Original Research

Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats

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Abstract

Mucositis resulting from cancer chemotherapy is characterized by intestinal inflammation and ulceration. Previously, emu oil (EO) improved intestinal architecture (*Br J Nutr*, 2010) in a rat model of chemotherapy-induced mucositis. We investigated EO for its further potential to promote intestinal repair in this mucositis model. Female Dark Agouti rats ($n = 8$/group) were gavaged with water, olive oil (OO) or EO once daily (1 mL), injected with 5-fluorouracil (5-FU) or saline on day 5 and euthanized on day 8, 9, 10 or 11. Intestinal villus height (VH) and crypt depth (CD), neutral mucin-secreting goblet cell (GC) count, myeloperoxidase (MPO) activity and selected cytokines were quantified; $P < 0.05$ was considered significant. In 5-FU-injected rats, only EO administration significantly increased VH in the ileum (day 8), jejunum and jejunum–ileum junction (days 8 and 9) compared to 5-FU controls ($P < 0.05$). GC count was significantly reduced by 5-FU (jejunum: days 8 and 9; ileum: day 8; $P < 0.05$) and EO increased ileal GC on days 10 and 11 compared to 5-FU controls. MPO activity was significantly increased in jejunum (days 8 and 9) and ileum (day 8) following 5-FU injection, compared to normal controls ($P < 0.05$). Both EO and OO significantly reduced jejunal MPO on days 8 and 9; however, only EO decreased ileal MPO on day 8. Cytokine levels were not significantly affected by either oil or 5-FU administration at the day 8 time point. Promotion of repair from injury could represent a new mechanism of action for EO, suggesting potential as an adjunct to conventional treatment approaches for cancer management.

Keywords: Chemotherapy, intestinal mucositis, emu oil, repair, rat

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Introduction

Mucositis is one of the most common and debilitating side effects of cancer treatment,[1] developing in approximately 40% of patients undergoing chemotherapy and up to 60% of patients undergoing high-dose chemotherapy.[2,3] This condition manifests due to the inability of chemotherapy agents to discriminate between normal and neoplastic cells,[4] resulting in ulcerating lesions, inflammation and deterioration of the mucosal membranes lining the alimentary tract.[5] The mucosa of the mouth (oral mucositis) and small intestine (gastrointestinal mucositis) is most commonly affected.

Symptoms of oral and gastrointestinal mucositis can be extremely debilitating and severely impact on quality of life for the patient. Incidence and severity can vary, depending on intensity, class of drug and radiation dosing protocol.[2] Sufferers of mucositis may experience nausea, bloating, diarrhoea and severe abdominal pain.[2,5]

The pathogenesis of mucositis is believed to be a five-stage process.[2,6–8] Two of the principles of mucositis development are the generation of reactive oxygen species which directly damage cells, tissue and blood vessels[7] and the up-regulation of pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β) and interleukin-6 (IL-6) eliciting further tissue damage.[6] Epithelial barrier disruption allows bacterial translocation from the lumen to the lamina propria, resulting in ulceration.[9] Accordingly, agents capable of neutralizing reactive oxygen species and decreasing inflammation could be valuable adjuncts to mucositis treatment.

Developing treatments for intestinal mucositis has proven problematic, as not all treatments are effective for both oral and gastrointestinal mucositis. Furthermore, potential anti-mucotoxic agents must protect the mucosa without interfering with effects of cytotoxins on the neoplasm. Thus far, considerable effort has been expended to find an effective method for treating mucositis; however, it continues to be a dose limiting complication of cancer therapy.[10] Recent studies into mucositis treatment have focused on hormones, growth factors and

cytokines which modulate immune responses, cell proliferation and apoptosis[1] and recognition of intestinal bacteria by pattern recognition receptors (Toll-like receptors-2 and -9).[9] Many naturally sourced compounds have suggested potential such as grape seed extract,[11] attributed to proanthocyanidin-mediated antioxidant and anti-inflammatory effects, certain probiotics[12,13] which modulate the microbiota and inhibit pro-inflammatory cytokine expression and even plant-extracts such as Iberogast.[14]

Efforts to ameliorate chronic inflammatory conditions, including ulcerative colitis and rheumatoid arthritis, have been directed towards increasing dietary levels of polyunsaturated fatty acids (PUFAs) which include omega-3 and omega-6 found in marine-derived oils including fish oil[15,16] and the New Zealand Green-Lipped mussel extract, Lyprinol.[17,18] Recently, attention has been directed towards animal-derived oils with high levels of PUFAs, for example, oil derived from the Australian ratite bird, the Emu (Dromaius novaehollandiae).

Traditionally endemic to Australia, the Emu is now farmed around the world for its meat, leather and most recently, its FA-rich oil.[19,20] Indigenous Australians first used emu oil (EO) to facilitate wound healing, pain alleviation and treatment of inflamed joints.[21,22] Topical application of EO to mice with adjuvant-induced inflammation decreased levels of TNF-α and other pro-inflammatory cytokines,[23] involved in the pathogenesis of mucositis.[6] In a preliminary study in rats, Abimosleh et al.[19] indicated that orally administered EO improved selected parameters associated with the manifestation of experimentally induced colitis, characterized by inflammation and ulceration of the large bowel. Furthermore, Lindsay et al.,[24] in a rat model of mucositis, proposed a potential mechanism of action for EO, speculating on the possibility of a more rapid rate of recovery during the recovery phase of mucositis.

Accordingly, we hypothesized that orally administered EO would accelerate recovery of the intestine following chemotherapy damage. To this end, we investigated the effects of EO on the time course of intestinal repair in a rat model of 5-fluorouracil (5-FU)-induced mucositis.

Materials and methods

General experimental procedures

Animal studies. Throughout acclimatization and the experimental period, Female Dark Agouti rats (120–140 g) were individually housed in metabolism cages (Tecniplast, Exton, PA, USA) at room temperature with a light:dark cycle of 12 h. All rats were given ad libitum access to food (standard 18% casein-based diet[25]) and water and were acclimatized for two days prior to experimentation. All animal studies were conducted in compliance with the Australian Code of Practice for the Care and Use of Animals, and were approved by the Animal Ethics Committees of the Children, Youth and Women's Health Service and The University of Adelaide.

Rats were randomly assigned to six groups (n = 8/group): Group 1: saline + water, Group 2: saline + olive oil (OO), Group 3: saline + EO, Group 4: 5-FU + water, Group 5: 5-FU + OO and Group 6: 5-FU + EO. Water, OO or EO (1 mL) was administered once daily via oro-gastric gavage from days 0 to 8, 9, 10 or 11. On day 5, all rats were intraperitoneally injected with a single dose of saline or the chemotherapeutic agent, 5-fluorouracil (5-FU, Mayne Pharma Pty Ltd, Mulgrave, Victoria, Australia; 150 mg/kg). 5-FU is a pyrimidine analogue belonging to the anti-metabolite group of drugs that has customarily required intravenous administration due to poor bioavailability when administered orally.[26]

Table 1 Major FA composition of emu and olive oils used in the current study including UFA and SFA ratios

Major FA composition of emu and olive oils			
Fatty acid	**Common name**	**Emu**	**Olive**
16:0	Palmitic acid	24	10.4
16:1*n*−7	Palmitoleic acid	4.3	0.7
18:0	Stearic acid	8.5	3.1
18:1*n*−9	Oleic acid	49.1	73.9
18:2*n*−6	Linoleic acid	9.5	8.4
18:3*n*−3	α-Linolenic acid	1.1	0.7
Saturated		32.5	13.5
MUFA		53.4	74.6
PUFA		10.6	9.1
UFA:SFA ratio		2	6.2

FA: fatty acid; UFA: unsaturated fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

Emu and OO preparation. EO, sourced from Emus farmed in North-Eastern South Australia, was prepared utilizing specific methodologies developed for Technology Investment Corporation by Emu Tracks (Marleston, Adelaide, South Australia). Briefly, these processes involved the rendering and filtration of Emu adipose tissue, with appropriate considerations for delivery of quality assurance and product consistency. Fatty acid analysis of EO and OO was carried out using gas chromatography, as described previously[27] (Table 1). OO (Conga Foods, Spain) was selected as the control oil due to its high level of oleic acid; similar to that of EO. The OO control was included to determine any non-specific oil-related effects for subsequent comparison with EO. Both EO and OO were individually stored at 4°C in 50 mL opaque containers.

Daily metabolic data and disease activity index

Body weight, food and water intake, and faecal and urine output were monitored and measured daily. The severity of mucositis was assessed daily, using a disease activity index (DAI), which scored body weight loss, rectal bleeding, stool consistency and overall general condition of the animal, increasing in severity on a scale of 0–3 for each parameter, which was totalled to achieve an overall DAI, as described previously.[28,29] Overall condition was determined by (1) mobility/agility: healthy rats were considered quite active, whereas rats affected by 5-FU characteristically became very weak and feeble, sitting hunched with very little movement and (2) fur: healthy rats were well-groomed with fur flat to the body,

whereas 5-FU-injected rats became scruffy in appearance, with ruffled fur.

^{13}C-sucrose breath test

Immediately prior to kill (day 8, 9, 10 or 11), the ^{13}C-sucrose breath test (SBT) was performed as a non-invasive assessment of the functional status of small intestinal health and to ensure safety of EO.[30] Briefly, following an overnight fast, a baseline breath sample was collected at t = 0. Rats were then gavaged with 1 mL of a 25% ^{13}C-labeled sucrose solution (BDH, Merck Pty Ltd, Kilsyth, Victoria, Australia); breath samples were collected every 15 min for 120 min and samples were analysed for $^{13}CO_2$ concentration using an isotope ratio mass spectrometer (Europa Scientific, Crewe, Cheshire, UK).[4] Data were expressed as mean percentage cumulative dose of ^{13}C recovered at 90 min post-sucrose administration (%CD90). This provides an indirect indication of the rate at which sucrose is cleaved by the enzyme sucrase in the small intestine, and therefore, the level of sucrase present on small intestinal enterocytes (brush border).

Tissue collection

On day 8, 9, 10 or 11, rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation. Cardiac puncture was performed to remove whole blood for subsequent cytokine analysis of serum. The gastrointestinal tract was removed, measured, emptied of contents and weighed. Segments of the small intestinal tract (jejunum, jejunum–ileum junction [JI] and ileum; 2 cm) were removed and placed in 10% buffered formalin for histological analyses. Additionally, segments (4 cm) directly adjacent to the corresponding histological samples were collected and snap-frozen in liquid nitrogen for biochemical analysis. Samples were stored at −80°C until prepared for analysis by homogenization in 10 mmol/L phosphate buffer. The remaining visceral organs (thymus, lungs, heart, liver, kidneys and spleen) were weighed and discarded.

Histological analyses

Mucosal measurements. Small intestinal samples were transferred from 10% buffered formalin to 70% ethanol 24 h post-collection. Specimens were then routinely processed and embedded in paraffin wax and sections (4 μm) were stained with haematoxylin and eosin (H&E). Measurements of villus height (VH) and crypt depth (CD) in the small intestine were determined for 40 well-orientated villi and crypts per small intestinal tissue section per rat and a mean value was then obtained.[31]

Neutral mucin-secreting goblet cell count. Following routine processing and embedding in paraffin wax, additional 4 μm sections were mounted on poly-L-lysine-coated slides. Sections were deparaffinized using Histolene (Fronine Laboratory Supplies Pty Ltd, Riverstone, New South Wales, Australia) and rehydrated in preparation for neutral mucin-secreting goblet cell (GC) staining. Sections were subjected to mild acid hydrolysis to eliminate the contribution of sialic acid residues prior to periodic acid-Schiff staining, which involved immersing sections in sulphuric acid for 60 min at 80°C in a water bath. After rinsing with tap and distilled water, sections were immersed in periodic acid solution (Sigma, St. Louis, MO, USA) for 20 min, washed and immersed in Schiff's reagent (Sigma, St. Louis, MO, USA) for a further 20 min. Sections were rinsed in tap water for 10 min and dehydrated. Neutral mucin-secreting GCs stained pink-purple and were counted in a blinded fashion on one side of 40 well-orientated villi per small intestinal tissue section per rat.

All histological analyses were performed in a blinded fashion, using an Olympus BH-2 light microscope (Tokyo, Japan) with Sony digital camera (Tokyo, Japan) and Image Pro Plus Software Package Version 4.5.1.27 (Media Cybernetics, Silver Spring, MD, USA) for villus and crypt measurements and Image J Software Version 1.44p (National Institutes of Health, USA) for neutral mucin-secreting GC counting.

Biochemical analyses

Myeloperoxidase activity. Myeloperoxidase (MPO) levels in the small intestine were determined as an indicator of neutrophil infiltration, and hence, acute inflammation, using techniques described by Howarth et al.[32] *Thawed, homogenized samples were centrifuged at 13,000 g for 12 min, after which the supernatant was discarded and the tissue homogenate was re-suspended in 200 μL of 0.5% hexadecyltrimethyl ammonium bromide buffer, a detergent (Sigma Chemicals, Castle Hill, NSW, Australia). After vortexing for 2 min, samples were again centrifuged at 13,000 g for 2 min. Background, negative and positive control samples (50 μL) and the supernatants of each test sample were then aliquoted into duplicate wells of a microtitre 96-well plate. Following the addition of a reaction solution (200 μL to each well; 4.2 mg of O-dianisidine dihydrochloride reagent, 12.5 μL H_2O_2, 2.5 mL potassium phosphate buffer [pH 6.0], 22.5 mL distilled water) the change in absorbance was measured at 450 nm at 1 min intervals for 15 min with a spectrophotometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore). Data were expressed as units of MPO per gram of tissue.*

Cytokine analyses. Whole blood collected via cardiac puncture during kill was immediately refrigerated at 4°C for approximately 2 h to allow for clotting. Blood samples collected on day 8 were centrifuged at 1000 g for 10 min at 4°C and serum was collected and stored at −80°C for subsequent cytokine analysis.

Multiplexed analyses of cytokines with the Bio-Plex system utilizes suspension array of 12 sets of beads (Bio-Plex Rat Cytokine 12-plex panel; Bio-Rad Laboratories, Hercules, CA, USA) internally dyed with different ratios of two spectrally distinct fluorochromes. Each set of beads is coupled with a monoclonal antibody raised against granulocyte macrophage-colony stimulating factor (GM-CSF), IFN-γ, interleukins (IL)-1α, 1β, 2, 4, 5, 6, 10, 12p70 and 13 and TNF-α. Briefly, the beads were successively incubated (1 h, shaking in the dark at room temperature) with duplicates of diluted standards and thawed serum samples (50 μL), followed by a further incubation (30 min, shaking in the dark at room temperature) with biotinylated detector antibodies (25 μL) and a

final incubation (10 min, shaking in the dark at room temperature) with phycoerythrin (PE)-conjugated streptavidin (50 μL; PE serves as the fluorescence indicator) in a 96-well plate. Between each incubation, beads were washed to remove unbound protein using wash buffer via magnetic separation (Bio-Plex Pro II wash station, Bio-Rad Laboratories, Hercules, CA, USA). Cytokine levels were measured on a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA). Principally, a red laser excites the beads, while a green laser excites PE to quantify the capture analyte. Eight-point standard curves were obtained and serum cytokine concentration values (expressed as pg/mL) were analysed by Bio-Plex Manager 6.0 Software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses

Statistical comparisons were conducted using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). All data sets were tested for normality of distribution using the Shapiro–Wilk statistic. Body weight and food intake data were analysed using a repeated measures analysis of variance (ANOVA) with LSD pair-wise comparisons post hoc test to compare the differences both among groups and within groups across the duration of the trial. All other metabolic data were analysed using a one-way ANOVA and Tukey's post hoc test to compare groups during each period; prior to (days 0–4) and post (days 5–8, 9, 10 or 11) saline or 5-FU injection. SBT, kill data, VH and CD, neutral mucin-secreting GC count, MPO activity and cytokines were analysed using a one-way ANOVA with Tukey's post hoc test. All parametric data were expressed as mean with their standard errors. DAI comparisons between groups each day were made using a Kruskal–Wallis test with Mann–Whitney U-tests and expressed as median (range). For all analyses, $P < 0.05$ was considered significant.

Results

Daily metabolic data

Prior to saline or 5-FU injection (days 0–4), all rats gained body weight ($P < 0.001$), with no significant differences among groups ($P > 0.05$; Supplemental Data 1). Following saline administration, all normal rats gained body weight on days 5–10, including those treated with OO and EO ($P < 0.001$). However, between days 6 and 10, EO treatment resulted in a reduced gain in body weight compared to normal controls (days 7 and 8; $P < 0.01$, days 6, 9 and 10; $P < 0.05$; Figure 1). A temporal effect was observed following 5-FU injection, such that body weight significantly decreased from days 6 to 8 and then began to increase from days 8 to 10 (6%; $P < 0.01$). Furthermore, in 5-FU-injected rats, body weight significantly decreased following OO and EO administration from days 6 to 9 (OO: 4%; EO: 5%) which then began to increase on day 10 ($P < 0.001$). Body weight was significantly lower in 5-FU-injected rats treated with EO compared to OO treatment on days 9 ($P < 0.05$) and 10 ($P < 0.05$; Figure 1).

During the period prior to saline or 5-FU injection (days 1–4), food intake was significantly lower ($P < 0.001$) following OO (23%) and EO (22%) administration compared to normal controls (Figure 2(a)). This effect was also observed throughout the post-saline injection period (days 5–10; $P < 0.001$; Figure 2(b)). 5-FU significantly reduced food intake between days 5 and 9 ($P < 0.001$) compared to normal controls (maximal decrease on day 8: 58%), which was normalized by day 10. In 5-FU-injected rats, OO and EO administration significantly decreased daily food intake compared to 5-FU controls (days 6–10; $P < 0.001$). A temporal effect was observed in all 5-FU groups, such that the lowest food intake occurred on day 8, which increased across days 8–10 ($P < 0.001$; Figure 2(b)).

Total water intake, urine and faecal output were not significantly affected by oil treatments or 5-FU across all kill groups for time periods prior to (days 0–4) and post (days 5–8, 9, 10 or 11) saline or 5-FU injection (data not shown).

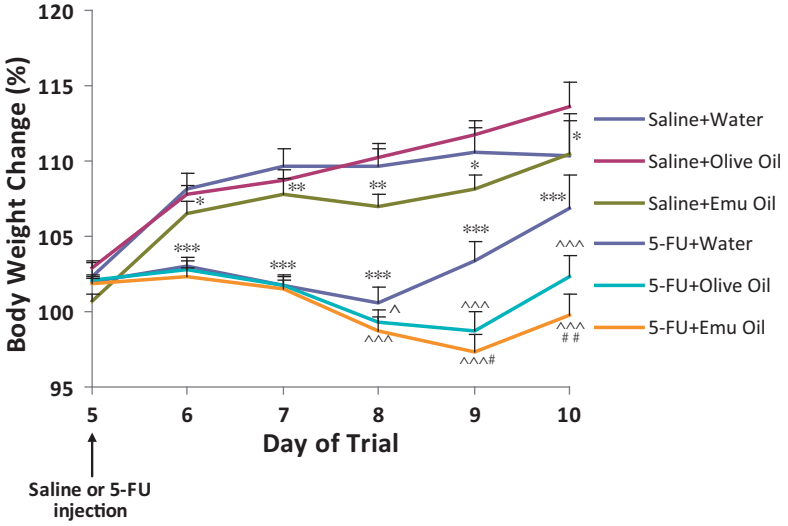

Figure 1 Daily body weight change of saline or 5-FU-injected rats on day 5, gavaged daily with 1 mL of water, olive oil or emu oil. Day 5 represents data from fasted rats. Body weight of fasted rats on kill days were not included in overall data analysis (kill days 8, 9, 10 and 11). Data are expressed as mean (body weight change from day 0 starting bodyweight; %) ± standard error of the mean. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared to saline + water; ^^^$P < 0.001$, ^$P < 0.05$ compared to 5-FU + Water; ##$P < 0.01$, #$P < 0.05$ compared to 5-FU + olive oil. (A color version of this figure is available in the online journal)

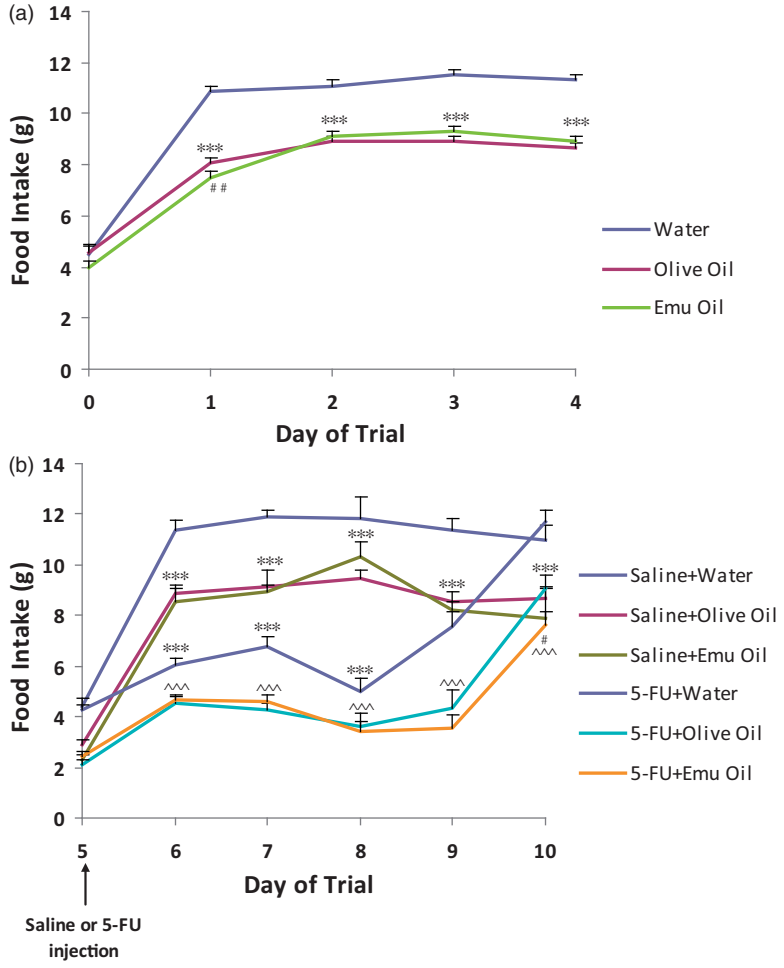

Figure 2 Daily food (18% casein-based diet) intake of (a) healthy and (b) saline or 5-FU-injected rats on day 5, gavaged daily with 1 mL of water, olive oil or emu oil. Days 0 and 5 represent data from fasted rats. Food intake of fasted rats on kill days was not included in overall data analysis (kill days 8, 9, 10 and 11). Data are expressed as mean (food intake; g) ±standard error of the mean. ***$P < 0.001$ compared to saline + water; ^^^$P < 0.001$ compared to 5-FU + Water; ##$P < 0.01$, #$P < 0.05$ compared to 5-FU + olive oil. (A color version of this figure is available in the online journal)

DAI

DAI was not significantly affected following OO or EO treatment in normal animals ($P > 0.05$; Table 2). 5-FU significantly increased DAI on days 7–11 compared to normal controls (days 7–10; $P < 0.001$, day 11; $P < 0.05$), with maximal disease activity on day 8 (median score 3 [range: 2–10]). In 5-FU-injected rats, OO significantly increased DAI on days 6 ($P < 0.05$) and 8–10 ($P < 0.001$) compared to 5-FU controls, attributed to increases in all DAI parameters (Table 2). EO administration in 5-FU-injected rats significantly increased DAI on days 6 ($P < 0.05$), 7 ($P < 0.01$) and 8 ($P < 0.05$) compared to 5-FU controls, primarily attributed to increased stool consistency scores. In 5-FU-injected rats, DAI was significantly lower following EO treatment compared to OO treatment on days 8 and 9 ($P < 0.001$).

^{13}C-SBT

Brush border sucrase activity was used as an indicator of the health and maturity of epithelial cells which line the small intestine (SI), assessed using the SBT. Oil treatment did not significantly affect overall functional SI health status in normal animals on any kill day ($P > 0.05$; Supplemental Data 2). On day 8, 5-FU significantly decreased percentage cumulative dose of ^{13}C at 90 min (%CD90) compared to healthy controls (67% decrease; $P < 0.05$); however, this was normalized by day 9 (Supplemental Data 2).

Visceral and gastrointestinal organ weights and lengths

Organ weights were expressed as a proportion of body weight. There were no significant differences in visceral organ weights including heart, lungs, left kidney, right kidney, spleen, liver, stomach, duodenum, caecum and colon ($P > 0.05$; data not shown). Thymus weight was significantly decreased in normal rats treated with OO (26%; $P < 0.01$) and EO (21%; $P < 0.05$) on day 10 and in 5-FU-injected rats (56% average; days 8 and 11; $P < 0.01$, days 9 and 10; $P < 0.001$) compared to normal controls (Supplemental Data 3).

SI (proximal jejunum to distal ileum) weight was significantly increased in normal rats treated with OO and EO compared to healthy controls on days 8 ($P < 0.001$) and 11 (OO;

Table 2 Disease activity index for each kill group (days 8, 9, 10 and 11) of rats gavaged daily with 1 mL of water, olive oil or emu oil and intraperitoneally injected with saline or 5-FU on day 5

	Saline			5-FU		
Day of trial	Water	Olive oil	Emu oil	Water	Olive oil	Emu oil
6	0 (0)	0 (0)	0 (0)	0 (0–1)	0 (0–2)^	0 (0–3)^
7	0 (0–2)	0 (0–1)	0 (0)	1 (0–3)***	1 (0–5)	1 (0–3)^^
8	0 (0–1)	0 (0–1)	0 (0–1)	3 (2–10)***	7 (0–11)^^^	4 (0–9)^###
9	0 (0–2)	0 (0–1)	0 (0)	2 (1–8)***	6.5 (0–10)^^^	3 (0–10)###
10	0 (0)	0 (0)	0 (0–1)	1 (0–5)***	3 (1–8)^^^	3 (0–6)
11	0 (0)	0 (0–1)	0 (0)	1 (0–2)*	2 (1–5)	2 (0–4)

Data are expressed as median disease activity index score (range).
***$P < 0.001$, *$P < 0.05$ compared to saline + water; ^^^$P < 0.001$, ^^$P < 0.01$, ^$P < 0.05$ compared to 5-FU + water; ###$P < 0.001$ compared to 5-FU + olive oil.

$P < 0.01$, EO; $P < 0.001$; Supplemental Data 4). 5-FU significantly increased SI weight on day 11 (28%; $P < 0.05$) compared to normal controls. Furthermore, SI weight was significantly greater in chemotherapy-injected rats administered OO (44%; day 11; $P < 0.001$) and EO (33%; days 8 and 10; $P < 0.001$, day 11; $P < 0.05$) compared to 5-FU controls (Supplemental Data 4).

SI length was significantly increased in normal rats treated with OO (day 8: 75.3 ± 2.2 cm and day 11: 77.3 ± 1.7 cm; $P < 0.05$) and EO (day 11: 77.5 ± 1.3 cm; $P < 0.01$) compared to healthy controls (day 8: 72 ± 0.9 cm and day 11: 73.3 ± 1.3 cm). EO treatment in 5-FU-injected rats resulted in an 8.5% increase in SI length compared to 5-FU controls on days 8 (74 ± 0.9 cm and 68.5 ± 1.3 cm respectively; $P < 0.05$) and 11 (82.6 ± 1.1 cm and 75.8 ± 1.5 cm respectively; $P < 0.001$). Furthermore, SI length was significantly greater in 5-FU-injected rats treated with OO (80 ± 1.1 cm), compared to 5-FU controls on day 11 ($P < 0.001$). Duodenum and colon lengths were not significantly affected by oil treatments or 5-FU ($P > 0.05$; data not shown).

VH and CD measurements

Day 8. VH was increased in the jejunum and ileum of normal rats following OO and EO administration, respectively, compared to healthy controls ($P < 0.05$; Figure 3(a)). 5-FU significantly decreased VH across all small intestinal sections compared to normal controls ($P < 0.001$). Only EO significantly increased VH in the ileum of 5-FU-injected rats (33%), compared to 5-FU controls and 5-FU-injected rats treated with OO (37%; $P < 0.05$; Figure 3(a)). In the mid-small intestine (JI), crypts were lengthened by both OO ($P < 0.001$) and EO ($P < 0.01$) treatment in healthy rats compared with normal controls. 5-FU significantly decreased CD in the jejunum (22%; $P < 0.01$) and JI (19%; $P < 0.05$), compared with normal controls (Figure 3(a)).

Day 9. EO increased VH in the jejunum ($P < 0.05$) and JI ($P < 0.001$) of healthy rats compared with normal controls (Figures 3(b) and 4). VH was decreased in the jejunum (15%) and JI (25%) following 5-FU administration compared with 5-FU controls ($P < 0.05$). This effect was normalized by EO in 5-FU-injected rats, leading to significantly lengthened jejunal (29%) and JI (45%) villi compared with 5-FU controls ($P < 0.01$; Figures 3(b) and 4). Furthermore, 5-FU-injected rats treated with EO resulted in significant lengthening of jejunal villi compared with OO treatment (21%; $P < 0.05$). JI crypts in healthy rats were significantly increased by EO compared to normal controls ($P < 0.05$). 5-FU significantly lengthened JI crypts compared with normal controls (20%; $P < 0.05$), which was further increased following OO ($P < 0.01$) and EO ($P < 0.001$) administration compared to 5-FU controls (Figure 3(b)). Moreover, 5-FU-injected rats administered EO exhibited significantly lengthened ileal crypts compared with 5-FU controls (25%; $P < 0.05$).

Day 10. VH was significantly increased following administration of both OO and EO in the jejunum and JI; and by EO in the ileum, compared with healthy controls ($P < 0.01$; Figure 3(c)). 5-FU administration in normal rats did not significantly affect VH on day 10 ($P > 0.05$). However, in 5-FU-injected rats, VH was greater following EO administration (17%; jejunum), both OO (21%) and EO treatment (24%; JI), and OO administration (29%; ileum), compared to 5-FU-controls ($P < 0.05$). JI CD was significantly increased following EO administration in healthy rats compared to controls ($P < 0.01$). On day 10, 5-FU significantly increased JI (26%; $P < 0.01$) and ileal (22%; $P < 0.05$) CD compared with normal controls, which was further increased following both OO (30%) and EO (35%; JI; $P < 0.01$) and OO (20%; ileum; $P < 0.05$) administration (Figure 3(c)).

Day 11. EO administration in normal rats significantly lengthened villi in the jejunum ($P < 0.01$) and JI ($P < 0.05$) compared to healthy controls (Figure 3(d)). Furthermore, villus lengthening was observed in the JI (27%) and ileum (34%) of 5-FU-injected rats following EO administration ($P < 0.01$). Both EO and OO significantly lengthened jejunal (OO; $P < 0.05$, EO; $P < 0.01$) and JI ($P < 0.05$) crypts in normal animals compared with controls. Jejunal crypt lengthening occurred following 5-FU-injection compared with normal controls (22%; $P < 0.01$), which was further lengthened by EO administration in 5-FU-injected rats compared to 5-FU controls (19%; $P < 0.01$). In 5-FU-injected rats, both OO ($P < 0.01$) and EO ($P < 0.001$) significantly increased JI

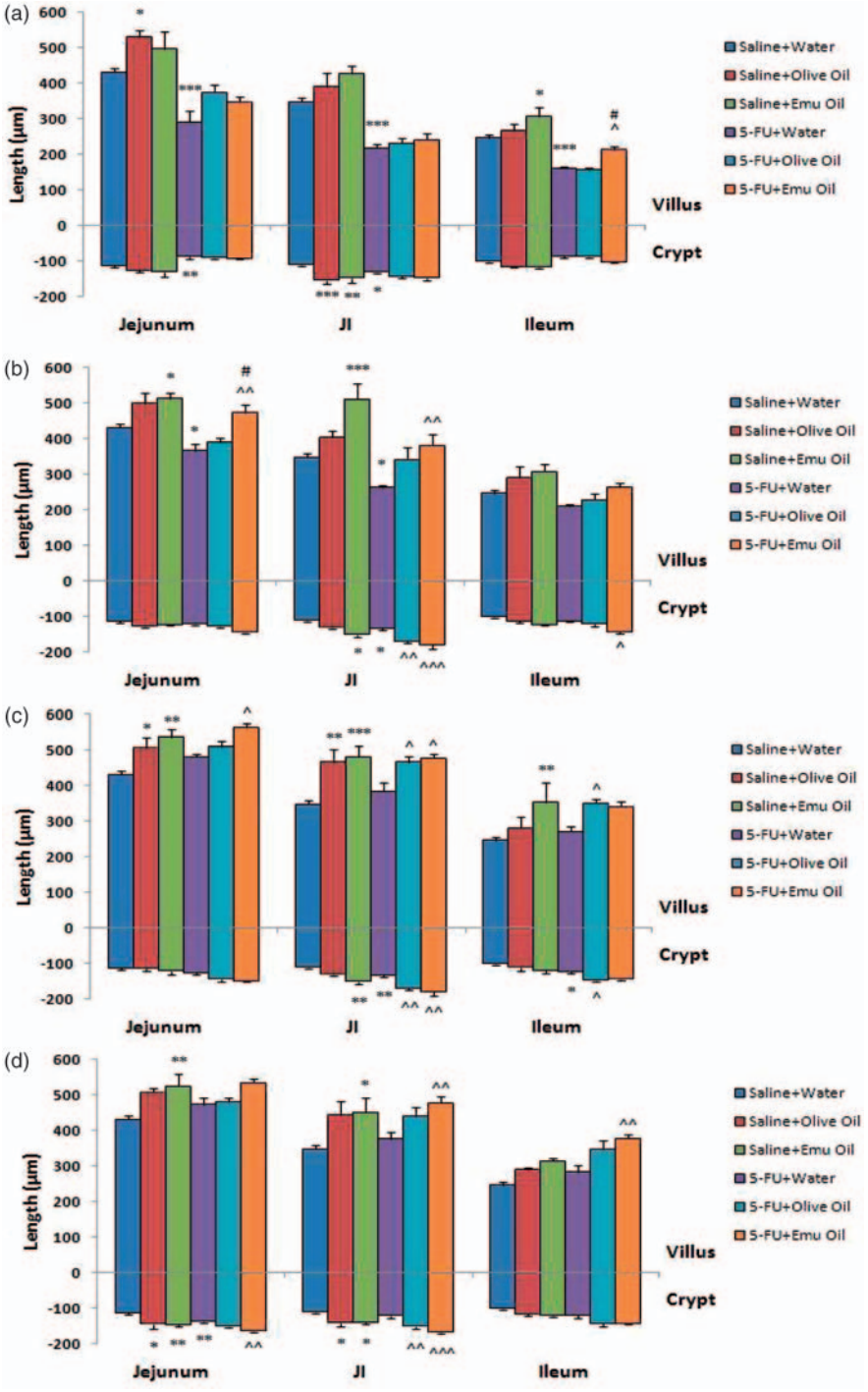

Figure 3 Villus height and crypt depth measurements in the rat jejunum, jejunum–ileum junction and the ileum for each kill group: (a) day 8, (b) day 9, (c) day 10 and (d) day 11. Rats were daily gavaged with 1 mL of water, olive oil or emu oil and intraperitoneally injected with saline or 5-FU on day 5. Data are expressed as mean (length; μm) ± standard error of the mean. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared to saline + water; ^^^$P < 0.001$, ^^$P < 0.01$, ^$P < 0.05$ compared to 5-FU + Water; #$P < 0.05$ compared to 5-FU + olive oil. (A color version of this figure is available in the online journal)

CD compared with chemotherapy-injected controls (Figure 3(d)).

Neutral mucin-secreting GCs

In the jejunum, 5-FU significantly decreased numbers of neutral mucin-secreting GCs on days 8 (41%; $P < 0.001$) and 9 (30%; $P < 0.05$) compared to normal controls (Figures 5(a) and 6). This decrease was also observed in the ileum on day 8 (47%; $P < 0.01$; Figure 5(b)). Both OO (75%) and EO (75%) significantly increased ileal GC count in 5-FU-injected rats on day 10 compared to 5-FU controls ($P < 0.01$). Moreover, on day 11, only EO significantly increased ileal GC numbers compared to 5-FU controls (54%; $P < 0.05$; Figure 5(b)).

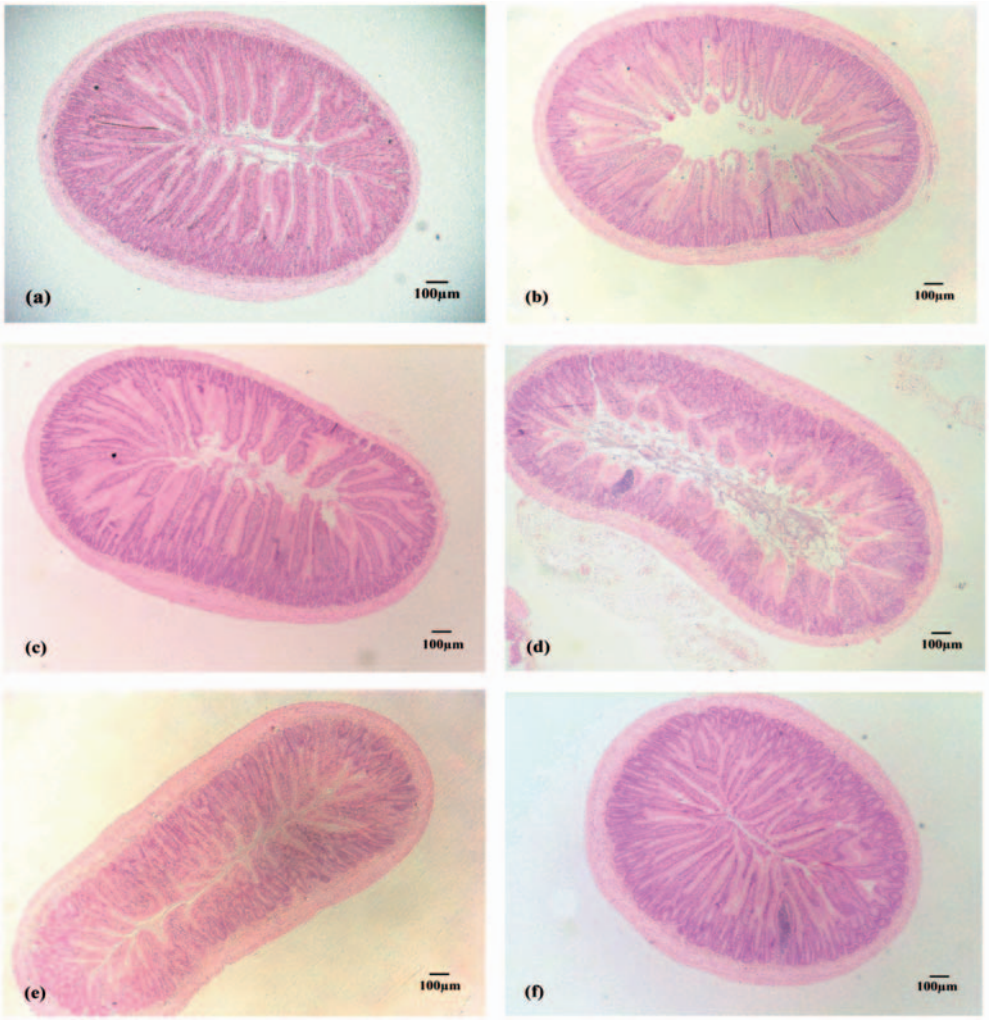

Figure 4 Representative photomicrographs of 4 μm rat jejunum sections on day 9 stained with haematoxylin and eosin (4x magnification): (a) saline + water, (b) saline + olive oil, (c) saline + emu oil, (d) 5-FU + water, (e) 5-FU + olive oil and (f) 5-FU + emu oil. (A color version of this figure is available in the online journal)

MPO activity

MPO activity, indicative of acute inflammation, was significantly elevated on days 8 (277%; $P < 0.001$) and 9 (137%; $P < 0.05$) in the jejunum following 5-FU administration compared to normal controls (Figure 7(a)). Both OO and EO treatment in 5-FU-injected rats significantly decreased MPO activity on days 8 (OO: 45%; $P < 0.05$, EO: 62%; $P < 0.001$) and 9 (OO: 71%; $P < 0.01$, EO: 83%; $P < 0.001$) compared to 5-FU controls. This effect was also observed on day 11 ($P < 0.05$; Figure 7(a)). In the ileum, 5-FU increased MPO levels 6-fold on day 8 compared to healthy controls ($P < 0.001$; Figure 7(b)). Only EO significantly reduced day 8 ileal MPO activity compared to 5-FU controls (55%) and 5-FU-injected rats treated with OO (50%; $P < 0.05$). Furthermore, in chemotherapy-injected rats, EO significantly decreased MPO levels on days 9 and 10 compared to 5-FU controls ($P < 0.05$; Figure 7(b)).

Cytokine analyses

Cytokine concentrations including GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70 and IL-13 and TNF-α were not significantly affected by either oil treatment or 5-FU administration at the day 8 time point ($P > 0.05$; Supplemental Data 5).

Discussion

Mucositis remains a significant problem faced by cancer patients undergoing chemotherapy and radiotherapy. The present study indicated a promotion of intestinal repair from chemotherapy-induced injury, through oral administration of EO.

Daily EO administration to normal rats resulted in decreased intake of 18% casein-based diet throughout the trial. This could have been accounted for by the lipid-rich constituents of EO and thus maintenance of overall caloric intake, as evidenced by the absence of changes in body weight compared to normal rats. Furthermore, OO elicited a similar reduction in food intake in normal animals, suggesting a non-specific oil effect. Consistent with a previous study by Abimosleh et al.,[19] EO increased SI weights in normal rats on days 8 and 10, relative to untreated controls, possibly

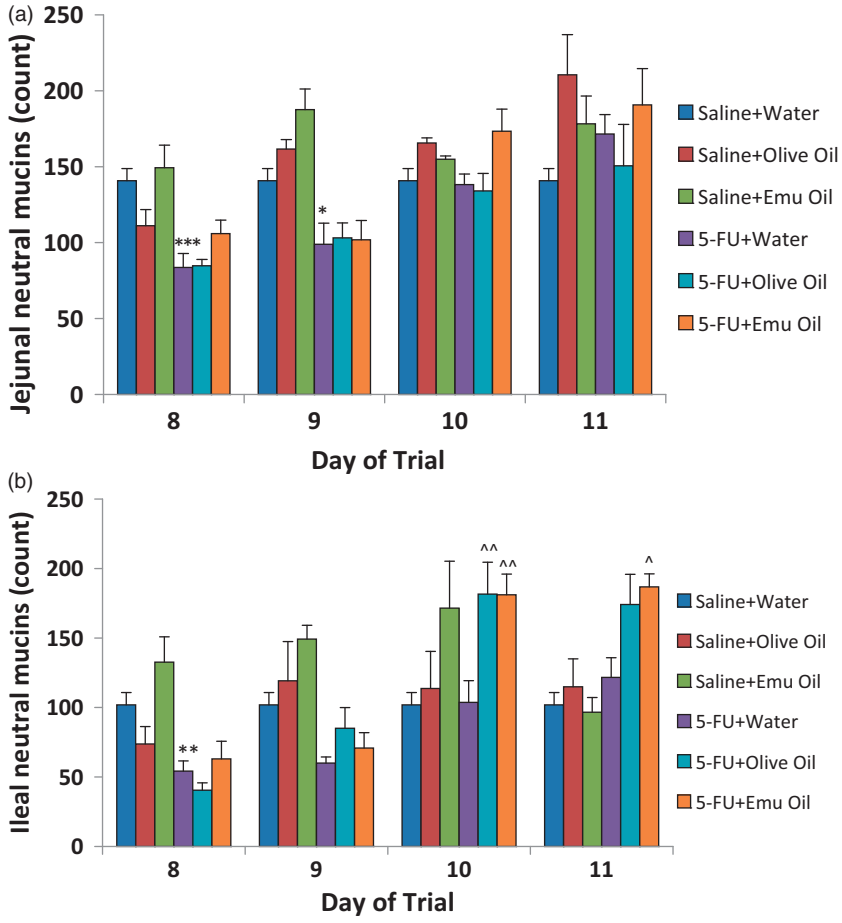

Figure 5 Neutral mucin-secreting goblet cell counts of rat (a) jejunum and (b) ileum for each kill group (days 8, 9, 10 and 11) following daily gavages of 1 mL water, olive oil or emu oil and intraperitoneal saline or 5-FU injections on day 5. Data are expressed as mean (goblet cell count) ± standard error of the mean. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared to saline + water; ^^$P < 0.01$, ^$P < 0.05$ compared to 5-FU + water. (A color version of this figure is available in the online journal)

attributed to concomitant increases in SI, villus and crypt lengthening. Additionally, the high oil viscosity could have resulted in adherence of EO to the luminal epithelial surface, thereby increasing SI weight.[19] Increases in SI weight on days 8 and 10 were observed following OO treatment, highlighting a generalized oil effect. In the current study, histological assessment revealed that EO consistently lengthened villi and crypts throughout the SI in normal rats. Although VH was increased in the ileum on days 8 and 10 following EO administration, the most prominent increase in overall mucosal thickening was evident in the more proximal (jejunum) to mid-SI (JI).

Villus and crypt lengthening could have resulted from increased crypt cell proliferation (hyperplasia) and hence villus elongation following differentiation and upward migration, a decrease in the rate of crypt cell apoptosis, cell hypertrophy or a combination of these factors.[19] Tazuke et al.[33] demonstrated an increase in small intestinal crypt cell proliferation following glutamine administration in a rat model of chemotherapy (cisplatin)-induced mucosal injury. In this study, the proliferative cell nuclear antigen, expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle (late G1- and S-phase), was assessed as an indicator of the proliferative state of the cell using immunostaining techniques. Furthermore, Gibson et al.[3] demonstrated that keratinocyte growth factor and the chemotherapeutic agent, methotrexate, synergistically increased apoptosis in intestinal crypts and breast cancer using tumour-bearing rats; assessed using the TUNEL assay. Accordingly, proliferation and apoptosis would be important parameters to define in future experimental studies of EO and chemotherapy, utilizing these well-established techniques.

Chang et al.[34] reported that diets containing a combination of fish oil (high in omega-3 FAs) and pectin (FO/P) resulted in a lower colonic tumour incidence in rats than diets rich in Corn oil (high in omega-6 FAs) and cellulose.FO/P was protective against colon cancer due to the induction of apoptosis and suppression of cell proliferation.[34,35] Moreover, pectin is a highly fermentable fibre that yields higher levels of the short-chain FA, butyrate, upon microbial fermentation, whereas cellulose is poorly fermented. It remains unclear whether the long-chain FAs present in EO would exert similar effects to butyrate. Although the omega-3 FAs present in EO (total approximately 1.1% [α-linolenic acid]; Table 1) are not as pronounced as fish oil (total approximately 30% [20:5 n−3: 18%, 22:6n−3: 12%][23]), future studies in the chemotherapy and cancer setting could benefit from further examination of EO in combination with pectin. The spontaneous tumour-

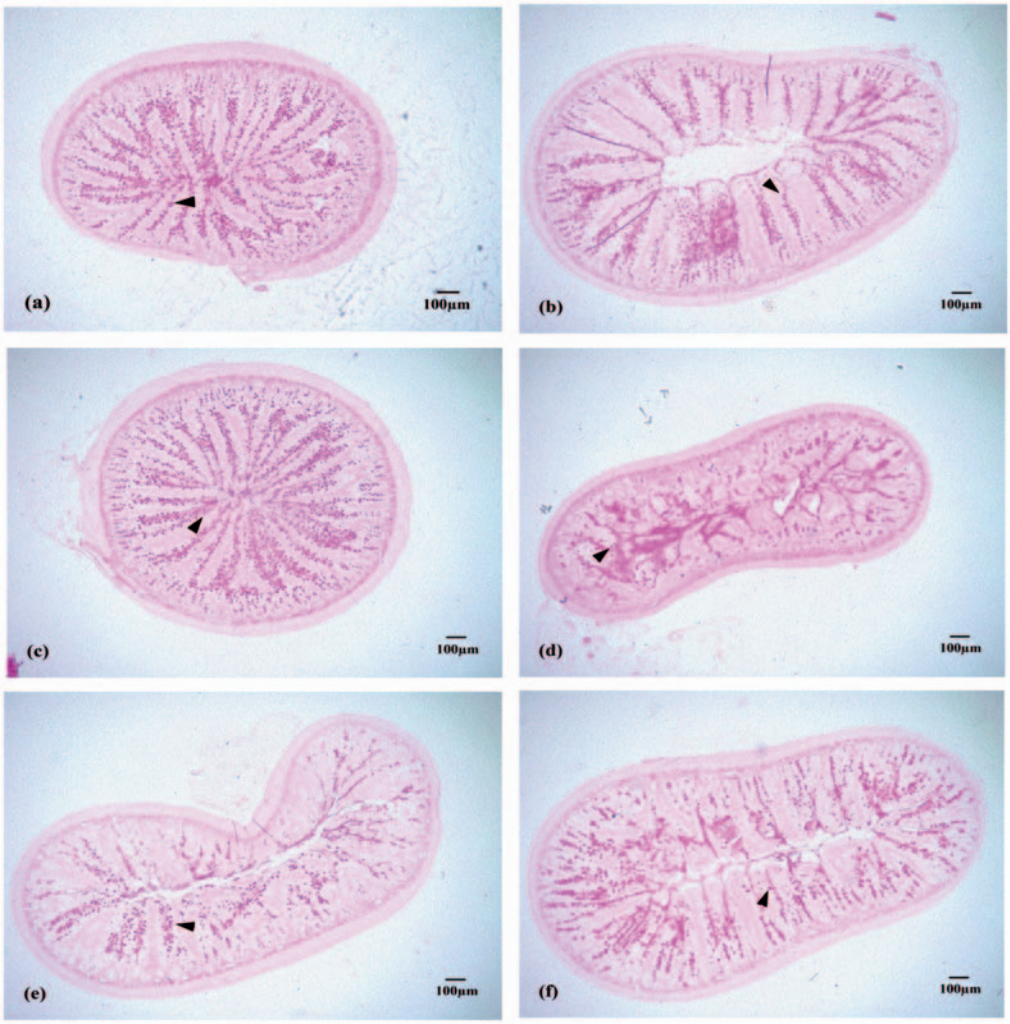

Figure 6 Representative photomicrographs of 4 μm rat jejunum sections on day 9 stained with periodic acid-Schiff reagent (4x magnification). Neutral mucin-secreting goblet cells stain pink-purple: (a) saline + water, (b) saline + olive oil, (c) saline + emu oil, (d) 5-FU + water, (e) 5-FU + olive oil and (f) 5-FU + emu oil. (A color version of this figure is available in the online journal)

bearing rat model (Dark Agouti mammary adenocarcinoma) would be appropriate to assess the impact of EO and pectin on breast tumour growth coincident with the enhancement of small intestinal repair following chemotherapy.

Proximal to mid-SI changes in mucosal thickness suggested that EO metabolism in the duodenum and subsequent bioactivity of EO downstream could have led to the observed jejunal and JI mucosal lengthening. However, Abimosleh et al.[19] demonstrated increased CD in the distal colon following EO administration to normal rats, highlighting its activity throughout the gastrointestinal tract. Despite these alterations of SI parameters, overall functional health status of the SI in the current study was maintained by EO as evidenced by the SBT, supporting the safety of EO as an oral administration.

As previously reported,[11,17,36,37] injection of 5-FU resulted in significant weight loss, reduction of food intake, increased DAI, decreased thymus weight and severe SI injury, three days post-mucositis induction (day 8). This damage was characterized by a reduction of brush border sucrase activity, as assessed indirectly by the SBT,[30] decreased protective barrier function via a reduction of neutral mucin-secreting GCs[38] and increased MPO activity indicative of acute inflammation.[39] Furthermore, chemotherapy-induced damage manifested histologically as shortened villi and crypts, due to reduced cellularity.[6,11] However, consistent with previous studies, during the recovery phase of mucositis (5 days post-5-FU injection; day 10), VH and CD elongated to a greater extent than normal, as a compensatory mechanism of stimulated cell proliferation to repair damage sustained from 5-FU administration.[24,40] Moreover, at this repair stage, SI injury subsided, as evidenced by the normalized SBT, GC count and MPO activity. Interestingly, in the present study, pro-inflammatory cytokines including TNF-α, IFN-γ, IL-1β and IL-6 known to be directly involved in the pathogenesis of mucositis, were not elevated in serum three days post-mucositis induction. Logan et al.[41] demonstrated that at this time point following 5-FU injection (150 mg/kg) in female Dark Agouti rats, alimentary tract (oral, jejunal and colonic) tissue expression of nuclear factor-κB, TNF-α, IL-1β and IL-6, as indicated by the intensity of immunohistochemical staining, was not altered.[41] This

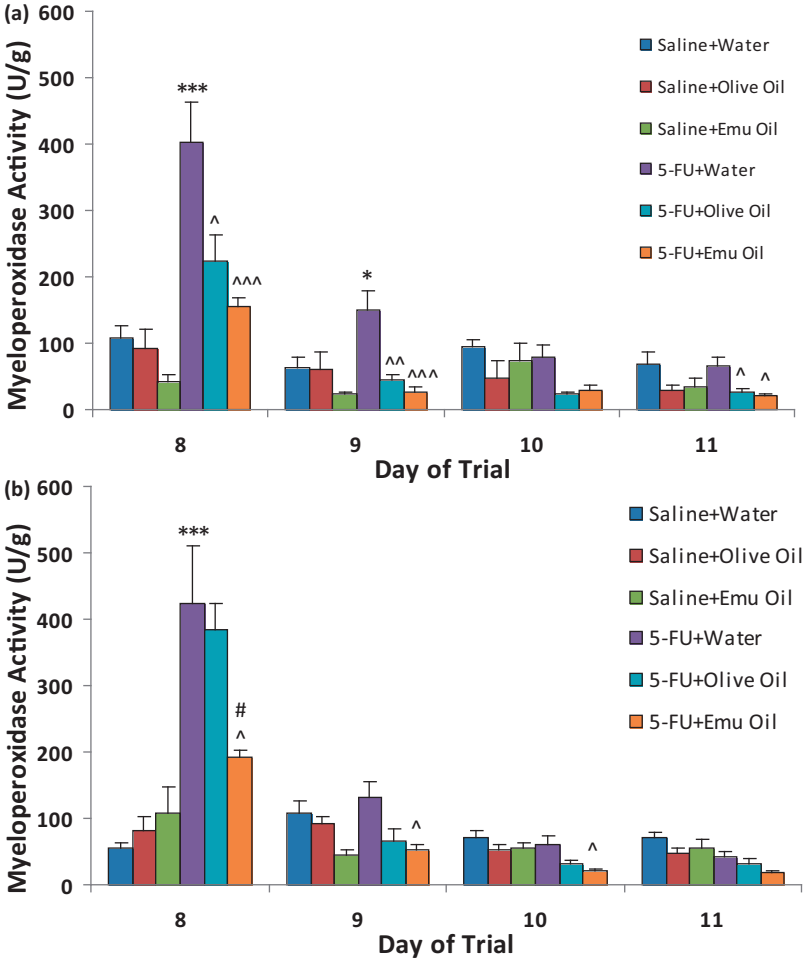

Figure 7 Myeloperoxidase activity indicative of acute inflammation in the rat (a) jejunum and (b) ileum for each kill group (days 8, 9, 10 and 11) following daily gavages of 1 mL water, olive oil or emu oil and intraperitoneal saline or 5-FU injections on day 5. Data are expressed as mean (myeloperoxidase activity; units per gram; U/g) ± standard error of the mean. ***$P < 0.001$, *$P < 0.05$ compared to saline + water; ^^^$P < 0.001$, ^^$P < 0.01$, ^$P < 0.05$ compared to 5-FU + Water; #$P < 0.05$ compared to 5-FU + olive oil. (A color version of this figure is available in the online journal)

may suggest that the T-helper 1 (Th1) and Th2 inflammatory response was not yet elicited three days post-mucositis induction (day 8). Future experimental studies could benefit from a complete time course of cytokine profiling within intestinal tissue samples.

In the present study, EO gave indications of accelerated repair in the chemotherapy-damaged intestine. On day 8 during maximal intestinal damage, only EO administration maintained VH in the ileum and by day 9, only EO was able to significantly increase jejunal and JI villi compared with untreated 5-FU controls. Previous studies have indicated an adaptive response of mucosal growth in an adjacent region following severe intestinal damage or small bowel resection (SBR).[42] Haxhija et al.[43] reported that proximal SBR resulted in ileal enterocytes becoming hypertrophic. However, these same authors noted that jejunal cells became hyperplastic in response to ileal resection.[43]

5-FU preferentially damages the proximal SI (jejunum) due to the higher cell turnover rate in this region.[44] Therefore, in the current study, the observed compensatory crypt and subsequent villus elongation in the JI and ileum may have represented cell hypertrophy which could potentially be quantified in further studies. Indeed, a compensatory mucosal thickening was evident in the jejunum by day 11. This effect was consistently enhanced by EO administration, suggesting an acceleration of the repair process. Although OO exerted similar effects, it failed to maintain mucosal thickness, unlike EO, further supporting a specific EO effect.

The increased acute inflammation, indicated by enhanced MPO activity in 5-FU controls on days 8 and 9, was significantly lowered following EO administration. Lindsay et al.[24] also reported decreased acute SI inflammation four days after 5-FU-injection with daily EO administration. Furthermore, the anti-inflammatory properties of topically applied EO have been well-documented.[22,23,45,46]

The mechanism by which EO exerts its anti-inflammatory effects remains unclear. One potential mechanism could be attributed to the omega-3 and -9 FAs present in EO. Omega-3 FAs typically reduce inflammation both directly (via down-regulation of the inflammatory eicosanoid pathways, which produce thromboxane B2, prostaglandin E2 and leukotriene B4) and indirectly (by altering the expression of inflammatory genes through effects on transcription factor activation),[47] whereas omega-9 FAs are believed to promote

anti-inflammatory effects by inhibition of macrophage migration. Moreover, Yoganathan et al.[23] indicated that the anti-inflammatory properties of EO are not fully accounted for by the FA profile alone.[23] Components in the non-triglyceride fraction of EO including carotenoids, flavones, tocopherols and skin-permeation enhancing factors may confer additional anti-inflammatory and antioxidant effects.[23,48] Bennett et al.[48] supported these antioxidant claims in vitro demonstrating that EO scavenges free radicals and exerts a protective role against oxidative damage. These antioxidants may have impacted on levels of damaging reactive oxygen species which are generated in the first stage of mucositis. MPO combines with hydrogen peroxide during the de-granulation process and produces hypochlorous acid and various radical oxygen species.[49] These toxic agents are responsible for the damage to cells, tissues and blood vessels[8] and contribute to the increase in levels of secondary messengers, transcription factors such as NF-κB[6] and subsequent pro-inflammatory cytokines.

Promotion of repair from injury could represent a new mechanism of action for EO, suggesting potential as an adjunct to conventional treatment approaches for cancer management. Future studies should focus on the identification of bioactive EO constituents to gain a more thorough understanding of its mechanism of action, thereby facilitating the development of more potent EO preparations.

Author contributions: SM conducted all animal trials, experiments and data analyses and prepared the final manuscript. CDT assisted with experimental planning and analysis of data. GSH contributed to the experimental design, analysis of data and manuscript preparation.

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