Preventative Effects of Lactulose in the Trinitrobenzenesulphonic Acid Model of Rat Colitis

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Aims: Lactulose is a drug used as a laxative that has been shown to promote the growth of lactobacilli and bifidobacteria, acting as a prebiotic and with a potential beneficial effect in inflammatory bowel disease. The present study describes the preventive antiinflammatory activity of lactulose in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis.

Methods: Rats were rendered colitic by a colonic instillation of 10 mg of TNBS dissolved in 0.25 mL of 50% ethanol. One group of colitic rats received lactulose, which was incorporated in the drinking water (2.5% wt/vol) for 2 weeks before TNBS instillation, and colonic damage was evaluated 1 week after colitis induction. Different biochemical markers of colonic inflammation were assayed: myeloperoxidase activity, glutathione content, tumor necrosis factor α, leukotriene B4 levels, and colonic inducible nitric oxide synthase expression. In addition, bacterial counts (for lactobacilli and bifidobacteria) were performed in colonic contents from colitic rats.

Results: The results show that lactulose exerted a preventive antiinflammatory effect in this model of rat colitis, as evidenced by a significant reduction of myeloperoxidase activity and by a decrease of both colonic tumor necrosis factor α and leukotriene B4 production. This effect was also characterized by an inhibition of colonic inducible nitric oxide synthase expression, which is unregulated as a consequence of the inflammatory status. This beneficial effect was associated with increased levels of lactobacilli and bifidobacteria species in colonic contents in comparison with untreated colitic rats.

Conclusion: In conclusion, the intestinal antiinflammatory effect of lactulose could be related to its prebiotic properties, supporting its potential use in human inflammatory bowel disease.

Key Words: bifidobacteria, lactobacilli, lactulose, nitric oxide synthase, rat colitis, tumor necrosis factor

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Inflammatory bowel disease (IBD) is a chronic disease of the digestive tract, and the name usually refers to 2 related conditions, namely, ulcerative colitis and Crohn’s disease, which are characterized by chronic and spontaneously relapsing inflammation. Although the etiology of IBD remains unknown, there is increasing experimental evidence to support a role for luminal bacteria in the initiation and progression of these intestinal conditions; this is probably related to an imbalance in the intestinal microflora, the relative predominance of aggressive bacteria, and an insufficient amount of protective species.1,2 This could justify the remission achieved in intestinal inflammation after treatment with antibiotics such as metronidazole or ciprofloxacin,3 or the fact that germfree animals may fail to develop experimental intestinal inflammation.4 In consequence, a possible approach to IBD therapy is to modify the intestinal microflora in these patients by the administration of probiotics. In fact, it has been reported that the administration of a mixture of Bifidobacterium and Lactobacillus species or of nonpathogenic, viable Escherichia coli,6 which have been proposed to act by preventing the colonization of the intestine by microbial pathogens, prolongs remission in ulcerative colitis.7–9 Another possible way to modify the intestinal microflora in these intestinal conditions may be through the administration of prebiotics, defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of limited bacteria in the colon, especially bifidobacteria.10 In fact, different prebiotics, including dietary fiber, germinated barley foodstuff, and inulin, have been reported to exert

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Copyright © 2005 by Lippincott Williams & Wilkins
beneficial effects in both human and experimental colitis.\textsuperscript{11–14} Lactulose is a drug that is mainly used as a laxative and for the treatment of portosystemic encephalopathy. Following oral administration, intact lactulose reaches the colon, where it is split by bacteria, leading to a reduction in fecal pH and promoting the growth of lactic acid-producing bacteria (lactobacilli) that may have a protective role against potential pathogens as a prebiotic and thus resulting in a beneficial effect on IBD. In fact, previous studies have shown the ability of lactulose to prevent enterocolitis in interleukin (IL)-10 gene-deficient mice\textsuperscript{15} and to abolish systemic endotoxemia in a hapten-induced rat model of colitis.\textsuperscript{16}

The aim of the present study was to test the preventative effects of lactulose in the trinitrobenzenesulphonic acid (TNBS) model of rat colitis, a well-established model of intestinal inflammation with some resemblance to human IBD.\textsuperscript{17} Special attention was paid to its effects on the production of some of the mediators involved in the inflammatory response, such as tumor necrosis factor (TNF) \( \alpha \), leukotriene \( \text{LTB}_4 \), and nitric oxide (NO). In addition, the correlation among the intestinal anti-inflammatory effect of lactulose and the modifications on colonic flora induced by this prebiotic was also studied.

**MATERIALS AND METHODS**

This study was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the National Institutes of Health.

**Reagents**

All chemicals were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. Glutathione reductase was provided by Boehringer Mannheim (Barcelona, Spain). Lactulose was provided by Solvay Pharma, S.A. (Parets del Valles, Barcelona, Spain).

**Experimental Design**

Female Wistar rats (180 to 200 g) obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) were housed individually in makrolon cages (Panlab, Barcelona, Spain) and maintained in an air-conditioned atmosphere with a 12-hour light-dark cycle, and they were provided with free access to tap water and food. The rats were randomly assigned to 3 groups (\( n = 10 \)): 2 groups (noncolitic and control groups) received tap water and the other group (treated group) received lactulose at 2.5\% (wt/vol) in their drinking water for 3 weeks. Two weeks after starting the experiment, the rats were fasted overnight, and those from the control and treated groups were rendered colitic by the method originally described by Morris et al.\textsuperscript{19} Briefly, they were anesthetized with halothane and given 10 mg of TNBS dissolved in 0.25 mL of 50\% ethanol (vol/vol) by means of a Teflon (Dupont, Wilmington, Del) cannula inserted 8 cm into the anus. During and after TNBS administration, the rats were kept in a head-down position until they recovered from the anesthetic and were then returned to their cages. Rats from the noncolitic group were administered 0.25 mL of phosphate-buffered saline solution intracolonically instead of TNBS. All rats were killed with an overdose of halothane 1 week after the induction of colitis.

**Assessment of Colonic Damage**

The body weight and water and food intake were recorded daily throughout the experiment. Once the rats were killed, the colon was removed aseptically, placed on an ice-cold plate, longitudinally opened, and the luminal contents were collected for the microbiological studies (see below). Afterward, the colonic segment was cleaned of fat and mesentry and blotted on filter paper; each specimen was weighed, and its length was measured under a constant load (2 g). The colon was scored for macroscopically visible damage on a scale of 0 to 10 by 2 observers who were unaware of the treatment, according to the criteria described by Bell et al\textsuperscript{19} (Table 1), which take into account the extent and the severity of colonic damage. Representative whole gut specimens were taken from a region of the inflamed colon corresponding to the segment adjacent to the gross macroscopic damage and were fixed in 4\% buffered formaldehyde. Cross sections were selected and embedded in paraffin. Equivalent colonic segments were also obtained from the noncolitic group. Full-thickness sections of 5 \( \mu \)m were obtained at different levels and were stained with hematoxylin and eosin. The histologic damage was evaluated by 2 pathologist observers (AN and AC), who were blinded to the experimental groups, according to the criteria described previously by Stucchi et al\textsuperscript{20} (Table 2). The colon was subsequently divided into 4 segments for biochemical determinations. Two fragments were frozen at \(-80^\circ\text{C}\) for myeloperoxidase (MPO) activity and NO synthase (NOS) expression, and another sample was weighed and

<table>
<thead>
<tr>
<th>TABLE 1. Criteria for Assessment of Macroscopic Colonic Damage</th>
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<tbody>
<tr>
<td><strong>Score</strong></td>
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<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>6–10</td>
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TABLE 2. Criteria for Assessment of Microscopic Colonic Damage

<table>
<thead>
<tr>
<th>Location</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal epithelium</td>
<td>Ulceration: none (0); mild surface (1); moderate (2); extensive-full thickness (3)</td>
</tr>
<tr>
<td>Crypts</td>
<td>Mitotic activity: lower third (0); mild mid third (1); moderate mid third (2); upper third (3)</td>
</tr>
<tr>
<td></td>
<td>Mucus depletion: none (0); mild (1); moderate (2); severe (3)</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>Mononuclear infiltrate: none (0); mild (1); moderate (2); severe (3)</td>
</tr>
<tr>
<td></td>
<td>Granulocyte infiltrate: none (0); mild (1); moderate (2); severe (3)</td>
</tr>
<tr>
<td></td>
<td>Vascularity: none (0); mild (1); moderate (2); severe (3)</td>
</tr>
<tr>
<td>Submucosal</td>
<td>Mononuclear infiltrate: none (0); mild (1); moderate (2); severe (3)</td>
</tr>
<tr>
<td></td>
<td>Granulocyte infiltrate: none (0); mild (1); moderate (2); severe (3)</td>
</tr>
<tr>
<td></td>
<td>Edema: none (0); mild (1); moderate (2); severe (3)</td>
</tr>
</tbody>
</table>

Maximum score: 27. Modified from Stucchi et al.20

frozen in 1 mL of 50 g/L trichloroacetic acid for determination of the total glutathione content. The remaining sample was immediately processed for the measurement of TNFα and LTB4 levels. All biochemical measurements were completed within 1 week from the time of sample collection and were performed in duplicate.

MPO activity was measured according to the technique described by Krawisz et al,21 and the results were expressed as MPO units per gram of wet tissue (1 unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide per minute at 25°C). Total glutathione content was quantified with the recycling assay described by Anderson,22 and the results were expressed as nanomoles per gram of wet tissue. Colonic samples for TNFα and LTB4 determinations were immediately weighed, minced on an ice-cold plate, and suspended in a tube with a 10 mM sodium phosphate buffer (pH 7.4; 1:5 wt/vol).23 The tubes were placed in a shaking water bath (37°C) for 20 minutes and centrifuged at 9000 g for 30 seconds at 4°C; the supernatants were frozen at −80°C until assayed. TNFα was quantified by enzyme-linked immunosorbent assay (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the results were expressed as picograms per gram of wet tissue. LTB4 was determined by enzyme immunoassay (Amersham Pharmacia Biotech), and the results expressed as nanograms per gram of wet tissue.

To study inducible NOS (iNOS) expression, the colonic samples obtained from rats were homogenized (1/5 wt/vol) in phosphate-buffered saline solution supplemented with 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate, 1% Triton X-100, and protease and phosphatase inhibitors (i.e., aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). A total of 100 μg of proteins were boiled at 95°C in Laemmli SDS loading buffer and were separated on 7.5% SDS-polyacrylamide gel electrophoresis. The proteins were then electrotransferred to nitrocellulose membranes (PROTAN; Schleicher & Schuell, Dassel, Germany). The membranes were blocked for at least 1 hour at room temperature in Tris buffered saline-0.1% Tween-20 (TBS-T) 5% nonfat dry milk and then were incubated with TBS-T containing 5% bovine serum albumin and a dilution (1:2000) of iNOS antibody (Transduction Laboratories, Becton Dickinson Biosciences, Madrid, Spain). After 3 washes of 5 minutes each with TBS-T, the membranes were incubated with 5% nonfat dry milk and peroxidase-conjugated antirabbit IgG antibody for 1 hour (1:5000). After 3 washes of 5 minutes with TBS-T, enhanced chemiluminescence detection was performed (NEN Life Science Products, Zaventem, Belgium). The control of protein loading and transfer was conducted by the detection of the β-actin levels.

Microbiological Studies

Luminal content samples were weighed, homogenized, and serially diluted in sterile peptone water. Serial 10-fold dilutions of homogenates were plated on specific media for Lactobacillus or Bifidobacterium and were incubated under anaerobic conditions in an anaerobic chamber for 24 to 48 hours at 37°C. After incubation, the final count of colonies was reported as log10 colony-forming units per gram of material.

Statistics

All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance and post hoc least significance tests. Differences between proportions were analyzed with the χ2 test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Rockville, Md), with statistical significance set at P < 0.05.

RESULTS

Lactulose administration for 2 weeks did not induce any symptoms of diarrhea or any effects in the weight evolution. However, once the colitis was induced, the lactulose-treated rats showed an overall lower impact of TNBS-induced colonic damage compared with the TNBS control group. The anti-inflammatory effect was evidenced macroscopically by a significantly lower colonic damage score than that of the control rats (P < 0.01), with a significant reduction in the extent of colonic necrosis and/or inflammation induced by the administration of TNBS/ethanol (Table 3). However, no modifications were observed in the colonic weight/length ratio between the colitic groups, which increased significantly as a consequence of the inflammatory process (Table 3). The
histologic studies confirmed the intestinal antiinflammatory effect exerted by lactulose (Fig. 1). Histologic assessment of colonic samples from the TNBS control group revealed severe transmural disruption of the normal architecture of the colon, extensive ulceration and inflammation involving all the intestinal layers of the colon, giving a mean score value of 19.0 ± 1.3 (mean ± SEM). Colonic samples were characterized by severe edema, interstitial microhemorrhages, and diffuse leukocyte infiltration, mainly composed of neutrophils in the mucosal layer and, to a lesser extent, lymphocytes and histocytes in the submucosa. Most of the rats showed epithelial ulceration of the mucosa affecting >50% of the surface. The inflammatory process was associated with crypt hyperplasia and dilation and with moderate goblet cell depletion. However, histologic analysis of the colonic specimens from rats treated with lactulose revealed a more pronounced recovery in the intestinal architecture than that in controls, with a mean score of 9.6 ± 0.3 (mean ± SEM). *P < 0.05.

FIGURE 1. Histologic sections of colonic mucosa from colitic rats 1 week after TNBS instillation stained with hematoxylin and eosin. A, noncolitic group showing the normal histology of the rat colon (original magnification, 20×). B, TNBS control group showing complete destruction of the mucosa, which has been substituted by inflammatory granulation tissue. There is evident edema and intense diffuse transmural inflammatory infiltrate (original magnification, 100×). C, lactulose-treated group showing amelioration in the inflammatory process and “restoration” of the mucosal tissue with the presence of mucin replenished goblet cells (original magnification, 100×).

<table>
<thead>
<tr>
<th>Group</th>
<th>Damage Score</th>
<th>Extent of Damage (cm)</th>
<th>Colon Weight (mg/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncolitic</td>
<td>0</td>
<td>0</td>
<td>54.2 ± 2.3</td>
</tr>
<tr>
<td>TNBS control</td>
<td>7.3 ± 0.4 (6–9)</td>
<td>4.40 ± 0.37</td>
<td>169.1 ± 20.4</td>
</tr>
<tr>
<td>TNBS lactulose</td>
<td>5.9 ± 0.3 (4–7)*</td>
<td>2.98 ± 0.33†</td>
<td>143.2 ± 8.7</td>
</tr>
</tbody>
</table>

Damage score for each rat was assigned according to the criteria described by Bell et al19 (see Table 1), and data are expressed as mean ± SEM (range). Extent of damage and colon weight data are expressed as mean ± SEM. *P < 0.05. †P < 0.01 versus TNBS control. All colitic groups differ significantly from noncolitic group (P < 0.01, not shown).
the colonic oxidative stress induced by the inflammatory process, as previously reported in this model of experimental colitis\textsuperscript{23} (Table 4). Finally, the colonic inflammation induced by TNBS was characterized by increased levels of colonic TNF\textalpha and LTB\textsubscript{4} (Table 4) and by a greater colonic iNOS expression (Fig. 2) in comparison with noncolitic animals. The treatment of colitic rats with lactulose resulted in a significant reduction of colonic TNF\textalpha and LTB\textsubscript{4} levels (Table 4), which did not show any statistical differences with normal rats. In addition, a lower colonic iNOS expression was observed in lactulose-treated colitic animals when compared with TNBS control animals (Fig. 2).

### Effects of Lactulose Administration on Colonic Bacterial Profile

No statistical difference in total fecal bacteria counts was found between normal rats and rats with TNBS colitis. However, lactulose-treated colitic rats showed significantly higher counts of \textit{Lactobacillus} and \textit{Bifidobacterium} species in colonic contents than those in control colitic rats ($P < 0.01$; Fig. 3). No statistical differences were observed in both \textit{Bifidobacterium} and \textit{Lactobacillus} counts between lactulose-treated colitic rats and normal rats ($P > 0.05$; Fig. 3).

### DISCUSSION

The results obtained in the present study reveal the efficacy of lactulose therapy in intestinal inflammation, confirming the results of previous studies that demonstrated the ability of this compound to attenuate the development of colonic injury in IL-10 gene-deficient mice.\textsuperscript{15} Thus, lactulose incorporation in drinking water (2.5% wt/vol) facilitated recovery from TNBS-induced colonic damage, as it was evidenced histologically, with a significant reduction in the extent and severity of inflamed tissue. This beneficial effect was also determined biochemically by a decrease in colonic MPO activity, a marker of neutrophil infiltration that has been previously described to be up-regulated in experimental colitis\textsuperscript{24} and is widely used to detect and follow intestinal inflammatory processes. In consequence, a reduction in the activity of this enzyme can be interpreted as a manifestation of the antiinflammatory activity of a given compound.\textsuperscript{25} The ability of lactulose to reduce granulocyte infiltration, demonstrated by MPO activity inhibition, was confirmed histologically because the level of leukocyte infiltrate in the colonic mucosa was lower in treated colitic animals than in the corresponding TNBS control groups. The inhibitory effect on the infiltration of inflammatory cells into the colonic mucosa might account for the beneficial effect of this prebiotic against tissue injury, because the margination and extravasation of circulating granulocytes contribute markedly to colonic injury in this model of IBD.\textsuperscript{26} These results confirm those of a previous study\textsuperscript{27} that described the attenuation exerted by lactulose in leukocyte-endothelial cell adhesion in an experimental model of rat ileitis induced by indomethacin.

### TABLE 4. MPO Activity, Total Glutathione Content, and TNF\textalpha and LTB\textsubscript{4} Levels in Colon Specimens From Noncolitic Rats, TNBS Control Colitic Rats, and TNBS Lactulose-treated Colitic Rats

<table>
<thead>
<tr>
<th>Group (n = 10)</th>
<th>MPO activity (units MPO/g)</th>
<th>GSH (nmol/g)</th>
<th>LTB\textsubscript{4} (ng/g)</th>
<th>TNF\textalpha (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-colitic</td>
<td>6.9 ± 1.2</td>
<td>2296 ± 41</td>
<td>2.50 ± 0.18</td>
<td>238.4 ± 16.6</td>
</tr>
<tr>
<td>TNBS control</td>
<td>195.5 ± 14.5*</td>
<td>1620 ± 111*</td>
<td>5.09 ± 0.60*</td>
<td>389.4 ± 36.4*</td>
</tr>
<tr>
<td>TNBS lactulose</td>
<td>135.7 ± 16.1*†</td>
<td>1688 ± 82*</td>
<td>3.12 ± 0.51‡</td>
<td>256.6 ± 48.5‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. GSH, glutathione.

* $P < 0.01$ versus noncolitic group.

† $P < 0.01$ versus TNBS control group.

‡ $P < 0.05$ versus TNBS control group.

**FIGURE 2.** Effects of lactulose treatment (2.5% in drinking water) on colonic NOS expression in TNBS experimental colitis in rats.

**FIGURE 3.** Effects of lactulose treatment on bacteria levels (\textit{Lactobacillus} and \textit{Bifidobacterium}) in TNBS experimental colitis in rats. * $P < 0.01$ versus the TNBS control group.
This effect can be the consequence of the inhibition of the synthesis and/or release of different mediators that participate in the recruitment and activation of these cells, such as LTB₄,²⁸ because lactulose treatment of colitic rats was associated with a decrease in colonic LTB₄ levels.

The intestinal antiinflammatory activity exerted by lactulose was also characterized by the down-regulation of the colonic TNFα, an important proinflammatory mediator that has been proposed to play a key role in colonic inflammation²⁹; in fact, different drugs that are capable of interfering with the activity of this mediator have been successfully developed for IBD therapy.³⁰,³¹

During the last decade, it has become increasingly clear that chronic colonic inflammation is associated with enhanced NO production, mainly by means of iNOS activity, in both humans and experimental rats.³²–³⁴ NO overproduction by iNOS has been suggested to be deleterious to intestinal function,³²,³³ thus contributing significantly to gastrointestinal immunopathology during the chronic inflammatory events that take place in IBD. The important role attributed to NO in these intestinal conditions prompted us to study whether the beneficial effects of lactulose on TNBS colitis could be related to an effect on colonic NO production. The results obtained in the present study reveal that colonic inflammation is associated with a higher iNOS expression in comparison with noncolitic rats. This agrees with previous observations reported for the same experimental model of rat colitis³³ and for human IBD,³⁴ which have described the enhanced NO production in the inflamed mucosa by colonic iNOS. The intestinal antiinflammatory effect exerted by lactulose was associated with a significant inhibition of colonic iNOS expression. As a consequence, an inhibition in NO production may also contribute to the beneficial effect, preventing, at least partially, the deleterious activity ascribed to NO when it is produced in high amounts by iNOS. Similar beneficial effects have been reported after NOS inhibition in different experimental models of intestinal inflammation.³²,³³,³⁵

Lactulose (1,4β-galactosido-fructose) is a compound that is neither absorbed nor metabolized during the transit through the upper intestine and that promotes favorable conditions for the growth of Lactobacillus species.³⁶ Recently, it has been reported that lactulose ingestion by healthy humans resulted in increased fecal bifidobacterial counts.³⁷ This prebiotic effect also has been noted in the present study, because lactulose-treated colitic rats showed a significant increase in lactobacilli and bifidobacteria counts in comparison with nontreated colitic rats, which could contribute to the beneficial effect exerted by lactulose in this model of experimental colitis. In fact, it has been previously described that the increase in the levels of Lactobacillus species reduces the concentration of adherent and translocated bacteria and attenuates the colitis in IL-10 gene-deficient mice,¹⁵ thus preventing the pathogenic effect of other species that may play an important role in the generation of the exacerbated immune response in intestinal inflammation, as proposed both in experimental models³⁸ and in humans.³⁹

In conclusion, lactulose supplementation facilitates the recovery of the inflamed tissue in the TNBS model of rat colitis, an effect that is associated with amelioration in the production of some of the mediators involved in the inflammatory response of the intestine, such as cytokines, including TNFα, and NO. This beneficial effect could be ascribed to its prebiotic effect, which would attenuate the exacerbated immune response evoked by the colonic instillation of the hapten TNBS in rats.

ACKNOWLEDGMENTS

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REFERENCES


