

## Antioxidative and immunomodulatory effects of tributyrin supplementation on experimental colitis

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### Abstract

Tributyryn (TBT) is a TAG composed of three butyric acids that has beneficial effects on ulcerative colitis due to its trophic, anti-inflammatory, pro-apoptotic and anti-carcinogenic properties. The goal of the present study was to evaluate the efficacy and mechanisms of action of TBT supplementation in the prevention of mucosal damage in experimental colitis. Mice received either a control diet or a TBT-supplemented diet for 15 d. Colitis was induced by dextran sodium sulphate administration during the last 7 d. Mucosal damage and the activation of immune cells and cytokines were determined by histological score, flow cytometry and ELISA. Leucocyte rolling and adhesion were assessed by intravital microscopy. Oxidative stress was determined by monitoring hydroperoxide concentration and evaluating superoxide dismutase (SOD) and catalase activities. Intestinal permeability was analysed using diethylenetriaminepentaacetate acid (<sup>99m</sup>Tc-DTPA). Compared with the colitis group, the animals in the colitis + TBT group had reduced mucosal damage and neutrophil and eosinophil mucosal infiltration, which were associated with a higher percentage of regulatory T cells (Treg) and higher levels of transforming growth factor  $\beta$  and IL-10 in the lamina propria. The level of *in vivo* leucocyte adhesion in the colon microvasculature was reduced after TBT supplementation. A lower level of hydroperoxide and higher levels of SOD and catalase activities were associated with TBT supplementation. TBT-supplemented mice showed reduced intestinal permeability to the levels intermediate between the control and colitis groups. In conclusion, the present results show that TBT has positive effects on colonic restructuring in experimental colitis. Additionally, TBT supplementation changes the immune response by controlling inflammation and regulating the expression of anti-inflammatory cytokines and Treg.

**Key words:** Tributyrin: Butyrate: Ulcerative colitis: Inflammation: Oxidative stress

Tributyryn (TBT) is a TAG present in butter. It is composed of three molecules of butyric acid which is a four-carbon SCFA produced from the fermentation of dietary fibres and undigested carbohydrates by intestinal microbiota. Butyrate is the preferential fuel of colonocytes and functions as a trophic, anti-inflammatory, pro-apoptotic and anti-carcinogenic agent<sup>(1–7)</sup>. All these properties make butyrate a potential adjuvant in the treatment of inflammatory bowel diseases, particularly ulcerative colitis (UC).

UC begins in the rectum and extends along the colon in the retrograde direction<sup>(3,8)</sup>. The active phase of UC is characterised by crypt abscesses and ulcerations that affect the

mucosa and submucosa. The ulcerations cause intense inflammatory infiltrate and oxidative stress in the colon. Although the aetiology of the disease is not fully understood, a combination of genetic, immune and environmental factors is involved in the disease onset<sup>(2,9–15)</sup>.

Butyrate enemas have been used in the treatment of UC with positive clinical results<sup>(16–18)</sup>. Oral butyrate supplementation effectively reduces inflammation in experimental studies of dextran sodium sulphate (DSS)-induced colitis<sup>(4)</sup>. However, oral butyrate use in clinical trials is limited due to its disagreeable odour and its unwanted side effects, which include nausea, headache and anorexia<sup>(2,3)</sup>. Despite these limitations,

**Abbreviations:** DSS, dextran sodium sulphate; DTPA, diethylenetriaminepentaacetate acid; TBT, tributyrin; TGF- $\beta$ , transforming growth factor  $\beta$ ; Treg, regulatory T cells; UC, ulcerative colitis.

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oral butyrate reduces inflammatory infiltrate and enhances mucosal regeneration<sup>(2,3)</sup>. TBT is not volatile and is odourless. Moreover, similar to other medium-chain TAG, TBT is hydrolysed more rapidly by lipases than long-chain fatty acids. TBT does not require bile salts for absorption because it is water-soluble, and can be absorbed as an intact TAG. Once inside the intestinal epithelial cell, it is rapidly hydrolysed into three molecules of butyrate by specific cellular lipases<sup>(2,19)</sup>. Thus, oral supplementation of TBT could potentially replace butyrate enemas in UC treatment. However, the faster absorption rate of butyrate along the proximal areas of the gastrointestinal tract could reduce the amount of butyrate released in the distal colon, where most of the UC lesions are located.

In the present study, we evaluated the efficacy of oral TBT supplementation in a model of DSS-induced UC. We investigated TBT action on mucosal trophism, the intestinal barrier, oxidative stress and the profiles of immune cells and cytokines.

## Methods

### Experimental protocol

The present study followed the institutional and national guidelines for the care and use of animals. All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the Federal University of Minas Gerais (#223/2008). Mice (C57BL/6, 8 weeks old) were divided into three groups. The control animals received water and the standard rodent diet AIN-93M<sup>(20)</sup>. The colitis animals received a DSS solution and the standard diet. The colitis + TBT animals received a DSS solution and a TBT-supplemented standard diet (5 g/kg diet). The animals were kept in collective cages with 12 h light–dark cycles, controlled temperature (22 ± 2°C) and free access to food and water. The animals' weight and their food and water intakes were evaluated weekly.

In the main experiment, the diet was offered for 2 weeks and DSS (molecular weight 36 000–50 000 Da; MP Biomedicals) was added to the drinking water (2.0%) on the 8th experimental day. On the 10th or 15th experimental day (3 or 7 d after the introduction of DSS, respectively), all animals were euthanised under anaesthesia for removal of blood and organs. The results were obtained after 7 d of DSS administration, unless otherwise stated.

The colon was removed and fixed in 4% formaldehyde for 4 h. The tissue was then embedded in paraplast and sectioned (10 µm thick) for haematoxylin and eosin staining. After image acquisition using Image Pro Plus software (Media Cybernetics), the images were analysed using a semi-quantitative score as described previously<sup>(21)</sup>.

The intestinal permeability experiments were performed on the day of killing (15th experimental day). The animals were given 100 µl diethylenetriaminepentaacetate acid (DTPA) labelled with 3.7 MBq of <sup>99m</sup>technetium by oral administration 4 h before killing. The determination of the level of radiation in the blood of animals was performed using an automatic scintillator (Mor; Abbott). The results are presented as the

percentage of the radiation dose, which was calculated using the following formula:

$$\% \text{ Dose} = (\text{CPM in blood} \times 100) / \text{CPM of the standard dose},$$

where CPM is counts per min.

The enzyme activities of myeloperoxidase, *N*-acetylglucosaminidase and eosinophil peroxidase were evaluated in the colon, as described previously<sup>(22)</sup>. Briefly, tissue samples were homogenised and centrifuged. The pellets were dissolved in 0.5% hexadecyl trimethyl ammonium bromide (HETAB) in phosphate buffer and, after homogenisation and freezing/thawing sessions, the suspensions were centrifuged and the supernatants were used for the quantification of the enzymes. For eosinophil peroxidase quantification, 75 µl of the supernatant were added to 75 µl *O*-phenylenediamine (Sigma-Aldrich), diluted in Tris–HCl and H<sub>2</sub>O<sub>2</sub>, and incubated at 37°C in the dark for 30 min. The reaction was stopped by adding 50 µl H<sub>2</sub>SO<sub>4</sub>, and then fluorescence was read at 492 nm. For myeloperoxidase quantification, 25 µl of the supernatant were added to 25 µl 3,3',5,5'-tetramethylbenzidine hylbenzidine (Sigma-Aldrich) in dimethyl sulphoxide (Sigma-Aldrich) and the mixture was incubated at 37°C for 5 min. Then, 100 µl of H<sub>2</sub>O<sub>2</sub> were added to the supernatant. The reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub>, and then fluorescence was read at 450 nm. For *N*-acetylglucosaminidase quantification, the precipitates were dissolved in Triton X-100 (0.1%; Sigma-Aldrich) and then centrifuged. Then, 100 µl of the supernatant were added to 100 µl *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine (Sigma-Aldrich) in citrate/phosphate buffer. After incubation, 100 µl of glycine buffer were used to stop the reaction, and fluorescence was read at 400 nm.

To isolate leucocytes for flow cytometry, the caecal lymph node and spleen were macerated in complete Roswell Park Memorial Institute medium and processed as described previously<sup>(23)</sup>. The leucocytes in the lamina propria were separated as described previously<sup>(24)</sup>. The cell suspensions were incubated with specific antibodies and then fixed (1% paraformaldehyde) and analysed by flow cytometry (FACScan; Becton Dickinson). Data acquisition was performed using CELLQuest™ and analysed using FlowJo software 7.6 (TreeStar). The analyses were performed in the specific gates for lymphocytes or monocytes/dendritic cell populations. We used the following antibody combinations (BD Pharmingen) to detect specific cell types: regulatory T lymphocytes (CD4<sup>+</sup>CD25<sup>+</sup>LAP<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells); T helper lymphocyte memory cells (CD4<sup>+</sup>CD44<sup>+</sup>); activated T helper lymphocytes (CD4<sup>+</sup>CD69<sup>+</sup>); B1 lymphocytes (CD5<sup>+</sup>CD19<sup>+</sup> cells); activated B lymphocyte (CD19<sup>+</sup>CD21<sup>+</sup> cells); monocyte and macrophage (MOMA<sup>+</sup> cells); activated macrophage (MOMA<sup>+</sup>CD80<sup>+</sup> cells); dendritic cells (CD11c<sup>+</sup> cells). For cytokine analyses (TNF-α, interferon γ, IL-4, IL-1β, IL-17, IL-10 and transforming growth factor β (TGF-β)), colons were homogenised in the extraction solution and centrifuged (10 000 rpm, 10 min, 4°C). The supernatant collected was used to detect cytokines by ELISA using commercial kits (R&D Systems) according to the manufacturer's instructions.

The total blood leucocyte counts were analysed in a Neubauer chamber after Turk staining (1:10 dilution).

The differential count was performed using the Quick Panoptic kit (LaborClin).

Intravital microscopy was performed in the colon of mice after overnight fasting. The procedure was performed while the animals were under anaesthesia. After intravenous rhodamine 6G (Sigma Chemical) injection (0.15 mg/kg), the colonic microcirculation was visualised by fluorescence microscopy (Nikon Eclipse). Rolling cells were defined as the number of leucocytes passing by a certain point during 1 min in a specific colonic venule. Adherent cells were defined as cells remaining in a same region for at least 30 s. The results were expressed as the number of adherent cells in a 100 µm region of a specific venule.

Oxidative stress in the colon was evaluated by analysing several factors including thiobarbituric acid-reactive species<sup>(25)</sup>, hydroperoxide concentration<sup>(26)</sup>, superoxide dismutase activity<sup>(27)</sup> and catalase activity<sup>(28)</sup>. All results were normalised to total protein concentration in the colon. Protein concentration was analysed using the Lowry method<sup>(29)</sup>.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad). Results were evaluated by the Kolmogorov–Smirnov test for normality, and Grubbs’ tests and box plot for outliers. Data with a normal distribution were tested using one-way ANOVA and Newman–Keuls *post hoc* test analysis, while non-parametric data were tested by Kruskal–Wallis ANOVA and Dunn’s tests. Results are expressed as means with their standard errors with a significance level of 5% ( $P < 0.05$ ).

**Results**

Weight gain and food and water intakes were similar among all groups before DSS administration. During the 7 d of DSS administration, as expected, there was weight loss in both

groups receiving DSS, regardless of whether they received TBT (Table 1).

Colitis was histologically confirmed in the colitis group by the presence of areas with intense ulceration, the depletion of goblet cells, the presence of abscesses in the crypts, the thickening of the muscle layers and an intense level of inflammatory infiltrate. The animals supplemented with TBT, compared with non-supplemented mice, presented an improved mucosal architecture (with more preserved areas), a reduced extension and intensity of the inflammatory infiltrate, and an absence of mucosal ulcerations (Fig. 1). In the colitis group, both the apical and basal regions of the mucosa were compromised. However, in the colitis + TBT group, the apical part of the mucosa had some architectural damage, whereas the basal mucosal cells were undamaged (Fig. 1(C)). The total histopathological score and the individual score items were improved with TBT supplementation (Fig. 1(D)–(F)).

Because the level of inflammatory cell infiltration was reduced in the colitis + TBT group, we analysed the intensity of macrophage, neutrophil and eosinophil infiltration. The results showed increased infiltration of both neutrophils and eosinophils in the colitis group. The level of neutrophil and eosinophil infiltration was significantly reduced after TBT supplementation (Fig. 2(A) and (B)). There were no differences observed in macrophage infiltration (Fig. 2(C)).

We also evaluated the distribution of lymphocyte subtypes, dendritic cells and activated macrophage in the lamina propria, caecal lymph node, spleen and blood. Compared with the colitis group, the percentages of these cell types were not significantly different in the colitis + TBT group. However, only the colitis + TBT group presented higher percentages of LAP + regulatory T cells (Treg) and activated macrophages in the lamina propria compared with the control group (Table 2). The changes in immune cells in the lymph nodes are similar to those seen in the lamina propria. The immune cell profile of the spleen and blood was not significantly affected by colitis

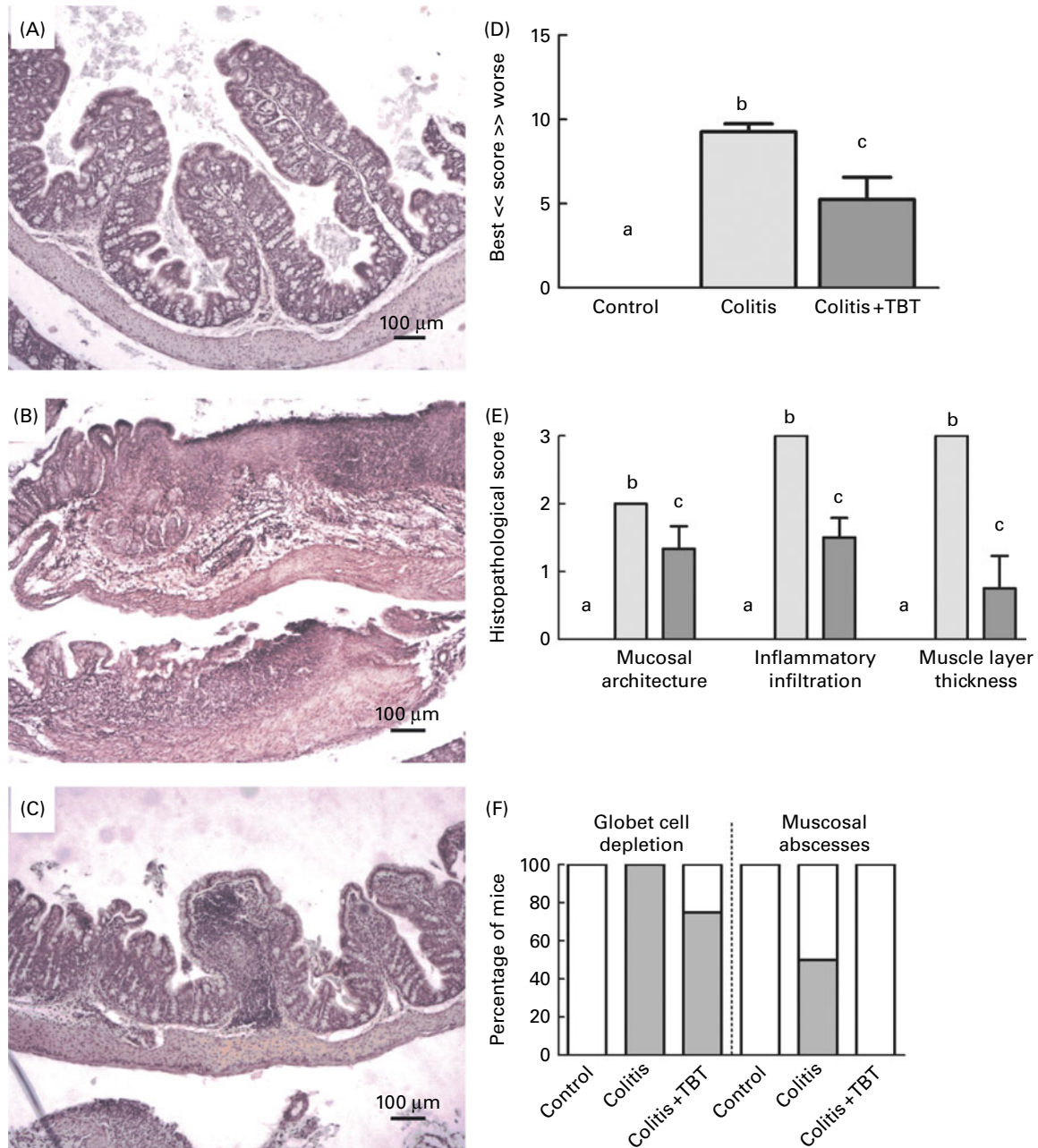
**Table 1.** Weight variation, food intake and water intake\* (Mean values with their standard errors)

|                                 | Before DSS administration |     |                  |     |                  |     | After DSS administration |     |                  |     |                   |     |
|---------------------------------|---------------------------|-----|------------------|-----|------------------|-----|--------------------------|-----|------------------|-----|-------------------|-----|
|                                 | Control                   |     | Colitis          |     | Colitis + TBT    |     | Control                  |     | Colitis          |     | Colitis + TBT     |     |
|                                 | Mean                      | SE  | Mean             | SE  | Mean             | SE  | Mean                     | SE  | Mean             | SE  | Mean              | SE  |
| <b>DSS administration (7 d)</b> |                           |     |                  |     |                  |     |                          |     |                  |     |                   |     |
| Weight variation (g)            | 1.4 <sup>a</sup>          | 0.1 | 1.6 <sup>a</sup> | 0.1 | 1.5 <sup>a</sup> | 0.1 | 1.1 <sup>a</sup>         | 0.1 | 0.1 <sup>b</sup> | 0.1 | -0.2 <sup>b</sup> | 0.1 |
| Food intake (g/d)               | 3.3                       | 0.2 | 3.5              | 0.1 | 3.6              | 0.1 | 3.9                      | 0.2 | 3.5              | 0.2 | 3.6               | 0.2 |
| Water intake (ml/d)             | 3.4 <sup>a</sup>          | 0.2 | 3.1 <sup>a</sup> | 0.2 | 3.1 <sup>a</sup> | 0.2 | 4.3 <sup>b</sup>         | 0.2 | 3.0 <sup>a</sup> | 0.1 | 3.0 <sup>a</sup>  | 0.1 |
| <b>DSS administration (3 d)</b> |                           |     |                  |     |                  |     |                          |     |                  |     |                   |     |
| Weight variation (g)            | 1.2                       | 0.4 | 1.1              | 0.4 | 1.0              | 0.4 | 0.5                      | 0.2 | 0.5              | 0.1 | 0.5               | 0.1 |
| Food intake (g/d)               | 3.3                       | 0.3 | 3.2              | 0.3 | 3.3              | 0.1 | 3.6                      | 0.4 | 3.4              | 0.6 | 3.2               | 0.3 |
| Water intake (ml/d)             | 3.2                       | 0.1 | 3.0              | 0.1 | 2.8              | 0.2 | 3.2                      | 0.3 | 3.1              | 0.2 | 2.7               | 0.3 |

Control, group receiving the standard diet; colitis, group receiving the standard diet and dextran sodium sulphate (DSS); colitis + TBT, group receiving the tributyrin-rich diet and DSS.

<sup>a,b</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ; one-way ANOVA and Newman–Keuls multiple comparison test).

\*  $n$  11 per group for food and water intake with 7 d of DSS administration;  $n$  3 per group for food and water intake with 3 d of DSS administration;  $n$  12/14 per group for weight variation with 7 and 3 d of DSS administration.



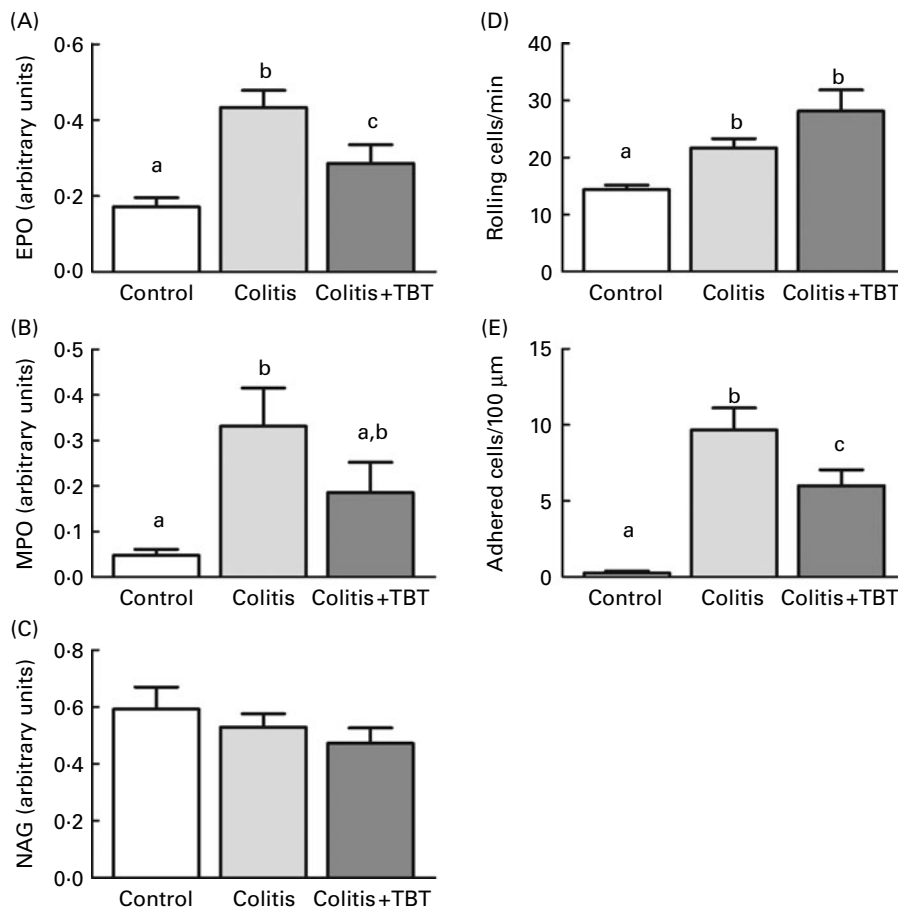
**Fig. 1.** Histopathological analysis of the colonic mucosa of animals from (A) the control group receiving the standard diet (□), (B) the colitis group receiving the standard diet and dextran sodium sulphate (DSS) (▣) and (C) the colitis + tributyrin (TBT) group receiving the TBT-rich diet and DSS (▤), and (D) the total histopathological score and components: (E) mucosal architecture, inflammatory infiltration and muscle layer thickness, (F) depletion of goblet cells and mucosal abscesses of colonic mucosa (*n* 4 per group). □, Present; ▣, absent. <sup>a,b,c</sup>Mean values with unlike letters were significantly different (*P*<0.05; one-way ANOVA and Newman–Keuls multiple comparison test). (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>)

because the cell distribution was similar among all groups (Table 2).

The balance between the pro-inflammatory and anti-inflammatory cytokines was also evaluated. There were no differences observed in the levels of TNF- $\alpha$ , interferon  $\gamma$ , IL-4 and IL-17 (Fig. 3(A)–(D)). However, IL-1 $\beta$  was significantly elevated in the colitis + TBT group compared with the other groups (Fig. 3(E)). The concentration of the anti-inflammatory cytokine TGF- $\beta$  was increased in

TBT-supplemented mice compared with the controls (Fig. 3(F)). Moreover, the reduction of IL-10 levels caused by colitis was partially reversed by TBT supplementation, as indicated by the fact that the IL-10 level in the colitis + TBT group was similar to that between the control and colitis levels (Fig. 3(G)).

These changes in mucosal inflammation were confirmed by *in vivo* evaluation of leucocyte rolling and adhesion in the colon microvasculature. The number of rolling cells was



**Fig. 2.** Evaluation of the enzyme activities of (A) eosinophil peroxidase (EPO), (B) myeloperoxidase (MPO) and (C) *N*-acetylglucosaminidase (NAG), as an indirect determination of eosinophil, neutrophil and macrophage infiltration, respectively. Intravital microscopy showing (D) the rolling and (E) the adhesion of leucocytes in the colons of animals from the control group receiving the standard diet, the colitis group receiving the standard diet and dextran sodium sulphate (DSS) and the colitis + tributyrin (TBT) group receiving the TBT-rich diet and DSS ( $n$  10 mice per group for EPO, MPO and NAG;  $n$  3/4 per group for adhesion and rolling). <sup>a,b,c</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ; one-way ANOVA and Newman–Keuls multiple comparison test).

augmented in both colitis groups compared with the control group. However, TBT supplementation partially reversed the increased cell adhesion observed in the colitis group (Fig. 2(D) and (E)).

According to the thiobarbituric acid-reactive species results, the level of lipid peroxidation showed no difference among the experimental groups (Fig. 4(A)). The increased hydroperoxide concentration after colitis induction was lower in TBT-supplemented mice (Fig. 4(B)). Associated with this partial improvement in hydroperoxide concentration, superoxide dismutase and catalase activities were also increased by TBT supplementation compared with the non-supplemented group (Fig. 4(C) and (D)).

To investigate the colon damage induced by colitis, we evaluated the  $^{99m}\text{Tc}$  recovery in the blood of a standard dose of  $^{99m}\text{Tc}$ -DTPA given by oral administration.  $^{99m}\text{Tc}$ -DTPA was minimally absorbed throughout the intestines in the control group (Fig. 5). The present results confirm the increase in permeability in the animals from the colitis group. The results of the present experiment show that TBT reduces the intestinal permeability to intermediate levels between the colitis and control groups.

We also evaluated colitis evolution by studying TBT action after 3 d of colitis induction. The animal weight gain and water and food intake were similar between the groups. These results suggest that the intensity of colitis was moderate and did not affect general metabolism (Table 1).

Although no weight loss was observed, it was possible to see areas with discrete ulcerations, goblet cell depletion and moderate inflammatory infiltrate in both colitis groups (Fig. 6). Additionally, the histological score did not show any improvement in the colitis + TBT group (Fig. 6(D)–(F)). An analysis of neutrophil and eosinophil infiltration showed increased neutrophil infiltration in both colitis groups. However, there was a small but significant reduction in TBT-supplemented mice (Fig. 7(B)). Eosinophil infiltration was similar among the groups (Fig. 7(A)).

An analysis of oxidative stress showed that the alteration of hydroperoxide concentration as well as catalase activity occurred earlier in the DSS-treated groups (Fig. 7(D) and (F)). Collectively, the results suggest that a reduction of neutrophil infiltration, with its consequent reduction of oxidative stress, were the earliest detectable events after TBT supplementation.

**Table 2.** Profile of leucocytes in the lamina propria, caecal lymph node, spleen and blood of mice from the control group receiving the standard diet, the colitis group receiving the standard diet and dextran sodium sulphate (DSS), the colitis + tributyrin (TBT) group receiving the TBT-rich diet and dextran sodium sulphate\* (Mean values with their standard errors, *n* 4–5 per group)

| Cell type (%)†                        | Control           |      | Colitis             |      | Colitis + TBT     |      |
|---------------------------------------|-------------------|------|---------------------|------|-------------------|------|
|                                       | Mean              | SE   | Mean                | SE   | Mean              | SE   |
| <b>Lamina propria</b>                 |                   |      |                     |      |                   |      |
| Dendritic cells                       | 1.5               | 0.3  | 1.6                 | 0.2  | 2.2               | 0.3  |
| Monocytes/macrophages                 | 8.7               | 0.8  | 19.1                | 5.7  | 19.5              | 1.7  |
| Macrophages                           | 49.2 <sup>a</sup> | 9.3  | 67.7 <sup>a,b</sup> | 3.9  | 72.6 <sup>b</sup> | 5.1  |
| B.1 cells                             | 2.0               | 0.03 | 2.7                 | 0.2  | 3.4               | 0.7  |
| Activated B cells                     | 94.7 <sup>a</sup> | 0.7  | 89.7 <sup>b</sup>   | 1.2  | 90.4 <sup>b</sup> | 1.1  |
| Memory T cells                        | 39.4 <sup>a</sup> | 0.9  | 67.6 <sup>b</sup>   | 3.7  | 68.9 <sup>b</sup> | 4.1  |
| Activated T cells                     | 23.9 <sup>a</sup> | 0.7  | 46.7 <sup>b</sup>   | 4.0  | 45.6 <sup>b</sup> | 3.2  |
| LAP <sup>+</sup> regulatory T cells   | 2.5 <sup>a</sup>  | 0.3  | 5.8 <sup>a,b</sup>  | 0.5  | 9.9 <sup>b</sup>  | 3.0  |
| FOXP3 <sup>+</sup> regulatory T cells | 8.4 <sup>a</sup>  | 0.4  | 17.6 <sup>b</sup>   | 2.6  | 16.3 <sup>b</sup> | 1.6  |
| <b>Caecal lymph node</b>              |                   |      |                     |      |                   |      |
| Dendritic cells                       | 1.5 <sup>a</sup>  | 0.2  | 0.6 <sup>b</sup>    | 0.2  | 0.8 <sup>b</sup>  | 0.3  |
| Monocytes/macrophages                 | 30.5 <sup>a</sup> | 3.3  | 33.6 <sup>b</sup>   | 2.5  | 37.3 <sup>b</sup> | 3.8  |
| Macrophages                           | 13.5              | 4.1  | 16.9                | 4.0  | 18.5              | 4.6  |
| B.1 cells                             | 5.4 <sup>a</sup>  | 1.6  | 3.6 <sup>b</sup>    | 0.7  | 2.7 <sup>b</sup>  | 0.3  |
| Activated B cells                     | 97.9 <sup>a</sup> | 0.5  | 98.6 <sup>b</sup>   | 0.3  | 98.9 <sup>b</sup> | 0.1  |
| Memory T cells                        | 11.7              | 4.5  | 8.8                 | 2.5  | 10.5              | 1.9  |
| Activated T cells                     | 17.4              | 3.0  | 15.8                | 2.9  | 18.2              | 1.9  |
| LAP <sup>+</sup> regulatory T cells   | 2.0               | 0.5  | 1.3                 | 0.2  | 1.2               | 0.5  |
| FOXP3 <sup>+</sup> regulatory T cells | 9.6               | 2.0  | 9.9                 | 0.6  | 9.9               | 1.9  |
| <b>Spleen</b>                         |                   |      |                     |      |                   |      |
| Dendritic cells                       | 5.2               | 0.8  | 5.8                 | 0.9  | 6.2               | 0.7  |
| Monocytes/macrophages                 | 21.7              | 1.7  | 21.2                | 2.8  | 22.6              | 3.4  |
| Macrophages                           | 52.5              | 6.9  | 51.6                | 5.0  | 53.3              | 6.1  |
| B.1 cells                             | 4.4 <sup>b</sup>  | 0.7  | 5.1 <sup>a</sup>    | 0.8  | 3.9 <sup>b</sup>  | 0.5  |
| Activated B cells                     | 91.5              | 1.7  | 89.7                | 1.3  | 90.1              | 1.8  |
| Memory T cells                        | 20.5              | 3.6  | 15.2                | 4.9  | 14.9              | 2.5  |
| Activated T cells                     | 10.2              | 1.4  | 10.5                | 2.5  | 9.5               | 1.6  |
| LAP <sup>+</sup> regulatory T cells   | 3.8               | 0.6  | 4.1                 | 1.6  | 5.4               | 0.9  |
| FOXP3 <sup>+</sup> regulatory T cells | 9.9               | 1.0  | 9.9                 | 1.5  | 9.8               | 1.0  |
| <b>Blood</b>                          |                   |      |                     |      |                   |      |
| Total number of cells                 | 17 446            | 3173 | 16 495              | 1040 | 17 250            | 1221 |
| Eosinophils                           | 0.8               | 0.4  | 0.5                 | 0.2  | 0                 | 0    |
| Neutrophils                           | 18.5              | 5.2  | 14.6                | 2.3  | 12.1              | 2.7  |
| Basophils                             | 5.6               | 0.8  | 6.1                 | 1.7  | 3.1               | 1.5  |
| Monocytes                             | 2.8               | 1.3  | 2.4                 | 0.4  | 3.1               | 0.8  |
| Lymphocytes                           | 72.1              | 5.5  | 75.1                | 2.4  | 81.5              | 3.4  |

<sup>a,b</sup> Mean values with unlike superscript letters were significantly different (*P* < 0.05; one-way ANOVA and Newman–Keuls multiple comparison test).

\* 7 d of DSS administration.

† All data represent percentage of cells in each respective gate, except for total number of blood cells, which are expressed as cells/mm<sup>3</sup> blood.

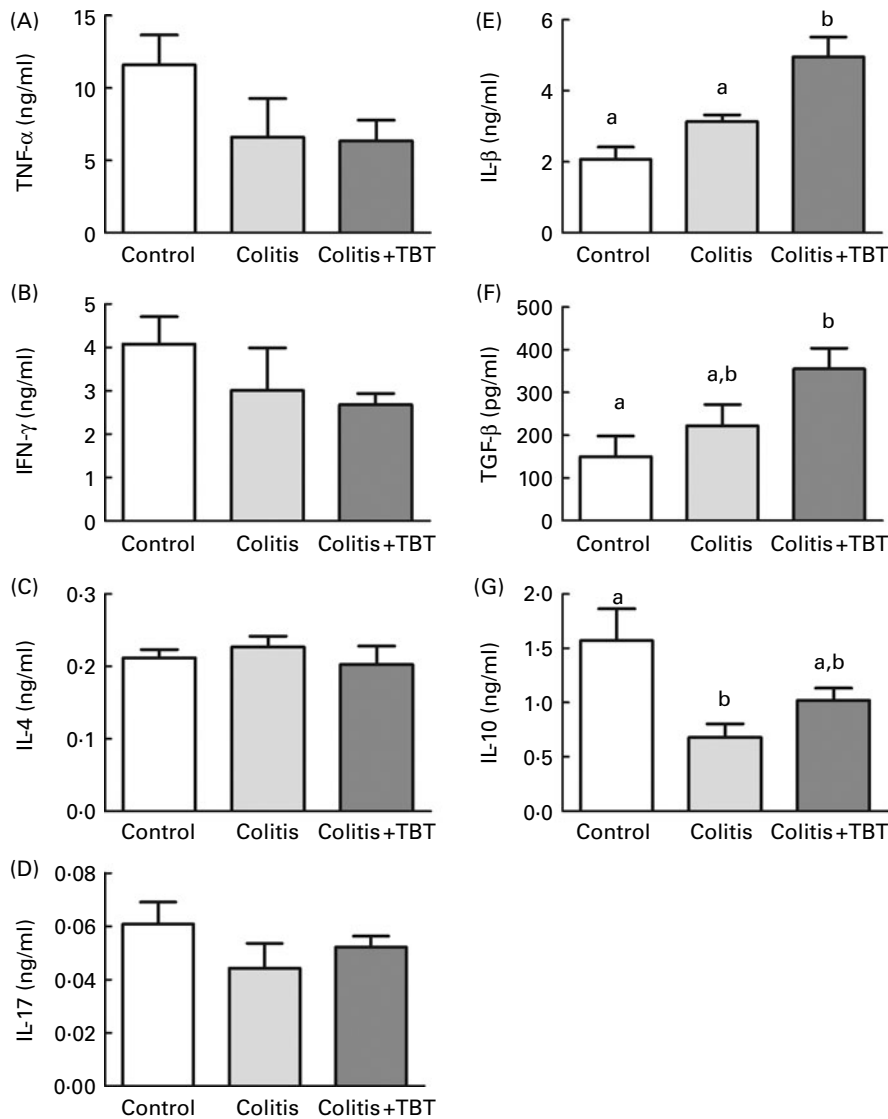
## Discussion

The present results show that TBT supplementation, acting pre-emptively, provides the same beneficial effects as butyrate in UC that were observed in our previous study<sup>(4)</sup>. The present results suggest that faster repair, instead of damage prevention, is the main mechanism of TBT action. In addition to the trophic effects evidenced by histology, TBT supplementation was able to control inflammation by reducing the migration of neutrophils and eosinophils. Additionally, TBT increased the population of Treg and the concentrations of TGF-β and IL-10 anti-inflammatory cytokines. The induction of oxidative stress, an important component of cellular damage, was also attenuated mainly by the up-regulation of catalase and superoxide dismutase activities. This modulation of inflammatory status was shown by the *in vivo*

demonstration of impaired leucocyte adhesion in the colonic microvasculature of TBT-supplemented mice.

The trophic effects of butyrate on colonocytes are well known<sup>(6,30,31)</sup> and are related to an increase in cell metabolism, cell differentiation and proliferation, and reconstruction of the damaged colonic mucosa. Similar to butyrate<sup>(4)</sup>, TBT supplementation was able to improve the mucosal architecture, reducing the size and severity of lesions. However, the present study goes beyond the histological aspects of such effects and reveals some of the mechanisms by which TBT modulates the inflammatory response related to colon damage.

We treated mice for 3 or 7 d with DSS to induce colitis. We chose these DSS administration times because intestinal epithelial renewal (from the basal to apical regions) occurs every 3–4 d. Therefore, all intestinal cells were renewed at



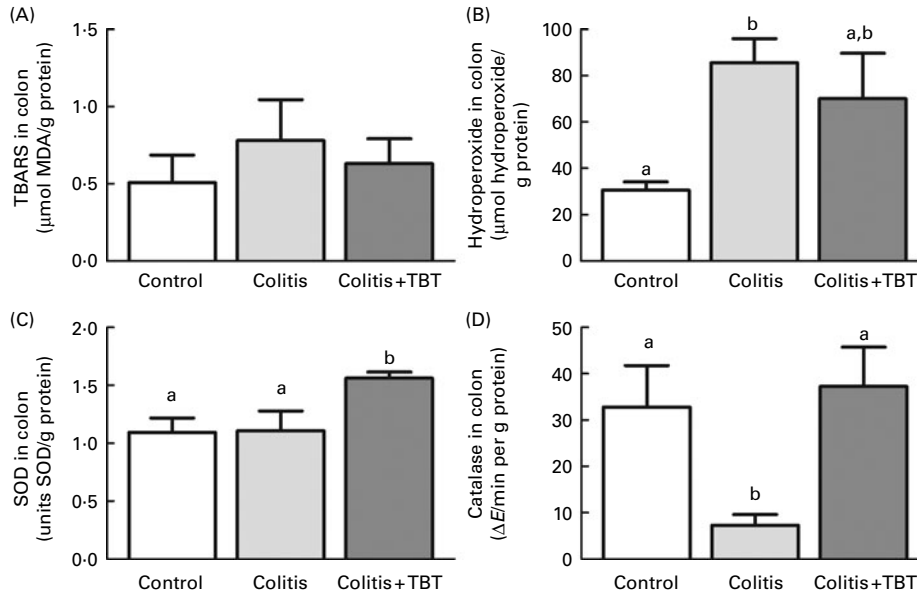
**Fig. 3.** Cytokine concentrations in the colons of animals from the control group receiving the standard diet, the colitis group receiving the standard diet and dextran sodium sulphate (DSS) and the colitis + tributyrin (TBT) group receiving the TBT-rich diet and DSS ( $n$  4–6 per group for each cytokine). <sup>a,b,c</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ; one-way ANOVA and Newman–Keuls multiple comparison test). IFN- $\gamma$ , interferon  $\gamma$ ; TGF- $\beta$ , transforming growth factor  $\beta$ .

least once after the beginning of DSS administration, offering a more homogeneous result. Additionally, considering the intestinal cells' renewal and also the differences between the apical and basal cells, we supplemented mice 7 d before DSS administration to guarantee that all cells had been exposed to TBT during their maturation period. The colon histology data after 3 or 7 d of colitis induction suggest that the effects of TBT start as soon as 3 d after colitis induction. Supplementation with TBT improves the mucosal architecture and neutrophil infiltration.

Associated with its trophic action, we observed that TBT supplementation improved the intestinal permeability. <sup>99m</sup>Tc-DTPA is used to determine the paracellular permeability in situations of discrete changes in the intestinal barrier. However, if the lesion is large enough to cause the loss of cellular integrity, <sup>99m</sup>Tc-DTPA will have access to the systemic

circulation by a transcellular route. Because we observed a complete loss of the mucosal architecture in the colitis group, both paracellular and transcellular permeability were increased. The improvement in mucosal lesions and the absence of ulcerations suggest that transcellular permeability is being repaired by TBT. However, paracellular permeability is still altered because the <sup>99m</sup>Tc-DTPA recovery in the blood of TBT-supplemented animals is between those of the control and colitis groups. The present study confirms in an experimental model the results obtained by several *in vitro* studies that show the effect of butyrate on permeability<sup>(6,7,30,32)</sup>.

Eosinophils and neutrophils are known inflammatory effectors in UC. The infiltration of these cell types can enhance mucosal damage and dysfunction that perpetuates colon inflammation<sup>(33–36)</sup>. Eosinophils can cause tissue damage by releasing lipid mediators such as platelet-activating factor,

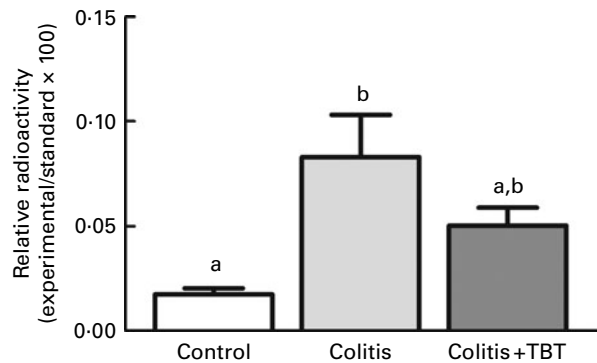


**Fig. 4.** (A) Analysis of thiobarbituric acid-reactive species (TBARS), measurement of (B) hydroperoxide level, (C) superoxide dismutase (SOD) activity and (D) catalase activity in the colon of animals from the control group receiving the standard diet, the colitis group receiving the standard diet and dextran sodium sulphate (DSS) and the colitis + tributyrin (TBT) group receiving the TBT-rich diet and DSS (*n* 5/6 per group for TBARS, *n* 4 per group for hydroperoxide and *n* 4/5 per group for superoxide dismutase and catalase). <sup>a,b</sup>Mean values with unlike letters were significantly different (*P* < 0.05; one-way ANOVA and Newman–Keuls multiple comparison test). MDA, malondialdehyde.

leukotrienes and toxic granule protein<sup>(33,37)</sup>. It has been suggested that eosinophils also act as antigen-presenting cells that stimulate T-cell proliferation and activation<sup>(38)</sup>. Neutrophil migration is responsible for extensive colon damage as well as the release of toxic components and free radicals<sup>(39,40)</sup>. Supplementation with TBT was also able to reduce neutrophil infiltration, the predominant inflammatory cell type in acute UC. This result further confirms the anti-inflammatory effects of TBT. Similar results were observed when intraluminal infusions of butyrate or SCFA were administered in DSS-induced colitis rats<sup>(41,42)</sup>. It should be noted that reduced neutrophil infiltration associated with TBT is the earliest effect detected by the present study. The reduction in neutrophils can be detected with only 3 d of colitis induction, which occurs before the histological evidence of mucosal damage and improvement of inflammatory score were observable. The effect on neutrophil infiltration may be due to a direct effect of TBT (or butyrate) on these inflammatory cells or may be due to a faster mucosal repair, which would reduce intraluminal inflammatory stimuli. Cell rolling and adhesion are initial steps for cell infiltration. Our intravital microscopy data suggest that the lower neutrophil infiltration observed in the present study was due to impaired cell adhesion in the colon microvasculature, slowing the access of inflammatory cells to the damaged mucosa. The present data are supported by others showing the effect of butyrate in reducing the expression of the adhesion molecule vascular cell adhesion molecule 1 and inhibiting the adhesion of leucocytes to the vascular endothelium<sup>(2,30)</sup>.

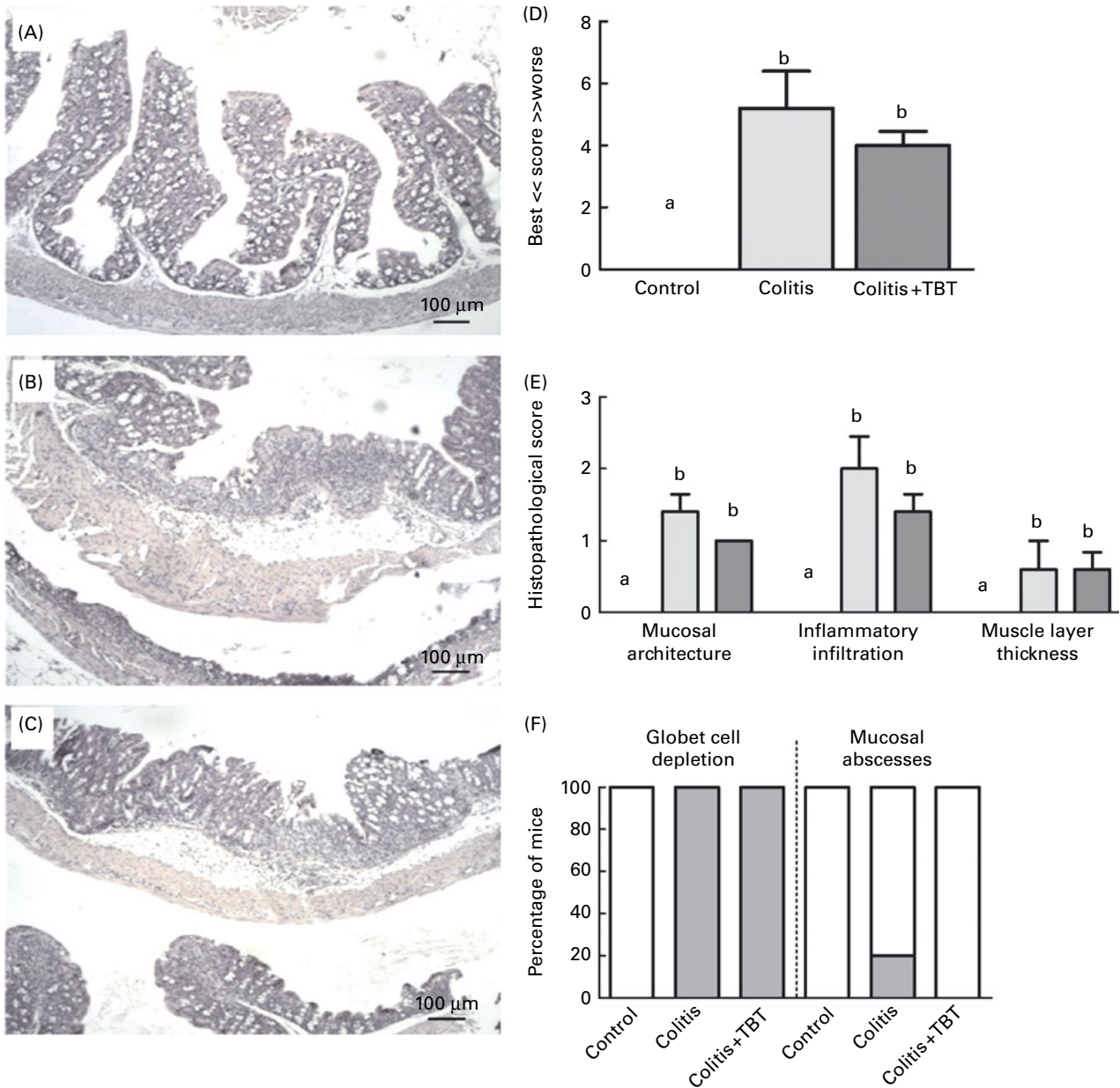
In addition to the enhanced inflammatory infiltrate, colitis is also linked to extensive mucosal injury. Mucosal injury increases the production and release of reactive oxygen

species such as superoxide and H<sub>2</sub>O<sub>2</sub> by neutrophils<sup>(10,35,43)</sup>. In the present study, the reduction of neutrophil infiltration after TBT supplementation resulted in a reduced production of hydroperoxides. A reduction of reactive oxygen species production should be accompanied by the down-regulation of superoxide dismutase and catalase activities due to a reduction of their substrates. However, the present experiments showed an increased activity of both enzymes after TBT supplementation. These data suggest that TBT has a direct effect on the activity of these antioxidant enzymes. A similar finding has been described previously for butyrate<sup>(44)</sup>. In addition to the influence on enzyme activity, butyrate also increases the expression of enzymes by acting as an inhibitor of the enzyme histone deacetylase<sup>(44–46)</sup>.



**Fig. 5.** Intestinal permeability of animals from the control group receiving the standard diet, the colitis group receiving the standard diet and dextran sodium sulphate (DSS) and the colitis + tributyrin (TBT) group receiving the TBT-rich diet and DSS (*n* 5 per group). <sup>a,b</sup>Mean values with unlike letters were significantly different (*P* < 0.05; one-way ANOVA and Newman–Keuls multiple comparison test).



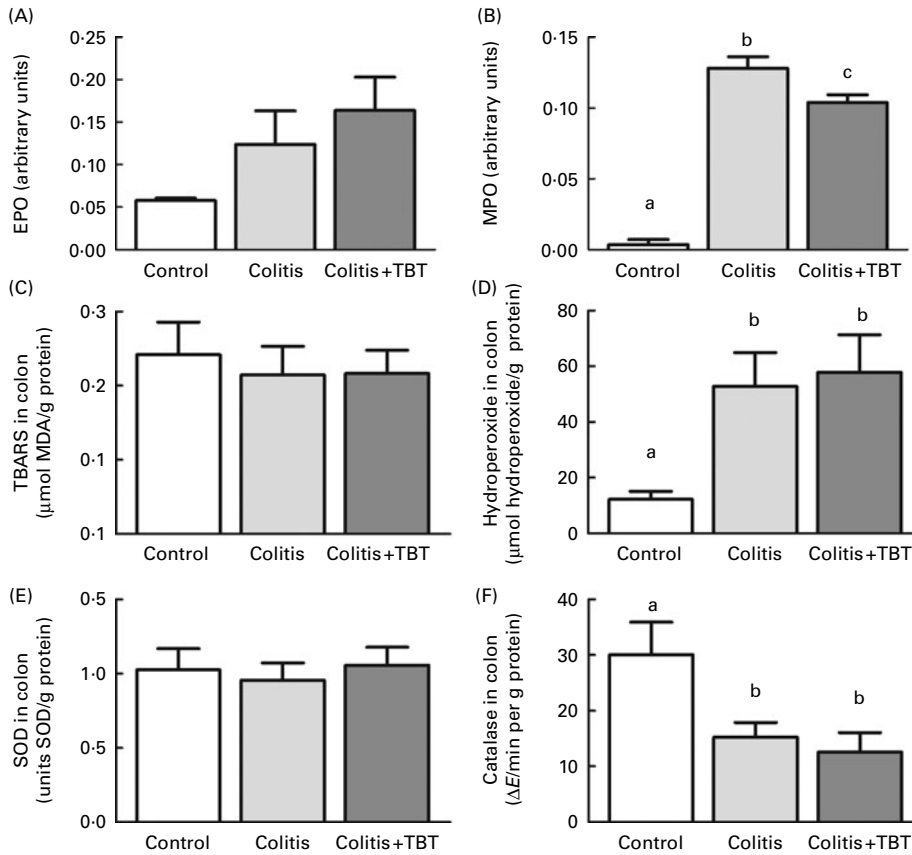


**Fig. 6.** Histopathological analysis of the colonic mucosa of animals from (A) the control group receiving the standard diet and dextran sodium sulphate (DSS) (□), (B) the colitis group receiving the standard diet and dextran sodium sulphate (DSS) (■) and (C) the colitis + tributyrin (TBT) group receiving the TBT-rich diet and DSS for 3 d (■), and (D) the total histopathological score and components: (E) mucosal architecture, inflammatory infiltration and muscle layer thickness, (F) depletion of goblet cells and mucosal abscesses of colonic mucosa (*n* 5 per group). □, Present; ■, absent. <sup>a,b</sup> Mean values with unlike letters were significantly different (*P* < 0.05; one-way ANOVA and Newman–Keuls multiple comparison test). (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>)

The level of macrophage infiltration was not different among any of the groups. However, TBT-supplemented mice had a significantly higher percentage of activated macrophages than the control group.

The concentration of IL-1 $\beta$  was elevated in animals supplemented with TBT compared with the control animals. IL-1 $\beta$  is produced by activated macrophages and is an important mediator of the pro-inflammatory response. Besides this effect, IL-1 $\beta$  regulates several important processes including cell proliferation, differentiation and apoptosis<sup>(47,48)</sup>. Thus, the increase in IL-1 $\beta$  levels in the colitis + TBT group is

associated with the improvement in the mucosal architecture. These results suggest that IL-1 $\beta$  has a cell-proliferative role and is important to mucosal regeneration, rather than a role as a pro-inflammatory effector. Supplementation with TBT was also linked to the elevation of the anti-inflammatory cytokines IL-10 and TGF- $\beta$ . IL-10 is an anti-inflammatory cytokine with a critical role in inflammatory diseases. IL-10 can decrease the production of pro-inflammatory cytokines and is associated with inflammatory intestinal diseases<sup>(21,49)</sup>. IL-10 knockout mice develop bowel inflammation characterised by discontinuous transmural lesions affecting the small



**Fig. 7.** Evaluation of the enzyme activities of (A) eosinophil peroxidase (EPO) and (B) myeloperoxidase (MPO), as an indirect determination of eosinophil and neutrophil infiltration, respectively; (C) analysis of thiobarbituric acid-reactive species (TBARS), and measurement of (D) hydroperoxide levels, (E) superoxide dismutase (SOD) activity and (F) catalase activity of colons of animals from the control group receiving the standard diet, the colitis group receiving the standard diet and dextran sodium sulphate (DSS) and the colitis + tributyrin (TBT) group receiving the TBT-rich diet and DSS for 3 d (*n* 3/6 per group for EPO and MPO, *n* 4/6 per group for TBARS and hydroperoxide and *n* 6/8 per group for SOD and catalase). <sup>a,b,c</sup>Mean values with unlike letters were significantly different (*P* < 0.05; one-way ANOVA and Newman–Keuls multiple comparison test). MDA, malondialdehyde.

and large intestines and by the dysregulated production of pro-inflammatory cytokines, indicating that endogenous IL-10 is a central regulator of the mucosal immune response<sup>(21)</sup>. Moreover, IL-10 deficiency in patients with UC contributes to persistent inflammation<sup>(49)</sup>. The present results are in agreement with the beneficial effect of IL-10 on the intestinal mucosa. The increased levels of IL-10 in colitis + TBT mice demonstrate that TBT is able to modulate inflammation and colonic homeostasis, which is consistent with previously described *in vitro* studies<sup>(3,50)</sup>.

In addition to regulating IL-10, TBT supplementation also increased TGF-β production. This cytokine and its subtypes are highly pleiotropic cytokines secreted by virtually all cell types. In the immune system, TGF-β has a modulator effect and can induce FOXP3-positive Treg. In the presence of IL-6, TGF-β induces IL-17-producing Th17 cells and a pro-inflammatory response. In the present study, the increase in TGF-β in the colitis + TBT group was associated with increases in Treg (LAP<sup>+</sup>) cells. However, the levels of IL-17<sup>+</sup> cells were not affected. These data indicate a regulatory, rather than an inflammatory, action for TGF-β.

Treg cells were significantly increased in both colitis groups compared with the control group, which is especially

important because in animal models, the infusion of Treg cells can cure ongoing colitis and is associated with a migration of injected Treg into the mesenteric lymph node and the colon<sup>(51,52)</sup>. Although FOXP3<sup>+</sup> Treg were increased in both colitis groups, only the colitis + TBT group had a statistically significant increase in LAP<sup>+</sup> Treg cells compared with the control group. In addition to FOXP3<sup>+</sup> Treg cells, the intestinal mucosa can also induce CD4<sup>+</sup>LAP<sup>+</sup>T cells, as observed in the present study. The surface expression of latency associated peptide (LAP) on Treg cells is induced by FOXP3<sup>(53)</sup>. TGF-β, a cytokine increased by TBT supplementation, is also responsible for LAP expression and LAP<sup>+</sup> Treg cells. Therefore, the analyses of FOXP3<sup>+</sup> and LAP<sup>+</sup> Treg cells expressed in the lamina propria suggest that LAP<sup>+</sup> Treg are the most important Treg induced by TBT supplementation.

It is interesting that oral administration of a short-chain TAG can control the immune response because it suggests that the supplementation of butyrate functions as more than a major fuel for colonocytes and may interfere with cell migration and interaction with the lamina propria.

In conclusion, the present results show that oral administration of TBT has positive effects on the colonic restructuring in experimental colitis. In addition to reducing mucosal

damage and intestinal permeability, TBT supplementation causes several changes in the immune response, mainly through inflammation control as a result of regulating the expression of anti-inflammatory cytokines and Treg cells.

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