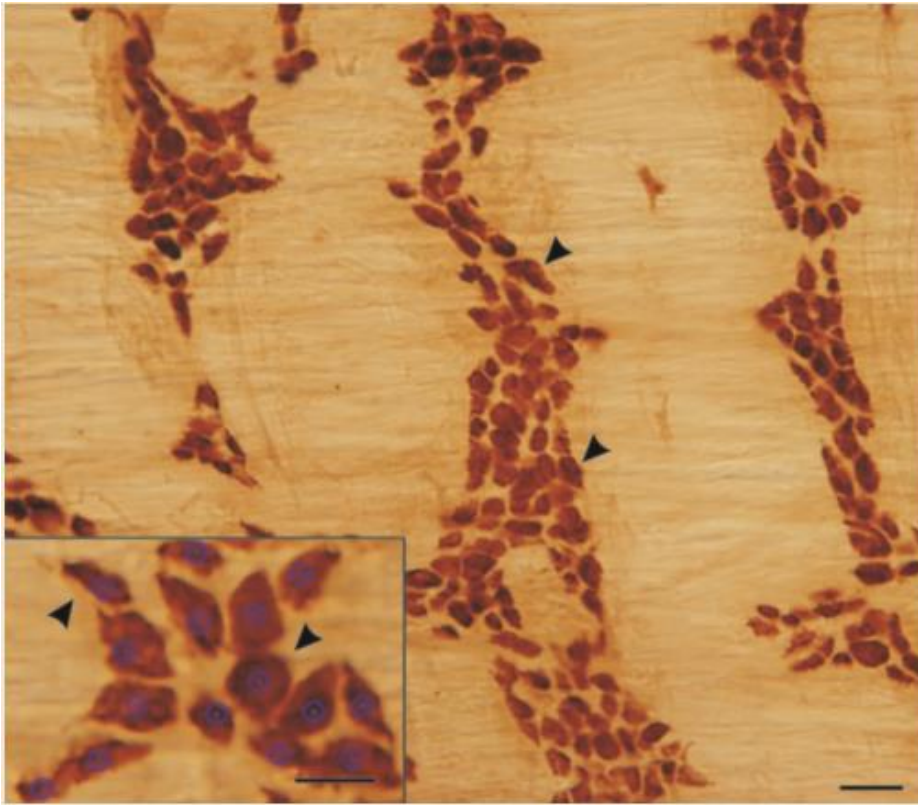
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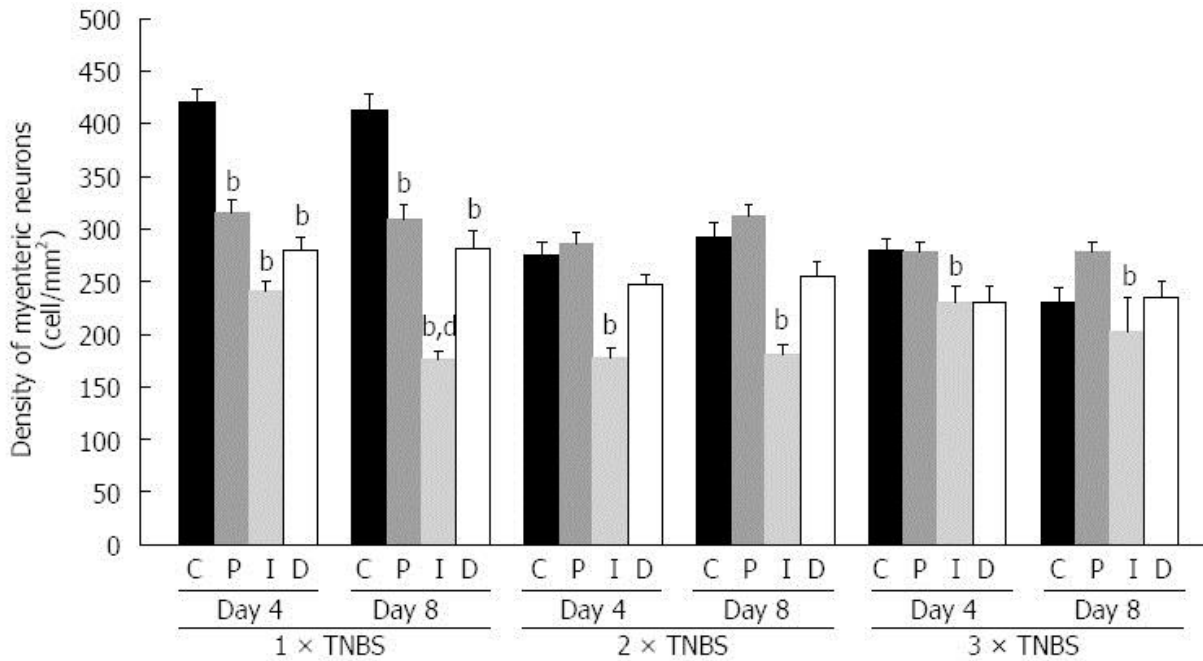
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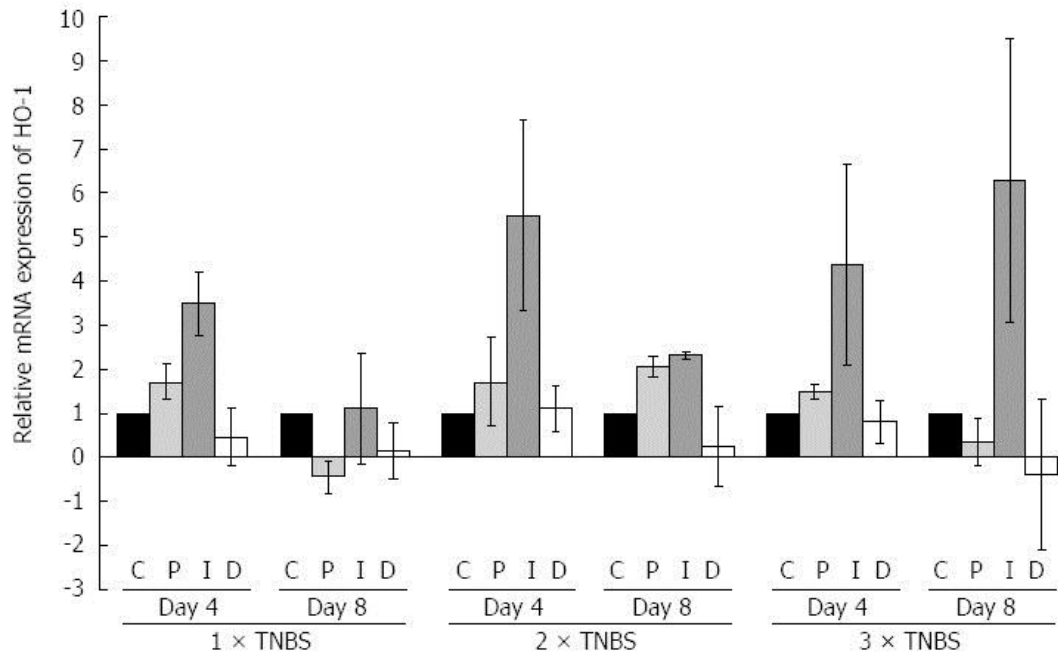
**Figure 1** Means of the ulcerated area per total area of colonic segments in rat descending colon two ( $n = 8$ ), four ( $n = 9$ ) and eight ( $n = 10$ ) days after the first and second 2,4,6-trinitrobenzenesulfonic acid treatments. The extension of the ulcerated area was reduced significantly after the second dose of TNBS compared to that in the animals treated only once. Data are expressed as mean  $\pm$  SE. <sup>b</sup> $P < 0.01$  vs once TNBS-treated group on day two. TNBS: 2,4,6-trinitrobenzenesulfonic acid.



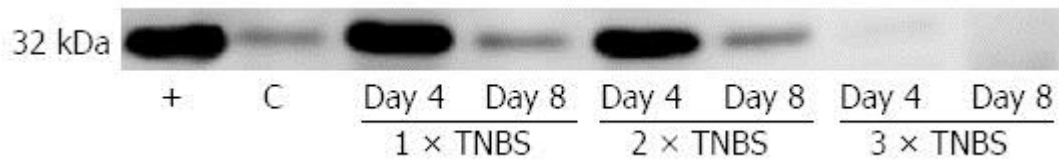
**Figure 2** Representative light micrographs of whole mount preparations of the rat descending colon after HuC/HuD immunohistochemistry. Arrowheads point to stained myenteric neurons. Density of neurons per field was determined with Plexus Pattern Analysis software, where the soma of the neurons was encircled (insert). Bars: 50  $\mu\text{m}$  and 25  $\mu\text{m}$  (insert).



**Figure 3** Density of HuC/HuD-immunoreactive myenteric neurons in the colonic segments of control (C) ( $n = 12$ ) and TNBS-treated rats four ( $n = 13$ ) and eight ( $n = 14$ ) days after colitis induction. In the 2, 4, 6-trinitrobenzenesulfonic acid -treated groups the inflamed segment (I) and the adjacent proximal (P) and distal (D) colonic segments were examined. Significant decrease in myenteric neuronal density was first detected on day four after each TNBS administration. When the rats were treated only once (1 x TNBS) the number of myenteric neurons decreased significantly in all three colonic segments, while after repeated treatments (2 x TNBS, 3 x TNBS), a significant decrease in neuronal number was demonstrated exclusively in the I segments. After the single dose of TNBS a further significant neuronal loss was detected until day eight post-induction, whereas after repeated treatments the number of myenteric neurons did not decrease further between days four and eight. Data are expressed as means  $\pm$  SE. <sup>b</sup> $P < 0.01$  vs age-matched control groups; <sup>d</sup> $P < 0.01$  vs once TNBS-treated group on day four. TNBS: 2,4,6-trinitrobenzenesulfonic acid.



**Figure 4** Relative mRNA expression of heme oxygenase-1 compared to the controls ( $n = 12$ ) (C) in the inflamed segment (I) and the adjacent proximal (P) and distal (D) colonic segments four ( $n = 13$ ) and eight ( $n = 14$ ) days after TNBS treatments. Irrespectively of the number of treatments (1 x TNBS, 2 x TNBS, 3 x TNBS), the HO-1 expression was always marked on day four in the I segments and a more limited expression was also detected in the P but not in the D colonic segments. After the first and second TNBS administrations the rate of HO-1 gene expression decreased, but never returned to baseline level up to day eight post-induction. Nevertheless, after the third TNBS treatment the high level of HO-1 expression was not merely sustained: a pronounced further increase was demonstrated until day eight post-induction. Data are expressed as means  $\pm$  SD. TNBS: 2, 4, 6-trinitrobenzenesulfonic acid; HO-1: Heme oxygenase-1.



**Figure 5 Protein expression of heme oxygenase-1 (32 kDa) in the inflamed segments of the colon four ( $n = 4$ ) and eight ( $n = 5$ ) days after 2, 4, 6-trinitrobenzenesulfonic acid treatments, compared to the controls ( $n = 4$ ) (C). Four days after the first and second treatment (1 × TNBS, 2 × TNBS) elevated HO-1 protein level was detected. Then the amounts of protein in both samples declined and eight days after the first treatment it reached already the baseline level. Following the third treatment (3 × TNBS) HO-1 protein expression could not be detected at all. TNBS: 2, 4, 6-trinitrobenzenesulfonic acid; HO-1: Heme oxygenase-1.**



**Table 1 Weight characteristics of the four experimental groups of rats before the 2, 4, 6-trinitrobenzenesulfonic acid treatments (initial) and eight days after the 2, 4, 6-trinitrobenzenesulfonic acid treatments (final)**

	Body weight (g) ± SE			
	Initial		Final	
Control	201.5 ± 1.6	<i>n</i> = 34	356 ± 20.9 <sup>b</sup>	<i>n</i> = 10
1 x TNBS	238.3 ± 3.6	<i>n</i> = 42	239 ± 10.9	<i>n</i> = 11
2 x TNBS	242.7 ± 3.3	<i>n</i> = 40	319.3 ± 8.2 <sup>b</sup>	<i>n</i> = 10
3 x TNBS	197.2 ± 3.5	<i>n</i> = 50	376.5 ± 11.3 <sup>b</sup>	<i>n</i> = 11

<sup>b</sup>*P* < 0.01, initial *vs* final. TNBS; 2, 4, 6-trinitrobenzenesulfonic acid.