Research Article

Systemic administration of a nanoemulsion with tributyrin reduces inflammation in experimental colitis

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Tributyrin (TBT) is a triacylglycerol formed by three molecules of butyrate that is able to improve colonic mucosal lesions and inflammation when given orally or as an enema. The effects of systemic TBT administration in inflammatory bowel disease (IBD) have been less well studied. We prepared a nanoemulsion containing tributyrin (nTBT) and studied its effects on a model of dextran sodium sulfate (DSS)-induced colitis. Mice were kept in the experiment for 15 days. Animals in the Colitis group received DSS (2.5% in their drinking water), whereas Control mice received only water during the last 7 experimental days. Animals were injected intraperitoneally (IP) with a nanoemulsion lacking TBT (Control and Colitis group) or containing TBT (Control nTBT and nTBT-Colitis) every other day for 15 days. Colonic mucosal damage and cytokine concentration were assessed by histopathological score and ELISA, respectively. Inflammatory infiltrates were assessed by enzymatic assay, and leukocyte rolling and adhesion in the colonic circulation were determined by intravital microscopy. Results revealed that the colonic architecture was not modified by nTBT administration. However, the colons of nTBT-Colitis mice presented reduced eosinophilic and neutrophilic infiltration associated with increased TGF-β concentrations compared to those of the Colitis group. Leukocyte rolling and adhesion were reduced in the nTBT-Colitis group, suggesting a systemic anti-inflammatory effect of nTBT. In conclusion, our results show that nTBT administered intraperitoneally is effective in reducing DSS-induced inflammation, whereas the trophic effects described with oral or local administration of TBT were not present when it was injected systemically.

Practical applications: In this study, we show that the intraperitoneal administration of a nanoemulsion with tributyrin (nTBT) is able to reduce the inflammation observed in DSS-induced colitis. Due to its anti-inflationary actions, nTBT could be used in the future as a potential auxiliary drug in the treatment of inflammatory bowel diseases as well as other disorders with a systemic (low-grade) inflammatory component such as atherosclerosis and obesity.

Keywords: Butyrate / Colitis / Fatty acid / Inflammatory bowel disease / Tributyrin

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Abbreviations: DSS, dextran sodium sulfate; **EPO,** eosinophil peroxidase; **HDAC,** Histone deacetylase; **IBD,** inflammatory bowel disease; **ICAM-1,** Intercellular Adhesion Molecule 1; **IL,** interleukin; **MPO,** myeloperoxidase; **NAG,** *N*-acetylglucosaminidase; **nTBT,** nanoemulsion containing tributyrin; **NFκB,** nuclear transcription factor kappa B; **TBT,** tributyrin; **TGF-β,** Tumor growth factor beta; **TNF,** tumor necrosis factor; **VCAM-1,** vascular cell adhesion molecule -1

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1 Introduction

Ulcerative colitis is characterized by crypt abscesses and ulcerations surrounded by prominent inflammatory infiltrate with areas of mucosal ulceration, apoptosis, and increased oxidative stress is present and related to disease severity [1, 2].

Butyrate, a 4-carbon fatty acid, is the main fuel of colonocytes and presents anticarcinogenic and anti-inflammatory properties [1, 3–6]. However, it has an unpleasant odor and taste that limits its clinical use. Butyrate microparticles have been tested because they are better tolerated and maintain butyrate concentration longer in the blood and because of its longer half-life; microparticles were more effective than butyrate in improving inflammation and oxidative stress [1, 5, 7]. Tributyrin (TBT) is the triacylglycerol composed of three molecules of butyrate that shares the same trophic, anti-inflammatory, and antioxidative effects with butyrate [8–10]. Although TBT is almost odorless and tasteless, it is rapidly absorbed in the stomach when given orally [11]. Oral TBT administration (3.1 g/kg by gavage) is able to keep blood concentration of butyrate above 0.1 mM only for 45 min [12]. On the other hand, parenteral injection of TBT can reach higher concentrations, but with a shorter half-life (about 6 min) [13]. We believed that parenteral administration of a nanoemulsion with TBT (nTBT) would keep systemic concentration of butyrate higher and longer compared to oral TBT administration. Moreover, since inner phase of tributyrin emulsion may provide a space for solubilizing lipid soluble drugs such as anti-inflammatory agents, drug loaded tributyrin emulsion may be an ideal carrier delivering two agents to treat colitis simultaneously [13]. However, it is not clear if nTBT could exert the same beneficial effects such as improvement of intestinal trophism and reduction of colitis induced inflammation seen after oral supplementation of butyrate or TBT [10, 14, 15]. In this study we aimed to observe whether all beneficial effects of oral administrations of butyrate were maintained when given systemically as nTBT.

2 Materials and methods

2.1 Preparation and analysis of nanoemulsion

Nanoemulsions (Table 1) were prepared by the hot melt homogenization method using an emulsification-ultrasound (Ultra-cell 750W, Sonics & Materials, Inc., Newtown, CT). First, the oil and aqueous phases were weighed separately and heated to 80 °C. Keeping the temperature at 80 °C, the aqueous phase was gently dropped on the oily phase with constant agitation, at 8000 rpm in an Ultra Turrax T-25 homogenizer (Ika Labortechnik, Germany). This emulsion was immediately submitted to high intensity probe sonication (20% amplitude), for 10 min, using a high intensity ultrasonic processor (Microprocessor controlled, 750 Watt model; Sonics Materials Inc., USA). The resultant nanoemulsion was then cooled to room temperature with manual agitation. The pH of the formulations was adjusted to 7.0 with a solution of 0.01 M HCl or 0.01 M NaOH (Digimed DM 20, Brazil).

Table 1. Composition of the nanoemulsion with or without tributyrin

Components	Nanoemulsion without tributyrin (%)	Nanoemulsion with tributyrin (%)
Oily phase (% w/w)		
Tributyrin	–	2
Soybean oil	4	2
Tween-80	0.75	0.75
Lecithin S 75-3	0.75	0.75
Aqueous phase (% w/w)		
Glycerol	2.25	2.25
Distilled water	q.s.t. 100	q.s.t. 100

The nanoemulsion was characterized immediately after production and after 100 days of preparation. It was therefore possible to demonstrate the stability of the formulations. The mean particle diameter of nanoemulsions in the dispersion was determined by dynamic light scaterring (DLS) using a Zetasizer Nano-ZS90 (Malvern Instruments, United Kingdom), at a fixed angle of 90° and temperature of 25 °C. The dispersions were diluted in distilled and filtered water (cellulose ester membrane, 0.45 mm, Millipore, USA) up to counts of 50–300 Kcps (1000 counts per second). The data were reported in terms of intensity as a function of particle size obtained from three repeated measurements of the dispersions. The maximum value acceptable for polydispersity index was 0.3.

ζ potential measurements were carried out using a Zetasizer Nano ZS90 (Malvern Instruments, United Kingdom) at temperature of 25 °C. Before the measurements, the dispersions were diluted in filtered 1 mM NaCl solution pH 7.0 (cellulose ester membrane, 0.45 μm, Millipore, USA) up to a counting of 100–1000 Kcps. All measurements were performed in triplicate. The ζ potential of particles is indirectly determined by measuring their electrophoretic mobility. ζ potential values are then calculated from these data using the Smoluchowski equation (Malvern Instruments, 2015).

2.2 Experimental protocol

The study was approved by the Ethics Committee for Animal Experimentation of UFMG (4/2013). Eight-week-old C57BL/6 mice receiving standard chow diet were divided into four groups: (i) No treatment control (CT) group: animals received water and nanoemulsion, with no nTBT; (ii) Treatment control (Colitis group): animals received DSS and nanoemulsion, with no TBT; (iii) nTBT group: animals received water and nTBT; and (iv) Colitis nTBT group: animals received DSS solution and nTBT. Animals were

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Table 2. Characterization of the nanoemulsion containing tributyrin at 0 and 100 days after production

	Nanoemulsion with tributyrin**	
Parameter*	Day 0	Day 100
Mean diameter (nm)	121 ± 2	114 ± 1
Polydispersion index (PI)	0.15 ± 0.03	0.13 ± 0.01
ζ potential (mV)	-35 ± 8	-24 ± 9

*Data expressed as mean ± sd, $n = 3$ different samples.
**Composition: tributyrin (2.0%), soybean oil (2.0%), Tween-80 (0.75%), lecithin S 75-3 (0.75%), glycerol (2.25%), distilled water (q.s.t. 100%).

kept in collective cages with 12-h light/dark cycles and controlled temperature (26 °C ± 2 °C), and they had free access to food and liquids. Body weight, and food and water intake were evaluated weekly. In the Colitis groups, DSS (MW = 36 000–50 000, MP Biomedicals) was added to the drinking water (2.0%) from the 8th until the 15th experimental day, according to the 7-days protocol, previously tested and validated [6, 16]. The nanoemulsions were injected (IP) every other day during the course of the experiment (15 days). The use of nTBT 7 days previously to DSS administration aimed to obtain an equilibrium of nTBT among tissues and optimize its effects. On the 15th experimental day (7 days after introduction of DSS), all animals were euthanized under anesthesia for the collection of blood and organs. Colons were removed and fixed in 4% formaldehyde for 4 h, embedded in paraplast and sectioned (10 μm thick) for hematoxilyn and eosin staining. After acquisition (Image Pro Plus software, Media Cybernetics, MD), images were analyzed using a semi-quantitative score as previously described [17].

Intestinal permeability assays were performed using ovalbumin on the day of euthanasia. The animals received 500 μL of ovalbumin solution (160 mg/mL) by oral gavage 75 min prior to euthanasia as previously described [18]. Serum ovalbumin concentrations were determined by ELISA.

Colonic myeloperoxidase (MPO), *N*-acetylglucosaminidase (NAG), and eosinophil peroxidase (EPO) activities were evaluated, as previously described [19]. Briefly, tissue samples were homogenized and centrifuged. The pellets were dissolved in HETAB 0.5% in phosphate buffer and after homogenization and freezing/thawing sessions. The suspensions were centrifuged, and the supernatants were used for the quantification of the enzymes. For EPO quantification, 75 μL of the supernatant was added to 75 μL of OPD (diluted in Tris–HCl and H_2O_2) and incubated at 37 °C in the dark for 30 min. The reaction was stopped by adding 50 μL H_2SO_4, and the fluorescence was then read at 492 nm. For MPO quantification, 25 μL of the supernatant was added to 25 μL of TMB in DMSO, and the mixture was incubated at 37 °C for 5 min. Then, 100 μL of H_2O_2 was added to the supernatant. The reaction was stopped by adding H_2SO_4, and then the fluorescence was read at 450 nm. For NAG quantification, the precipitates were dissolved in Triton X-100 (0.1) and centrifuged. Then, 100 μL of supernatant was added to 100 μL of p-nitrophenyl-*N*-acetyl-β-D-glucosamine in citrate/phosphate buffer. After incubation, 100 μL of glycine buffer was used to stop the reaction, and the fluorescence was read at 400 nm.

For cytokine (IL-4, IL-6, IL-10, TGF-β) analyses, colons were homogenized in the extraction solution and centrifuged (10 000 rpm, 10 min, 4 °C). The supernatants were collected for cytokine detection by ELISA, using commercial kits (R&D Systems, USA) according to the manufacturer's instructions.

Total blood leukocyte counts were analyzed in a Neubauer chamber after Turk staining (1:10 dilution). The differential count was performed using the Quick Panoptic kit (LaborClin, Brazil).

For intravital microscopy, mice were fasted overnight, anesthetized and injected IV with 0.15 mg/kg of rhodamine 6G (Sigma Chemical Co., USA) to label leukocytes. The

Table 3. Weight variation, food intake and liquid intake of mice from the Control group receiving water and nanoemulsion without TBT, the Control nTBT group receiving water and nanoemulsion with tributyrin (nTBT), the Colitis group receiving DSS and nanoemulsion without tributyrin, and the Colitis-nTBT group receiving DSS and nTBT.

	Control	nTBT	Colitis	Colitis-nTBT
Before DSS administration (Days 1–7)				
Weight variation (g)*	1.3 (0,2)[a]	2.0 (0,5)[a]	1.3 (0,3)[a]	2.3 (0,4)[a]
Food intake (g/day)**	2.9 (0,2)	3.0 (0,3)	3.1 (0,2)	3.2 (0,4)
Liquid intake (mL/day)**	5.9 (0,1)[a]	5.6 (0,4)[a]	5.7 (0,1)[a]	5.5 (0,1)[a]
After DDS administration (Days 8–15)				
Weight variation (g)*	1.1 (0,2)[a]	1.0 (0,2)[a]	−0.6 (0,4)[b]	−0.4 (0,4)[b]
Food intake (g/day)**	2.6 (0,1)	2.7 (0,1)	2.5 (0,1)	2.4 (0,1)
Liquid intake (mL/day)**	6.0 (0,2)[a]	6.0(0,4)[a]	4.5 (0,2)[b]	4.4 (0,3)[b]

ANOVA + Newman–Keuls multiple comparisons test. Different letters in a same line = statistical difference ($p < 0.05$).
*$n = 15$/group.
**$n = 3$/group.

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colon was exposed and the rhodamine-labeled leukocytes visualized in microcirculation by fluorescence microscopy (Nikon Eclipse, Japan). Rolling cells were defined as leukocytes passing by a certain point during one minute 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/100 μm [20].

Statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA). The results were evaluated by the Kolmogorov–Smirnov test for normality, and the Grubbs and Box–Plot tests were used for outliers. Data with a normal distribution were tested using a one-way ANOVA and a Newman–Keuls post-test analysis, whereas nonparametric data were tested by Kruskal–Wallis ANOVA and Dunn's tests. The results are expressed as the mean and standard error with significance level of 5% ($p < 0.05$).

3 Results

Measurements of the particle diameter of the nanoemulsion indicated that the nanoTBT was in the adequately nanomeric scale and was suitable for intraperitoneal administration. Regarding the polydispersion index, a value of 0.15 is sufficient to affirm that that the nanoemulsion is monodispersed. The ζ potential showed that nTBT had electrostatic repulsion sufficient to maintain the nanoparticle stability. The results obtained immediately after preparation (Table 2) were similar to those obtained after 100 days of preparation, confirming the stability of the nanoemulsion.

Food intake was similar among the groups. As expected, liquid intake and weight gain were reduced after introduction of DSS solution. However, neither parameters differed between the Colitis and nTBT + Colitis groups (Table 3).

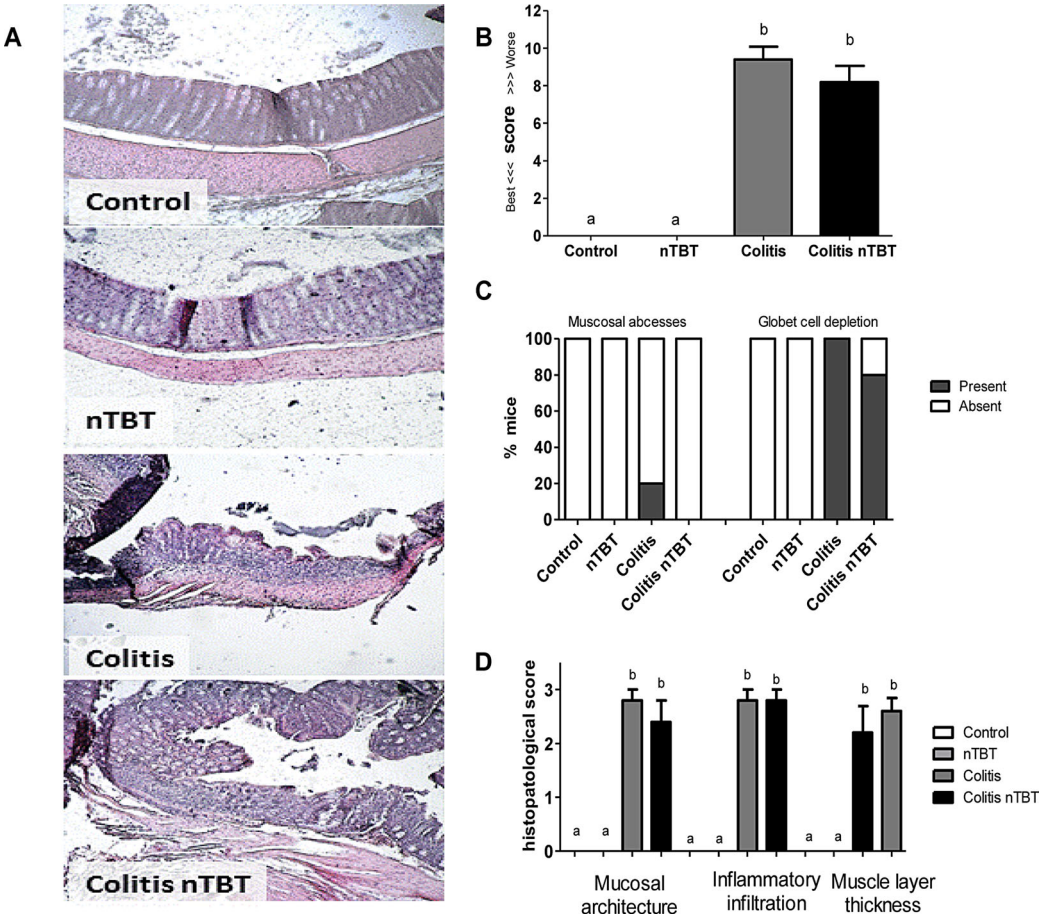


Figure 1. Colon assessment of mice from the Control group, receiving an IP administration of nanoemulsion without tributyrin (TBT); the Control nTBT group, receiving an IP administration of nanoemulsion with TBT; the Colitis group, receiving DSS and an IP administration of nanoemulsion without TBT; and the Colitis nTBT group, receiving DSS and an IP administration of nTBT. (A) Histological illustration of colon histology – HE staining; (B) Global histopathological score; (C) Score of depletion of goblet cells and mucosa abscesses; (D) Score of mucosal architecture, inflammatory infiltration and muscle layer thickness. $n = 6$ mice/group. Statistical tests: One-way ANOVA and Newman-Keuls multiple comparisons test. Different letters indicate $p < 0.05$.

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Histological analysis revealed the effective induction of colitis in both Colitis groups. In the Colitis group, areas of ulceration, goblet cell depletion, crypt abscesses, thickening of the muscle layer, and inflammatory infiltrate were observed. However, nTBT administration did not efficiently improve colon morphology, as evidenced by histopathological score (Fig. 1).

To evaluate the inflammatory infiltrate, macrophages, neutrophils, and eosinophils were assessed by measuring the activity of NAG, MPO, and EPO, respectively. Macrophage infiltration was not altered by the induction of colitis and was similar in all groups. Eosinophil and neutrophil infiltrates were increased in the Colitis group, but nTBT treatment reduced the infiltration of both cells to the levels seen in the Control group (Fig. 2).

We could not detect differences in IL-6 concentration among the groups (Fig. 3A). IL-4 and IL-10 concentrations were lower in the Colitis groups compared to the Control groups. In mice receiving nTBT, the levels of both cytokines

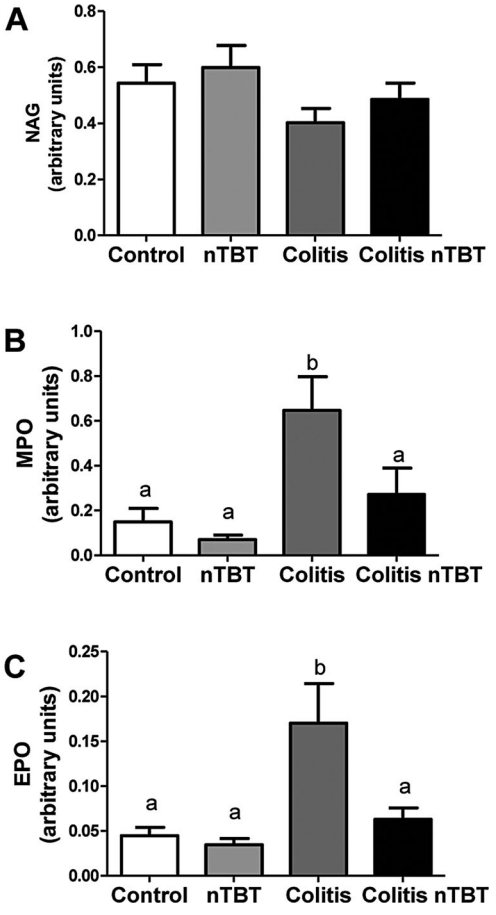


Figure 2. Evaluation of (A) *N*-acetylglucosaminidase (NAG), (B) myeloperoxidase (MPO), and (C) eosinophil peroxidase (EPO) activities, as an indirect determination of macrophage, neutrophil and eosinophil infiltration, respectively, in colon of animals from the Control group, receiving an IP administration of nanoemulsion without tributyrin (TBT); the Control nTBT group, receiving intraperitoneal administration of nanoemulsion with TBT (nTBT); Colitis group, receiving DSS and intraperitoneal administration of nanoemulsion without TBT; Colitis nTBT group, receiving DSS and intraperitoneal administration of nTBT. ($n = 7/10$ mice per group). Statistical tests: One-way ANOVA and Newman–Keuls multiple comparisons test. Different letters indicate statistical difference among groups ($p < 0.05$).

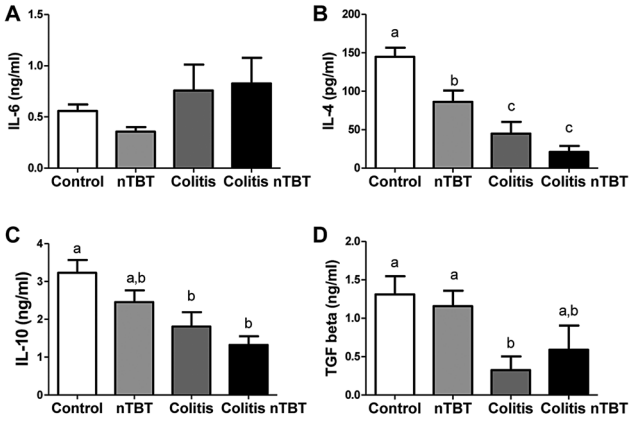


Figure 3. Cytokine concentrations in the colon of animals from Control group, receiving an IP administration of nanoemulsion without tributyrin (TBT); the Control nTBT group, receiving an IP administration of nanoemulsion with TBT (nTBT); the Colitis group, receiving DSS, and an IP administration of nanoemulsion without TBT; the Colitis nTBT group, receiving DSS and an IP administration of nTBT. ($n = 3/5$ per group for each cytokine). Statistical tests: One-way ANOVA and Newman–Keuls multiple comparisons test. Different letters indicate statistical difference among groups ($p < 0.05$).

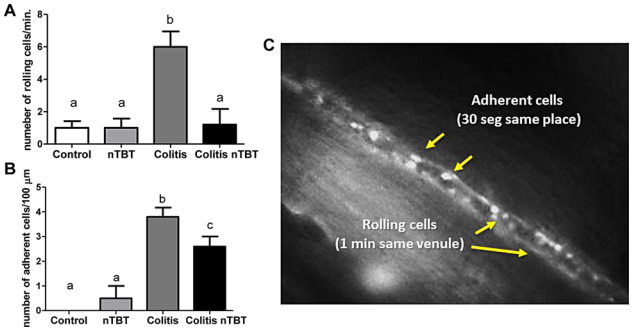


Figure 4. Rolling (A) and adhesion (B) of leukocytes in the colonic microvasculature of animals from the Control group, receiving an IP administration of nanoemulsion without tributyrin (TBT); the Control nTBT group, receiving an IP administration of nanoemulsion with TBT (nTBT); the Colitis group, receiving DSS and an IP administration of nanoemulsion without TBT; and the Colitis nTBT group, receiving DSS and an IP administration of nTBT ($n = 5$ per group). (C) Image illustrating intravital microscopy of a colonic venule from DSS induced colitis animal. Statistical tests: One-way ANOVA and Newman–Keuls multiple comparisons test. Different letters indicate $p < 0.05$.

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tended to be lower (Fig. 3B and C). Nevertheless, the concentration of TGF-β, which was reduced in the Colitis group, was increased to intermediate levels between the Control and Colitis groups after nTBT administration (Fig. 3).

To investigate whether the decrease in eosinophil and neutrophil concentration was due to a reduction in leukocyte migration, we observed, in real time, the adhesion and rolling of leukocytes in the colon vasculature, using intravital microscopy. As expected, leukocyte adhesion and rolling were increased in colitis animals. In nTBT-Colitis mice, both parameters were reduced compared to the Colitis group (Fig. 4).

4 Discussion

The goal of producing a nanoemulsion of tributyrin was to increase the half-life of butyrate in the blood, inducing a longer systemic butyrate effect. The administration of a TBT lipid emulsion to rats increased the bioavailability of butyrate and/or TBT up to four times compared with the administration of pure TBT [21, 22]. We also aimed to observe whether all beneficial effects of topic or oral administrations of butyrate were maintained when given systemically as nTBT. We successfully produced nTBT that was suitable for IP injection and stable for at least 3 months when stored under refrigeration (8 °C).

Our results demonstrated that nTBT was able to reproduce similar anti-inflammatory effects to those obtained with the oral administration of butyrate, thereby reducing DSS-induced colitis. However, the trophic effect characterized by increased villi length, absence of ulcerations and normal goblet cell population [6, 10, 23] was not observed with the use of the nanoemulsion. These results suggest that the trophic but not the anti-inflammatory action of butyrate is dependent on its presence in the colon. The trophic effect of butyrate is based on its capacity to serve as preferential fuel for colonocyte growth [24]. As butyrate is systemically dispensed in nTBT administration, the concentration of this fatty acid in the colon does not reach the levels necessary to induce colonocyte proliferation. We believe that nTBT acts indirectly (systemically) in enterocytes, reducing inflammatory cell migration to sites of intestinal lesion due to butyrate action as histone deacetylase inhibitor, down-regulating expression of the transcription factor NFκB and, consequently, inflammation [25]. This idea is reinforced by our previous study using EA.hy926 endothelial cells pre-cultivated with butyrate, showing a reduced concentration of VCAM1, CCL2, TNF, IL1β, and IL6 as well as the increased IL10 production in butyrate pre-cultivated cells. The results also showed that the inhibition of activation of NFκB was consequent to lower concentration and nuclear translocation of the p65 subunit. [7]. Those data reinforce MPO, EPO, cytokines, and intravital microscopy results, demonstrating the protective effect of nTBT on colitis-related inflammation.

Regarding cytokine concentrations, our results showed that nTBT did not improve the profile of IL-6, IL-4, and IL-10 concentrations seen after DSS treatment. These results are similar to those found in our previous study, which did not find any improvement on IL-4 and IL-10 intestinal concentrations in DSS-treated mice receiving TBT orally [10]. nTBT induced a relative increase of TGF-β concentration. This result is also in agreement with that found after oral TBT administration [10]. TGF-β is an inhibitory cytokine recognized as a key regulator of immunological homeostasis and inflammatory responses. Reduced TGF-β activity is considered to be responsible for the development of autoimmune disorders in several pathologic conditions including intestinal bowel diseases [26]. TGF-β is related to the suppression of Th1 and cytotoxic lymphocytes, and the inhibition of CD4 T-cell proliferation and differentiation [27, 28]. Although lymphocytes were not assessed in this study, the relative increase of TGF-β after nTBT treatment can suggest that the T cell infiltration may also have been reduced in the colons of Colitis nTBT mice. Evidences suggest that TGF-β can act in concert with epidermal, insulin-like, fibroblast growth factors, as well as VEGF to protect host tissue from luminal challenges and facilitate repair of mucosal injury in IBD [29]. All of these actions contributed to the prevention and control of colitis in our model.

An increase in eosinophil numbers has been reported in subsets of IBD patients, and eosinophils are significantly increased in the mucosa and submucosa of the colon [30–32]. The presence of eosinophils in the colonic mucosa has been associated with weight loss, malabsorption, changes in mucosal architecture, and reduced flattening of the villi and crypts in the colon [33–35]. DSS-induced colitis is also linked to the colonic migration of neutrophils, which triggers extensive tissue damage due to the release of free radicals [36–38]. Accordingly, the reduction in colonic neutrophils is related to a better disease prognosis [10, 37, 39, 40]. In this study, nTBT reduced the infiltration of eosinophils and neutrophils observed in the Colitis group. Our results are in agreement with several previous studies showing the beneficial effect of butyrate and tributyrin in these cell types [6, 9, 10, 14]. These data suggest an improvement of inflammation and signal an improved potential course of the disease due to nTBT treatment.

The migration of leukocytes to the inflammatory site is dependent on the rolling and adhesion of inflammatory cells in the vascular endothelium. Leukocyte adhesion to the molecules selectin, VCAM-1 and ICAM-1 permit cells to transmigrate forward to the focus of inflammation. Animals from the Colitis-nTBT group showed a reduction in both rolling and adhesion of leukocytes to the colonic vasculature. The increase in leukocyte adhesion in colitis is due to the higher number of adhesion molecules present. It has been demonstrated that TGF-β down-regulates IL-6-induced tyrosine phosphorylation of STAT1 and STAT3 and ICAM-

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1 expression which could contribute to the reduction of leukocyte rolling and adhesion seen in Colitis-nTBT group [41]. Furthermore, studies have demonstrated that the action of butyrate is related to its ability to decrease the expression of adhesion molecules as VCAM-1 and ICAM-1 maybe via histone deacetylase inhibition [4, 42–44]. Our results of intravital microscopy agree with results of eosinophil and neutrophil infiltration as well as TGF-β concentration, thus reinforcing the beneficial role of nTBT on inflammation.

5 Conclusions

Our results show that the systemic administration of nanoemulsion of tributyrin was effective in DSS-induced colitis by limiting mucosal leukocyte migration and activation. However, the trophic effects characteristic of local administration of butyrate and TBT could not be observed. These results suggest that the trophic effect of butyrate/TBT is dependent on its presence in the colon, whereas the anti-inflammatory effect can be reached systemically.

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The authors declare no conflicts of interest.

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