Butyrate enema therapy stimulates mucosal repair in experimental colitis in the rat

J D Butzner, R Parmar, C J Bell, V Dalal

Abstract

Background—The short chain fatty acid (SCFA) butyrate provides energy for colonocytes, stimulates colonic fluid and electrolyte absorption and is recognised as an effective treatment for multiple types of colitis.

Aim—To examine the impact of butyrate enema therapy on the clinical course, severity of inflammation, and SCFA stimulated Na^+ absorption in a chronic experimental colitis.

Methods—Distal colitis was induced in rats with a trinitrobenzenesulphonic acid (TNBS) enema. Five days after induction, rats were divided into groups to receive: no treatment, saline enemas, or 100 mM Na-butyrate enemas daily. On day 24, colonic damage score and tissue myeloperoxidase (MPO) activity were evaluated. Colon was mounted in Ussing chambers and Na^+ transport and electrical activities were measured during a basal period and after stimulation with 25 mM butyrate.

Results—In the untreated and the saline enema treated TNBS groups, diarrhoea and extensive colonic damage were seen, associated with increased tissue MPO activities and absent butyrate stimulated Na^+ absorption. In contrast, in the butyrate enema treated TNBS group, diarrhoea ceased, colonic damage score improved, and tissue MPO activity as well as butyrate stimulated Na^+ absorption recovered to control values.

Conclusion—Butyrate enema therapy stimulated colonic repair, as evidenced by clinical recovery, decreased inflammation, and restoration of SCFA stimulated electrolyte absorption.

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Keywords: short chain fatty acids, Na^+ absorption, diarrhoea, butyrate, colon transport.

Gastrointestinal Research Group, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada
J D Butzner
R Parmar
C J Bell
V Dalal

Correspondence to:
Dr J Decker Butzner,
Department of Pediatrics,
3330 Hospital Drive NW,
University of Calgary,
Calgary, Alberta, Canada
T2N 4N1.

Short chain fatty acids (SCFAs) produced by the bacterial fermentation of carbohydrate and fibre play an important physiological role in the maintenance of the health and integrity of the colonic epithelium.[1–3] Of all the SCFAs, butyrate provides the primary energy source for the colonic epithelium, with 60–80% undergoing metabolism.[4 5] The colonocyte's dependence on SCFA metabolism increases from the proximal to the distal colon.[6] Butyrate also regulates epithelial proliferation and differentiation of the colonic mucosa.[3 7–9] In addition, SCFAs stimulate sodium and fluid salvage by the colon.[1–3]

A growing body of evidence links deficiency, decreased absorption, and impaired metabolism of SCFAs to the pathophysiology of multiple types of diarrhoeal disease. Clinical findings suggest SCFA deficiency causes diversion colitis, an inflammatory reaction of the colorectum that occurs after surgical diversion of the colonic contents.[10] Daily colonic perfusion with SCFAs stimulates both clinical and endoscopic remission of diversion colitis after only two weeks. Relapse can be prevented for prolonged periods with continued SCFA treatment.[10] An acute infectious ileocolitis inhibits both SCFA stimulated electrolyte absorption and absorption of physiological concentrations of SCFAs in the rabbit. These transport abnormalities are associated with increased luminal concentrations of SCFAs.[11] Ulcerative colitis inhibits both in vivo and in vitro SCFA oxidation by colonic epithelial cells.[12–14] Furthermore, in the rat, metabolic inhibition of colonic epithelial SCFA oxidation causes colitis.[15] Despite evidence for impaired SCFA oxidation in distal ulcerative colitis, enema therapy with combinations of SCFAs has been used successfully to treat this disease.[16 17] Furthermore, two clinical studies showed that enema therapy with 80–100 mM butyrate as the sole SCFA, stimulated clinical, endoscopic, and histological improvement in patients with refractory distal ulcerative colitis.[18 19]

Because of the success of butyrate enema therapy in distal ulcerative colitis, the aims of these experiments were: (a) to examine the effects of a chronic experimental colitis on in vitro SCFA stimulated electrolyte absorption; (b) to correlate changes in transport with other parameters of colonic damage and inflammation; and (c) to assess the effects of treatment with daily butyrate enemas given in a pharmacological concentration (100 mM) on recovery of colonic structure and function. Experimental colitis was induced in rats with trinitrobenzenesulphonic acid (TNBS) and the resulting injury was examined for a short (five days) and a long (24 days) time period. TNBS colitis exhibits clinical, histological, and microscopic similarities to inflammatory bowel disease and the course of colonic injury has been well characterised.[20–23]

Methods

Animals

Male Wistar rats (230-270 g) were purchased from Life and Environment Sciences Animal

Resource Centre (Calgary, AB, Canada) and fed standard laboratory chow and tap water ad libitum. A constant photoperiod (14 h light, 10 h dark) and constant temperature of 20°C were maintained. Procedures for the care and handling of animals used in this study were approved by the University of Calgary Animal Care Committee and carried out in accordance with guidelines established by the Canadian Council on Animal Care. For 24 hours before the induction of colitis, rats were observed to ensure appropriate feeding behaviour and health. Weights as well as the presence and severity of diarrhoea were recorded daily.

Induction of colitis

Colonic inflammation was induced by a modification of the method described by Morris et al.[20] On day zero, rats received 0·75 ml of an enema containing 60 mg of 2, 4, 6 TNBS (Fluka Chemical, Ronkonkoma, NY) dissolved in 30% ethanol (vol/vol), through a side hole catheter inserted into the colon with the tip 8–10 cm proximal to the anus. A control group received a single saline enema (0·75 ml of 0·9% saline) or no enema. Data on both sets of animals compromising this control group did not differ and are combined. Randomly selected animals from the TNBS colitis and control groups were evaluated on day five as outlined below. The remaining animals from both the TNBS colitis and control groups were randomly divided into three 'treatment' regimens. In the first treatment group, both controls and TNBS colitis animals received daily saline enemas (140 mM NaCl, pH 7·0) for 19 days from day 5 to 23. Controls and TNBS colitis animals in the second group received daily SCFA enemas (100 mM Na-butyrate, 40 mM NaCl, pH 7·0) from day 5 to 23. The third group of controls and rats with TNBS colitis received no further treatment. This resulted in six treatment groups on day 24 of the study: saline enema – control, saline enema – TNBS colitis, butyrate enema – control, butyrate enema – TNBS colitis, untreated – control, and untreated – TNBS colitis.

Assessment of damage and inflammation

Rats were killed by cervical dislocation; the distal colon excised and luminal contents flushed with HCO_3^--free HEPES buffer at 37°C. The colon was opened by an incision along the mesenteric border and laid flat. An assessment of damage was scored on the segment of distal colon 5 cm in length proximal to the colorectal junction, using the criteria outlined in Table I adapted from J L Wallace et al.[21] This scoring system assesses the extent and severity of visible colonic damage; the presence and severity of adhesions; and as markers of diarrhoea, the presence of loose, watery stool or perianal fur soiling at the time of study. In the TNBS treated groups, injury was also seen 1–2 cm above this distal 5 cm segment. The 1 cm proximal end of this segment was excised, immediately frozen on dry ice, and stored at −70°C. Myeloperoxidase

TABLE I *Criteria for scoring of colonic damage*

Feature	Score
Ulceration	
Normal appearance	0
Focal hyperaemia, no ulcers	1
Ulceration without hyperaemia or bowel wall thickening	2
Ulceration with inflammation at one site	3
⩾ Two sites of ulceration and inflammation	4
Major sites of damage extending >1 cm along the length of the colon	5
Damage extended to >2 cm along the length of the colon, increase the score by one for each additional cm of damage	6–10
	plus
Adhesions	
No adhesions to surrounding tissue(s)	0
Minor adhesions (colon can be separated from other tissues with little effort)	1
Major adhesions	2
Diarrhoea	
Absence of	0
Presence of	1
	Total score

(MPO) activity was determined as described by Morris et al and modified by Wallace et al within seven days of obtaining tissue.[20][21] MPO is an enzyme found in cells of myeloid origin, especially neutrophils. It provides a quantitative index of granulocyte infiltration in gastrointestinal tissues.[24]

Na^+ transport and electrical activities

For transport studies, a 4 cm section of the distal colonic segment was stripped of the serosa and underlying muscle layers with forceps. Adjacent pieces of the stripped colon were mounted in Ussing chambers with an exposed surface area of 0·4 cm^2 and colonic transport as well as electrical activities were examined as previously described.[11] Both the serosal and the mucosal sides of the tissue were bathed in 9 ml of HCO_3^- free HEPES buffer at pH 6·8 and 37°C. These conditions maximise in vitro butyrate stimulated Na^+ absorption in rat distal colon.[25] The buffer contained (in mM): 120 Na, 10 K, 1·1 Mg, 1·25 Ca, 134·7 Cl, 2 H_2PO_4, 25 HEPES, and 10 glucose. The solutions were maintained at 37°C with heated water jackets and circulated by a bubble gas lift mechanism containing 100% O_2.[11]

The transepithelial potential difference (PD) was determined and the tissue clamped at zero voltage by continuously introducing the appropriate short circuit current (I_{sc}) with an automatic voltage clamp (DVC 1000; World Precision Instruments, New Haven, CT). Every five minutes open circuit PD was measured and tissue conductance (G) was calculated from PD and I_{sc} according to Ohm's law.

When electrical parameters reached a steady state, 5 μCi of ^{22}Na (New England Nuclear, Montreal, QC, Canada) were added to either the mucosal or the serosal side of the paired chambers. After addition of isotope, reservoirs were allowed to equilibrate for 20 minutes. Steady state fluxes for the basal period were calculated from four samples obtained at time zero and five minute intervals for a total of 15 minutes. The samples were replaced with

equal amounts of the HCO_3^- free HEPES buffer. Immediately after the basal period, 25 mM Na-butyrate (final concentration) was added to the mucosal side while the serosal side received 25 mM Na-isethionic acid to maintain osmolarity and volume. After a 10 minute equilibration period, a second set of flux measurements were performed over a 15 minute period as described above. The final concentrations of butyrate and isethionic acid in the bathing solutions remained constant throughout the experiment by replacement with buffer containing 25 mM Na-butyrate or Na-isethionic acid. If at any time during flux measurements the conductances of paired tissues differed by >25%, the experiment was rejected.

Calculations and statistics

Unidirectional mucosal to serosal (J_{ms}) and serosal to mucosal (J_{sm}) fluxes were calculated from aliquots at three consecutive five minute flux intervals and one overall 15 minute interval. The net flux (J_{net}) for each period was calculated as the difference between oppositely directed unidirectional fluxes of tissue pairs. By subtracting J_{net} in the basal period from J_{net} in the stimulated period, the net effect of butyrate on sodium transport (ΔJ_{net}) was derived [$\Delta J_{net} = J_{net}$ (stim)$-J_{net}$ (basal)]. Data are expressed as means (SEM). A one way analysis of variance was used for multiple comparisons of data and unpaired t tests used for comparisons within a group. p Values of $\leqslant 0.05$ were considered significant.

Results

Clinical

On day zero, mean body weights did not differ between controls (241 (4) g, n=32) and the group to be treated with TNBS (253 (5), n=33). All rats that received TNBS on day zero developed diarrhoea within 24 hours. By day five, when the first colonic studies were

performed, all TNBS treated rats had persistent diarrhoea and developed significant (p<0.001) weight loss (−7 (2) g) compared with the weight gain (31 (5)) seen in controls. Diarrhoea persisted in most animals with TNBS colitis that received either no treatment (7 of 9) or 19 days of saline enemas (7 of 8). In contrast by day 24, diarrhoea ceased in seven of eight animals with TNBS colitis that were treated with butyrate enemas for 19 days. No diarrhoea was seen throughout the study period in controls that received no treatment or 19 days of either saline or butyrate enemas.

Weight gain from day 5 to 23 did not differ between the untreated (137 (11) g, n=9), saline enema treated (120 (8), n=7) or butyrate enema treated (130 (7), n=6) control groups. The weight gains of the untreated (73 (11) g, n=9) and saline enema treated (72 (10), n=8) groups with TNBS colitis were significantly decreased (p<0.01) compared with their respective control groups. In contrast, the weight gain (98 (9) g, n=8) from day 5 to 23 of the butyrate enema treated TNBS group recovered to that of control groups.

Colonic damage score

In all groups of controls examined, either on day 5 (n=10) or day 23 (n=22), no colonic damage was seen and colonic damage score was zero. Colonic damage scores of rats with TNBS colitis, including the untreated group examined on day five (10·0 (0·7), n=8) as well as the untreated (8·0 (0·8)) and saline enema treated (7·8 (0·6)) groups examined on day 24, were significantly higher (p<0.01) compared with the butyrate enema treated TNBS group examined on day 24 (3·7 (0·6)). Two animals in the butyrate enema treated TNBS group displayed focal hyperaemia of the colon and in six rats, one small area of ulceration was seen, associated with a minor adhesion in three. Severity of colonic damage did not differ between the untreated TNBS colitis group examined on day five and either the untreated or the saline enema treated TNBS colitis groups evaluated on day 24 of the study. Ulcers in these three groups were increased both in number and size compared with the butyrate enema treated TNBS group. Also, both minor and major adhesions were seen.

Tissue MPO activity

Tissue MPO activities of the respective control groups were all less than 3 units/mg tissue and did not differ between treatment groups (Fig 1). On day five of the study, colonic MPO activity was significantly increased (p<0.001) in the TNBS group compared with untreated controls (Fig 1). On day 24 of the study, colonic MPO activities in the untreated and the saline enema treated TNBS groups were significantly decreased (p<0.01) compared with the TNBS group on day five, but remained significantly increased (p<0.01) compared with values of their respective control groups on day 24 (Fig 1). In contrast with the persistent increases in tissue MPO
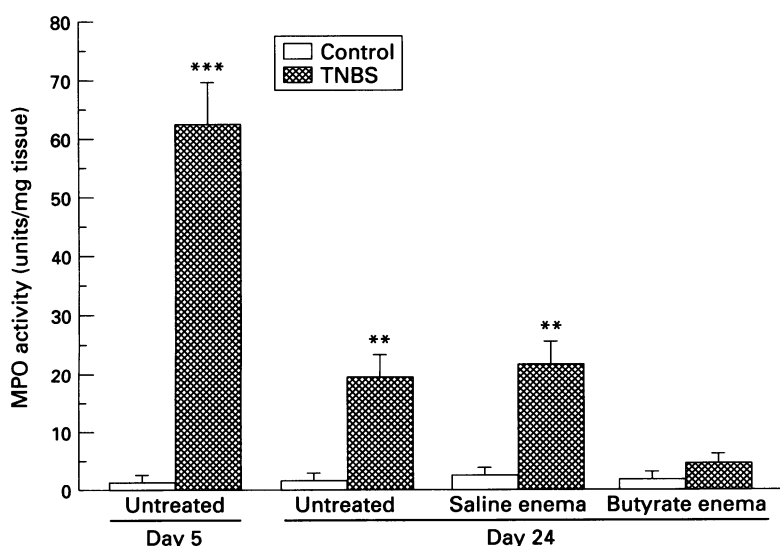

Figure 1: Tissue myeloperoxidase (MPO) activities in units/mg tissue for control and TNBS groups on day five or 24 for each treatment category. **p<0·01, ***p<0·001 compares the TNBS colitis group with the respective control group.

TABLE II   *Na^+ Transport and electrical activities in rat distal colon*

Study period	Number	J_{ms}	J_{sm}	J_{net}	I_{sc}	G
Day 5						
Untreated – control						
Basal	10	11·4 (0·8)	5·1 (0·5)	6·3 (0·9)	1·0 (0·2)	10 (1)
Butyrate		15·9 (0·9)	5·3 (0·5)	10·5 (0·9)	0·6 (0·1)	11 (1)
p Value		<0·01	NS	<0·01	NS	NS
Untreated – TNBS						
Basal	8	13·4 (0·8)	11·9 (0·9)	1·5 (0·8)	0·6 (0·3)	52 (6)
Butyrate		11·6 (0·6)	12·0 (1·0)	−0·4 (1·0)	0·7 (0·2)	38 (4)
p Value		NS	NS	NS	NS	NS
Day 24						
Untreated – control						
Basal	9	11·8 (0·8)	5·6 (0·4)	6·1 (0·9)	0·9 (0·1)	13 (1)
Butyrate		15·9 (0·7)	5·9 (0·4)	10·0 (1·0)	0·9 (0·2)	14 (1)
p Value		<0·01	NS	<0·01	NS	NS
Untreated – TNBS						
Basal	8	12·9 (1·9)	7·9 (1·1)	4·9 (0·9)	0·7 (0·4)	23 (3)
Butyrate		14·3 (1·6)	10·4 (0·9)	3·8 (1·2)	0·4 (0·2)	30 (4)
p Value		NS	NS	NS	NS	NS
Saline enemas – control						
Basal	7	12·8 (1·3)	6·4 (0·7)	6·4 (1·0)	0·5 (0·2)	14 (2)
Butyrate		17·5 (1·3)	6·9 (0·6)	10·6 (1·0)	0·3 (0·1)	20 (5)
p Value		<0·05	NS	<0·02	NS	NS
Saline enemas – TNBS						
Basal	7	17·0 (1·1)	7·3 (0·7)	9·7 (0·8)	0·8 (0·2)	19 (2)
Butyrate		17·0 (1·4)	8·6 (0·6)	8·5 (1·4)	0·3 (0·1)	24 (2)
p Value		NS	NS	NS	<0·05	NS
Butyrate enemas – control						
Basal	6	10·7 (0·6)	4·5 (0·2)	6·2 (0·6)	0·8 (0·2)	14 (3)
Butyrate		15·2 (0·9)	5·7 (0·5)	9·5 (0·8)	0·4 (0·2)	22 (4)
p Value		<0·01	NS	<0·01	NS	NS
Butyrate enemas – TNBS						
Basal	8	17·0 (0·6)	7·7 (0·6)	9·3 (0·7)	1·0 (0·1)	22 (4)
Butyrate		20·0 (0·8)	7·8 (0·2)	12·2 (0·8)	0·8 (0·1)	28 (5)
p Value		<0·01	NS	<0·02	NS	NS

Values are mean (SEM) for rats on day 5 or 24 of the study in the following groups: non-manipulated controls±a single saline enema on day 0 (control); TNBS enema given on day 0 (TNBS); daily saline enemas given from day 5 to 24 (saline enemas); daily butyrate enemas given from day 5 to 24 (butyrate enemas); and no daily enema therapy (untreated). The number of animals studied in each group is given. J_{ms}, J_{sm}, J_{net} and I_{sc} are given in $\mu Eq/cm^{-2}/h^{-1}$; G is given in millisiemens/cm^2. The p value compares the basal state with the stimulated state within each group.

the addition of butyrate, confirming electroneutral stimulation of Na^+ absorption. Similarly, in the control groups on day 24 that received either daily saline or SCFA enemas from day 5 to 23 and were not subjected to TNBS colitis, the addition of butyrate stimulated J_{ms} and J_{net} Na^+ fluxes above basal activities and did not change J_{sm} Na^+ flux, I_{sc} or G (Table II).

In contrast, the distal colons of both the day 5 and day 24 untreated groups with TNBS colitis as well as the day 24 saline enema treated group failed to show stimulation of J_{ms} or J_{net} Na^+ absorption after the addition of 25 mM butyrate (Table II). J_{sm} Na^+ flux and G in these three groups and I_{sc} in the day 5 and day 24 untreated TNBS colitis groups were not changed after the addition of butyrate. Also on day five, TNBS colitis caused an increase (p<0·01) in J_{sm} Na^+ flux and G and a decrease (p<0·01) in J_{net} Na^+ absorption during the basal period compared with day five controls. After the addition of butyrate, I_{sc} in the day 24 saline enema treated TNBS colitis group was reduced.

Unlike the other TNBS colitis groups on day 24, distal colon of the butyrate enema treated TNBS group responded to butyrate stimulation, as evidenced by increases in J_{ms} and J_{net} Na^+ absorption above basal values (Table II). Similar to the findings in the control groups, J_{sm} Na^+ flux, Isc or G did not differ between the basal and stimulated periods in the butyrate enema treated TNBS group on day 24.

An examination of ΔJ_{net} Na^+ transport summarises these data (Fig 2). The addition of butyrate to the distal colon of all control groups stimulated ΔJ_{net} Na^+ absorption above basal values to a similar degree (Fig 2). Na^+ absorption was replaced by mild Na^+ secretion after the addition of butyrate to the mucosa of the day five untreated, the day 24 untreated, and the day 24 saline enema treated groups with TNBS colitis (Fig 2). In contrast in the day 24 butyrate enema treated TNBS colitis group, the addition of a physiological concentration of butyrate once against stimulated ΔJ_{net} Na^+ absorption to values similar to those seen in all control groups not subjected to TNBS colitis.

activities seen at 24 days in untreated or saline enema treated rats with TNBS colitis, MPO activity in the butyrate enema treated TNBS group fell to control values by day 24 (Fig 1).

Na^+ transport and electrical activities

The addition of 25 mM butyrate to the distal colons of the untreated control groups on days five and 24 of the study period stimulated J_{ms} and J_{net} Na^+ fluxes above basal values but did not alter J_{sm} Na^+ flux (Table II). Tissue electrical activities, I_{sc} and G, were not changed by
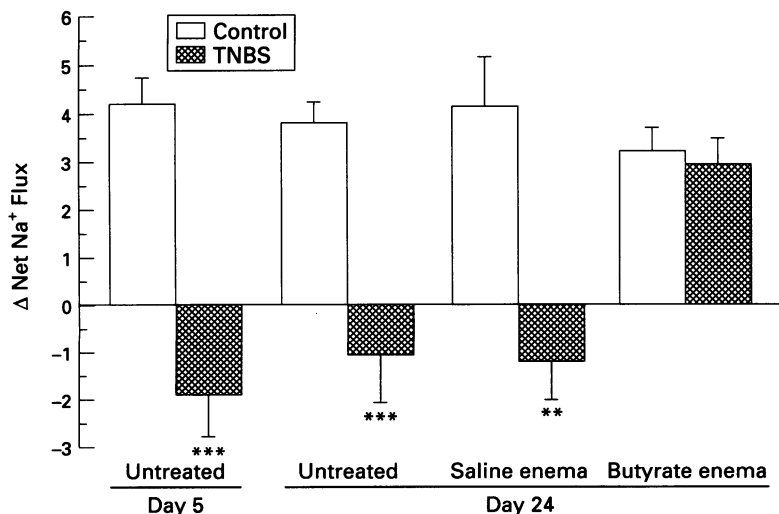
Discussion

In these in vitro experiments, the addition of a physiological concentration of butyrate (25 mM) to the mucosal side of the distal rat colon of untreated controls stimulated electroneutral Na^+ absorption, confirming previous findings of Binder and Mehta.[25] In the rat, butyrate absorption from the lumen by distal colonic epithelial cells occurs via an apical butyrate-HCO_3 exchanger.[25][26] Exchange of the strong base, HCO_3, for the weak base, SCFA, decreases intracellular pH, which in turn stimulates apical electroneutral Na-H exchange.[27] The apical extrusion of H^+ from the cell corrects intracellular pH and the absorption of Na^+ with its subsequent basolateral transport from the cell via Na-K-ATPase stimulates colonic

Figure 2: Δ Net Na^+ Flux = (J_{net} (butyrate) − J_{net} (basal)) in $\mu Eq/cm^{-2}/h^{-1}$ in rat distal colon of control and TNBS groups on day five or 24 for each treatment category.
**p<0·01, ***p<0·001 compares the TNBS group with the respective control groups.

water absorption.[25-28] Thus, SCFA stimulated Na$^+$ absorption plays an integral part in the colonic salvage of fluid and electrolytes.

In these experiments, untreated controls gained weight and displayed no evidence of colonic damage or inflammation. Similarly, 19 days of either saline or butyrate enema therapy given to controls did not cause colonic damage, trigger inflammation or change epithelial function. In contrast, rats subjected to a TNBS induced colonic injury developed acute weight loss and diarrhoea. Five days after TNBS administration, an extensive colonic injury was seen, as evidenced by persistent diarrhoea and pronounced inflammation. The multiple ulcers overlying a thickened mucosa and fibrous adhesions were similar to previous observations of the TNBS induced colonic injury in rats.[20-23] Five days after the induction of injury, net absorption of Na$^+$ was decreased before the addition of butyrate and butyrate failed to stimulate Na$^+$ absorption. This was associated with increases in serosal to mucosal Na$^+$ flux and electrical conductance, both manifestations of increased epithelial permeability. Our findings confirm recent observations that correlated basal colonic electrolyte transport with morphological injury in this model of experimental colitis.[23] Four days after injury, these investigators observed a pseudomembrane overlying an extensive area of epithelial necrosis and inflammation. This was associated with abolition of electrolyte absorption and increased tissue leakiness. During this acute phase of injury, diarrhoeal fluid losses are most probably caused by extensive epithelial damage associated with increased tissue leakiness as well as a decreased SCFA induced colonic salvage of electrolytes and fluid.

By day 24, in the untreated and saline enema treated groups with TNBS colitis, Na$^+$ absorption in the basal period recovered to control values despite the persistence in inflammation. Addition of the SCFA butyrate, however, once again failed to stimulate the colonic salvage of Na$^+$ in these two groups. This probably contributed to persistent diarrhoeal fluid losses. Bell *et al* also showed a recovery of basal electrolyte transport associated with persistent inflammation and diarrhoea over this time period.[23] They suggested the continued diarrhoea was caused by other mechanisms, but did not explore its pathophysiology.

The important finding of this study is the group with colitis that received butyrate enema therapy for 19 days displayed both a clinical and functional recovery. Diarrhoea ceased and weight gain recovered to control values. Colonic damage was minimal and inflammation, as measured by tissue MPO activity, decreased to the level of controls. As seen with the other TNBS groups at day 24, Na$^+$ absorption in the basal period recovered to control values. Unlike the other TNBS groups, however, SCFA stimulated Na$^+$ absorption recovered in the group that received daily butyrate enema therapy. Recently D'Argenio *et al* showed that butyrate enema therapy promotes histological repair and reduces

inflammation of TNBS induced colitis in the rat.[22] This study extends D'Argenio's findings by correlating structural repair and reduced inflammation with recovery of colonic epithelial transport function. In the TNBS model of chronic colitis, these experiments show that butyrate enema therapy reduces diarrhoea by stimulating tissue repair, which results in recovery of SCFA stimulated electrolyte absorption, an important contributor to the colonic salvage of fluid and electrolytes. Similarly, in humans, enema treatment with SCFAs including butyrate has been used successfully to treat diversion colitis, refractory distal ulcerative colitis, and radiation proctitis.[10 16-19 29] Hove *et al* recently showed that patients with active ulcerative colitis displayed rectal butyrate absorption equivalent to healthy controls, when provided with a pharmacological concentration (70 mM) of butyrate.[30]

Butyrate enema therapy probably stimulates epithelial repair by several mechanisms. In multiple species, including the rat, butyrate serves as the primary metabolic fuel of colonocytes.[4-6 15] In addition, butyrate induces colonic trophism by stimulating proliferation of immature epithelial cells at the base of colonic crypts.[7 8] Interestingly, when this proliferation zone expands to involve the upper 40% of the crypt, which may be seen in response to epithelial injury, butyrate has a different role. It inhibits further proliferation and stimulates differentiation, which improves colonic function, including improved electrolyte absorption.[1-3 9] In summary, enema therapy with the SCFA, butyrate, reduces inflammation, stimulates colonic repair, and restores colonic function in this experimental model of chronic colitis. These results support continued investigations of the role for SCFAs, especially butyrate, in the treatment of multiple types of colonic injury.

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