Emu Oil Promotes Intestinal Repair in Rat Models

of Enteric Inflammation

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ABSTRACT

Several disorders of the gastrointestinal (GI) tract including ulcerative colitis, chemotherapy-induced mucositis and non-steroidal anti-inflammatory drug (NSAID)-induced enteropathy, are characterised by inflammation, ulceration, mucosal damage and malabsorption. Treatment options are variably effective, highlighting the need to broaden therapeutic approaches, including adjunctive strategies. Emu Oil, derived from subcutaneous and retroperitoneal Emu adipose tissue, is a rich source of fatty acids (FA). Despite limited rigorous scientific studies, topically applied Emu Oil has demonstrated potent anti-inflammatory properties *in vivo*. Previously, orally administered Emu Oil improved intestinal architecture in a rat model of mucositis, with early indications of enhanced intestinal repair. Accordingly, this thesis investigated the effects of orally administered Emu Oil in rat models of colitis (colonic damage), NSAID-enteropathy (small intestinal [SI] damage) and on the time course of SI repair in chemotherapy-induced mucositis.

In the current study, Emu Oil improved colonic tissue damage associated with dextran sulphate sodium-induced colitis in Sprague Dawley rats and facilitated the repair process **(Chapter 2)**. Improvements were indicated histologically by reduced intestinal damage severity scores and enhanced crypt compensatory elongation in the colon. These findings suggested the potential for Emu Oil to augment conventional treatment approaches for colitis. The effectiveness of Emu Oil in the colon provided impetus to further investigate Emu Oil action proximally, in the SI. In a rat model of chemotherapy (5-Fluorouracil; 5-FU)-induced mucositis, Emu Oil maintained SI villus height and crypt depth during the phase of maximal damage **(Chapter 3)**. This was followed by an enhanced compensatory mucosal thickening, suggesting an acceleration of the repair process. Furthermore, Emu

Oil significantly decreased myeloperoxidase (MPO) activity, indicative of acute inflammation, in the jejunum and ileum of 5-FU-injected rats. Potent anti-inflammatory properties of Emu Oil were reaffirmed in NSAID (Indomethacin)-induced enteropathy, whereby MPO activity in the jejunum and ileum of Indomethacin-treated rats was markedly decreased following Emu Oil administration **(Chapter 4)**.

Treatments for diseases such as coronary artery disease and GI disorders seek to minimise oxidative damage by free radicals through the use of antioxidants. Oils derived from ratites (flightless birds) predominantly comprise FA varying in composition between ratite species. The influence of farm location, rendering method, duration and storage mode was investigated for free radical scavenging activity (RSA) against 2,2-diphenyl-1-picryl hydracyl and primary oxidation status of Ratite Oils **(Chapter 5)**. Emu Oil conferred the greatest RSA compared to Ostrich and Rhea Oil, potentially attributed to its high unsaturated FA: saturated FA ratio and non-triglyceride fraction minor constituents. Rendering and storage variables impacted on Emu Oil RSA and primary oxidation.

This thesis identified Emu Oil as a safe, renewable and economical means to augment pharmaceutical options for GI disorders. A new mechanism of action for Emu Oil could represent a promotion of repair from injury together with decreased SI inflammation. This suggests potential for Emu Oil as an adjunct to conventional treatment approaches for colitis, cancer management and long-term NSAID usage.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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(6) R Raghu Nadhanan, **SM Abimosleh**, YW Su, MA Scherer, GS Howarth, CJ Xian. Dietary emu oil supplementation suppresses 5-fluorouracil chemotherapy-induced inflammation, osteoclast formation, and bone loss. *American Journal of Physiology Endocrinology and Metabolism* 2012 Jun;302(11):E1440-9

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Suzanne Mashtoub Abimosleh   **Date**

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My beautiful mum, Ann Asma Mashtoub, to whom I am eternally indebted; without her unconditional love, continual encouragement to strive for the best, strength (and delicious food!), I would be forever lost. To my dad, Steve Zouhair Mashtoub, my amazing siblings Nawal, Joanne, Kamahl, Leanne, brother-in-law Samir and my niece, princess Eva; their love, support and friendship have been my guiding light.

~ In loving memory of my dear friend Pat ~

"If we never had a cloudy day with wind and rain and thunder,
we would never see a rainbow and rejoice in all its wonder…" Pat Westin

PUBLICATIONS ARISING FROM THIS THESIS

SM Abimosleh, CD Tran, GS Howarth. Emu Oil: a novel therapeutic for disorders of the gastrointestinal tract? *Journal of Gastroenterology and Hepatology* 2012 May;27(5):857-61

SM Abimosleh, RJ Lindsay, RN Butler, AG Cummins, GS Howarth. Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. *Digestive Diseases and Sciences* 2012 Apr;57(4):887-96

R Raghu Nadhanan, **SM Abimosleh**, YW Su, MA Scherer, GS Howarth, CJ Xian. Dietary emu oil supplementation suppresses 5-fluorouracil chemotherapy-induced inflammation, osteoclast formation, and bone loss. *American Journal of Physiology Endocrinology and Metabolism* 2012 Jun;302(11):E1440-9

AK Samanta, VA Torok, NJ Percy, **SM Abimosleh**, GS Howarth. Microbial fingerprinting detects unique bacterial communities in the faecal microbiota of rats with experimentally-induced colitis. *Journal of Microbiology* 2012 Apr;50(2):218-2

KY Cheah, SEP Bastian, TMV Acott, **SM Abimosleh**, KA Lymn, GS Howarth. Grape Seed Extract Reduces Selected Disease Markers in the Proximal Colon of Dextran Sulphate Sodium-Induced Coltits in Rats. *Digestive Diseases Sciences* 2012 Nov (epub ahead of print) DOI 10.1007/s10620-012-2464-1

CD Tran, R Katsikeros, **SM Abimosleh**. Current and Novel Treatments for Ulcerative Colitis. *Ulcerative Colitis from Genetics to Complications* InTech 2012: 189-211

SM Abimosleh, DC Bennett, CD Tran, GS Howarth. Processing of Ratite Oils Affects Primary Oxidation Status, Resulting in Greater Radical Scavenging Properties for Emu Oil Compared to Rhea and Ostrich Oils. *Journal of Food Science* 2012; Under review

SM Abimosleh, CD Tran, GS Howarth. Emu Oil Expedites Small Intestinal Repair Following 5-Fluorouracil-Induced Mucositis in Rats. *Experimental Biology and Medicine* 2012; Under review

SM Abimosleh, CD Tran, GS Howarth. Emu Oil Reduces Small Intestinal Inflammation in the Absence of Clinical Improvement in a Rat Model of Indomethacin-Induced Enteropathy. *Evidence-Based Complementary and Alternative Medicine* 2012; Under review

Publications arising from conferences

SM Abimosleh, Z Lotfollahi, J Papini, L Hoyle, D Stomaci, E Penascoza, CD Tran, KA Lymn, RN Butler and GS Howarth. Orally-administered Emu Oil maintains intestinal goblet cell numbers and improves barrier function in a rat model of chemotherapy-induced mucositis.

1. Multinational Association of Supportive Care in Cancer (MASCC), New York, USA. *Supportive Care in Cancer* 2012; 20(1):627

2. Australian Gastroenterology Week (AGW), Adelaide, South Australia. *Journal of Gastroenterology and Hepatology* 2012; 27(4):17

AK Samanta, VA Torok, NJ Percy, **SM Abimosleh** and GS Howarth. Microbial fingerprinting detects unique bacterial communities in the faecal microbiota of rats with experimentally-induced colitis. AGW, Adelaide, South Australia. *Journal of Gastroenterology and Hepatology* 2012; 27(4):28

SM Abimosleh, CD Tran and GS Howarth. Emu Oil enhances histological repair in a rat model of chemotherapy-induced intestinal mucositis. Joint Annual Scientific Meeting of the Nutrition Society of New Zealand and the Nutrition Society of Australia, Queenstown, New Zealand. *Australasian Medical Journal* 2011; 4(12):104-716

2ⁿᵈ Prize for Best Oral Presentation

SM Abimosleh, CD Tran, GS Howarth. Emu Oil Promotes Repair From Chemotherapy-Induced Intestinal Mucositis In Rats

1. MASCC, Athens, Greece. *Supportive Care in Cancer* 2011; 19(2):67-370

2. AGW, Brisbane, Queensland. *Journal of Gastroenterology and Hepatology* 2011; 26:15-21

SM Abimosleh, RN Butler, GS Howarth. Effects of emu oil during the recovery phase of chemotherapy-induced intestinal mucositis in rats.

1. AGW, Gold Coast, Queensland. *Journal of Gastroenterology and Hepatology* 2010; 25:A2-A17

2. Nutrition Scoiety of Australia, Perth, WA. *Australasian Medical Journal* 2010; 3

S Mashtoub, RJ Lindsay, KA Lymn, MS Geier, RN Butler, GS Howarth. Emu Oil Improves Parameters of Intestinal Integrity in Rat Models of Inflammatory Bowel Disease and Intestinal Mucositis. MASCC, Rome, Italy. *Supportive Care in Cancer* 2009; 17(7):857-1039

M Vu, B Hoang, XD Ding, M Sultani, **S Mashtoub**, RJ Lindsay, KA Lymn, RN Butler, GS Howarth. A safety study of emu oil as a potential adjunctive treatment for ulcerative colitis and mucositis. AGW, Sydney, NSW. *Journal of Gastroenterology and Hepatology* 2009; 24:A239-A247

Poster of Merit Prize

S Mashtoub, RJ Lindsay, KA Lymn, TWV Acott, R Yazbeck, AG Cummins, RN Butler, GS Howarth. Emu oil increases crypt depth but only minimally affects other indicators of colonic integrity in a rat model of colitis. AGW, Sydney, NSW. J Gastro Hepatol 27: A239-A247 (2009)

THESIS STRUCTURE

This thesis is presented as a 'Thesis by Publication' and includes a combination of published manuscripts and papers under review. Each manuscript is formatted according to the journal specifications.

Furthermore, additional publications arising from the research described in this thesis are included as Appendices.

CHAPTER 1

REVIEW OF THE LITERATURE

CONTEXT STATEMENT

The work contained in this chapter has been published in the *Journal of Gastroenterology and Hepatology.*

SM Abimosleh, CD Tran, GS Howarth. Emu Oil: a novel therapeutic for disorders of the gastrointestinal tract? *Journal of Gastroenterology and Hepatology* 2012 May;27(5):857-61

Chapter 1 provides an overview of intestinal disorders characterised by inflammation and identifies the need to broaden therapeutic approaches to attenuate the inflammatory response and facilitate mucosal healing. Furthermore, this chapter explores Emu Oil literature, leading to identification of Emu Oil as potential treatment option for inflammatory intestinal disorders.

STATEMENT OF AUTHORSHIP

Emu Oil: A novel therapeutic for disorders of the gastrointestinal tract?

Suzanne Mashtoub ABIMOSLEH (Candidate)

Conducted a thorough review of the literature, prepared manuscript.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12

Cuong D. Tran

Contributed to manuscript preparation.

I hereby certify that the statement of contribution is accurate.

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Gordon S. Howarth

Contributed to manuscript preparation and revision.

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Signed   Date
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Abimosleh, S.M., Tran, C.D. & Howarth, G.S. (2012) Emu oil: a novel therapeutic for disorders of the gastrointestinal tract?
Journal of Gastroenterology and Hepatology, v. 27(5), pp. 857-861

CHAPTER 2

EMU OIL INCREASES COLONIC CRYPT DEPTH IN A RAT

MODEL OF ULCERATIVE COLITIS

CONTEXT STATEMENT

The work contained in this chapter has been published in *Digestive Diseases and Sciences.*

SM Abimosleh, RJ Lindsay, RN Butler, AG Cummins, GS Howarth. Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. *Digestive Diseases and Sciences* 2012 Apr;57(4):887-96

Previous literature identified topically applied Emu Oil as a potent anti-inflammatory agent in a mouse model of Croton Oil-induced auricular swelling (Yoganathan *et al*. 2003) and experimentally-induced polyarthritis (Snowden and Whitehouse 1997). More recently, orally administered Emu Oil improved small intestinal damage associated with chemotherapy-induced mucositis (Lindsay *et al*. 2010). This prompted further investigation into the effects of orally administered Emu Oil in the context of colitic damage, as described in **Chapter 2**.

The work contained in this chapter formed the basis of my Honours research, awarded in 2008. This work was completed and the manuscript was published in 2012 during the tenure of my PhD. It was important to include this manuscript as **Chapter 2** of my thesis to identify dose-dependent effects of Emu Oil and for subsequent applications in **Chapters 3 and 4**. Furthermore, this manuscript provides greater insight into the potential efficacy of Emu Oil in the colon, highlighting its bioactivity in the distal GI tract.

Further publications arising from this chapter included **Appendices 1, 2 and 3**. **Appendix 1** describes the partial protective properties of grape seed extract assessed histologically, utilising the same rat model of colitis to that employed in **Chapter 2**. Furthermore,

Appendix 2 identified the changes in microbiota associated with colitis in rats, using faecal microbial profiling. Restoring microbial homeostasis by targeting colitis-associated bacteria was implicated as a novel therapeutic strategy for colitis. **Appendix 3** is a published book chapter which provides a thorough literature review on the current and novel treatments for ulcerative colitis, including animal models.

Appendix 1

KY Cheah, SEP Bastian, TMV Acott, **SM Abimosleh**, KA Lymn, GS Howarth. Grape Seed Extract Reduces Selected Disease Markers in the Proximal Colon of Dextran Sulphate Sodium-Induced Coltits in Rats. *Digestive Diseases Sciences* 2012 Nov (epub ahead of print) DOI 10.1007/s10620-012-2464-1

Appendix 2

AK Samanta, VA Torok, NJ Percy, **SM Abimosleh**, GS Howarth. Microbial fingerprinting detects unique bacterial communities in the faecal microbiota of rats with experimentally-induced colitis. *Journal of Microbiology* 2012 Apr;50(2):218-2

Appendix 3

CD Tran, R Katsikeros, **SM Abimosleh**. Current and Novel Treatments for Ulcerative Colitis. *Ulcerative Colitis from Genetics to Complications* InTech 2012: 189-211

STATEMENT OF AUTHORSHIP

Emu oil Increases Colonic Crypt Depth in a Rat Model of Ulcerative Colitis

Digestive Diseases and Sciences 2012; 57: 887-96

Suzanne Mashtoub ABIMOSLEH (Candidate)

Conducted all animal trials and experimental work, analysed data, interpreted results, prepared figures and manuscript.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12

Ruth J. Lindsay

Assisted with animal trials, experimental work and data analyses.

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Ross N. Butler

Contributed to experimental conceptualisation and design, data interpretation.

I hereby certify that the statement of contribution is accurate.

Signed   Date 27/11/2012

Adrian G. Cummins

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Signed   Date ⟶5/11/2012

Gordon S. Howarth

Contributed to experimental conceptualisation and design, data interpretation, critical manuscript evaluation.

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Signed   Date 27/11/12

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Abimosleh, S.M., Lindsay, R.J., Butler, R.N., Cummins, A.G. & Howarth, G.S. (2012) Emu oil increases colonic crypt depth in a rat model of ulcerative colitis.
Digestive Diseases and Sciences, v. 57(4), pp. 887-896

CHAPTER 3

EMU OIL EXPEDITES SMALL INTESTINAL REPAIR FOLLOWING

5-FLUOROURACIL-INDUCED MUCOSITIS IN RATS

CONTEXT STATEMENT

The work contained in this chapter is currently under review in *Experimental Biology and Medicine.*

SM Abimosleh, CD Tran, GS Howarth. Emu Oil Expedites Small Intestinal Repair Following 5-Fluorouracil-Induced Mucositis in Rats. *Experimental Biology and Medicine* 2012; Under review

The effectiveness of Emu Oil in the colon **(Chapter 2)** provided impetus to further investigate Emu Oil action proximally, in the SI. Furthermore, Lindsay *et al.* (2010) in a rat model of mucositis, proposed a potential mechanism of action for Emu Oil, speculating on the possibility of a more rapid rate of recovery. **Chapter 3** therefore describes the effect of orally-administered Emu Oil on the time course of intestinal repair in a rat model of chemotherapy-induced mucositis.

The experimental model of mucositis employed in **Chapter 3** was also utilised in a jointly-authored manuscript published in *Cancer, Biology and Therapy.*

Wright TH, Yazbeck R, Lymn KA, Whitford EJ, Cheah KY, Butler RN, Feinle-Bisset C, Pilichiewicz AN, **Mashtoub S**, GS Howarth. The herbal extract, Iberogast, improves jejunal integrity in rats with 5-Fluorouracil (5-FU)-induced mucositis. *Cancer, Biology and Therapy* 2009 May;8(10):923-9

Appendix 4 used bone extracted from the Chapter 4 animal trial. The manuscript demonstrated that Emu Oil could counter chemotherapy (5-FU)-induced inflammation in

bone, preserved osteoblasts and suppressed osteloclast formation. Overall, Emu Oil was identified as a useful agent in preventing chemotherapy-induced bone loss.

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Appendix 4

R Raghu Nadhanan, **SM Abimosleh**, YW Su, MA Scherer, GS Howarth, CJ Xian. Dietary emu oil supplementation suppresses 5-fluorouracil chemotherapy-induced inflammation, osteoclast formation, and bone loss. *American Journal of Physiology Endocrinology and Metabolism* 2012 Jun;302(11):E1440-9

STATEMENT OF AUTHORSHIP

Emu Oil Expedites Small Intestinal Repair Following 5-Fluorouracil-Induced Mucositis in Rats

Experimental Biology and Medicine 2012; Under review

Suzanne Mashtoub ABIMOSLEH (Candidate)

Conducted all animal trials, experimental work, analysed data, interpreted results, prepared figures and manuscript.

I hereby certify that the statement of contribution is accurate.

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Cuong D. Tran

Contributed to experimental conceptualisation and design, data interpretation.

I hereby certify that the statement of contribution is accurate.

Signed   Date
                                                                    27/11/12

Gordon S. Howarth

Contributed to experimental conceptualisation and design, data interpretation, critical manuscript evaluation.

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Emu Oil Expedites Small Intestinal Repair Following 5-Fluorouracil-Induced Mucositis in Rats

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Short Title: Emu Oil expedites intestinal repair in mucositis

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 (1ml), 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 JI (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 Emu Oil, suggesting potential as an adjunct to conventional treatment approaches for cancer management.

Keywords: chemotherapy, intestinal mucositis, Emu Oil, repair, rat

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, diarrhea and severe abdominal pain [2, 5].

The pathogenesis of mucositis is believed to be a 5 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 tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β) and interleukin-6 (IL-6) eliciting further tissue damage[6]. 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[9]. Recent studies into mucositis treatment have focused on hormones, growth factors and cytokines which modulate immune responses, cell proliferation and apoptosis[1]. Many naturally-sourced compounds have suggested potential such as grape seed extract,[10] attributed to proanthocyanidin-mediated antioxidant and anti-inflammatory effects, certain probiotics[11, 12] which modulate the microbiota and inhibit pro-inflammatory cytokine expression and even plant-extracts such as Iberogast[13].

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[14, 15] and the New Zealand Green-Lipped mussel extract, Lyprinol[16, 17]. 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[18, 19]. Indigenous Australians first used Emu Oil to facilitate wound healing, pain alleviation and treatment of inflamed joints[20, 21]. Topical application of Emu Oil to mice with adjuvant-induced inflammation decreased levels of TNF-α and other pro-inflammatory cytokines[22], involved in the pathogenesis of mucositis[6]. In a preliminary study in rats, Abimosleh *et al.*[18] indicated that orally-administered Emu Oil improved selected parameters associated with the manifestation of experimentally-induced colitis, characterized by inflammation and ulceration of the large bowel. Furthermore, Lindsay *et al.,*[23] in a rat model of mucositis, proposed a potential mechanism

of action for Emu Oil, speculating on the possibility of a more rapid rate of recovery during the recovery phase of mucositis.

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Accordingly, we hypothesized that orally-administered Emu Oil would accelerate recovery of the intestine following chemotherapy damage. To this end, we investigated the effects of Emu Oil 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 (120-140g) were individually housed in metabolism cages (Tecniplast, Exton, PA, USA), at room temperature with a light:dark cycle of 12 hours. All rats were given *ad libitum* access to food (standard 18% casein-based diet[24]) and water, and were acclimatized for 2 days prior to experimentation. Animal experimentation was conducted across several identical trials in which lower numbers of rats from all treatment groups (usually 2-3) were represented. 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, Group 3: Saline+Emu Oil, Group 4: 5-FU+Water, Group 5: 5-FU+Olive Oil and Group 6: 5-FU+Emu Oil. Water, Olive Oil or Emu Oil (1ml) 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; 150mg/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[25].

Emu and Olive Oil Preparation. Emu Oil, 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 Emu Oil and Olive Oil was carried out using gas chromatography, as described previously[26] (Table 1). Olive Oil (Conga Foods, Spain) was selected as the control oil due to its high level of oleic acid; similar to that of Emu Oil. The Olive Oil control was included to determine any non-specific oil-related effects for subsequent comparison with Emu Oil. Both Emu Oil and Olive Oil were individually stored at 4^0C in 50ml opaque containers.

Daily Metabolic Data and Disease Activity Index

Body weight, food and water intake, and fecal 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 totaled to achieve an overall DAI, as described previously[27, 28]. 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 Emu Oil[29]. Briefly, following an overnight fast, a baseline breath sample was collected at t=0. Rats were then gavaged with 1ml of a 25% ^{13}C-labeled sucrose solution (BDH, Merck Pty Ltd, Kilsyth, Victoria, Australia); breath samples

collected every 15 minutes for 120 minutes, and samples were analyzed 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 minutes 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; 2cm) were removed and placed in 10% buffered formalin for histological analyses. Additionally, segments (4cm) directly adjacent to the corresponding histological samples were collected and snap-frozen in liquid nitrogen for biochemical analysis. Samples were stored at -80^0C until prepared for analysis by homogenization in 10mM 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 hours post-collection. Specimens were then routinely processed and embedded in paraffin wax and sections (4μm) 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[30].

Neutral Mucin-Secreting Goblet Cell Count. Following routine processing and embedding in paraffin wax, additional 4μm 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 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 minutes at 80^0C 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 minutes, washed and immersed in Schiff's reagent (Sigma, St. Louis, MO, USA) for a further 20 minutes. Sections were rinsed in tap water for 10 minutes and dehydrated. Neutral mucin-secreting goblet cells 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 goblet cell 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*[31]. Thawed, homogenised samples were centrifuged at 13,000g for 12 minutes, after which the supernatant was discarded, and the tissue homogenate was re-suspended in 200µL of 0.5% hexadecyltrimethyl ammonium bromide buffer, a detergent (Sigma Chemicals, Castle Hill, NSW, Australia). After vortexing for 2 minutes, samples were again centrifuged at 13,000g for 2 minutes. Background, negative and positive control samples (50µL) 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 (200µL to each well; 4.2mg of O-dianisidine dihydrochloride reagent, 12.5µL H_2O_2, 2.5ml potassium phosphate buffer [pH 6.0], 22.5ml distilled water) the change in absorbance was measured at 450 nm at 1 minute intervals for 15 minutes 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 4^0C for approximately 2 hours to allow for clotting. Blood samples collected on day 8 were centrifuged at 1,000g for 10 minutes at 4^0C, serum collected and stored at -80^0C 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), interferon-γ (IFN-γ), interleukins (IL)-1α, 1β, 2, 4, 5, 6, 10, 12p70 and 13 and tumor necrosis factor-α (TNF-α). Briefly, the beads were successively incubated (1 hour, shaking in the dark at room temperature) with duplicates of diluted standards and thawed serum samples (50µL), followed by a further

incubation (30 minutes, shaking in the dark at room temperature) with biotinylated detector antibodies (25μL) and a final incubation (10 minutes, shaking in the dark at room temperature) with phycoerythrin (PE)-conjugated streptavidine (50μL; 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 whilst 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 amongst 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, villus height and crypt depth, neutral mucin-secreting goblet cell 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 amongst groups ($p>0.05$; Figure 1a). Following saline administration, all normal rats gained body weight on days 5-10, including those treated with Olive Oil and Emu Oil ($p<0.001$). However, between days 6-10, Emu Oil 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 1b). A temporal effect was observed following 5-FU injection, such that body weight significantly decreased from days 6-8 and then began to increase from days 8-10 (6%; $p<0.01$). Furthermore, in 5-FU-injected rats, body weight significantly decreased following Olive Oil and Emu Oil administration from days 6-9 (Olive Oil: 4%; Emu Oil: 5%) which then began to increased on day 10 ($p<0.001$). Body weight was significantly lower in 5-FU-injected rats treated with Emu Oil compared to Olive Oil treatment on days 9 ($p<0.05$) and 10 ($p<0.05$; Figure 1b).

During the period prior to saline or 5-FU injection (days 1-4), food intake was significantly lower ($p<0.001$) following Olive Oil (23%) and Emu Oil (22%) administration compared to normal controls (Figure 2a). This effect was also observed throughout the post-saline injection period (days 5-10; $p<0.001$; Figure 2b). 5-FU significantly reduced food intake between days 5-9 ($p<0.001$) compared to normal controls (maximal decrease on day 8: 58%), which was normalized by day 10. In 5-FU-injected rats, Olive Oil and Emu Oil 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 2b).

Total water intake, urine and fecal 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).

Disease Activity Index (DAI)

DAI was not significantly affected following Olive Oil or Emu Oil 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, Olive Oil 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). Emu Oil 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 Emu Oil treatment compared to Olive Oil treatment on days 8 and 9 ($p<0.001$).

^{13}C- Sucrose Breath Test

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 ($p>0.05$; Figure 3). On day 8, 5-FU significantly decreased percentage cumulative dose of ^{13}C at 90 minutes (%CD90) compared to healthy controls (67% decrease; $p<0.05$), however, this was normalised by day 9 (Figure 3).

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 Olive Oil (26%; $p<0.01$) and Emu Oil (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 (Figure 4).

SI (proximal jejunum to distal ileum) weight was significantly increased in normal rats treated with Olive Oil and Emu Oil compared to healthy controls on days 8 ($p<0.001$) and 11 (Olive Oil; $p<0.01$, Emu Oil; $p<0.001$; Figure 5). 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 Olive Oil (44%; day 11; $p<0.001$) and Emu Oil (33%; days 8 and 10; $p<0.001$, day 11; $p<0.05$) compared to 5-FU controls (Figure 5).

SI length was significantly increased in normal rats treated with Olive Oil (day 8: 75.3±2.2cm and day 11: 77.3±1.7cm; $p<0.05$) and Emu Oil (day 11: 77.5±1.3cm; $p<0.01$) compared to healthy controls (day 8: 72±0.9cm and day 11: 73.3±1.3cm). Emu Oil treatment in 5-FU-injected rats resulted in an 8.5% increase in SI length compared to 5-FU controls on days 8 (74±0.9cm and 68.5±1.3cm respectively; $p<0.05$) and 11 (82.6±1.1cm and 75.8±1.5cm respectively; $p<0.001$). Furthermore, SI length was significantly greater in 5-FU-injected rats treated with Olive Oil (80±1.1cm), 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).

Villus Height (VH) and Crypt Depth (CD) Measurements

Day 8

VH was increased in the jejunum and ileum of normal rats following Olive Oil and Emu Oil administration, respectively, compared to healthy controls ($p < 0.05$; Figure 6a). 5-FU significantly decreased VH across all small intestinal sections compared to normal controls ($p < 0.001$). Only Emu Oil significantly increased VH in the ileum of 5-FU-injected rats (33%), compared to 5-FU controls and 5-FU-injected rats treated with Olive Oil (37%; $p < 0.05$; Figure 6a). In the mid-small intestine (JI), crypts were lengthened by both Olive Oil ($p < 0.001$) and Emu Oil ($p < 0.01$) 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 6a).

Day 9

Emu Oil increased VH in the jejunum ($p < 0.05$) and JI ($p < 0.001$) of healthy rats compared with normal controls (Figures 6b and 7). 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 normalised by Emu Oil in 5-FU-injected rats, leading to significantly lengthened jejunal (29%) and JI (45%) villi compared with 5-FU controls ($p < 0.01$; Figure 6b and 7). Furthermore, 5-FU-injected rats treated with Emu Oil resulted in significant lengthening of jejunal villi compared with Olive Oil treatment (21%; $p < 0.05$). JI crypts in healthy rats were significantly increased by Emu Oil 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 Olive Oil ($p < 0.01$) and Emu Oil ($p < 0.001$) administration compared to 5-FU controls (Figure 6b). Moreover, 5-FU-injected rats administered Emu Oil exhibited significantly lengthened ileal crypts compared with 5-FU controls (25%; $p < 0.05$).

Day 10

VH was significantly increased following administration of both Olive Oil and Emu Oil in the jejunum and JI; and by Emu Oil in the ileum, compared with healthy controls ($p < 0.01$; Figure 6c). 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 Emu Oil administration (17%; jejunum), both Olive Oil (21%) and Emu Oil treatment (24%; JI), and Olive Oil administration (29%; ileum), compared to 5-FU-controls ($p < 0.05$). JI crypt depth was significantly increased following Emu Oil 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 Olive Oil (30%) and Emu Oil (35%; JI; $p < 0.01$) and Olive Oil (20%; ileum; $p < 0.05$) administration (Figure 6c).

Day 11

Emu Oil administration in normal rats significantly lengthened villi in the jejunum ($p < 0.01$) and JI ($p < 0.05$) compared to healthy controls (Figure 6d). Furthermore, villus lengthening was observed in the JI (27%) and ileum (34%) of 5-FU-injected rats following Emu Oil administration ($p < 0.01$). Both Emu Oil and Olive Oil significantly lengthened jejunal (Olive Oil; $p < 0.05$, Emu Oil; $p < 0.01$) 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 Emu Oil administration in 5-FU-injected rats compared to 5-FU controls (19%; $p < 0.01$). In 5-FU-injected rats, both Olive Oil ($p < 0.01$) and Emu Oil ($p < 0.001$) significantly increased JI CD compared with chemotherapy-injected controls (Figure 6d).

Neutral mucin-secreting goblet cells

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

Myeloperoxidase (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 10a). Both Olive Oil and Emu Oil treatment in 5-FU-injected rats significantly decreased MPO activity on days 8 (Olive Oil: 45%; p<0.05, Emu Oil: 62%; p<0.001) and 9 (Olive Oil: 71%; p<0.01, Emu Oil: 83%; p<0.001) compared to 5-FU controls. This effect was also observed on day 11 (p<0.05; Figure 10a). In the ileum, 5-FU increased MPO levels 6-fold on day 8 compared to healthy controls (p<0.001; Figure 10b). Only Emu Oil significantly reduced day 8 ileal MPO activity compared to 5-FU controls (55%) and 5-FU-injected rats treated with Olive Oil (50%; p<0.05). Furthermore, in chemotherapy-injected rats, Emu Oil significantly decreased MPO levels on days 9 and 10 compared to 5-FU controls (p<0.05; Figure 10b).

Cytokine Analyses

Cytokine concentration 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; Table 3).

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 Emu Oil.

Daily Emu Oil 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 Emu Oil and thus maintenance of overall caloric intake, as evidenced by the absence of changes in body weight compared to normal rats. Furthermore, Olive Oil 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.*[18], Emu Oil increased SI weights in normal rats on days 8 and 10, relative to untreated controls, possibly attributed to concomitant increases in SI, villus and crypt lengthening. Additionally, the high oil viscosity could have resulted in adherence of Emu Oil to the luminal epithelial surface, thereby increasing SI weight[18]. Increases in SI weight on days 8 and 10 were observed following Olive Oil treatment, highlighting a generalized oil effect. In the current study, histological assessment revealed that Emu Oil consistently lengthened villi and crypts throughout the SI in normal rats. Although villus height was increased in the ileum on days 8 and 10 following Emu Oil 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[18]. Tazuke *et al.*[32] 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 tumor-bearing rats; assessed using the TUNEL assay. Accordingly, proliferation and apoptosis would be important parameters to define in future experimental studies of Emu Oil and chemotherapy, utilizing these well established techniques.

Change *et al.*[33] reported that diets containing a combination of Fish Oil (high in omega-3 FAs) and pectin (FO/P) resulted in a lower colonic tumor 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[33, 34]. Moreover, pectin is a highly fermentable fiber 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 Emu Oil would exert similar effects to butyrate. Although the omega-3 FAs present in Emu Oil (total approximately 1.1% [α-linolenic acid]; Table 1) are not as pronounced as Fish Oil (total approximately 30% [20:5n-3: 18%, 22:6n-3: 12%][22]), future studies in the chemotherapy and cancer setting could benefit from further examination of Emu Oil in combination with pectin. The spontaneous tumor-bearing rat model (Dark Agouti mammary adenocarcinoma) would be appropriate to assess the impact of Emu Oil and pectin on breast tumor growth coincident with the enhancement of small intestinal repair following chemotherapy.

Proximal to mid SI changes in mucosal thickness suggested that Emu Oil metabolism in the duodenum and subsequent bioactivity of Emu Oil downstream, could have led to the observed jejunal and JI mucosal lengthening. However, Abimosleh et al.[18] demonstrated increased crypt depth in the distal colon following Emu Oil 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 Emu Oil as evidenced by the SBT, supporting the safety of Emu Oil as an oral administration.

As previously reported[10, 16, 35, 36], injection of 5-FU resulted in significant weight loss, reduction of food intake, increased disease activity index, decreased thymus weight and severe SI injury, 3 days post-mucositis induction (day 8). This damage was characterized by a reduction of brush border sucrase activity, as assessed indirectly by the SBT[29], decreased protective barrier function via a reduction of neutral mucin-secreting goblet cells[37] and increased MPO activity indicative of acute inflammation[38]. Furthermore, chemotherapy-induced damage manifested histologically as shortened villi and crypts, due to reduced cellularity[6, 10]. However, consistent with previous studies, during the recovery phase of mucositis (5 days post-5-FU injection; day 10), villus height and crypt depth elongated to a greater extent than normal, as a compensatory mechanism of stimulated cell proliferation to repair damage sustained from 5-FU administration[23, 39]. Moreover, at this repair stage, SI injury subsided, as evidenced by the normalised SBT, goblet cell 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 3 days post-mucositis induction. Logan et al.[40] demonstrated that at this time point following 5-FU injection (150mg/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[40]. This may suggest that the T-helper 1 (Th1) and Th2 inflammatory response was not yet elicited 3 days post-mucositis induction (day 8). Future experimental studies could benefit from a complete time course of cytokine profiling in serum and intestinal tissue samples.

In the present study, Emu Oil gave indications of accelerated repair in the chemotherapy-damaged intestine. On day 8 during maximal intestinal damage, only Emu Oil administration maintained villus height in the ileum and by day 9, only Emu Oil 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)[41]. Haxhija *et al.*[42] reported that proximal SBR resulted in ileal enterocytes becoming hypertrophic. However, these same authors noted that jejunal cells became hyperplastic in reponse to ileal resection[42].

5-FU preferentially damages the proximal SI (jejunum) due to the higher cell turnover rate in this region[43]. 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 Emu Oil administration, suggesting an acceleration of the repair process. Although Olive Oil exerted similar effects, it failed to maintain mucosal thickness, unlike Emu Oil, further supporting a specific Emu Oil effect.

The increased acute inflammation, indicated by enhanced MPO activity in 5-FU controls on days 8 and 9, was significantly lowered following Emu Oil administration. Lindsay *et al.*[23] also reported decreased acute SI inflammation 4 days after 5-FU-injection with daily Emu Oil administration. Furthermore, the anti-inflammatory properties of topically applied Emu Oil have been well documented[21, 22, 44, 45].

The mechanism by which Emu Oil exerts its anti-inflammatory effects remains unclear. One potential mechanism could be attributed to the omega-3 and -9 FAs present in Emu Oil. 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)[46], whereas omega-9 FAs are believed to promote anti-inflammatory effects by inhibition of macrophage migration. Moreover, Yoganathan *et al.*[22] indicated that the anti-inflammatory properties of Emu Oil are not fully accounted for by the FA profile alone[22]. Components in the non-triglyceride fraction of Emu Oil including carotenoids, flavones, tocopherols and skin-permeation enhancing factors may confer additional anti-inflammatory and antioxidant effects[22, 47]. Bennett *et al.*[47] supported these antioxidant claims *in vitro* demonstrating that Emu Oil 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[48]. 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.

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

STATEMENT OF AUTHOR CONTRIBUTIONS AND ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Daily body weight change of (a) healthy and (b) saline or 5-FU-injected rats on day 5, gavaged daily with 1ml water, Olive Oil or Emu Oil. Days 0 and 5 represent 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. *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$ compared to Saline+Water; ^^^ indicates $p < 0.001$, ^ indicates $p < 0.05$ compared to 5-FU+Water; ## indicates $p < 0.01$, # indicates $p < 0.05$ compared to 5-FU+Olive Oil.

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 1ml 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. *** indicates $p < 0.001$ compared to Saline+Water; ^^^ indicates $p < 0.001$ compared to 5-FU+Water; ## indicates $p < 0.01$, # indicates $p < 0.05$ compared to 5-FU+Olive Oil.

Figure 3. Overall functional status of small intestinal health in rats assessed utilizing the ^{13}C sucrose breath test on day of kill (days 8, 9, 10 and 11). Rats were gavaged daily with 1ml water, Olive Oil or Emu Oil and intraperitoneally injected with saline or 5-FU on day 5. Data are expressed as mean (% cumulative dose of ^{13}C at 90 minutes; %CD90) ± standard error of the mean. * indicates $p < 0.05$ compared to Saline+Water.

Figure 4. Thymus weights of rats from each kill group (days 8, 9, 10 and 11) following body weight adjustment. Rats were gavaged daily with 1ml water, Olive Oil or Emu Oil

and intraperitoneally injected with saline or 5-FU on day 5. Data are expressed as mean (% relative to bodyweight; BW) ± standard error of the mean. *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$ compared to Saline+Water.

Figure 5. Small intestinal (SI; proximal jejunum to distal ileum) weights of rats from each kill group (days 8, 9, 10 and 11) following body weight adjustment. Rats were gavaged daily with 1ml water, Olive Oil or Emu Oil and intraperitoneally injected with saline or 5-FU on day 5. Data are expressed as mean (% relative to body weight; BW) ± standard error of the mean. *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$ compared to Saline+Water; ^^^ indicates $p<0.001$, ^ indicates $p<0.05$ compared to 5-FU+Water.

Figure 6. Villus height and crypt depth measurements in the rat jejunum, jejunum-ileum junction (JI) 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 1ml 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. *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$ compared to Saline+Water; ^^^ indicates $p<0.001$, ^^ indicates $p<0.01$, ^ indicates $p<0.05$ compared to 5-FU+Water; # indicates $p<0.05$ compared to 5-FU+Olive Oil.

Figure 7. Representative photomicrographs of 4μm 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.

Figure 8. 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 1ml 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. *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$ compared to Saline+Water; ^^ indicates $p < 0.01$, ^ indicates $p < 0.05$ compared to 5-FU+Water.

Figure 9. Representative photomicrographs of 4μm 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.

Figure 10. 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 1ml 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. *** indicates $p < 0.001$, * indicates $p < 0.05$ compared to Saline+Water; ^^^ indicates $p < 0.001$, ^^ indicates $p < 0.01$, ^ indicates $p < 0.05$ compared to 5-FU+Water; # indicates $p < 0.05$ compared to 5-FU+Olive Oil.

Table 1. Major fatty acid (FA) composition of Emu and Olive Oils used in the current study including unsaturated fatty acid (UFA) and saturated fatty acid (SFA) ratios. Monounsaturated fatty acid (MUFA); polyunsaturated fatty acid (PUFA).

Major FA Composition of Emu and Olive Oils			
Fatty Acid	**Common Name**	**Emu**	**Olive**
16:0	Palmitic acid	24	10.4
16:1n-7	Palmitoleic acid	4.3	0.7
18:0	Stearic acid	8.5	3.1
18:1n-9	Oleic acid	49.1	73.9
18:2n-6	Linoleic acid	9.5	8.4
18:3n-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

Table 2. Disease activity index (DAI) for each kill group (days 8, 9, 10 and 11) of rats gavaged daily with 1ml of water, Olive Oil or Emu Oil and intraperitoneally injected with saline or 5-FU on day 5. Data are expressed as median disease activity index score (range). *** indicates $p < 0.001$, * indicates $p < 0.05$ compared to Saline+Water; ^^^ indicates $p < 0.001$, ^^ indicates $p < 0.01$, ^ indicates $p < 0.05$ compared to 5-FU+Water; ### indicates $p < 0.001$ compared to 5-FU+Olive Oil.

Day of trial	Saline			5-FU		
	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)

Table 3. Analysis of cytokine concentration from rat serum on day 8. Rats were gavaged daily with of 1ml water, Olive Oil or Emu Oil and intraperitoneal saline or 5-FU injections on day 5. Data are expressed as mean (concentration; pg/ml) ± standard error of the mean. Granulocyte macrophage-colony stimulating factor (GM-CSF); interferon-γ (IFN-γ); interleukins (IL)-1α, 1β, 2, 4, 5, 6, 10, 12p70 and 13; tumor necrosis factor-α (TNF-α).

Cytokine conc (pg/ml)	GM-CSF	IFN-γ	IL-1α	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12p70	IL-13	TNF-α
Saline+Water	72 ± 6	129 ± 14	100 ± 9	514 ± 22	603 ± 29	145 ± 11	101 ± 4	301 ± 37	828 ± 47	44 ± 4	61 ± 4	29 ± 3
5-FU+Water	68 ± 10	132 ± 14	97 ± 13	408 ± 38	553 ± 36	137 ± 17	99 ± 7	292 ± 32	676 ± 54	41 ± 7	55 ± 6	24 ± 2
5-FU+Olive Oil	78 ± 5	145 ± 8	111 ± 7	541 ± 43	681 ± 34	158 ± 10	108 ± 3	348 ± 22	873 ± 54	52 ± 4	67 ± 4	33 ± 3
5-FU+Emu Oil	76 ± 9	135 ± 15	108 ± 12	566 ± 32	717 ± 31	155 ± 15	105 ± 6	319 ± 39	886 ± 46	50 ± 5	65 ± 6	33 ± 3
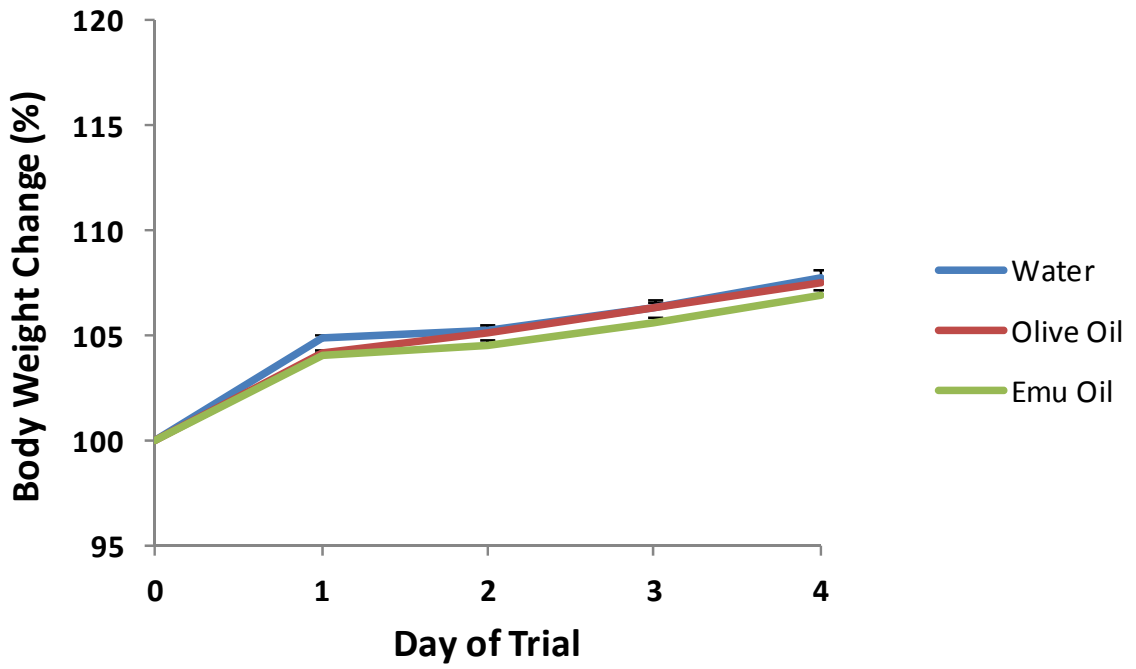
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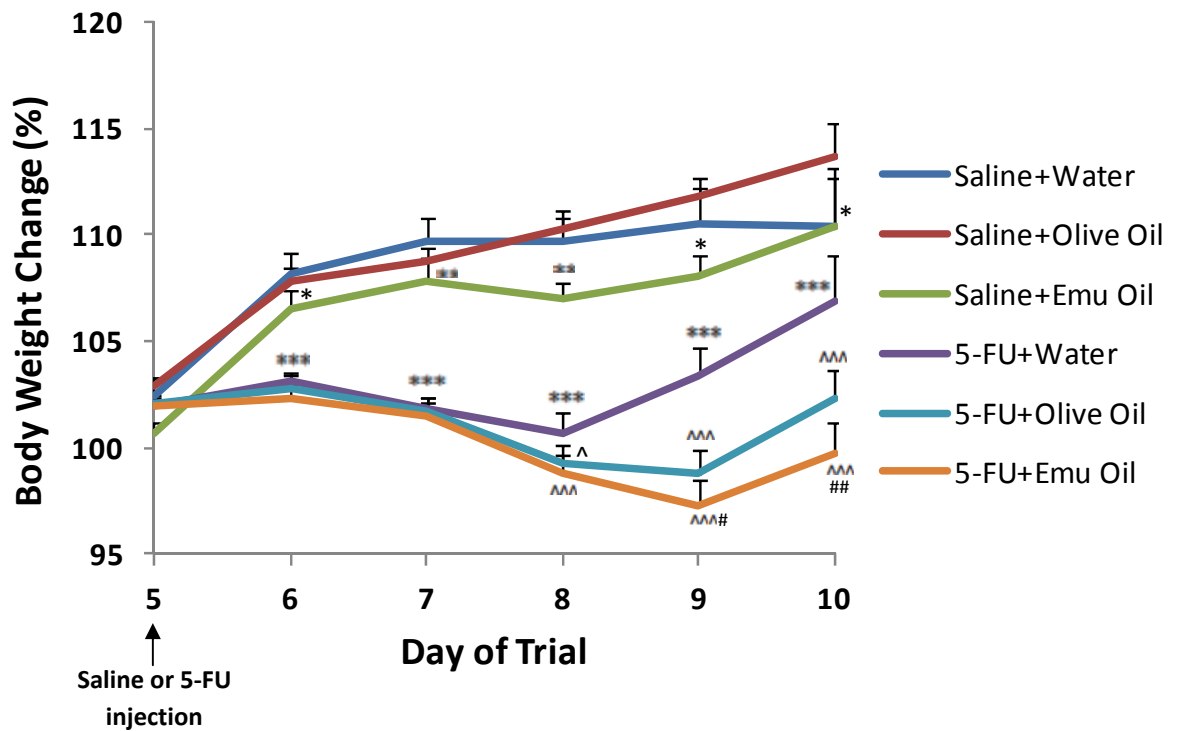
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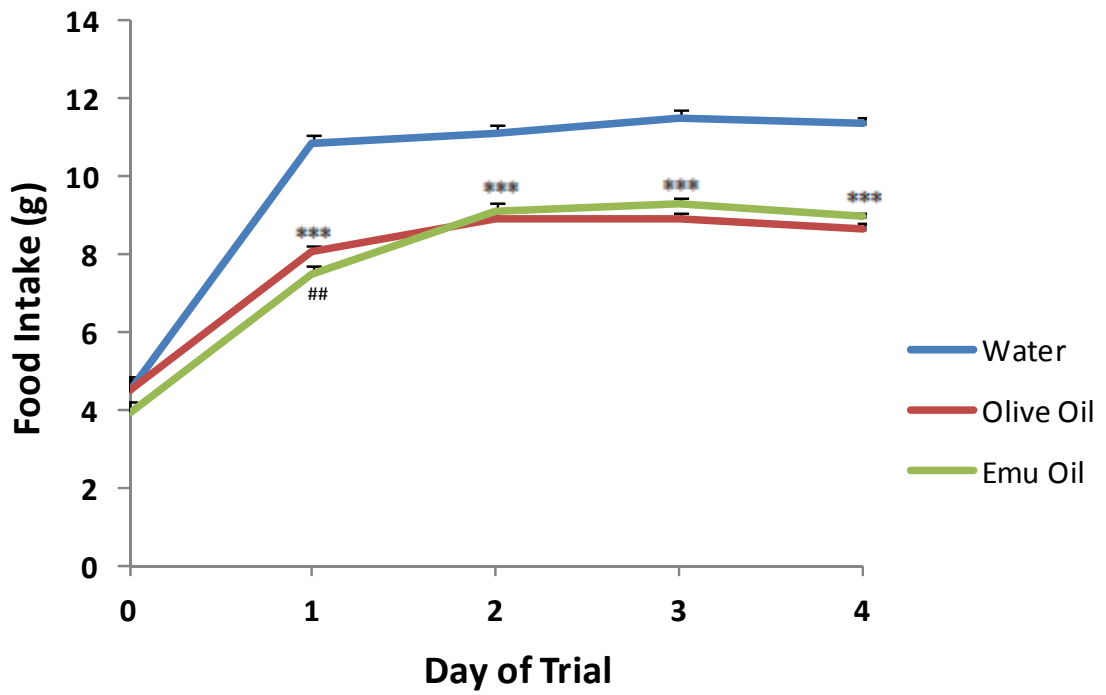
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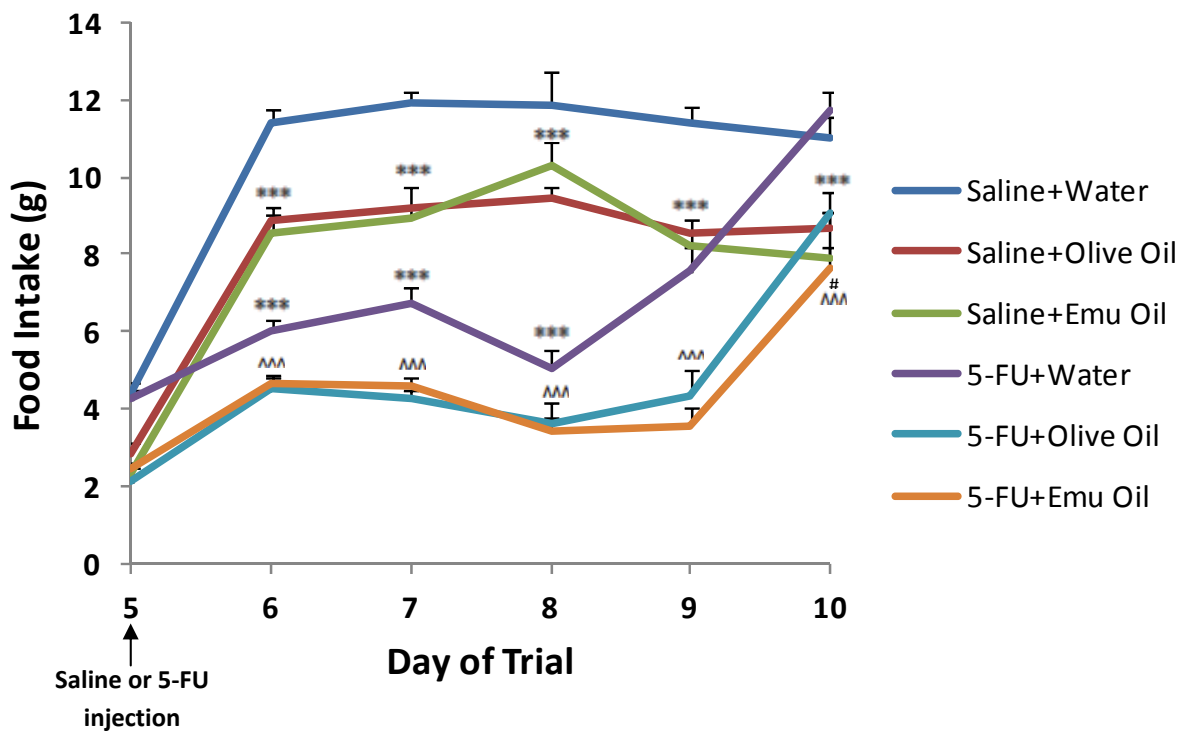
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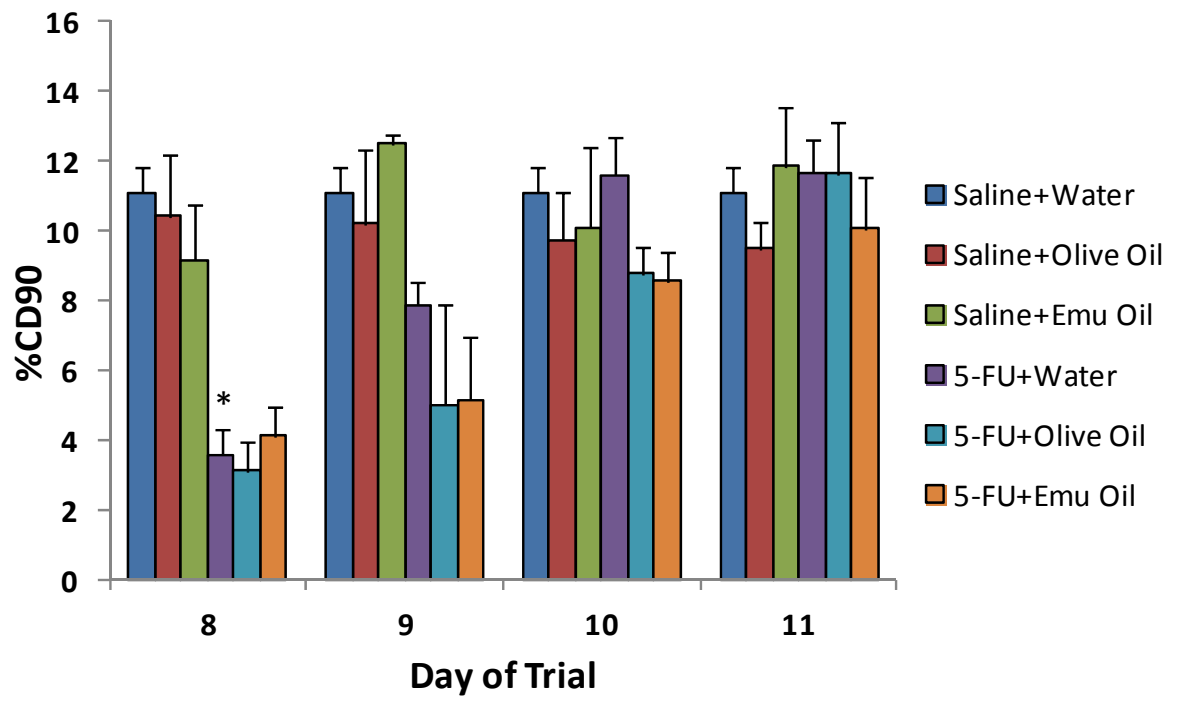
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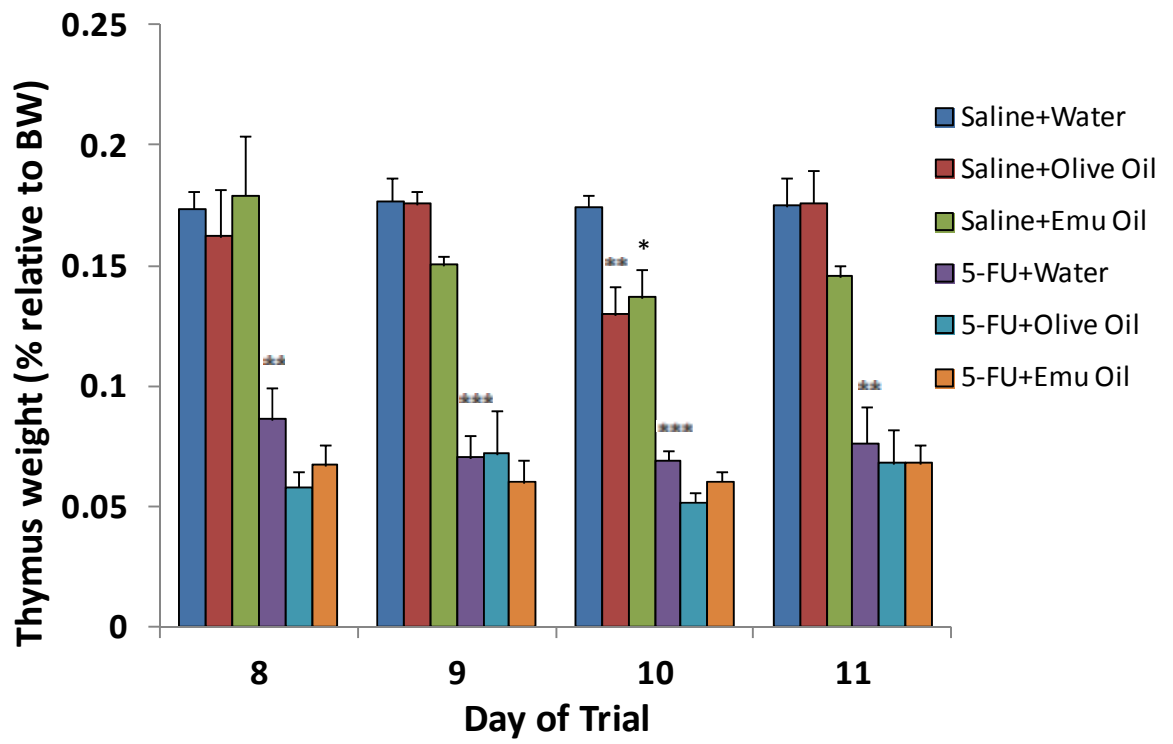
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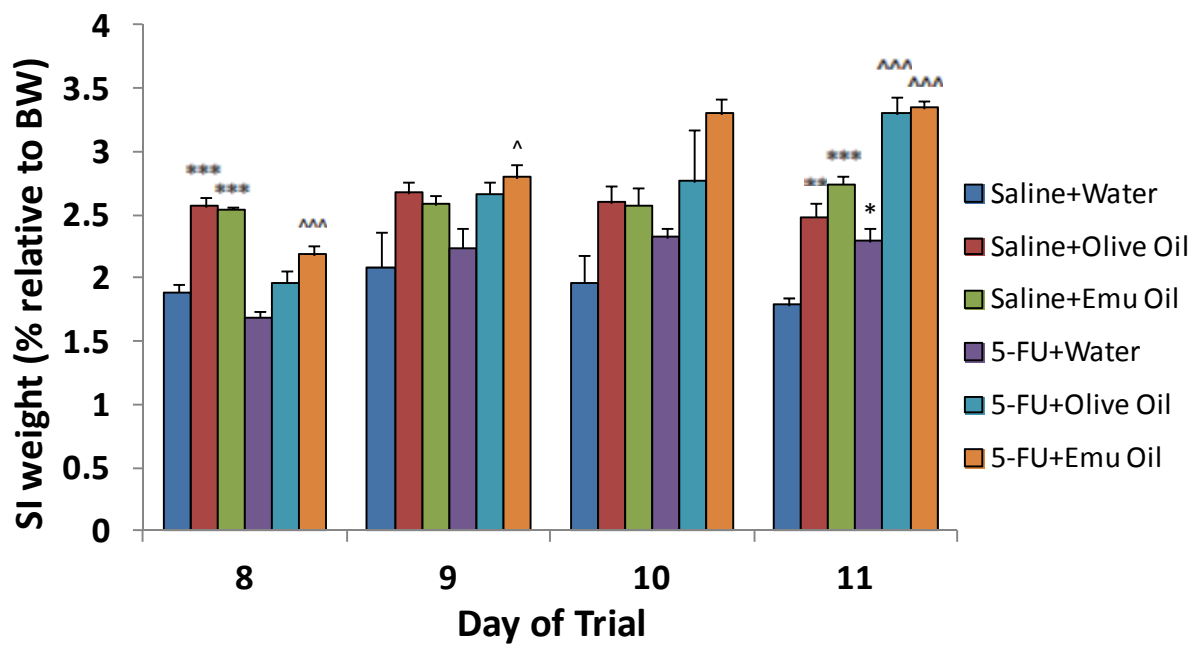
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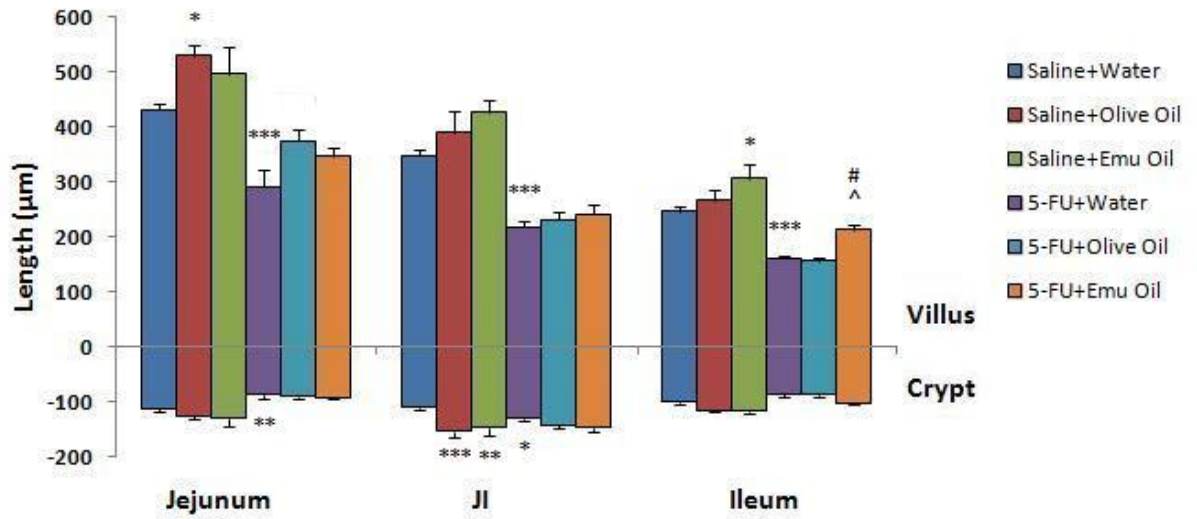
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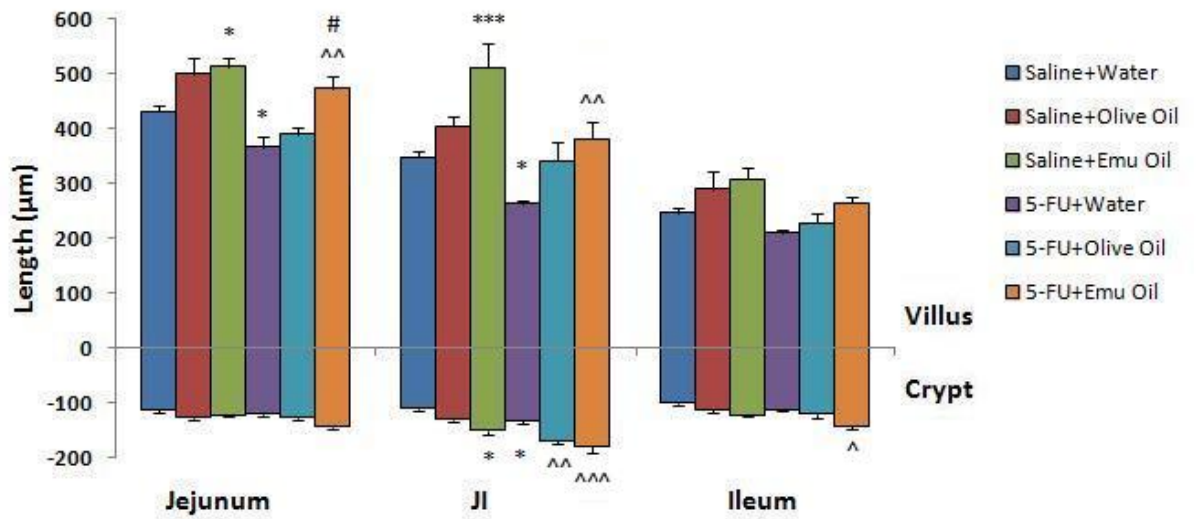
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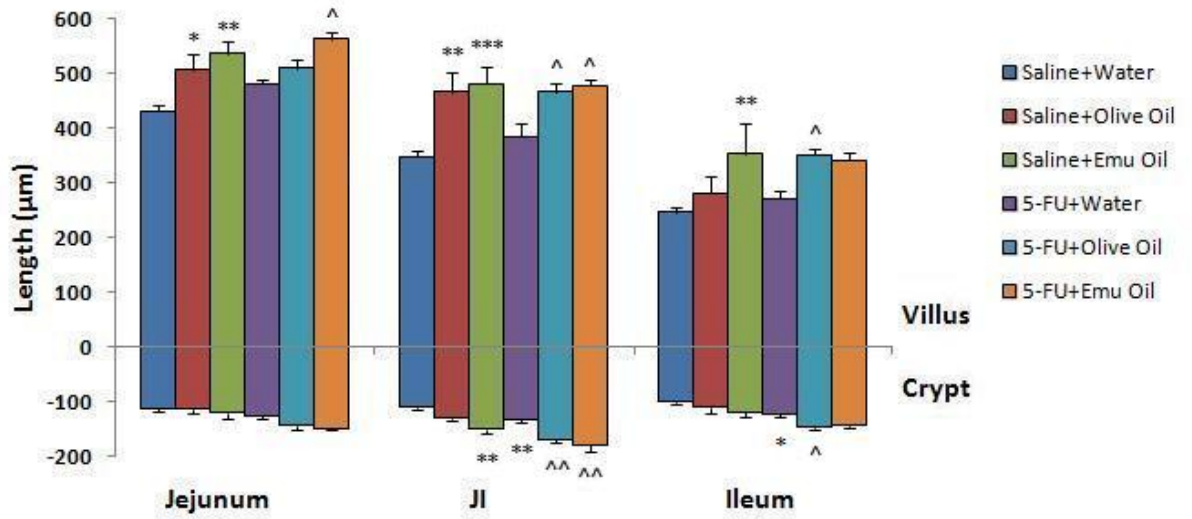
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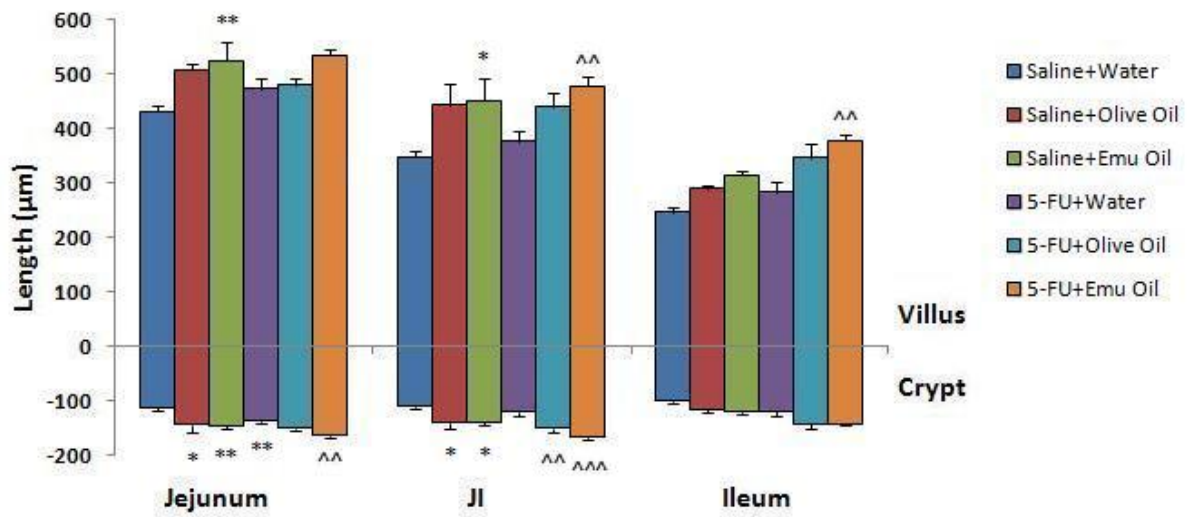
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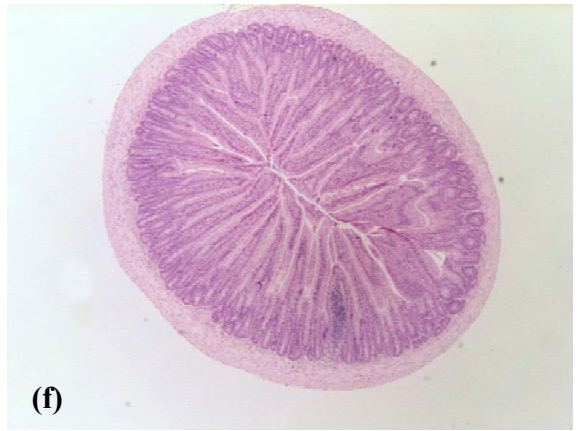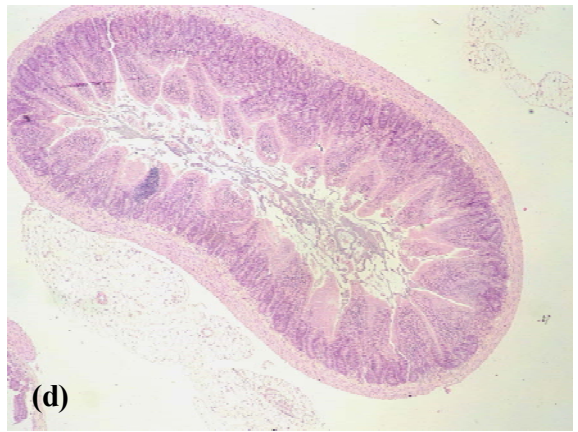
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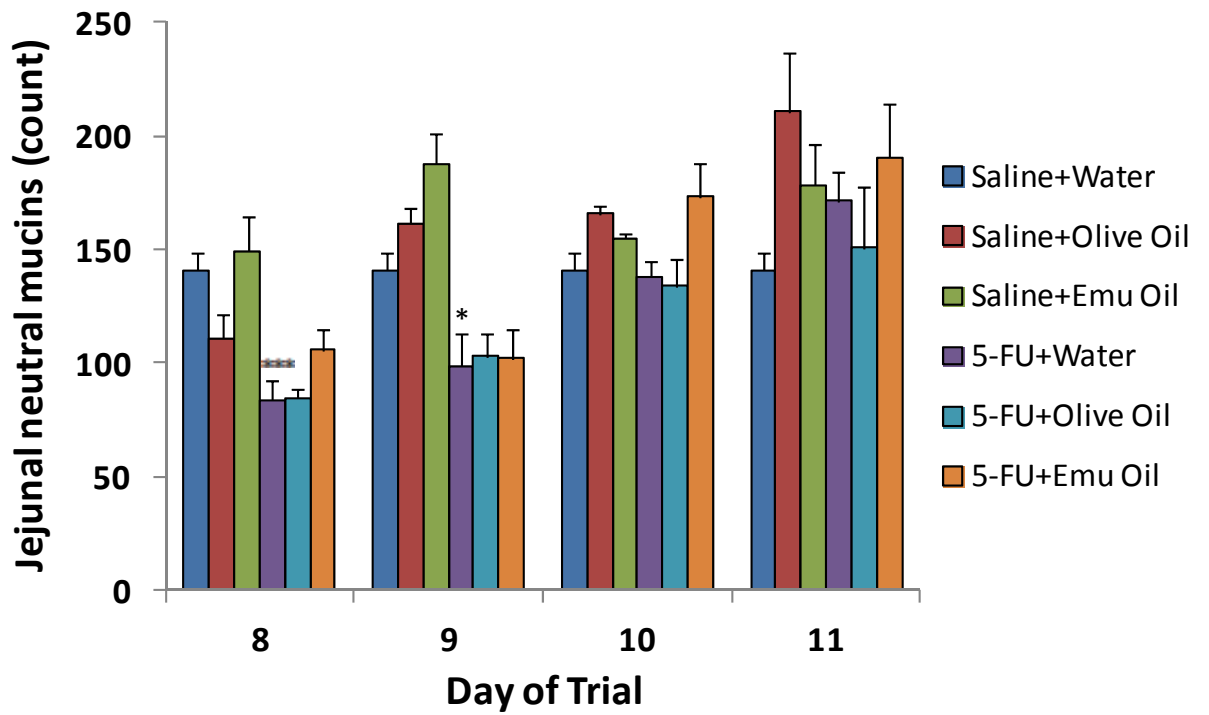
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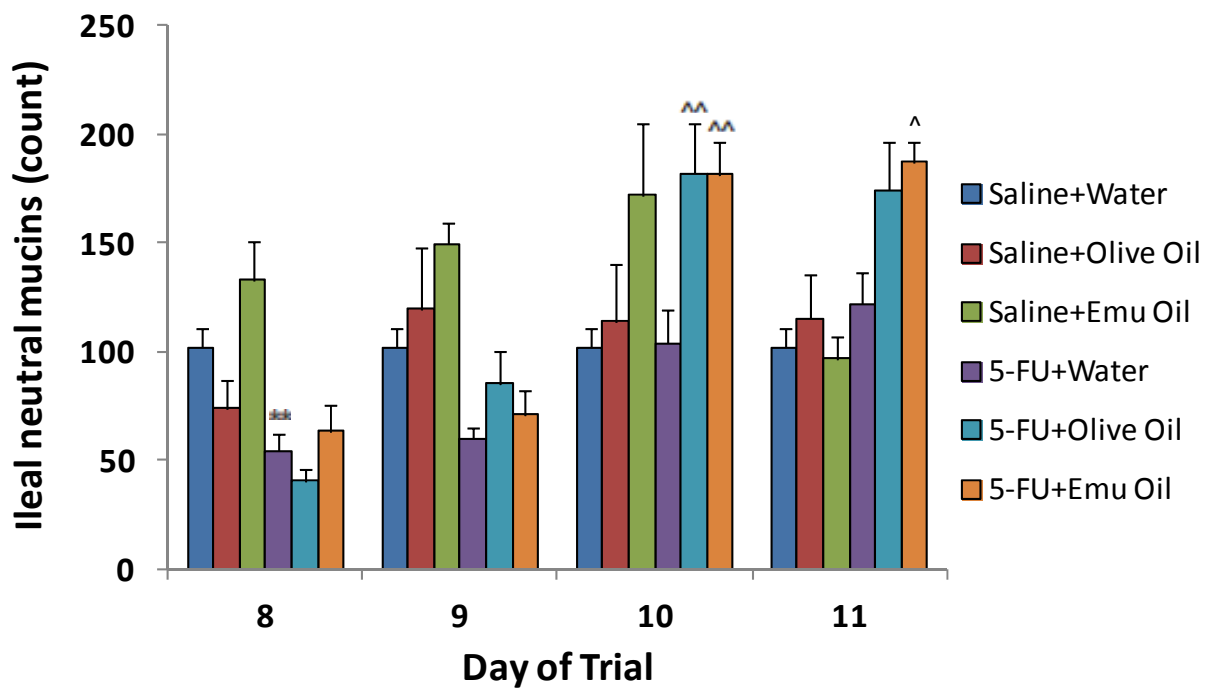
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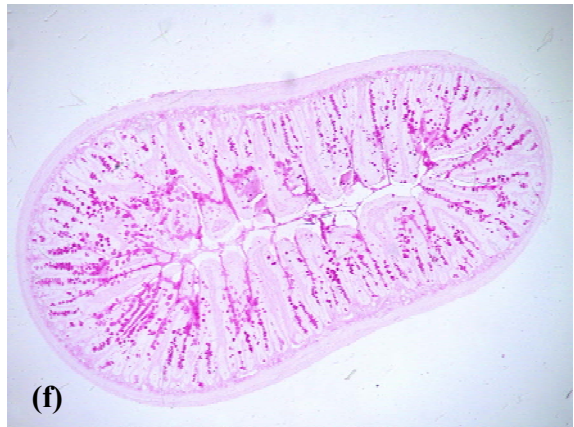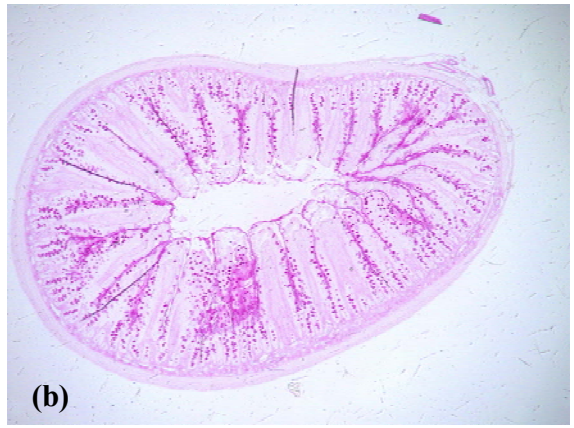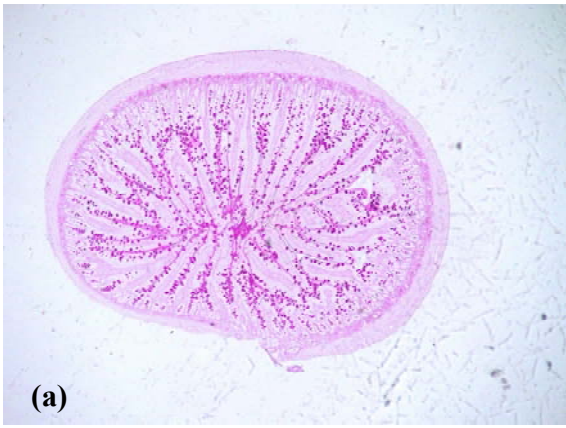
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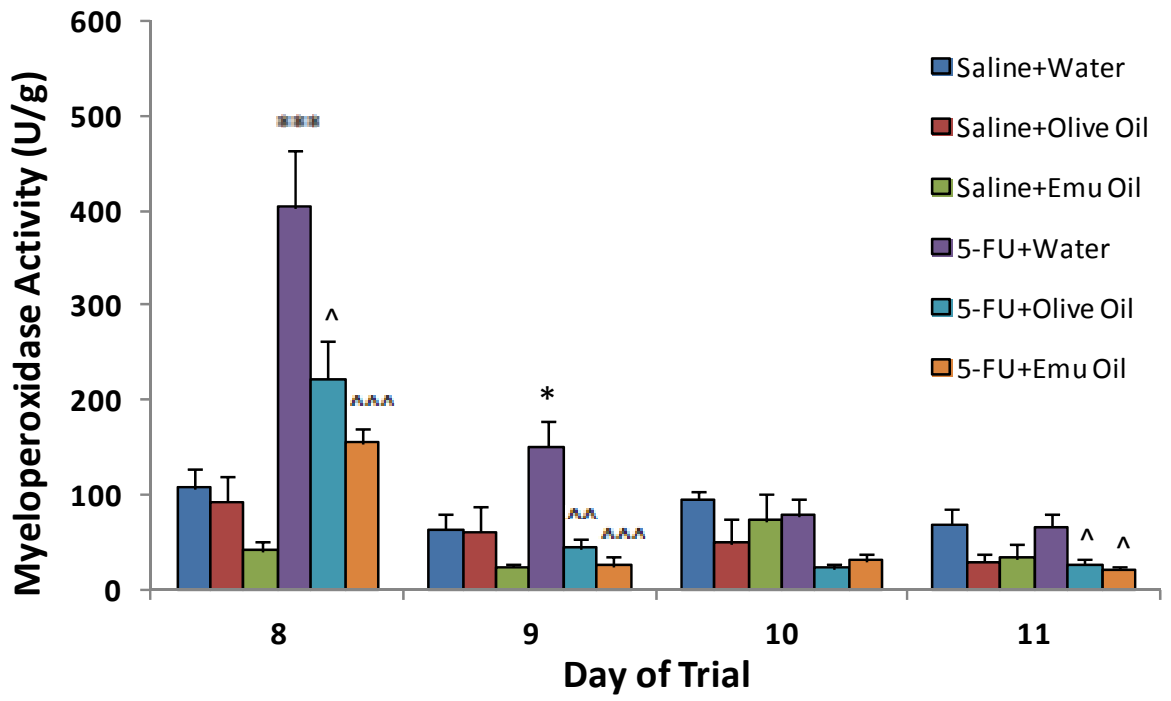
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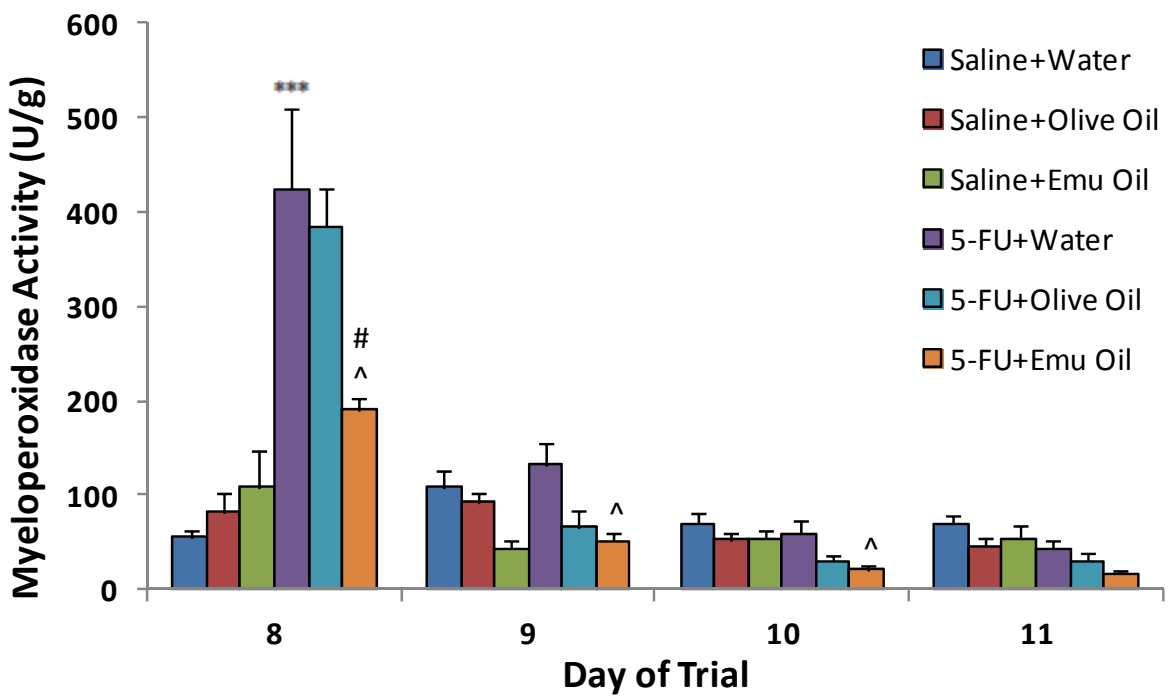
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Figure 10.

CHAPTER 4

EMU OIL REDUCES SMALL INTESTINAL INFLAMMATION IN THE ABSENCE OF CLINICAL IMPROVEMENT IN A RAT MODEL OF INDOMETHACIN-INDUCED ENTEROPATHY

CONTEXT STATEMENT

The work contained in this chapter is currently under review in *Evidence-Based Complementary and Alternative Medicine.*

SM Abimosleh, CD Tran, GS Howarth. Emu Oil Reduces Small Intestinal Inflammation in the Absence of Clinical Improvement in a Rat Model of Indomethacin-Induced Enteropathy. *Evidence-Based Complementary and Alternative Medicine* 2012; Under review

Chapter 4 describes the effects of orally-administered Emu Oil in a rat model of NSAID-induced enteropathy, characterised by SI inflammation and ulceration. This study was undertaken to ensure consistency of Emu Oil efficacy in the SI using a different model of intestinal damage compared to **Chapter 3**.

STATEMENT OF AUTHORSHIP

Emu Oil Reduces Small Intestinal Inflammation in the Absence of Clinical Improvement in a Rat Model of Indomethacin-Induced Enteropathy

Evidence-Based Complementary and Alternative Medicine 2012; Under review

Suzanne Mashtoub ABIMOSLEH (Candidate)

Conducted all animal trials, experimental work, analysed data, interpreted results, prepared figures and manuscript.

I hereby certify that the statement of contribution is accurate.

Signed   Date 4/12/12

Cuong D. Tran

Contributed to experimental conceptualisation and design, data interpretation.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12

Gordon S. Howarth

Contributed to experimental conceptualisation and design, data interpretation, critical manuscript evaluation.

I hereby certify that the statement of contribution is accurate.

Signed   Date 4/12/12

# Emu Oil Reduces Small Intestinal Inflammation in the Absence of Clinical Improvement in a Rat Model of Indomethacin-Induced Enteropathy

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ABSTRACT

Background. Non-steroidal anti-inflammatory drug (NSAID)-induced enteropathy is characterized by small intestinal (SI) damage and inflammation. Emu Oil (EO) has previously been reported to reduce intestinal inflammation in experimental models of mucositis and inflammatory bowel disease. ***Aim.*** We investigated EO for its potential to attenuate NSAID-induced enteropathy in rats. ***Methods.*** Male Sprague Dawley rats (n=10/group) were gavaged with Water, Olive Oil (OO) or EO (0.5ml; days 0-12) and with 0.5ml Water or the NSAID, Indomethacin (8mg/kg; days 5-12) daily. Disease activity index (DAI), ^{13}C-sucrose breath test (SBT), organ weights, intestinal damage severity (IDS) and myeloperoxidase (MPO) activity were assessed. $p < 0.05$ was considered significant. ***Results.*** In Indomethacin-treated rats, DAI was elevated (days 10-12) and SBT values (56%) and thymus weight (55%) were decreased, relative to normal controls. Furthermore, Indomethacin increased duodenum (68%), colon (24%), SI (48%), caecum (48%), liver (51%) and spleen (88%) weights, IDS scores and MPO levels (jejunum: 195%, ileum: 104%) compared to normal controls. Both EO and OO decreased (64%) jejunal MPO levels; however, only EO decreased ileal MPO levels (50%), compared to Indomethacin controls. ***Conclusions.*** EO reduced acute intestinal inflammation, although other parameters of Indomethacin-induced intestinal injury were not affected significantly.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed pharmaceutical agents, with approximately 30 million patients worldwide ingesting NSAIDs daily[1, 2]. NSAIDs have been indicated as an effective treatment option for rheumatic and musculoskeletal conditions[3] and to potentially lower the risk of cardiovascular and cerebrovascular insults. Indeed, recent studies have indicated efficacy for the treatment of colon cancer[2, 4]. NSAIDs represent a highly effective class of drug; however, their use is often associated with a broad spectrum of adverse effects, predominantly within the gastrointestinal (GI) tract[2, 3]. The adverse events in the stomach, duodenum, jejunum and ileum are collectively termed NSAID-enteropathy[5].

NSAID-associated intestinal toxicity has several manifestations including increased mucosal permeability, inflammation and ulceration, and in severe cases, bowel perforation[5]. Clinically evident features include anaemia, bleeding, mucosal diaphragms, strictures and chronic bowel inflammation[6]. Serious injury to the small intestine (SI) has been estimated to account for one-third of all NSAID-associated complications[6].

The pathogenesis of NSAID-enteropathy is proposed to commence via a direct intestinal insult together with its enterohepatic and consequent systemic effects[3, 5, 7]. Entrance of the acidic NSAID into enterocytes induces severe stress in the endoplasmic reticulum of mitochondria leading to cell death[3, 8]. An increase in mucosal permeability follows, which facilitates the translocation and action of luminal factors such as bile acids, dietary macromolecules, components of pancreatic juice and bacteria, thereby activating the inflammatory cascade[8, 9]. Within the mucosa, tumor necrosis factor-α (TNF-α) expression promotes auto-regulation and expression of other pro-inflammatory cytokines including

interleukin-1β (IL-1β) and IL-6[10] . Furthermore, neutrophils are recruited and infiltrate at the area of ulceration[3, 5, 10].

Commonly prescribed NSAIDs include Aspirin, Ibuprofen, Naproxen, Ketoprofen and Indomethacin[4]. The enzyme, cyclooxygenase (COX), which exists as two isoforms, is the target for NSAID action. COX converts arachidonic acid (AA) to prostaglandin G_2 (PGG$_2$), the common precursor of major prostanoids which include PGD$_2$, PGE$_2$, prostacyclin (PGI$_2$) and thromboxane (TXA$_2$)[11]. The two isoforms of COX generally have distinct functions; COX-1-derived PGs maintain mucosal integrity whereas COX-2 is rapidly induced in response to pro-inflammatory stimuli leading to PG production at inflammatory sites[12].

Historically, GI injury has been primarily associated with non-selective COX-1/2 inhibitors. On the basis of these findings, selective COX-2 inhibitors including Celecoxib and Meloxicam were developed. These were initially reported to elicit analgesic properties accompanied by minimal GI inflammatory side-effects[13]. However, a number of recent reports have suggested that the incidence of jejunal and ileal injury induced by chronic use of selective COX-2 inhibitors may be as high as non-selective therapies[5, 14-16]. The development of new therapies is therefore imperative to ameliorate the side-effects of NSAID use, particularly in patients undergoing long-term NSAID-treatment.

Favorable effects of diets high in n-3 polyunsaturated fatty acids (PUFAs) have been well documented in inflammatory conditions including rheumatoid arthritis[17] and inflammatory bowel disease (IBD)[18]. Cell culture studies with n-3 PUFAs have shown inhibition of COX-2 production and pro-inflammatory cytokines including TNF-α, interleukin-1 (IL-1), IL-6, IL-8 and IL-12[19-21]. Furthermore, animal studies with marine

oils rich in n-3 PUFAs support the *in vitro* observations. For example, Fish Oil reduced levels of TNF-α, IL-12 and IL-1β in a mouse model of IBD[22]. Recently, attention has been directed towards animal-derived oils with high levels of PUFAs, such as the oil derived from the Australian ratite bird, the Emu *(Dromaius novaehollandiae)*[23, 24].

Indigenous Australians first used Emu Oil to facilitate wound healing, pain alleviation and treatment of inflamed joints[23, 25, 26]. Topical application of Emu Oil decreased levels of TNF-α and IL-1α in a mouse model of adjuvant-induced inflammation[27]. These cytokines are involved in the pathogenesis of NSAID-enteropathy[5]. Abimosleh *et al.*[24] reported that orally-administered Emu Oil improved selected parameters associated with the manifestation of experimental IBD in rats. Furthermore, Lindsay *et al.*[28] demonstrated that Emu Oil reduced ileal myeloperoxidase activity indicative of neutrophil infiltration and improved mucosal architecture., in a rat model of intestinal mucositis.

Therefore, we hypothesized that orally-administered Emu Oil would protect against Indomethacin-induced enteropathy in rats.

MATERIALS AND METHODS

General Experimental Procedures

Animal Studies. Throughout acclimatization and the experimental period, male Sprague Dawley rats (140-170g) were individually housed in metabolism cages (Tecniplast, PA, USA) at room temperature with a light:dark cycle of 12 hours. All rats were given *ad libitum* access to food (standard 18% casein-based diet[29]) and water, and were acclimatized for 2 days prior to experimentation. Animal experimentation was conducted across several identical trials in which lower numbers of rats from all treatment groups (usually 2-3) were represented. 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 four groups (n=10/group); Group 1: Water+Water, Group 2: Indomethacin+Water, Group 3: Indomethacin+Olive Oil and Group 4: Indomethacin+Emu Oil. Water, Olive Oil or Emu Oil (0.5ml) was administered once daily via oro-gastric gavage from days 0 to 12. Between days 5 and 12, rats were administered Water (0.5ml) or Indomethacin (Sigma-Aldrich, MO, USA; 8mg/kg) via oro-gastric gavage.

The dose of Indomethacin (8mg/kg) was determined from a pilot study using male Sprague Dawley rats, which was performed immediately prior to the current study. Indomethacin dosages (6mg/kg, 8mg/kg and 10mg/kg) were tested and 8mg/kg was deemed to be the most appropriate for the current investigation, based upon metabolic parameters, disease activity index (DAI) and kill data.

Emu Oil and Olive Oil Preparation. Emu Oil, 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 Emu Oil and Olive Oil was carried out using gas chromatography, as described previously[30] (Table 1). Olive Oil (Conga Foods, Spain) was selected as the control oil due to its high level of oleic acid; similar to that of Emu Oil. The Olive Oil control was included to determine any non-specific oil-related effects for subsequent comparison with Emu Oil. Both Emu Oil and Olive Oil were individually stored at 4^0C in 50ml opaque containers.

Daily Metabolic Data and Disease Activity Index

Body weight, food and water intake, and fecal and urine output were monitored and measured daily. The severity of NSAID-induced enteropathy was assessed daily, using a 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 totaled to achieve an overall DAI, as described previously[31, 32]. Overall condition was determined by (1) mobility/agility: healthy rats are considered quite active, whereas rats affected by Indomethacin characteristically become very weak and feeble, sitting hunched with very little movement and (2) fur: healthy rats are well-groomed with fur flat to the body, whereas rats administered Indomethacin become scruffy in appearance, with ruffled fur.

^{13}C- Sucrose Breath Test

Immediately prior to kill (day 12), the ^{13}C-sucrose breath test (SBT) was performed as a non-invasive assessment of the functional status of SI health and to ensure safety of Emu Oil[33]. Briefly, following an overnight fast, a baseline breath sample was collected at t=0. Rats were then gavaged with 1ml of a 25% ^{13}C-labeled sucrose solution (BDH, Merck Pty Ltd, Victoria, Australia); breath samples collected every 15 minutes for 120 minutes, and samples were analyzed for $^{13}CO_2$ concentration using an isotope ratio mass spectrometer (Europa Scientific, Crewe, UK)[34]. Data were expressed as mean percentage cumulative dose of ^{13}C recovered at 90 minutes post-sucrose administration (%CD90). This provides an indirect indication of the rate at which sucrose is cleaved by the enzyme sucrase in the SI, and therefore, the level of sucrase present on SI enterocytes (brush border).

Tissue Collection

On day 12, rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation. The GI tract was removed, measured, emptied of contents and weighed. Furthermore, the SI tract (jejunum, jejunum-ileum junction [JI] and the ileum) was opened for intestinal damage severity assessment. Segments of the SI tract (jejunum, and ileum; 4cm) were collected and snap-frozen in liquid nitrogen for biochemical analysis. Samples were stored at -80^{0}C until prepared for analysis by homogenization in 10mM phosphate buffer. The remaining visceral organs (thymus, lungs, heart, liver, kidneys and spleen) were weighed and discarded.

Intestinal Damage Severity Scoring

Intestinal damage severity (IDS) was assessed utilizing a quantitative scoring system based on thirteen parameters. Opened intestinal sections (jejunum, JI and ileum) were inspected and quantified for erythema (small: <2cm, large: ≥2cm; Figure 1b), mild hemorrhage

(small: <2cm, large: ≥2cm; Figure 1b), severe hemorrhage (small: <2cm, large: ≥2cm; Figure 1c), single ulcers (small: <0.5cm, medium: ~0.5cm, large: >0.5cm; Figure 1d), cluster of ulcers (small: <4, large: ≥5; Figure 1e) and perforations (small: <0.5cm, large: ≥0.5cm; Figure 1f), in which number, size and location were recorded on a schematic intestinal diagram during kill. Assessment was performed in a blinded fashion using a dissecting microscope (Leica Microsystems, Wetzlar, Germany). Raw parameter data were totaled to achieve an overall score for each intestinal section from each rat.

Biochemical Analysis

Myeloperoxidase Activity. Myeloperoxidase (MPO) levels in the SI were determined as an indicator of neutrophil infiltration, and hence, acute inflammation, using techniques described by Howarth *et al*[35]. Thawed, homogenised samples were centrifuged at 13,000g for 12 minutes, after which the supernatant was discarded, and the tissue homogenate was re-suspended in 200μL of 0.5% hexadecyltrimethyl ammonium bromide buffer, a detergent (Sigma Chemicals, Sydney, Australia). After vortexing for 2 minutes, samples were again centrifuged at 13,000g for 2 minutes. Background, negative and positive control samples (50μL) 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 (200μL to each well; 4.2mg of O-dianisidine dihydrochloride reagent, 12.5μL H_2O_2, 2.5ml potassium phosphate buffer [pH 6.0], 22.5ml distilled water) the change in absorbance was measured at 450 nm at 1 minute intervals for 15 minutes with a spectrophotometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore). Data were expressed as units of MPO per gram of tissue.

Statistical Analyses

Statistical comparisons were conducted using SPSS version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA). All data sets were tested for normality of distribution using the Shapiro-Wilk statistic. 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 during (days 5-12) Indomethacin administration. SBT, kill data, intestinal damage severity and MPO activity 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

During the period prior to commencement of Indomethacin administration (days 0-4), Olive Oil and Emu Oil did not significantly affect body weight gain, total food and water intake and total urine output (p>0.05; Table 2) compared to normal, healthy controls. However, Olive Oil significantly reduced total fecal output in normal rats, compared to normal controls (25%; days 0-4; p<0.05).

Body weight was determined as a mean percentage change from the commencement of Indomethacin or water administration (days 5-11). Water-gavaged rats gained approximately 25% body weight from day 5 to 11, whereas Indomethacin administration resulted in significantly reduced weight gain in normal rats compared to normal controls (p<0.001; Figure 2). Neither oil treatment increased body weight in Indomethacin-treated rats compared to Indomethacin-treated controls (p>0.05; Figure 2).

Indomethacin significantly decreased total food intake (41%) and increased water intake (72%) in normal rats compared to healthy controls during days 5-12 (p<0.001; Figures 3a and 3b). Olive Oil and Emu Oil did not significantly affect total food or water intake in Indomethacin treated rats compared to Indomethacin controls (p>0.05). Indomethacin significantly decreased (21%; p<0.01) total fecal output during days 5-12 compared to healthy controls, which was subsequently normalized following Olive Oil and Emu Oil treatment (p<0.05; Figure 3c). Neither Indomethacin nor the oil treatments significantly affected total urine output (p>0.05; Figure 3d).

Disease Activity Index (DAI)

Indomethacin significantly increased DAI in normal rats on day 10 [median score 1 (range: 0-7); p<0.01], 11 [2 (1-7); p<0.001] and 12 [2 (1-8); p<0.001] compared to healthy controls [0 (0); Table 3]. Oil treatments did not significantly affect DAI in Indomethacin-treated rats compared to Indomethacin controls (p>0.05).

^{13}C- Sucrose Breath Test (SBT)

Indomethacin significantly decreased percentage cumulative dose of ^{13}C at 90 minutes (%CD90) compared to healthy controls (56% decrease; p<0.001; Figure 4). However, both oil treatments failed to significantly increase %CD90 in Indomethacin-administered animals compared to Indomethacin controls (p>0.05; Figure 4).

Visceral and Gastrointestinal Organ Weights and Lengths

Organ weights were expressed as a proportion of body weight. Significant increases in duodenum (68%; p<0.05), SI (48%; p<0.001), caecum (48%; p<0.001) and colon (24%; p<0.05) weights were evident in normal rats administered Indomethacin compared to healthy controls (Table 4). Oil treatments did not significantly affect duodenum, SI, caecum or colon weights in Indomethacin-treated rats (p>0.05). There were no significant differences in visceral organ weights including heart, lungs, left kidney, right kidney, and stomach following Indomethacin or oil treatments (p>0.05; Table 4).

Indomethacin significantly increased (51%) liver weight in normal rats compared to healthy controls (p<0.001; Figure 5). Thymus weight in Indomethacin treated rats was decreased (55%) compared to healthy controls (p<0.001; Figure 6). Indomethacin significantly increased spleen weight in normal rats compared to healthy controls (88%; p<0.001; Figure 6). Neither Olive Oil nor Emu Oil treatment significantly affected liver,

thymus or spleen weights in rats administered Indomethacin compared to Indomethacin controls ($p>0.05$; Figures 5 and 6).

Neither Indomethacin nor oil treatments significantly affected duodenum or colon length ($p>0.05$; Table 5). SI length in rats administered Indomethacin was significantly decreased compared to normal controls (41%; $p<0.001$; Table 5), however, neither Emu Oil nor Olive Oil affected SI length compared to Indomethacin controls.

Intestinal Damage Severity (IDS) Scoring

IDS scores in the jejunum, JI and ileum were significantly greater in rats administered Indomethacin, compared to normal controls ($p<0.001$; Figure 7). Neither oil treatment decreased the overall IDS in rats administered Indomethacin, compared to Indomethacin controls ($p>0.05$; Figure 7).

Myeloperoxidase (MPO) Activity

Jejunal MPO activity, indicative of acute inflammation, was increased by 195% following Indomethacin administration, compared to healthy controls ($p<0.01$; Figure 8a). In the jejunum, both Olive Oil and Emu Oil significantly decreased MPO activity in Indomethacin-treated rats compared to Indomethacin controls (64%; $p<0.01$). Ileal MPO activity was significantly elevated in normal rats administered Indomethacin compared to healthy controls (104%; $p<0.01$; Figure 8b). However, in the ileum, only Emu Oil significantly reduced MPO activity in Indomethacin-administered rats compared to Indomethacin controls (50%; $p<0.05$; Figure 8b). Olive Oil did not significantly affect ileal MPO activity ($p>0.05$).

DISCUSSION

NSAIDs have been widely used to achieve analgesic, anti-nociceptive and anti-inflammatory effects; however, a major limitation of NSAID usage has been related to GI side-effects. Emu Oil has been reported to reduce the severity of experimentally-induced inflammatory GI disorders including IBD[24] and chemotherapy-induced intestinal mucositis[28]. The current study indicated that Emu Oil reduced SI neutrophil infiltration and normalized total fecal output in a rat model of Indomethacin-induced enteropathy. Furthermore, Emu Oil treatment significantly increased liver and spleen weight and decreased thymus weight relative to Indomethacin controls. However, Emu Oil treatment failed to improve most other parameters associated with Indomethacin-induced SI damage.

In the current study, an 8mg/kg Indomethacin dose was employed, resulting in clinical manifestations which included significantly decreased food intake accompanied by weight loss and reduced fecal output and increased water intake, with no changes in urine output. Furthermore, elevated DAI, reduced brush border sucrase activity, as assessed indirectly by the SBT and elevated IDS were observed. In a study by Kamil et al.[2], rats were administered Indomethacin daily at 6mg/kg for 7 days to induce enteropathy. Interestingly, no clinical signs of enteropathy were observed, highlighting the clinical significance of minor dosage increases of Indomethacin. Clinical manifestations of Indomethacin-enteropathy were not improved by Olive Oil or Emu Oil treatment. Moreover, fecal output was increased following oil treatment relative to Indomethacin controls, consistent with the study by Abimosleh et al.[24] in a rat model of IBD. This may have represented a normalization of fecal output compared to Indomethacin controls. Alternatively, increased water intake coincident with an absence of changes in urine output may have indicated water loss in stools and subsequent dehydration.

In the current study, Indomethacin decreased SI length, a phenomenon reported to be a compensatory mechanism in response to intestinal damage to increase mucosal surface area[36] and potentially protect the intestine from pathogenic invasion. Furthermore, Indomethacin increased duodenum, SI and colonic weight. Although not quantified, these weight increases may have represented mucosal, submucosal or muscularis hyperemia and oedema, potentially combined with inflammatory cell infiltration and the associated exudate. This would likely cause an increase in GI weight. Emu Oil did not improve Indomethacin-enteropathy manifestations associated with intestinal weights.

Recent studies have hypothesized that neutrophil activation is an important primary event preceding Indomethacin-induced SI injury[37]. Fukumoto et al.[10] demonstrated that tissue-associated neutrophil accumulation, assessed by MPO activity, and keratinocyte chemoattractant (KC) mRNA expression, involved in chemotaxis and neutrophil activation, were significantly increased in Indomethacin-treated SI mucosa[10]. Furthermore, MPO levels and KC mRNA expression were reduced in TNF-α-deficient mice treated with Indomethacin compared to wild-type. This indicated a protective role of TNF-α deficiency in decreasing Indomethacin-induced SI mucosal injury[10]. In the current study, Indomethacin increased MPO activity in the jejunum and ileum. Both Olive Oil and Emu Oil treatment significantly decreased MPO activity, indicative of neutrophil infiltration, in the Indomethacin-damaged jejunum. Importantly, only Emu Oil decreased MPO levels in the ileum, relative to Indomethacin controls. Emu Oil has previously been shown to reduce levels of TNF-α in a croton-oil induced model of auricular swelling[27]. Therefore, the observed decrease in MPO levels following Emu Oil administration in the current study may have been the result of suppressed TNF-α expression.

PUFAs influence cytokine production, lymphocyte proliferation and macrophage activation state through modulation of AA (common n-6 PUFA) metabolism[38]. n-3 PUFAs replace AA from phospholipid membranes, thereby reducing the availability of AA to COX and thus reducing the production of PGs involved in the inflammatory cascade such as the 2-series eicosanoids. Furthermore, n-3 PUFAs can be directly metabolized by COX, resulting in the production of anti-inflammatory 3-series eicosanoids[38]. Cell culture studies with n-3 PUFAs eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to inhibit lipopolysaccharide-induced production of COX-2, and pro-inflammatory cytokines including TNF-α, IL-1, IL-6, IL-8 and IL-12 in endothelial cells[19, 20]. Furthermore, animal feeding studies with Fish Oil, a source of EPA and DHA, supported in vitro observations of decreased cytokine production[22, 39, 40]. Further studies of Emu Oil could benefit from a complete time-course of cytokine profiling to identify potential effects on pro-inflammatory cytokine antagonism.

Liver weight was significantly increased in the current study following Indomethacin (8mg/kg), compared to normal controls. In a previous rat model of Indomethacin-enteropathy, daily administration of Indomethacin at 6mg/kg did not result in significant liver weight changes. The impact of Indomethacin on liver weight therefore appears to be dose-dependent as Ilic et al.[41], consistent with the current findings, demonstrated liver weight increases in male albino Wistar rats injected daily for three days with 12.5mg/kg of diclofenac (NSAID). The authors observed pronounced parenchymal necrosis, elevated eosinophilic hepatocytes, extensive microvesicular steatosis and sinusoidal dilation[41]. Moreover, TNF-α, cytokine directly linked to the pathogenesis of enteropathy, binds to TNF-RI cell death receptors leading to hepatocyte apoptosis followed by accumulation of neutrophils[42]. Future studies would benefit from liver enzyme analyses including gamma-glutamyl transpeptidase (GGT), alanine aminotransaminase (ALT), alkaline phosphotase

(ALP) and bilirubin, which elevate in NSAID-induced toxicity[43]. Furthermore, histological assessment of Kupffer cells, resident liver macrophages[44], hepatotoxic lymphocytes including CD4^{+}, CD8^{+} T cells and natural killer cells and apoptotic hepatocytes[45] would provide greater insight into the hepatic impact of Indomethacin.

In the current study, Olive Oil and Emu Oil treatment to rats administered Indomethacin did not significantly affect liver weight, relative to Indomethacin controls. Abimosleh *et al.*[24] examined liver weight in healthy Sprague Dawley rats, which was not significantly affected by Olive Oil or Emu Oil. This indicated that the current findings were directly related to Indomethacin administration with no effect of oil administration. Valenzuela *et al.*[46] demonstrated that n-3 long-chain PUFA supplementation induced an antioxidant response preventing liver steatosis in mice fed a high fat diet. This was further supported by a study in which Fish Oil in combination with Indomethacin decreased the severity of liver steatosis in a mouse model of familial hypercholesterolemia[38]. Based upon the n-3 PUFAs present in Emu Oil and its reported antioxidant activity [47], liver steatosis seems less likely in the current study. However, a high n-6/n-3 PUFA ratio has been implicated in non-alcoholic FA liver disease[48] and therefore it would be beneficial to determine the effects of FA ratio and n-9 FA levels on liver function in future studies.

Thymus weight was significantly decreased following administration of 8mg/kg Indomethacin in the current study, relative to normal controls. Kamil *et al.*[2] did not report any thymus weight changes using 6mg/kg of oral Indomethacin. However, a single subcutaneous injection of Indomethacin at 35mg/kg significantly decreased thymus weight in rats after three days in a study by Filaretova *et al.*[36], consistent with the current study. Furthermore, the increased spleen weight evident following Indomethacin administration in the current study should be pursued in relation to the thymus weight decreases. Such

studies could determine the contribution of T ($CD4^+$ and $CD8^+$) and B lymphocyte upregulation or suppression in the thymus and spleen, respectively. However, neither Olive Oil nor Emu Oil significantly affected thymus or spleen weight.

CONCLUSIONS

In summary, Emu Oil significantly reduced SI MPO activity associated with Indomethacin-induced enteropathy. However, EO failed to improve other clinical manifestations of enteropathy. It remains unclear whether the reduction in acute inflammation would translate to improved clinical manifestation if Emu Oil dose was increased. Increased Emu Oil dose or frequency of administration could further decrease the extent of small bowel inflammation in NSAID-enteropathy and potentially achieve demonstrable clinical benefit.

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FIGURE LEGENDS

Figure 1. Macroscopic aspects of opened rat small intestinal sections representing (a) no damage (b) erythema and hemorrhage (c) severe hemorrhage (d) large single ulcer (e) large cluster of ulcers and (f) perforations. These parameters were utilized in the intestinal damage severity scoring system.

Figure 2. Percentage change of body weight in rats from day 5 to day 11 (during Indomethacin administration). Rats were gavaged daily with water, Olive Oil or Emu Oil (0.5ml) throughout the experimental period and commenced daily gavage with water or Indomethacin (8mg/kg) on day 5. Rats were fasted overnight on day 11 and therefore day 12 body weight data was not included. Data are expressed as mean (% change in body weight) ± standard error of the mean. *** indicates $p<0.001$ compared to Water+Water.

Figure 3. Total (a) food intake (b) water intake (c) fecal output and (d) urine output in rats during Indomethacin administration (days 5-12). Rats were gavaged daily with water, Olive Oil or Emu Oil (0.5ml). Data are expressed as mean (g or ml) ± standard error of the mean. *** indicates $p<0.001$, ** indicates $p<0.01$ compared to Water+Water; ^ indicates $p<0.05$ compared to Indomethacin+Water.

Figure 4. Overall functional status of small intestinal health in rats assessed utilizing the ^{13}C sucrose breath test on day 12. Rats were gavaged daily with water, Olive Oil or Emu Oil (0.5ml) throughout the experimental period and commenced daily gavage with water or Indomethacin (8mg/kg) on day 5. Data are expressed as mean (% cumulative dose of ^{13}C at 90 minutes; %CD90) ± standard error of the mean. *** indicates $p<0.001$ compared to Water+Water.

Figure 5. Liver weights following adjustment for rat body weight on day 12. Rats were gavaged daily with 0.5ml of water, Olive Oil or Emu Oil throughout the experimental period (days 0-12) and administered water or Indomethacin (Indo; 8mg/kg) daily from day 5. Data are expressed as mean (% relative to bodyweight; BW) ± standard error of the mean. *** indicates $p < 0.001$ compared to Water+Water.

Figure 6. Thymus and spleen weights following adjustment for rat body weight on day 12. Rats were gavaged daily with 0.5ml of water, Olive Oil or Emu Oil throughout the experimental period (days 0-12) and administered water or Indomethacin (Indo; 8mg/kg) daily from day 5. Data are expressed as mean (% relative to bodyweight; BW) ± standard error of the mean. *** indicates $p < 0.001$ compared to Water+Water.

Figure 7. Intestinal damage severity score in the rat jejunum, jejunum-ileum junction (JI) and ileum on day 12. Rats were gavaged daily with 0.5ml of water, Olive Oil or Emu Oil throughout the experimental period (days 0-12) and administered water or Indomethacin (Indo; 8mg/kg) daily from day 5. Data are expressed as mean (severity score) ± standard error of the mean. *** indicates $p < 0.001$ compared to Water+Water.

Figure 8. Myeloperoxidase activity indicative of acute inflammation in the rat (a) jejunum and (b) ileum on day 12. Data are expressed as mean (myeloperoxidase activity; units per gram; U/g) ± standard error of the mean. Rats were gavaged daily with 0.5ml of water, Olive Oil or Emu Oil throughout the experimental period (days 0-12) and administered water or Indomethacin (Indo; 8mg/kg) daily from day 5. ** indicates $p < 0.01$ compared to Water+Water; ^^ indicates $p < 0.01$, ^ indicates $p < 0.05$ compared to Indomethacin+Water.

Table 1. Major fatty acid (FA) composition of Emu and Olive Oils used in the current study including unsaturated fatty acid (UFA) and saturated fatty acid (SFA) ratios. Monounsaturated fatty acid (MUFA); polyunsaturated fatty acid (PUFA).

Major FA Composition of Emu and Olive Oils			
Fatty Acid	**Common Name**	**Emu**	**Olive**
16:0	Palmitic acid	24	10.4
16:1n-7	Palmitoleic acid	4.3	0.7
18:0	Stearic acid	8.5	3.1
18:1n-9	Oleic acid	49.1	73.9
18:2n-6	Linoleic acid	9.5	8.4
18:3n-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

Table 2. Body weight (% change from starting body weight), total food (g) and water (ml) intake and total fecal (g) and urine (ml) output in normal rats during the period prior to Indomethacin administration (days 0-4). Rats were gavaged daily with water, Olive Oil or Emu Oil (0.5ml). Data are expressed as mean (%, g or ml) ± standard error of the mean. * indicates $p < 0.05$ compared to Water group.

	Water	Olive Oil	Emu Oil
Body weight (%)	14.8 ± 0.5	14.8 ± 0.6	14.4 ± 0.6
Food Intake (g)	85.1 ± 1.5	83.4 ± 1.9	84.6 ± 2
Water Intake (ml)	353.6 ± 38.9	429.4 ± 50.6	425.6 ± 58.6
Fecal Ouput (g)	7.8 ± 0.4	6.1 ± 0.5*	7.1 ± 0.3
Urine Ouput (ml)	65.2 ± 3.6	66.9 ± 6.5	68.2 ± 4.2

Table 3. Disease activity index (DAI) of rats gavaged daily with 0.5ml of water, Olive Oil or Emu Oil throughout the experimental period (days 0-12) and administered water or Indomethacin (Indo; 8mg/kg) daily on day 5. Data are expressed as median disease activity index score (range). *** indicates $p < 0.001$, ** indicates $p < 0.01$ compared to Water+Water.

Day of trial	Water+Water	Indo+Water	Indo+Olive Oil	Indo+Emu Oil
6	0 (0)	0 (0)	0 (0)	0 (0)
7	0 (0)	0 (0)	0 (0)	0 (0)
8	0 (0)	0 (0-2)	0 (0)	0 (0)
9	0 (0)	0 (0-6)	0 (0-1)	0 (0-3)
10	0 (0)	1 (0-7)**	1 (0-3)	0 (0-4)
11	0 (0)	2 (1-7)***	2 (0-4)	1 (0-5)
12	0 (0)	2 (1-8)***	3 (0-5)	2 (0-7)

Table 4. Organ weight following adjustment for rat body weight. Rats were gavaged daily with 0.5ml of water, Olive Oil or Emu Oil throughout the experimental period (days 0-12) and administered water or Indomethacin (Indo; 8mg/kg) daily from day 5. Data are expressed as mean (% relative to body weight) ± standard error of the mean. All values x10^{-2}. *** indicates $p < 0.001$, * indicates $p < 0.05$ compared to Water+Water.

Weight (%)	Water+Water	Indo+Water	Indo+Olive Oil	Indo+Emu Oil
Heart	42 ± 0	41 ± 2	42 ± 2	40 ± 1
Lungs	62 ± 3	59 ± 4	61 ± 4	57 ± 3
L Kidney	40 ± 1	43 ± 1	41 ± 1	41 ± 2
R Kidney	44 ± 4	42 ± 1	52 ± 6	42 ± 2
Stomach	56 ± 2	66 ± 7	59 ± 11	57 ± 4
Duodenum	22 ± 1	37 ± 5*	49 ± 5	39 ± 6
SI	178 ± 5	263 ± 38***	330 ± 17	270 ± 40
Caecum	29 ± 1	43 ± 2***	46 ± 3	46 ± 3
Colon	38 ± 1	47 ± 1*	50 ± 2	49 ± 3

Table 5. Gastrointestinal organ length of rats gavaged daily with 0.5ml of water, Olive Oil or Emu Oil throughout the experimental period (days 0-12) and administered water or Indomethacin (Indo; 8mg/kg) daily from day 5. Data are expressed as mean (cm) ± standard error of the mean. *** indicates $p < 0.001$ compared to Water+Water.

Length (cm)	Water+Water	Indo+Water	Indo+Olive Oil	Indo+Emu Oil
Duodenum	6.5 ± 0.3	5.6 ± 0.3	6.6 ± 0.4	6.2 ± 0.4
SI	78.5 ± 2.2	46.2 ± 2.1***	51.0 ± 3.4	51.0 ± 3.4
Colon	12.9 ± 0.4	11.8 ± 0.6	12.0 ± 0.4	12.2 ± 0.5
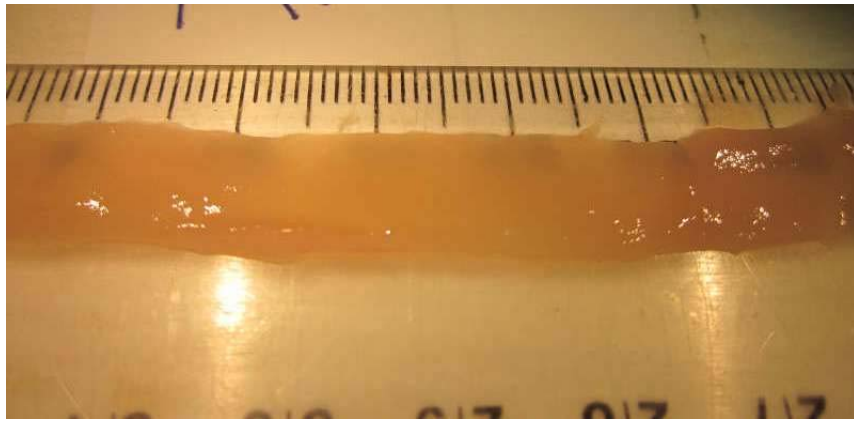
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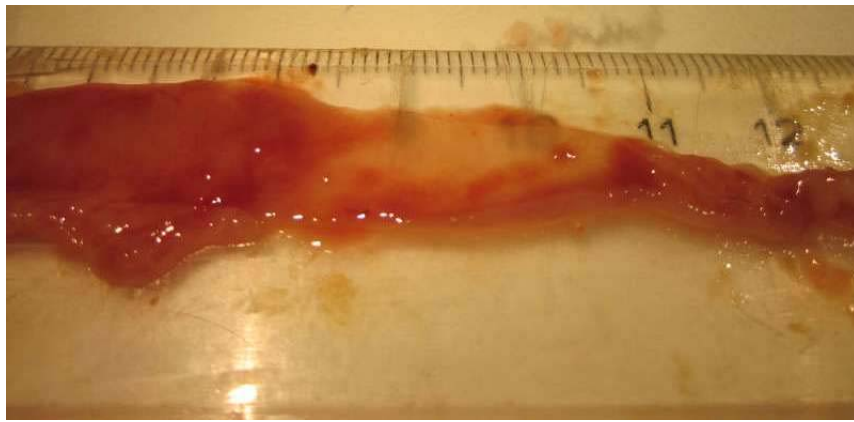
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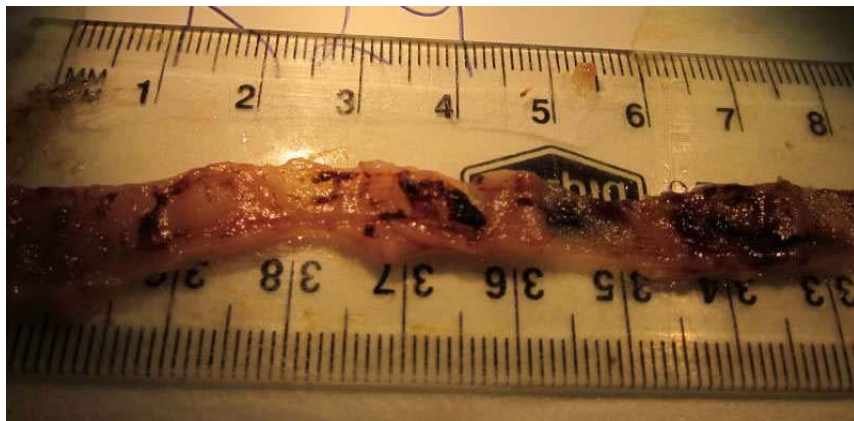
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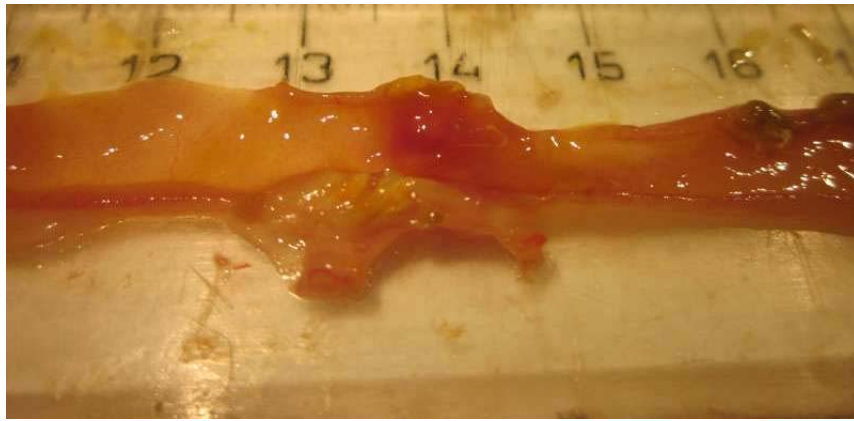
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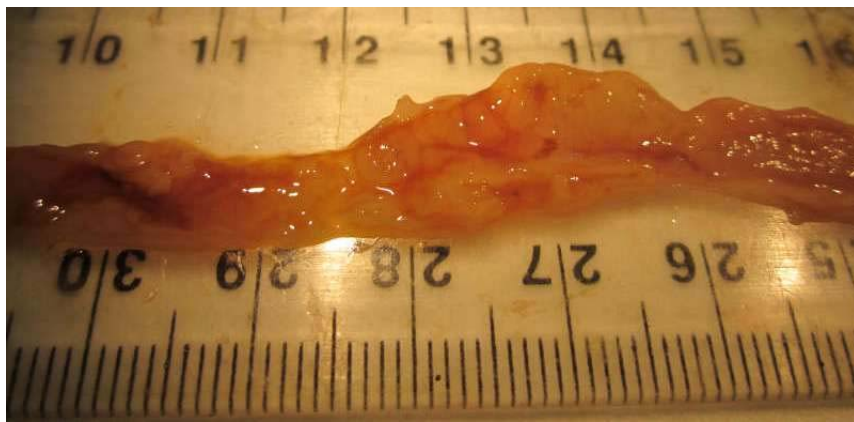
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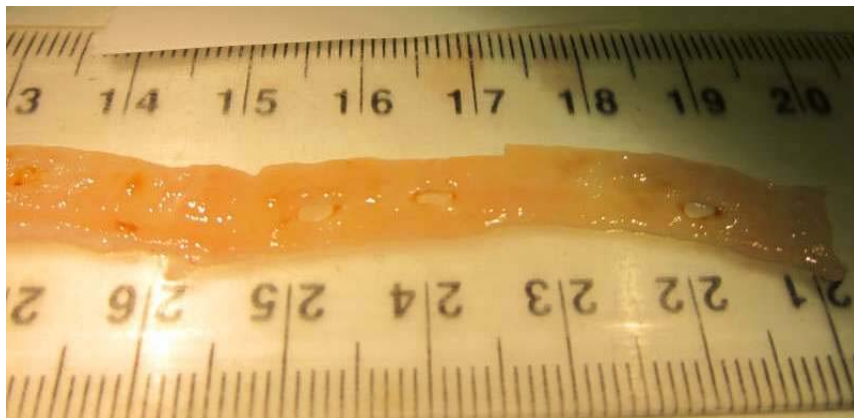
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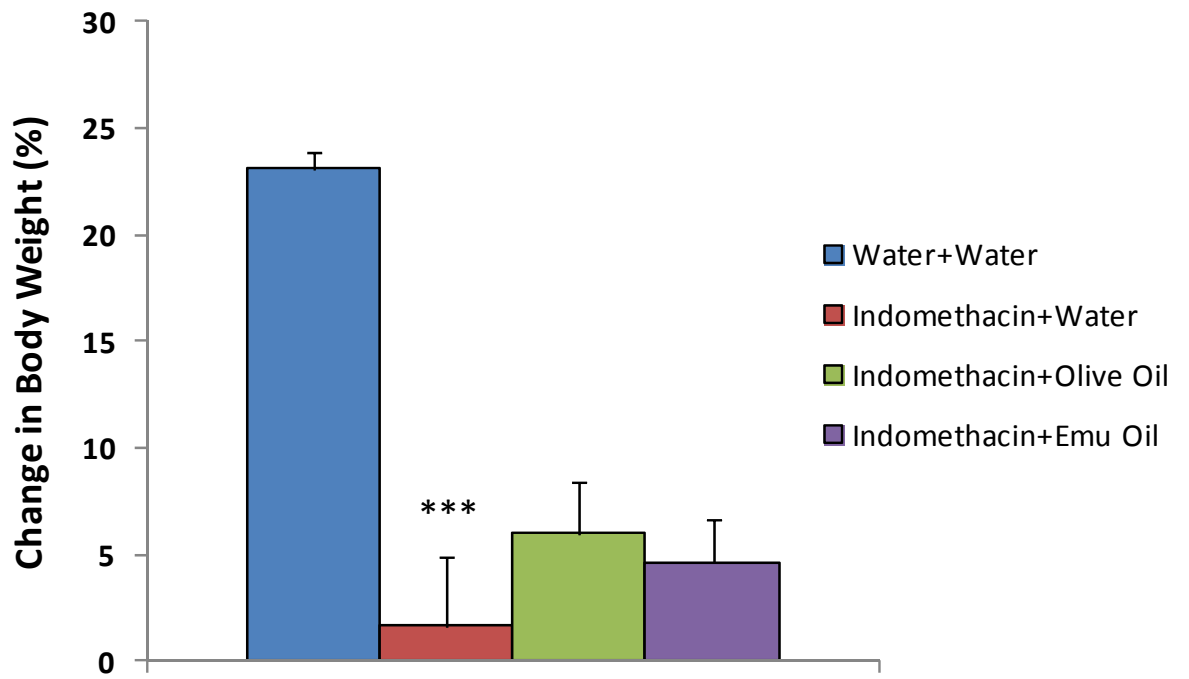
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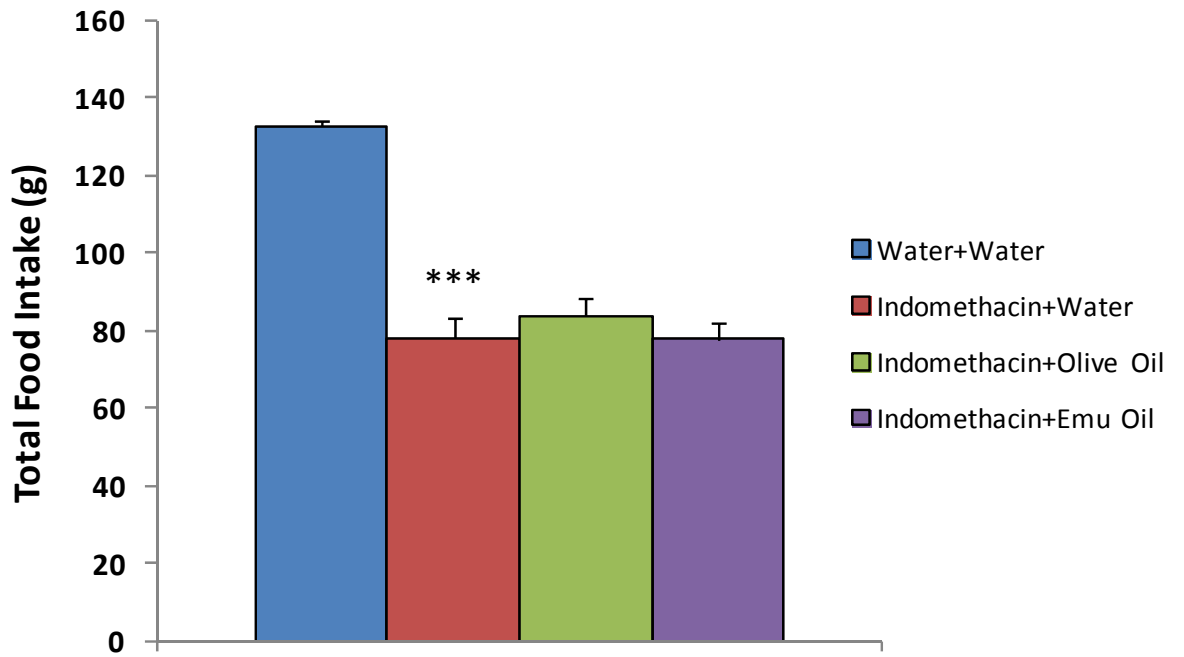
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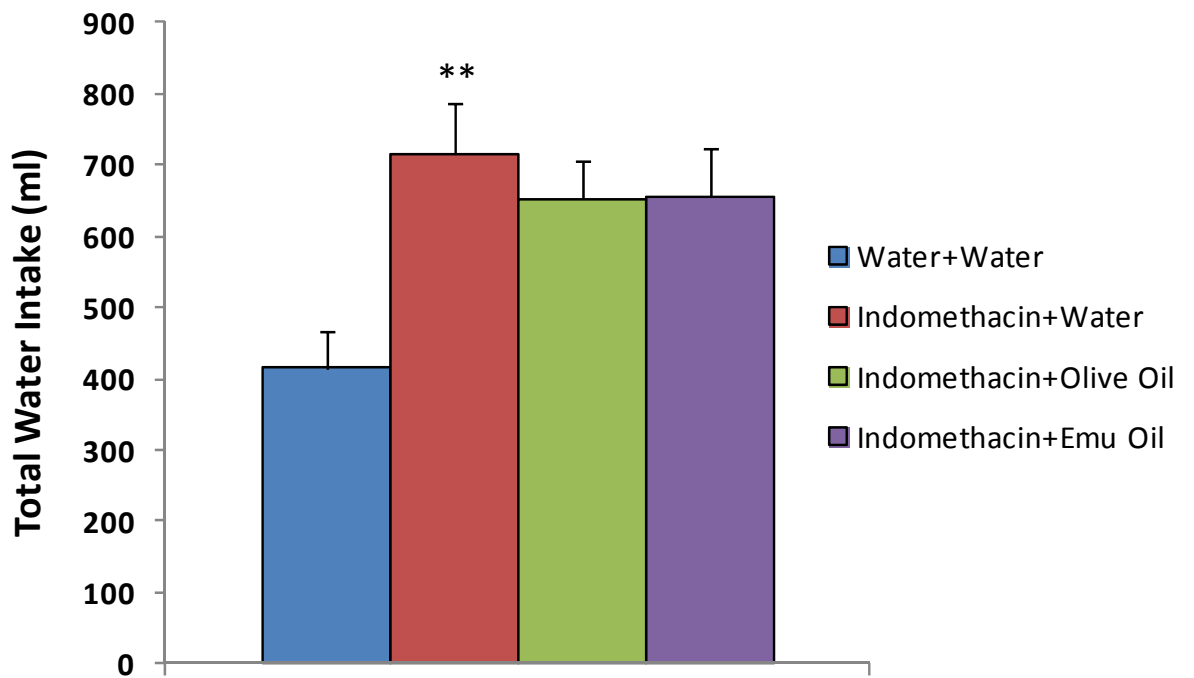
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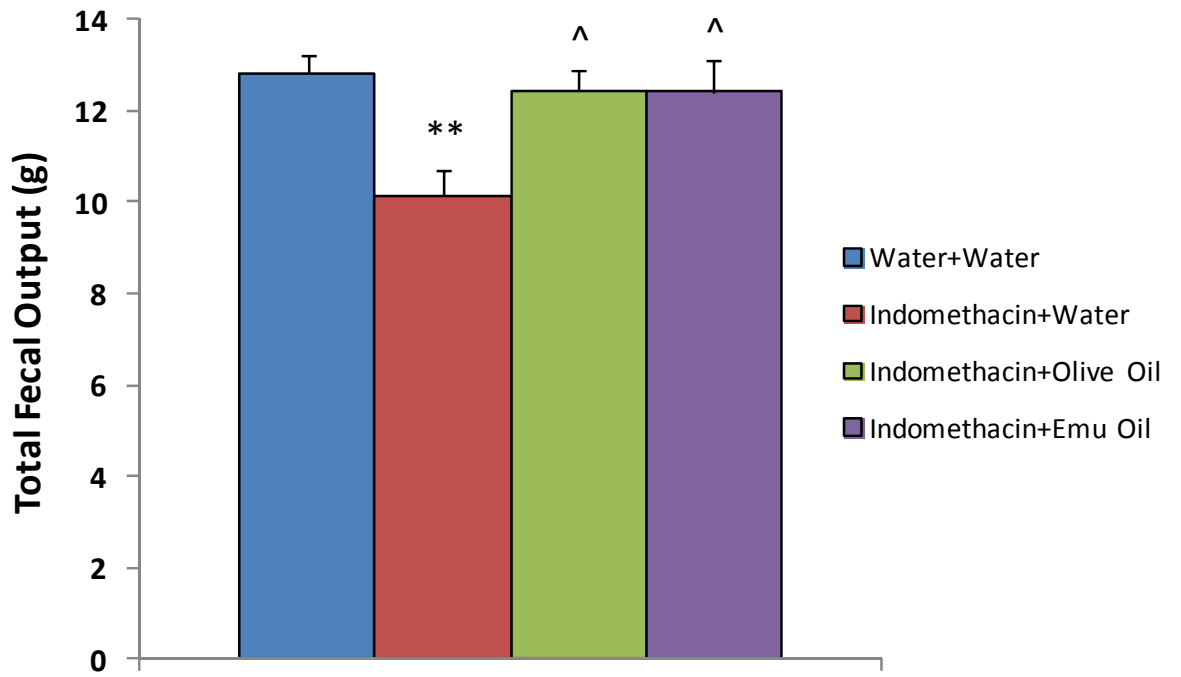
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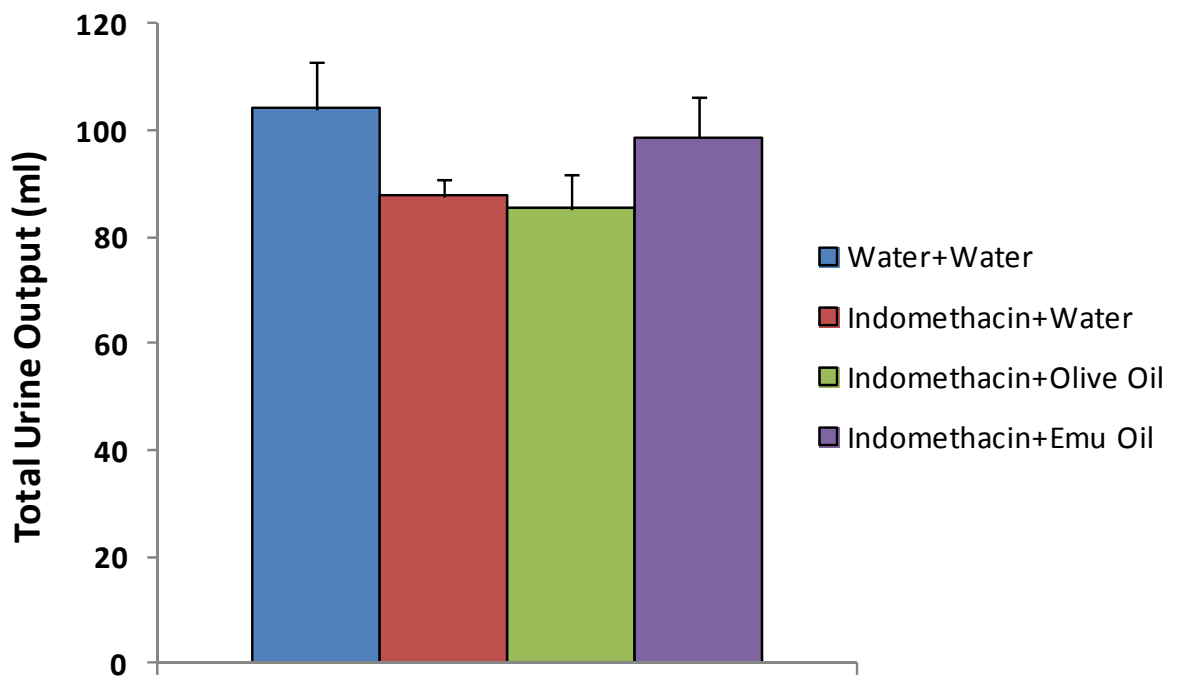
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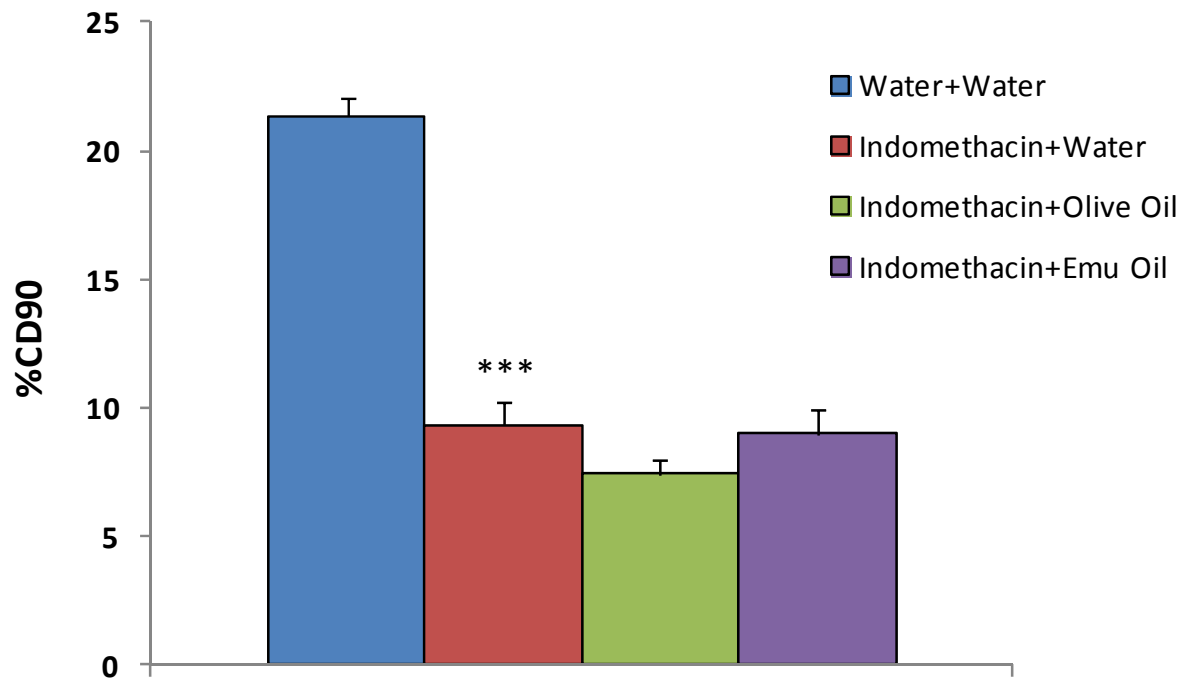
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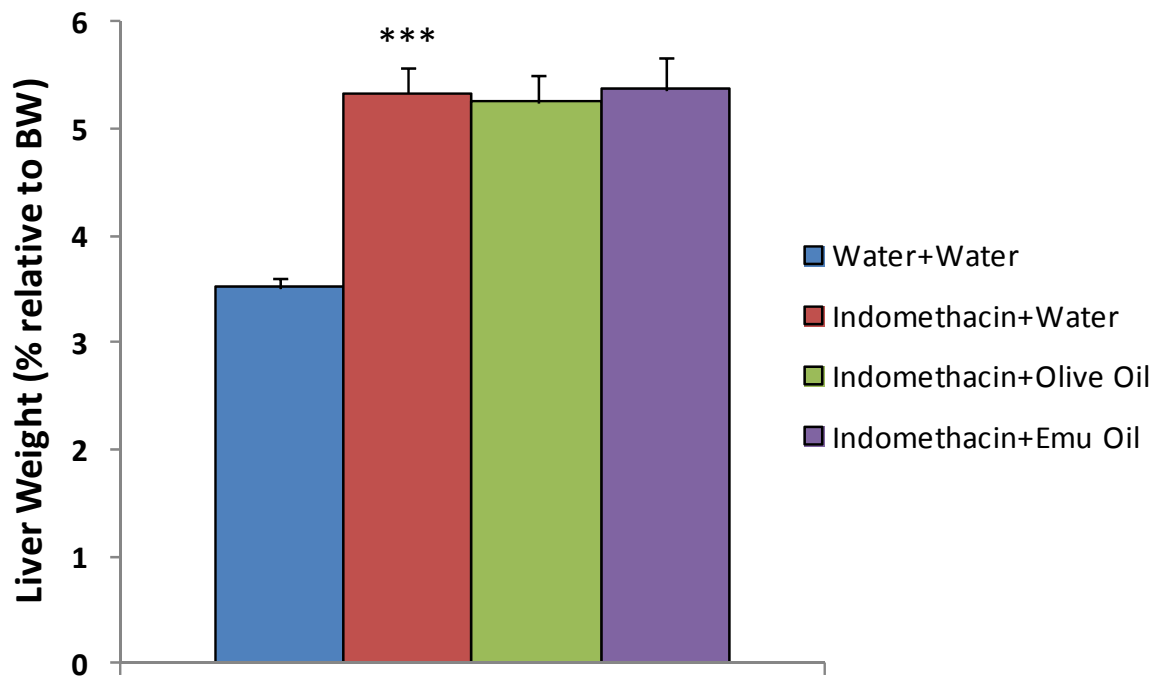
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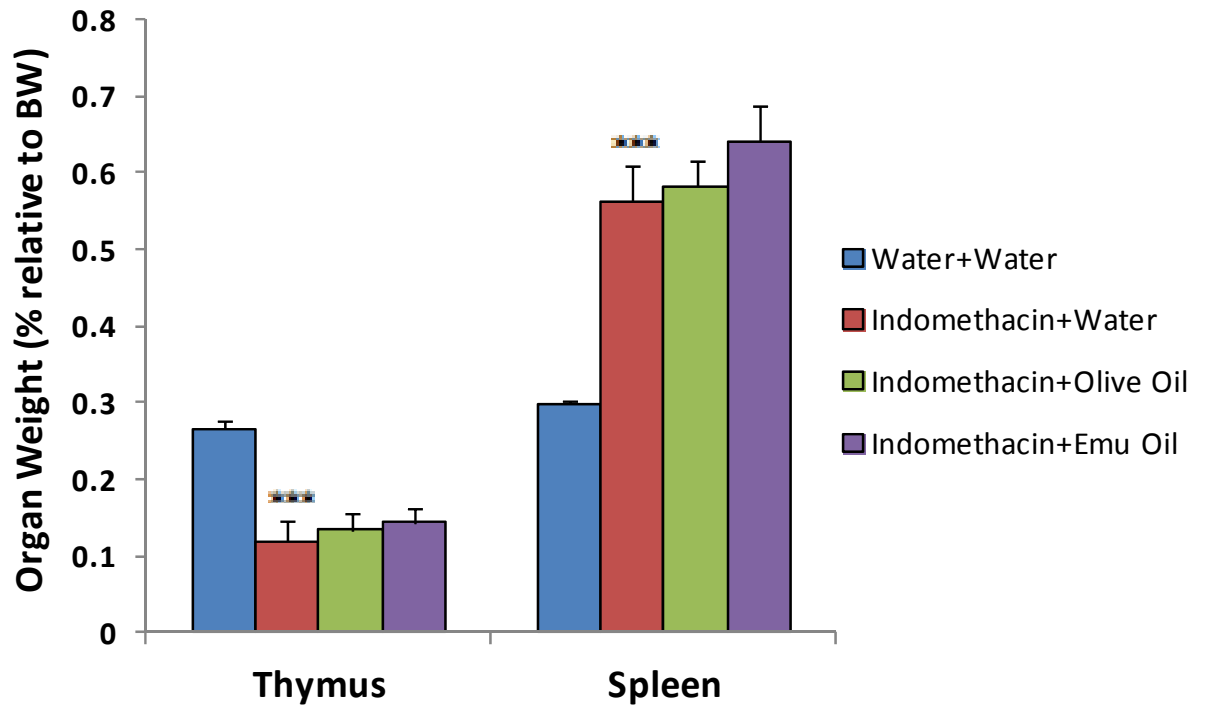
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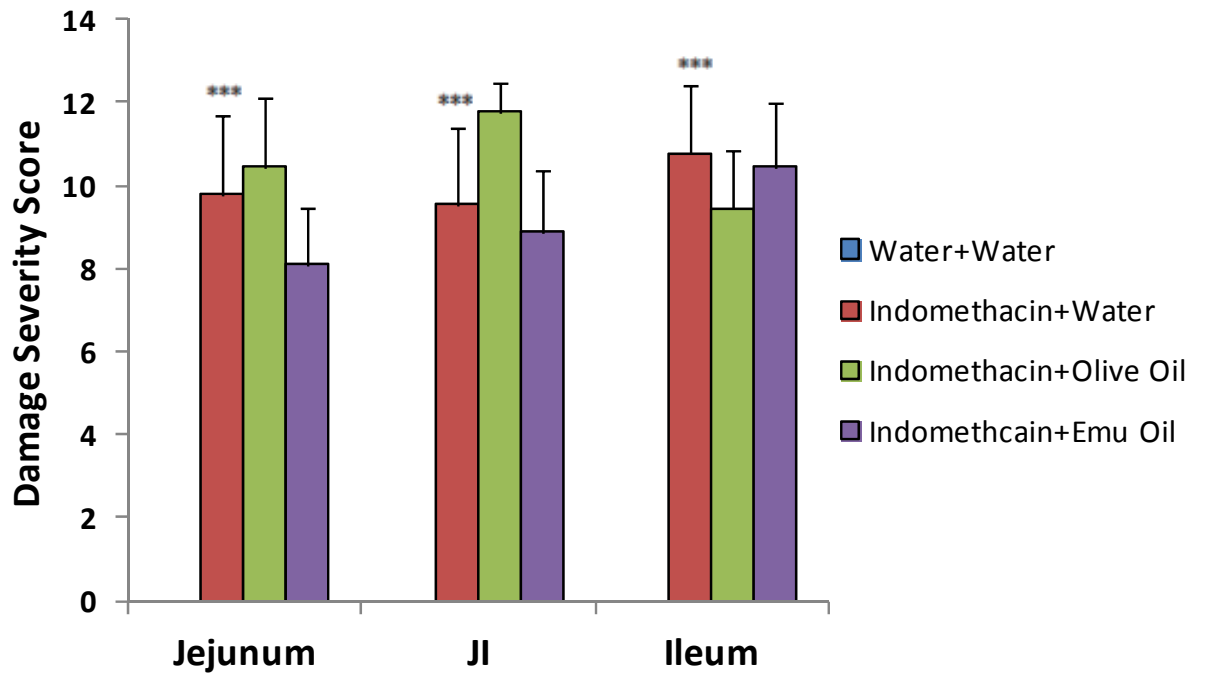
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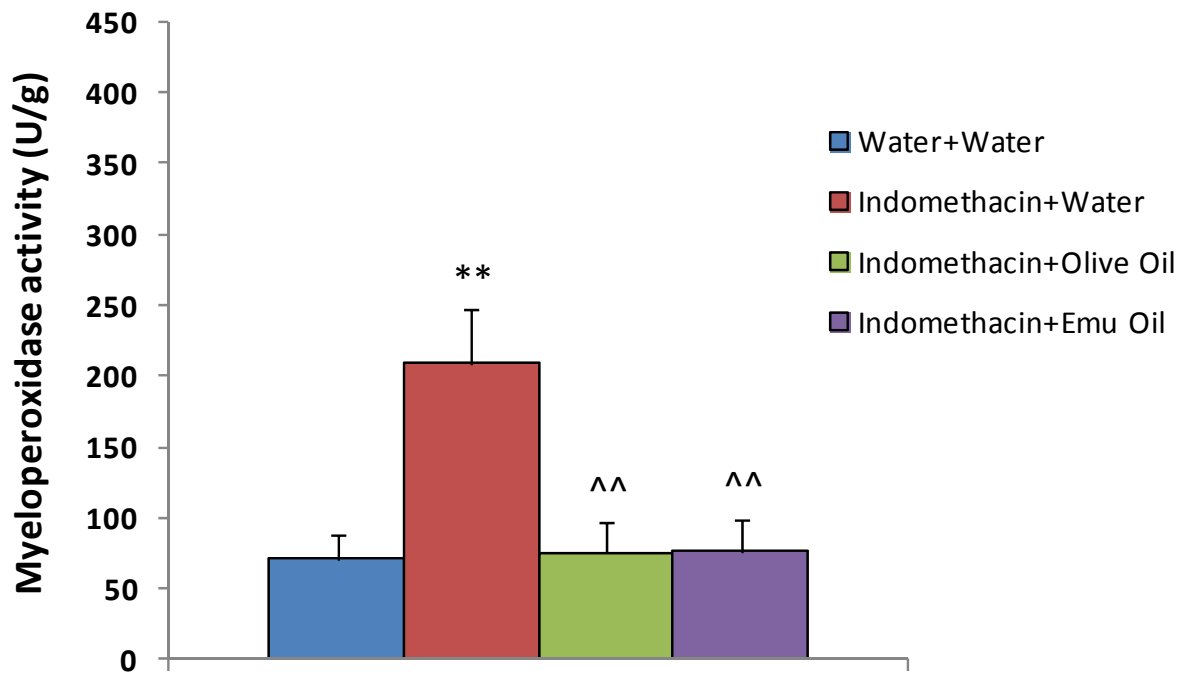
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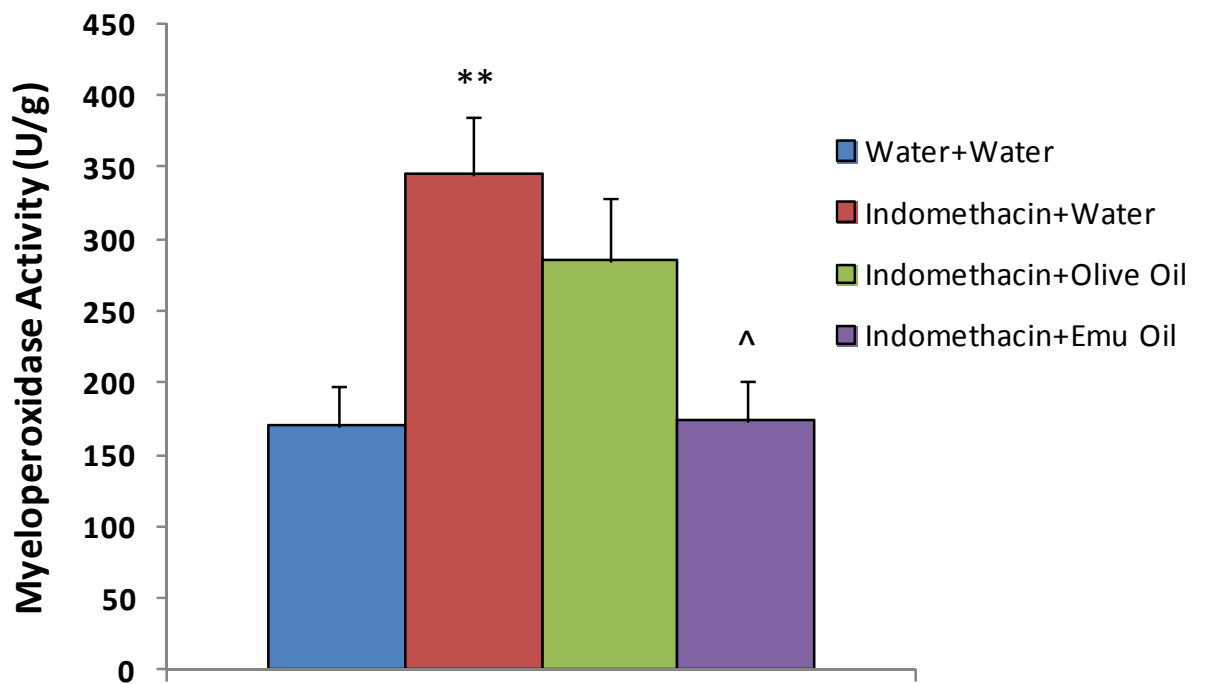
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Figure 8.

CHAPTER 5

PROCESSING OF RATITE OILS AFFECTS PRIMARY OXIDATION STATUS, RESULTING IN GREATER RADICAL SCAVENGING PROPERTIES FOR EMU OIL COMPARED TO RHEA AND OSTRICH OILS

CONTEXT STATEMENT

The work contained in this chapter is currently under review in the *Journal of Food Science.*

SM Abimosleh, DC Bennett, CD Tran, GS Howarth. Processing of Ratite Oils Affects Primary Oxidation Status, Resulting in Greater Radical Scavenging Properties for Emu Oil Compared to Rhea and Ostrich Oils. *Journal of Food Science* 2012; Under review

Chapter 5 describes the influence of farm location, rendering method, duration and storage mode on free radical scavenging activity and primary oxidation status of Ratite Oils. Different batches of Emu Oil were compared with Ostrich Oil, Rhea Oil and Olive Oil.

STATEMENT OF AUTHORSHIP

Processing of Ratite Oils Affects Primary Oxidation Status, Resulting in Greater Radical Scavenging Properties for Emu Oil Compared to Rhea and Ostrich Oils

Journal of Food Science 2012; Under review

Suzanne Mashtoub ABIMOSLEH (Candidate)

Conducted all experimental work, analysed data, interpreted results, prepared figures and manuscript.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12

Darin C. Bennett

Contributed to experimental conceptualisation and design, data analyses and interpretation of results.

I hereby certify that the statement of contribution is accurate.

Signed   Date 22 Nov 2012

Cuong D. Tran

Contributed to experimental conceptualisation and design, data interpretation.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12

Gordon S. Howarth

Contributed to experimental conceptualisation and design, data interpretation, critical manuscript evaluation.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12.

Processing of Ratite Oils Affects Primary Oxidation Status, Resulting in Greater Radical Scavenging Properties for Emu Oil Compared to Rhea and Ostrich Oils

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Abstract

Background. Treatments for diseases such as coronary artery disease and gastrointestinal disorders seek to minimise oxidative damage by free radicals through the use of antioxidants. Oils derived from ratites (flightless birds) predominantly comprise fatty acids varying in composition between ratite species. *Aim.* The current study investigated the influence of farm location, rendering method, duration and storage mode on radical scavenging activity (RSA) and primary oxidation status of Ratite Oils. *Methods.* Emu Oil (n=8; EO1, EO2a/b, EO3-7; varying processing and storage factors), Ostrich Oil (OsO), Rhea Oil (RO) and Olive Oil (OlO) were tested for free RSA against 2,2-diphenyl-1-picryl hydracyl (expressed as 1/IC50 g/ml) and primary oxidation (peroxide $mEqO_2/Kg$). $p<0.05$ was considered significant. *Results.* RSA (g/ml) of EO1 (558±22) was greater than EO2a (8±0.6), EO5 (413±26), EO6 (16±0.3) and EO7 (2±0.2), OsO (313±12), RO (32±12) and OlO (196±4), and less than EO3 (717±32; $p<0.001$). Antioxidant properties of OsO were more pronounced than RO ($p<0.001$). Primary oxidation ($mEqO_2/Kg$) of EO1 (97±0.6) was greater than EO2a (57±0.6), EO2b (28±0.2), EO5 (11±0.6), OsO (50±0.9) and OlO (61±0.9). *Conclusions.* Emu Oil conferred the greatest RSA compared to Ostrich and Rhea Oil, possibly attributed to its high UFA:SFA ratio and non-triglyceride fraction minor constituents. Rendering and storage variables impacted on Emu Oil RSA and primary oxidation.

Keywords: Ratite Oil, Emu Oil, primary oxidation, radical scavenging activity

Practical Application

The current research highlights the importance to standardise industrial rendering and storage procedures for Ratite Oils, to ensure consistent consumer health benefits.

Introduction

The pathology of a broad spectrum of chronic diseases and disorders, including coronary artery disease, gastrointestinal ulcers, inflammatory bowel disease, cancer and the side-effects of cancer therapy (mucositis), is characterised by damage to cellular components via reactive oxygen species (ROS)(Abimosleh and others 2012a; Lindsay and others 2010; Marnett 2000; Uchida 2000; Brzozowski and others 2001). Preventative therapies and treatments for these conditions are aimed at minimising oxidative damage by free radicals through the use of antioxidants(Aruoma 1998). Natural antioxidants are defined as compounds that function as free radical scavengers, reducing agents, chelators of pro-oxidant metals or as quenchers of singlet oxygen(Miraliakbari and Shahidi 2008). Antioxidants such as carotenoids, flavanoids, polyphenols, phytosterols and vitamin E, are widely found in beverages (red wine(Ghiselli and others 1998) and grape juices) and plant extracts (grape seed(Cheah and others 2009) and elderberry(Dubey and others 2012)), plant-based foods (fruits and vegetables, whole grains, nuts and seeds)(Hu 2003; Kalt and others 1999), plant-based oils (flaxseed, canola, tree nut(Miraliakbari and Shahidi 2008), olive(Owen and others 2000) and other vegetable oils) and marine-based oils (Fish Oil(Kris-Etherton and others 2003)). However, less attention has been directed towards the antioxidant potential of animal- and avian-derived oils.

Polyunsaturated fatty acids (PUFAs) found in many oils, such as Fish Oil and Olive Oil, react with oxygen and form peroxides during the initial step of oxidation, known as primary oxidation(Ottestad and others 2011). Primary oxidation determines a subsequent series of chain reactions (accelerated by high temperature and light and oxygen exposure) that generate the production of more stable secondary oxidation products including aldehydes and ketones(Choe and Min 2006; Ottestad and others 2011). Oxidative stability and deterioration of oils depend upon oil composition including concentration of

antioxidants, degree of processing and storage conditions. The unfavourable consequences of oxidation determine the rancidity of oils and degradation of functional and nutritional properties(Crapiste and others 1999). However, it remains unclear whether oil FA oxidation significantly affects radical-scavenging activity of antioxidants.

Ratites are flightless birds with a raft-like breastbone devoid of a keel and vestigial to non-existent breast muscles(Angel 1996). The principal members of the ratite family are the Australian Emu (*Dromaius novaehollandiae)*, the African Ostrich (*Struthio camelus*), the three New Zealand Kiwi species (*Apterix sp.*), the New Guinea Cassowary (*Casuarius sp.*), the Rhea or South American Ostrich (*Rhea americana*) and the South American Choique (*Pterocnemia pennata*). Certain ratites are farmed globally for commercialization of their meat, skin, feathers, eggs and more recently, their oil(Grompone and others 2005). Ratite Oil is derived from subcutaneous and retroperitoneal adipose tissue of the bird, by first rendering the macerated tissue and then passing the liquefied fat through a series of filters to extract a purified oil(Beckerbauer and others 2001). However, the composition of Rhea, Ostrich and Emu Oils is not identical(Grompone and others 2005; Bennett and others 2008).

Emu Oil was first used by Native Australian Aboriginals and early white settlers to facilitate wound healing, pain alleviation and treatment of inflamed joints(Whitehouse and others 1998; Snowden and Whitehouse 1997; Abimosleh and others 2012b). FAs represent the predominating component of Emu Oil, with a lipid content of 98.8% for subcutaneous adipose tissue, and 98.0% for retroperitoneal adipose tissue(Beckerbauer and others 2001). It has been suggested that the remaining 1-2% undefined non-triglyceride fraction comprises variable levels of compounds including carotenoids, flavones, polyphenols, tocopherols and phospholipids, which may confer therapeutic benefits including

antioxidant properties(Bennett and others 2008; Whitehouse and others 1998; Abimosleh and others 2012b). Other factors impacting on clinical efficacy of Emu Oil could include farm location, diet composition, rendering procedures, time of render and duration of storage.

We sought to investigate the influence of farm location, rendering method, duration and method of storage on radical scavenging activity and primary oxidation status of Ratite Oils.

Methods

Oil samples

Emu Oil samples (n=8) supplied by Emu Oil manufacturers from Australia and Canada were utilised. Emu Oil 1 (EO1), EO2 (EO2a and EO2b; differing in storage procedure [see below]) and EO3 were extracted from Emus farmed in North-Eastern South Australia. EO4 was extracted from a South-Eastern Emu farm in South Australia. EO1 and EO4 were rendered in 2009, whilst EO2 was rendered in 2011 and EO3 in 2010. EOs1-4 were rendered by an Emu Oil manufacturer in South Australia. EO5 was extracted from Emus farmed in Victoria, Australia and processed in 2011 by a Victorian manufacturer. EO6 and EO7 were extracted from Emus farmed in Duncan, British Columbia, Canada and both oils were rendered onsite in 2001. Ostrich Oil (OsO) and Rhea Oil (RO) adipose tissues were extracted in South Africa and Argentina, respectively, and were also rendered in Duncan, British Columbia, Canada in 2001. Olive Oil (OlO) was rendered in 2009 using olives grown in Spain.

Although general processing procedures varied amongst manufacturers, briefly, the process involved the rendering and filtration of Emu adipose tissue (for example, Emu Oil manufacturer of EOs 1-4 filtered at $0.2\mu m$ to remove bacteria including Salmonella and Listeria), with appropriate considerations for delivery of quality assurance and product consistency. Furthermore, storage of oil samples varied such that EOs1, 2a, 5 and 6 and OlO were individually stored at 4^0C in 50ml opaque containers, EO2b was stored at -20^0C for 6 months following render and left thawed for 2 months in a large 10L opaque bucket, and EOs 3 and 4, OsO and RO were stored in opaque containers (EOs 3 and 4: 100ml containers and OsO and RO: large 10L containers) at room temperature following render.

2,2-diphenyl-1-picryl hydracyl radical (DPPH) assay

The free radical scavenging activity (RSA) of oil samples against the 2,2-diphenyl-1-picryl hydracyl radical (DPPH) was determined by colorimetric absorbance. Principally, ionised DPPH produces a deep purple colour, which changes to pale yellow when the radicals are reduced by an antioxidant (hydrogen donor; Figure 1). Briefly, 5mL of each oil sample was weighed and dissolved in 25mL of toluene (Fisher Scientific, Ottawa, Canada) using a volumetric flask and allowed to mix for 24 hours. To obtain the required test concentrations, increasing volumes of the oil-toluene mixture were adjusted to 3.6mL with toluene. Finally, 0.4mL of a freshly prepared toluenic solution of 1mM DPPH (Sigma-Aldrich Canada Ltd, Oakville, Canada) was added to the oil-toluene mixture and vortexed for 20 seconds at room temperature. Absorbance of the test solutions at 517nm was measured after 15 mins using a spectrophotometer (Shimadzu, Kyoto, Japan). The RSA was calculated as the differences in absorbance of the DPPH radical solution with and without (control) sample:

Percentage inhibition = (Absorbance $_{control}$-Absorbance $_{sample}$)/Absorbance $_{control}$ x 100.

Overall, the RSA of the oil sample was expressed as reciprocal of the oil concentration that provided 50% inhibition using linear regression (1/IC50 g/ml). This analysis was repeated three times for each oil sample to obtain an average reading during month 1.

Oxidation analysis

Primary oxidation of oils, and hence rancidity, was assessed through determinations of peroxide values by colorimetric absorbance. Principally, R-O-O-R peroxides oxidize Fe^{2+} ions. The Fe^{3+} ions resulting from oxidation are grouped and form a red complex. Briefly, oil samples (volume depended upon dilution) were transferred into individual pre-filled cuvettes, vortexed and incubated in the spectrophotometer (cdr Foodlab Fat, Florence,

Italy), according to the manufacturer's instructions. Colorimetric intensity, measured at 505 nm, is directly proportional to the concentration of peroxides in the sample. Results were expressed as milli equivalents of oxygen per kilogram of oil sample ($mEqO_2/Kg$). Initially, four oil samples (EOs 1-4; 2.5μl of each sample) were analysed once during month 1, using the peroxide reference range of 1-50$mEqO_2/Kg$ in a quality control study. During month 9, the peroxide levels of these four samples were determined once again. EOs 1, 3 and 4 were diluted to achieve an appropriate reference range of 4-275$mEqO_2/Kg$ (100μl of each sample was pipetted into a cuvette pre-filled with diluent and 5μl of this mixture was then transferred to another pre-filled cuvette for analysis). EO5, OlO, OsO and EO2 top and bottom layers separated naturally at room temperature) were also diluted and analysed utilising a 4-275$mEqO_2/Kg$ reference range according to the manufacturer's instructions (cdr Foodlab Fat, Florence, Italy). This analysis was repeated three times for each oil sample to obtain an average reading during month 9.

Statistical Analyses

Statistical comparisons were performed using SPSS version 16.0 for Windows software (SPSS Inc. Chicago, Illinois, USA). The IC50 value for scavenging activity of the DPPH radical was expressed as the reciprocal of the mean ± standard error of the mean (SEM). Furthermore, peroxide values indicative of primary oxidation were expressed as mean ± SEM. Data from all analyses were analysed using a one-way ANOVA with a Tukey's *post hoc* test. For all analyses, $p < 0.05$ was considered statistically significant.

Results

Free radical scavenging activity (RSA)

The reciprocal concentration of EO1 in the test solution that produced 50% neutralisation (1/IC50) of the DPPH radical was significantly greater than EOs 2a, 5, 6 and 7 ($p < 0.001$; Figure 2). Compared to EO3, the RSA of EO1 was significantly less ($p < 0.001$). Furthermore, the RSA value for EO7 was not significantly different to EO2a nor EO6 ($p > 0.01$). Moreover, the antioxidant properties of OsO were more pronounced than RO ($p < 0.001$). However, the RSA values of both ratite-derived oils OsO and RO and OlO were significantly less than EOs 1, 3, 4 and 5 ($p < 0.001$).

Primary oxidation

Primary oxidation, assessed through peroxide value determinations, was greatest in EOs 1, 3 and 4 ($49.72 mEqO_2/kg$) compared to EO2a and OlO (4.5 and $31.35 mEqO_2/kg$, respectively), during month 1. The peroxide value of EO1, assessed during month 9, was significantly greater than EO2a, EO2b and EO5 ($p < 0.001$; Figure 3). However, primary oxidation was significantly less in EO1 compared to EO4 ($p < 0.001$). The peroxide value of EO2a was significantly greater than EO2b, and interestingly, upon separation, primary oxidation of the top layer of EO2a was significantly greater than the bottom layer ($p < 0.001$). Furthermore, the peroxide value of EO2a was significantly less than its top layer ($p < 0.001$) and significantly greater than its bottom layer ($p < 0.05$) upon separation. Amongst all EO samples, EO5 possessed the lowest peroxide value ($p < 0.001$). Compared to EO1, the primary oxidation values for OsO and OlO were significantly less pronounced ($p < 0.001$; Figure 3).

Correlation between primary oxidation and radical scavenging activity

Based upon EOs 1-4 and OlO, a highly significant correlation was evident (R^2=0.9914) relating peroxide value with radical scavenging capabilities for oil samples analysed during month 1 (Figure 4). The significantly correlating power trend-line indicated an ability for oils with increasing primary oxidation to act as antioxidants (Figure 4).

Discussion

Ratite Oil comparisons

Bennett and others (2008) have demonstrated that Emu Oil exhibits both antioxidant properties *in vitro* (radical scavenging activities) and a protective role against oxidative damage (assessed by measuring the ability to inhibit lipid peroxidation of erythrocytes) in a biological membrane model system. Furthermore, these investigators reported that Emu Oil afforded greater protection than Ostrich and Rhea Oils against oxidative damage *in vitro*(Bennett and others 2008).

In the current study, Emu Oil samples 1, 3, 4 and 5 possessed a greater ability to scavenge free radicals *in vitro* compared to oils derived from the Ostrich and Rhea. Furthermore, this antioxidant property was more pronounced in Emu Oil than Olive Oil. These differences in radical scavenging ability may have been attributed to the varying composition of oils sourced from these flightless birds (Table 1). It has been proposed that a high UFA:SFA ratio may confer protection against oxidative damage(Bennett and others 2008; Abimosleh and others 2012b). The high ratio of 2.0 in Emu Oil compared to lower ratios of 1.4 in Ostrich and Rhea Oils, may therefore have accounted for the greater antioxidant properties of Emu Oil detected in this setting. However, in the current study, although the UFA:SFA ratio of Olive Oil (6.2) was greater than for Emu Oil, it did not confer greater radical scavenging ability compared to Emu Oils 1, 3, 4 and 5. This implied that the antioxidant properties of Emu Oil may have resided in the 1-2% non-triglyceride fraction proposed to contain variable levels of antioxidant compounds including carotenoids, flavones, polyphenols, tocopherol and phospholipids(Whitehouse and others 1998; Abimosleh and others 2012b; Bennett and others 2008).

Time period since oil render

Oxidation of UFAs is the primary reaction responsible for the degradation of lipids. Indeed, the oxidation level of biologically-sourced oil is an important quality criterion for the food industry. Oxidation of oils not only produces rancid flavours but can also decrease nutritional quality and safety by the formation of oxidation products, which may play a role in the pathogenesis of atherosclerosis and even cancer(Staprans and others 1996; Kanazawa and others 2002). It is therefore imperative to determine the effect of oxidation and 'shelf-life' of oils on their ability to act as antioxidants.

In the current study, primary oxidation of Emu Oil was time-dependent, as indicated by the greater peroxide values of oils rendered at earlier stages. This was also evident with Ostrich Oil and Olive Oil, highlighting a non-specific oxidation process. Although the primary oxidation of Emu Oil 2a was not pronounced due to later rendering time, separation of the two oil layers at room temperature indicated significant levels of peroxide in the top layer, whilst peroxide levels in the bottom layer remained low. This suggests that the UFAs are concentrated in the top layer, based upon their high susceptibility to oxidation, whilst the more stable SFAs remain in the bottom layer. Furthermore, the peroxide value of EO2a was greater than its bottom layer upon separation, highlighting the greater stability of SFAs. This could be considered a useful determinant for oxidative stability and hence shelf-life extension. However, the radical scavenging properties and overall functional benefits of each layer remain unclear, warranting further investigation. Additionally, it is well documented that PUFAs such as omega-3 confer favourable health effects which range from reduced cardiovascular disease risk through to immunomodulatory effects in rheumatoid arthritis and even a role in brain development(Ruxton and others 2004). Therefore, it would be undesirable to eliminate these fatty acids from oil preparations.

Interestingly, a striking correlation was evident between radical scavenging activity and peroxide values which indicated an ability for oils with increasing primary oxidation to act as more effective antioxidants.

Emu Oil inter-batch variations and manufacturer comparisons

Ratite Oil composition can significantly differ depending upon diet, age of bird, farm location, methods and procedures used to render the fat into oil and the degree of processing and refining. Currently, there is no global standardized procedure to counteract these varying factors, which was reflected in the current study. Emu Oils 1-4 significantly varied in radical scavenging abilities and primary oxidation. However, considerations of rendering times minimised this variation, as evidenced by Emu Oils 1 and 4. Interestingly, there was a significant difference between Emu Oil derived from different manufacturers within the same year. Although extracted and processed within the same year, Emu Oil 5 possessed significantly greater radical scavenging ability than Emu Oil 2a; oils processed by differing manufacturing procedures. Furthermore, primary oxidation of Emu Oil 5 was significantly less pronounced than Emu Oil 2a. This highlights the importance of standardised extraction and processing procedures to optimize Emu Oil efficacy and ensure consistent therapeutic benefits for a range of medical conditions.

Storage procedure

Lipid oxidation is the cause of deteriorative modifications in edible oils as the chemical, sensory and nutritional properties are affected. Hence, lipid oxidation is often a decisive factor determining useful storage life(Martin-Polvillo and others 2004). However, rigorous scientific studies are limited to determine the optimal storage conditions for improvement of shelf-life. In the current study, various storage techniques were employed including freezing, refrigerating and duration at room temperature, small or large containers and a

combination of these factors. When comparing the same Emu Oil sample, there were notable differences in primary oxidation of samples stored refrigerated in small volumes (2a) compared to samples stored frozen and thawed in a large volume (2b). The freeze and thaw method proved most successful to minimise primary oxidation. Moreover, Crapiste *and others* (1999) described a dependent relationship between oil oxidation rate and oil surface area exposed to air; with sample volume contributing further to oxidation rate. Based upon these factors, auto-oxidation was significantly higher for a capped flask with 22.5g of sunflower oil than for an open flask with 45g of oil at the same temperature (47^0C). In the current study, the dissolution and diffusion of oxygen and dilution of oxidation compounds in the bulk oil may have accounted for the differences in peroxide values observed for the two storage methods of Emu Oil 2. However, the significant correlation between increasing peroxide values and improved radical scavenging abilities (Figure 4) suggests a fine balance in the determination of oxidation status versus antioxidant activity.

Conclusions

In the current study, Emu Oil conferred the greatest radical scavenging ability compared to Ostrich and Rhea Oil, possibly attributed to its higher UFA:SFA ratio and minor constituents in the non-triglyceride fraction. Other factors influencing Emu Oil radical scavenging activity and primary oxidation include farm location, rendering time, rendering methods and storage techniques. Method of storage was a critical factor for primary oxidation, whereby freeze-thaw technique and storage in larger volumes proved to be most effective. Furthermore, the rendering protocol was identified as an important factor in radical scavenging abilities and oil stability. It is recommended that future studies should carefully consider oxidation and radical scavenging activity of oil samples prior to *in vitro* and *in vivo* studies in order to standardise bioactivity. Moreover, the minor non-FA

constituents of Emu Oil possessing antioxidant properties should be identified and quantified. Such further studies would facilitate standardisation of Emu Oil bioactivity to ensure consistent health benefits.

Acknowledgements

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Table 1. Major fatty acid composition of Emu, Ostrich, Rhea and Olive Oils including unsaturated fatty acid (UFA): saturated fatty acid (SFA) ratios. The Emu Oil and Olive Oil presented in this table were used in the current study (EO1 and OlO respectively). Ostrich Oil and Rhea Oil values were obtained from Grompone *and others,* 2005. Monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA).

Major FA Composition of Emu, Ostrich, Rhea and Olive Oils					
Fatty Acid	**Common Name**	**Emu**	**Ostrich**	**Rhea**	**Olive**
16:0	Palmitic acid	24	34.9	34.4	10.4
16:1n-7	Palmitoleic acid	4.3	7.4	4.5	0.7
18:0	Stearic acid	8.5	5.7	5.4	3.1
18:1n-9	Oleic acid	49.1	30.5	30.6	73.9
18:2n-6	Linoleic acid	9.5	16	21	8.4
18:3n-3	α-Linolenic acid	1.1	2.1	1.9	0.7
Saturated		32.5	40.6	41.1	13.5
MUFA		53.4	37.9	35.1	74.6
PUFA		10.6	18.1	22.9	9.1
UFA:SFA ratio		2	1.4	1.4	6.2

$$\text{DPPH}^{\text{o}} + \text{AH} \longrightarrow \text{DPPH-H} + \text{A}^{\text{o}}$$
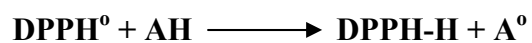
Figure 1. The reduction of the 2,2-diphenyl-1-picryl hydracyl stable radical (DPPH^{o}) by an antioxidant (AH). In its radical form, DPPH^{o} absorbs at 517nm and produces a deep violet colour, however, absorption diminishes (decolourises) upon reduction by an antioxidant (hydrogen donor).
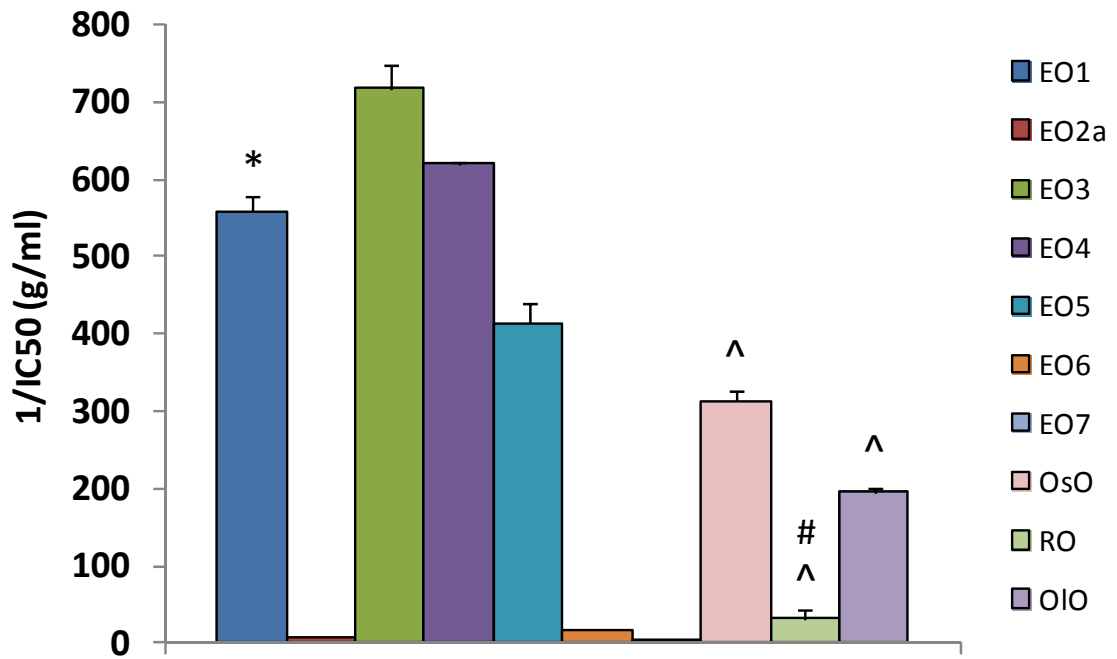
Figure 2. Reciprocal concentrations of oils producing 50% inhibition (1/IC50; g/ml) for scavenging activity of the DPPH radical (n=3 replicates), assessed during month 1 [Emu Oil (EO); Ostrich Oil (OsO); Rhea Oil (RO); Olive Oil (OlO)]. Data are expressed as mean ($1/IC_{50}$ g/ml) ± SEM. * indicates $p<0.001$ compared to all test samples except EO4; # indicates $p<0.001$ compared to OsO; ^ indicates $p<0.001$ compared to EOs 1, 3, 4 and 5.
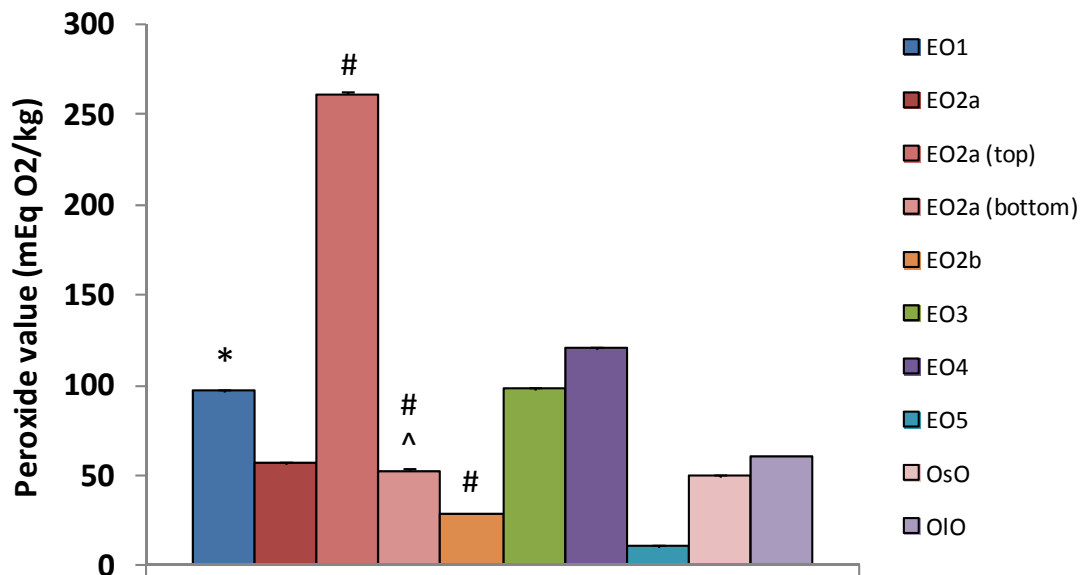
Figure 3. Peroxide values of oil samples indicative of primary oxidation (n=3 replicates), assessed during month 9 [Emu Oil (EO); Ostrich Oil (OsO); Olive Oil (OlO)]. Data are expressed as mean (Peroxide value mEqO$_2$/kg) ± SEM. * indicates $p<0.001$ compared to all test samples except EO3; [#] indicates $p<0.05$ compared to EO2a; [^] indicates $p<0.001$ compared to EO2a (top).
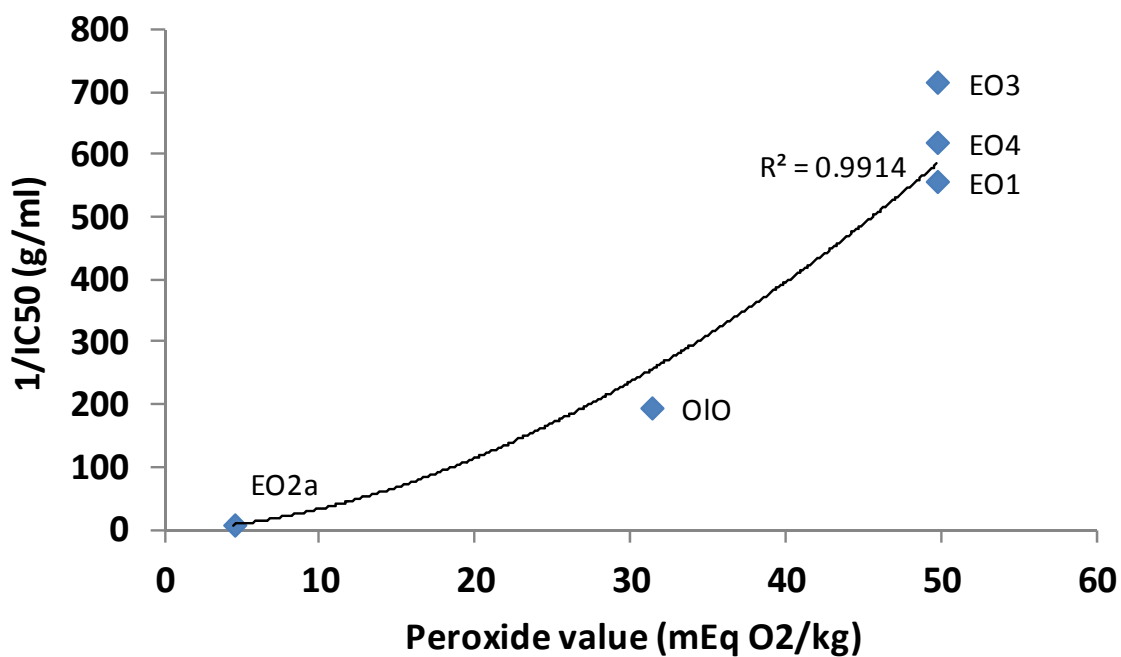
Figure 4. Correlation between oil peroxide value (mEqO$_2$/kg) and its radical scavenging capability (1/IC50 g/ml) during month 1 [Emu Oil (EO); Olive Oil (OlO)]. R^2=0.9914 for power trendline.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

In support of Emu Oil as a potential orally-administered nutraceutical, Emu Oil reduced colonic damage associated with dextran sulphate sodium-induced colitis in rats and accelerated the repair process **(Chapter 2)**. Improvements were indicated histologically by reduced intestinal damage severity scores and enhanced compensatory crypt elongation in the colon. Furthermore, distal colonic crypts were elongated by Emu Oil in normal rats, which could have represented a modulation of the proliferation and apoptosis balance. Therefore, proliferation and apoptosis would be important parameters to define in this setting to determine the mechanism of Emu Oil action. In future studies, assessment of cell proliferation in paraffin-embedded tissue sections could be determined by 5-bromodeoxyuridine (BrdU) immunohistochemistry. BrdU, injected intraperitoneally, is a thymidine analogue incorporated into the DNA of proliferating cells during the S phase of the cell cycle and is detected via an anti-BrdU antibody. Additionally, immunostaining of proliferative cell nuclear antigen, expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle (late G1- and S-phase), could be assessed as an indicator of crypt cell proliferative state. Furthermore, TUNEL assay and immunohistochemistry for caspase-3, gatekeeper in the caspase cascade, could be undertaken to establish the degree of epithelial cell apoptosis. Moreover, a high Bax/Bcl-2 ratio of tissue mRNA expression has been correlated with enhanced apoptosis. Techniques to define this ratio could therefore be considered for future mechanistic determinations of Emu Oil action.

Continual stimulation of mucosal thickening by Emu Oil beyond the experimental days assessed in the current study could potentially, yet improbably, be a safety concern. For example, proliferation and apoptosis imbalance could lead to neoplasia. Further investigations are warranted to assess mucosal thickening beyond the experimental period

of the current study. Future studies could determine whether Emu Oil elicits any changes in cellular pathways associated with colonic neoplasia such as P13-K signalling pathway. Nevertheless, although untested, continual crypt lengthening would appear unlikely based upon the decreased histological damage severity observed in the colon, indicating that rats administered Emu Oil suffered a lesser degree of tissue damage. Rat models of azoxymethane-induced colon cancer or mammary adenocarcinoma in spontaneous tumor-bearing Dark Agouti rats would be appropriate to assess the impact of Emu Oil on tumor growth, coincident with the enhancement of small intestinal (SI) repair following chemotherapy.

Animal models have contributed greatly to the understanding of colitis pathogenesis, revealing that intestinal inflammation occurs only in the presence of the microbiota, as animals raised under germ free conditions do not develop colitis. Restoring microbial homeostasis by targeting colitis-associated bacteria such as *Bacteriodes* spp, has been implicated as a novel therapeutic strategy for colitis. We identified microbiota changes associated with colitis in rats in **Appendix 2**. The effect of Emu Oil in this context is yet to be defined. In future studies, terminal restriction fragment length polymorphism analysis would be useful to quantify fecal bacterial communities from colitic rats treated with Emu Oil. This would define whether the anti-inflammatory properties of Emu Oil were related to its direct effect on the inflammatory response, or indirectly through restoration of the gut microbiota.

Clinical manifestations of 5-Fluorouracil (5-FU)-induced mucositis included decreased body weight and food intake and elevated disease activity index together with reduced SI functional health status **(Chapter 3)**. These parameters and reduced thymus weight were not improved following Emu Oil treatment. However, in the 5-FU-damaged SI, Emu Oil

maintained villus height and crypt depth during maximal damage followed by an enhanced compensatory elongation, suggesting an acceleration of the repair process. Similar to **Chapter 2**, Emu Oil administration to normal rats resulted in SI mucosal thickening. Further studies are warranted to determine the kinetic mechanism of Emu Oil action in regards to enterocyte apoptosis and proliferation. SI and colonic changes in mucosal thickness described in **Chapters 2 and 3** suggested that Emu Oil maintains its bioactivity throughout the gastrointestinal tract. Future studies could employ radio-labelled Emu Oil constituents to determine the rate of degradation with passage through the gastrointestinal tract. Moreover, intestinal permeability changes following Emu Oil treatment could be quantified in future studies utilizing tests such as serum citrulline levels or urinary lactulose, rhamnose and mannose excretion.

Emu Oil decreased MPO activity, indicative of acute inflammation, in the jejunum and ileum of 5-FU-injected rats **(Chapter 3)**. These findings further support the bioavailability of Emu Oil throughout the SI, evidenced by measurable anti-inflammatory effects in the distal SI. In future studies, increased Emu Oil dose or frequency of administration could further decrease the extent of small bowel inflammation in mucositis, potentially achieving clinical benefit.

The mechanism by which Emu Oil exerts its anti-inflammatory effects remains unclear. Potential mechanisms could be attributed to the omega-3 and -9 FAs present in Emu Oil or constituents in the non-triglyceride fraction including carotenoids, flavones, tocopherols and skin-permeation enhancing factors, which may confer additional anti-inflammatory and antioxidant effects. Further studies of Emu Oil could benefit from a complete time-course of cytokine profiling, including TNF-α, IFN-γ, IL-1β, IL-6 and IL-10, in serum and tissue samples, to identify potential effects on pro-inflammatory cytokine antagonism or

agonism of anti-inflammatory cytokines in the current inflammatory intestinal disorders. More specifically, upon fractionation of Emu Oil, individual constituents could be examined to determine effects on cyclooxygenase 1 or 2 pathways and pro-inflammatory cytokine levels *in vitro,* thereby facilitating the development of more potent Emu Oil preparations.

Indomethacin-induced enteropathy resulted in increased liver, spleen, duodenum, SI, caecum and colon weights **(Chapter 4)**. Emu Oil treatment did not improve these parameters. Together with **Chapters 2 and 3**, this highlighted the overall absence of systemic improvements by Emu Oil. MPO activity in the jejunum and ileum of Indomethacin-treated rats was decreased following Emu Oil administration. **Chapter 3 and 4** reaffirm the anti-inflammatory properties of Emu Oil, confirming its therapeutic application in intestinal disorders characterised by inflammation.

In all of the *in vivo* experimental studies conducted within this thesis, Emu Oil did not significantly impact daily metabolic parameters which included body weight, water intake, faecal output and urine output in normal rats. However, food intake was decreased, presumably the result of lipid-rich constituents of Emu Oil. Consequently, overall caloric intake was maintained, as evidenced by the absence of changes in body weight compared to normal rats **(Chapter 3)**. ^{13}C-sucrose breath test results confirmed the maintenance of SI functional health by Emu Oil and contributed to the data supporting its safety for oral administration. High oil viscosity could have resulted in adherence of Emu Oil to the luminal epithelial surface, contributing to the increased SI weight described in **Chapters 2 and 3**. Furthermore, concomitant increases in SI, villus and crypt lengthening could have contributed to SI weight increases in experimentally-induced mucositis **(Chapter 3)**.

However, Emu Oil did not significantly affect other visceral organ weights highlighting its safety for oral administration.

Based on the current findings, further studies could focus on the potential for Emu Oil to combat other intestinal conditions characterised by inflammation. For example, as Emu Oil is gluten-free, it could represent an adjunctive treatment-strategy for celiac disease, especially in refractory disease. Moreover, anti-inflammatory effects of Emu Oil should be investigated in other bowel conditions characterised by low-grade inflammation such as irritable bowel syndrome and infective gastroenteritis. Furthermore, it would be useful to quantify the coincident benefits of Emu Oil in arthritis and the associated co-morbidity of NSAID-induced enteropathy.

Radical scavenging activity and primary oxidation of Emu Oil were influenced by farm location, rendering time, rendering methods and storage techniques **(Chapter 5)**. Method of storage was determined to be a critical factor for primary oxidation, whereby freeze-thaw technique and storage in larger volumes proved to be most effective. Future studies should investigate the influence of oxidation and radical scavenging activity of oil samples using intestinal epithelial cell lines (IEC-6) and organ culture systems. Furthermore, clinical efficacy of different Emu Oil preparations should be explored using the currently employed rat models of intestinal damage. Such studies would facilitate standardisation of Emu Oil bioactivity to ensure consistent health benefits.

Emu Oil conferred greater radical scavenging ability than Ostrich and Rhea Oil, likely attributed to its higher unsaturated fatty acid (FA): saturated FA ratio together, with minor constituents in the non-triglyceride fraction **(Chapter 5)**. Comparisons of these constituents between Ratite Oils would assist in identifying the antioxidant properties of

Emu Oil. This could be achieved utilising the solid phase extraction technique for fractionation of antioxidant compounds in the oils.

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In conclusion, Emu Oil could represent a safe, renewable and economical means to augment pharmaceutical options for a range of intestinal conditions, not necessarily restricted to colitis, NSAID-enteropathy or mucositis.

APPENDICES

APPENDIX 1

GRAPE SEED EXTRACT REDUCES THE SEVERITY OF

SELECTED DISEASE MARKERS IN THE PROXIMAL COLON OF

DEXTRAN SULPHATE SODIUM-INDUCED COLITIS IN RATS

STATEMENT OF AUTHORSHIP

Grape Seed Extract Reduces the Severity of Selected Disease Markers in the Proximal Colon of Dextran Sulphate Sodium-Induced Colitis in Rats

Digestive Diseases and Sciences 2012; Nov 10: DOI 10.1007/s10620-012-2464-1

Ker Y. Cheah

Analysed data, interpreted results, prepared manuscript.

I hereby certify that the statement of contribution is accurate.

Signed   Date 28/11/2012

Susan E.P. Bastian

Contributed to experimental conceptualisation and design, data interpretation and manuscript preparation.

I hereby certify that the statement of contribution is accurate.

Signed   Date 27.11.12

Thomas M.V. Acott

Conducted animal trials and experimental work, analysed data, interpreted results.

I hereby certify that the statement of contribution is accurate.

Signed   Date 26/11/12

Suzanne Mashtoub ABIMOSLEH (Candidate)

Conducted animal trials, experimental work and data analyses.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12

Kerry A. Lymn

Conducted animal trials.

I hereby certify that the statement of contribution is accurate.

Signed   Date 30/11/2012

Gordon S. Howarth

Contributed to experimental conceptualisation and design, data interpretation, critical manuscript evaluation.

I hereby certify that the statement of contribution is accurate.

Signed   Date 3/12/12

APPENDIX 2

MICROBIAL FINGERPRINTING DETECTS UNIQUE BACTERIAL COMMUNITIES IN THE FAECAL MICROBIOTA OF RATS WITH EXPERIMENTALLY-INDUCED COLITIS

STATEMENT OF AUTHORSHIP

Microbial Fingerprinting Detects Unique Bacterial Communities in the Faecal Microbiota of Rats with Experimentally-Induced Colitis

The Journal of Microbiology 2012; 50 (2): 218-25

Ashis K. Samanta

Conducted experimental work, analysed data, interpreted results.

I hereby certify that the statement of contribution is accurate.

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APPENDIX 3

CURRENT AND NOVEL TREATMENTS FOR ULCERATIVE

COLITIS

STATEMENT OF AUTHORSHIP

Current and Novel Treatments for Ulcerative Colitis

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Conducted a thorough review of the literature, prepared manuscript.

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Current and Novel Treatments for Ulcerative Colitis

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1. Introduction

Ulcerative colitis and Crohn's disease are defined by a common term of inflammatory bowel disease. These chronic diseases result in significant morbidity and mortality. While there are no cures for these diseases, the last two decades have been a period of major advancement in our understanding of the biology of intestinal inflammation. This can be attributed to a steadily increasing number of experimental animal models with some clinical manifestation similar to those observed in human inflammatory bowel disease. These experimental animal models have also contributed greatly to our current understanding of the immunological, pathological and physiological features of chronic intestinal inflammation. However, specific causes of ulcerative colitis and Crohn's disease remain unknown. Conventional treatments for the disease include corticosteroids and immunosuppressives, however treatments in many patients are not entirely effective with many therapies associated with significant adverse effects. Thus, treatments that are effective and have little or no side effects remain an unmet need. There are numerous emerging therapeutic strategies which may be useful in the alleviation of chronic intestinal inflammation and this chapter will focus on novel therapies that may be effective for ulcerative colitis in the future.

2. Etiology of ulcerative colitis

While the precise etiology of inflammatory bowel disease is unknown, genetic susceptibility, environmental factors, impaired barrier function, imbalances or disruption to the commensal host microflora and an abnormal intestinal immune response are thought to play an important role in its manifestation.

2.1 The immune response

It is clear that not one single component of inflammatory bowel disease pathogenesis can trigger and maintain the disease. Understanding mucosal immunity in Crohn's disease and ulcerative colitis is fundamental in unraveling the complex mechanisms of chronic gut inflammation which can then provide some insight into the treatment of inflammatory bowel disease. The immune response is divided into two components, innate immunity and adaptive immunity.

2.2 Innate immunity

In the normal intestine, macrophages are conditioned by the mucosal microenvironment to express a non-inflammatory phenotype which is translated by a down-regulated expression of innate immunity receptors and constrained production of pro-inflammatory cytokines (1). In contrast, in inflammatory bowel disease-affected intestinal tissue, macrophages newly recruited from the peripheral blood still express monoctyic CD14 markers but are primed for the production of various pro-inflammatory cytokines such as interleukin (IL)1α, IL1β, and tumour necrosis factor (TNF)α (2-3). It has been reported that in Crohn's disease these CD14^{+} pro-inflammatory macrophages are increased and subsequently result in more IL23 and TNFα production compared to controls and ulcerative colitis and contribute to the production of interferon (IFN)γ by T cells (4).

Intestinal dendritic cells (DC) are antigen-presenting cells involved in the initiation and regulation of local innate immune response but also play a role in adaptive immunity (5). Similar to macrophages, their function is dependent on the mucosal microenvironment and function to provide protection and defense, induce tolerance or mediate inflammation (6). It has been shown that in inflammatory bowel disease, intestinal DC is activated, increasing the expression of microbial receptors and production of pro-inflammatory cytokines like IL12 and IL6 (7).

2.3 Adaptive immunity

B cell immunity

There is limited focus given to B cell immunity in inflammatory bowel disease even though in active inflammatory bowel disease there is antibody production and secretion of immunoglobulin (Ig)M, IgG and IgA, by both peripheral blood and mucosal mononuclear cells (8). The patterns of antibody class production differ in ulcerative colitis and Crohn's disease; in ulcerative colitis there is a disproportional increase in IgG1 secretion, whereas in Crohn's disease IgG1, IgG2 and IgG3 are increased compared to control cells (9).

T cell immunity

There has been a considerable increase in our understanding of adaptive immunity since the identification of CD4^{+} T helper 1 (Th1) and T helper 2 (Th2) subsets in mice (10) and humans (11). The T cell immunity field is still evolving and in addition to IFNγ-producing Th1 cells and IL4, IL5 and IL13-producing Th2 cells, new Th subsets have been identified including IL17 producing Th17 cells (12) and dual IFNγ- and IL17-producing Th17 cells (13). More recently, two new subsets of CD4^{+} effector Th cells have been described, Th9 and Th22, however, their function are not clearly understood (14). Furthermore, Cosmi et al. (15) reported that Th cells can produce both IL17 and IL4 which is a dual Th17 and Th2-mediated immune response.

In addition to Th cells, another major subset is made up of T regulatory (Treg) cells whose function is to monitor the immune response and prevent an excessive and potentially harmful immune response (16-17). It has been speculated that Th17 and Treg cells share common pathways, suggesting developmental and functional links between Treg and Th17 cells (18-19). Regulatory T cells are accepted to be key players in the maintenance of tolerance and prevention of autoimmunity (20). Of particular interest are the CD4^{+} CD25bright Foxp3^{+} Regulatory T cell (Treg), where mutations of the transcription factor Foxp3, crucial in the development and function of Tregs, manifest in multiple autoimmune

diseases in both mice and humans (21). In both cases a severe early onset of inflammatory bowel disease is observed as part of the pathology. A deficiency of number and/or function of Tregs are also seen in other autoimmune diseases including multiple sclerosis and systemic lupus, and the transfer of Tregs has been shown to treat experimental murine colitis and type 1 diabetes.

The Th17 effector cell is a relatively new effector cell lineage distinct from the Th1/Th2 dichotomy, and is driven by the transcription factor retinoic acid-related orphan receptor-γt, (RORγt). Th17 cells secrete predominately IL17 and potentially provide critical protection against fungi and extra cellular bacteria which are not covered by Th1/Th2 immunity (22). There is good evidence that Crohn's disease has a dominant Th1 component as shown by an elevated production of IFNγ and IL12 by lamina propria mononuclear cells (23-24). As well there is an increased production of IL-17 by Th17 cells and dual IFNγ- and IL17-producing mucosal Th cells (13, 25). In contrast, ulcerative colitis is considered an atypical Th2 response based on studies demonstrating increased IL5 and IL13 production of Th cells and also IL13 by natural killer (NK) T cells in the inflamed mucosa (26). The increased production of IL13 has been shown to induce cytotoxicity and apoptosis and impair mucosal barrier function (27), which may be a contributor to the overall pathogenesis of ulcerative colitis.

3. Characteristics of ulcerative colitis

The remainder of the chapter will focus on animal models of ulcerative colitis and novel therapeutic approaches in treating this condition. The clinical symptoms of ulcerative colitis consist of severe abdominal pain and increased frequency of bloody diarrhoea. Unlike Crohn's disease, ulcerative colitis is characterized by inflammation contained to the large intestine, which affects only the mucosal layer and is superficial in comparison to the inflammation seen in Crohn's disease. Inflammation commonly extends proximally from the rectum, and extensive superficial ulceration is typical (28). Inflammation is accompanied by ulceration, edema, and hemorrhage along the length of the colon. Complications of ulcerative colitis differ from Crohn's disease, with increased risk of perforation, toxic megacolon and a higher incidence of bowel cancer. Histopathological features include the presence of neutrophil infiltrates which form crypt abscesses (29).

4. Animal models of experimental ulcerative colitis

Even with a wealth of information on the etiology of ulcerative colitis there is still no cure. As a consequence numerous animal models of ulcerative colitis have been established to elucidate the potential mechanism of ulcerative colitis and to develop therapeutic strategies within the preclinical phase.

5. Chemical-induced colitis

5.1 Dextran Sulphate Sodium(DSS)-induced colitis

Features of DSS-induced colitis

The DSS model of experimental colitis (30) is one of the most popular and widely utilized and characterized animal models of ulcerative colitis. DSS is a synthetic sulphated polysaccharide composed of dextran and sulphated anhydroglucose unit (31).

Supplementing the drinking water of rodents with low molecular weight DSS (54,000 mol. wt.) results in histopathological and symptomatic features resembling ulcerative colitis (31-32). Histologically, DSS-induced colitis resembles the damage manifested in human ulcerative colitis patients with an inflammatory response consistent with human inflammatory bowel disease (33). DSS administration also produces visual signs of disease activity including rectal bleeding, weight loss and diarrhea (32), all common features of ulcerative colitis.

The DSS-induced ulcerative colitis model has been well characterized morphologically and biochemically (34-35). After a four-day treatment with 3% DSS in the drinking water, mice show signs of acute colitis including weight loss, bloody stools, and diarrhoea (34). Histologically, DSS produces submucosal erosions, ulceration, inflammatory cell infiltration and crypt abscesses as well as epithelioglandular hyperplasia (35). The luminal bacteria in the colon induce the production of the inflammatory cytokines, IL6 and TNFα, which cause colitis. The damage induced by DSS has been reported to affect the distal colon and caecum preferentially, with lesser damage evident in the proximal colon (32). This model is particularly useful to study the contribution of the innate immune mechanism towards colitis as well as for the study of epithelial repair mechanisms.

Mechanism of action

The mouse model of colitis induced by DSS histologically resembles human ulcerative colitis, and although the exact mechanism of DSS-induced mucosal injury is not fully understood, a topical toxic effect of DSS on the colonic epithelial cells has been proposed (36). This results in loss of barrier function which would likely result in an increased uptake of luminal antigens (bacteria and bacterial products) as well as activation of lamina propria immune cells and the inflammatory response (37). It has been reported that DSS-induced colitis alters the tight junction complex resulting in the loss of barrier function thereby facilitating the development of the inflammatory infiltrate and development of intestinal inflammation (38).

Chronic model of DSS-induced colitis

DSS is commonly administered in a dose range of 3-10% for 7-10 days to induce an acute inflammation depending on the susceptibility of the species or the molecular weight of DSS (39). The DSS-induced acute colitis model may be extrapolated to a chronic colitis model by simply prolonging the administration of DSS. It has been suggested that to induce chronicity, DSS is normally administered in three to five cycles with a 1- to 2-week rest between cycles (40-41). This is useful in understanding disease progression as well as pathological inflammatory changes observed in ulcerative colitis. Interestingly, in inbred rats administered 5% DSS for 215 days, intestinal tumors (adenomas, adenocarcinomas as well as papillomas) were seen (42) predominantly in the colon and caecum.

5.2 2, 4, 6-Trinitrobenzene sulfonic acid (TNBS)-induced colitis

Features of TNBS-induced colitis

Another model that has been used widely is the well characterized haptene reagent TNBS-induced colitis. This model of chronic colitis also resembles human ulcerative colitis in its various histological features including infiltration of colonic mucosa by neutrophils and macrophages. There is also increased production of inflammatory mediators including Th1 profile of cytokines (IFNγ, TNFα and IL12) resulting in substantial inflammation and tissue

injury (43). Studies (44-45) have indicated that the TNBS-induced colitis model is useful for testing therapeutic strategies for humans. More specifically, when TNBS is introduced into the colon of susceptible mice it induces a T cell-mediated immune response within the colonic mucosa, leading to dense infiltration of T cells and marcophages throughout the entire wall of the large bowel (46). In addition, this histopathologic characteristic is accompanied by clinical features of progressive weight loss, bloody diarrhoea, rectal prolaspe and large bowel wall thickening (47). The TNBS-induced colitis model has been very useful in studying many important aspects of gut inflammation, including cytokine secretion patterns, mechanisms of oral tolerance, cell adhesion and immunotherapy.

Induction of TNBS colitis

In 2001, Scheiffele and Fuss (48) described the induction of TNBS colitis in mice. Colitis is induced by the administration of TNBS through a trocar needle using a rubber catheter inserted via the anus (49). Scheiffele and Fuss (48) recommended using 0.5 to 4.0 mg TNBS in 45% to 50% ethanol intra-rectally. Inherent in this model and other similar models is the need for ethanol at high concentrations as a vehicle for intra-colonic administration of the hapten. It seems that ethanol is a prerequisite since it acts as a barrier breaker, and allows TNBS to enter the mucosa to induce colitis (50). Ethanol by itself causes severe inflammation in the intestinal mucosa therefore it is difficult to distinguish between the ethanol-induced inflammation and hapten-induced inflammation (51). There are no standard practices for this model subsequently a critical appraisal of the various studies using TNBS colitis and a recommendation for future use of this model has been extensively reviewed by te Velde et al. (52). Intra-rectal administration of TNBS results in ulceration and thickening of the bowel wall which may persist for at least 8 weeks (53). Furthermore, granulomas and Langhans-type giant cells were also observed at the site of ulceration and inflammation. The inflammation was characterized by high myeloperoxidase and decreased glutathione levels (53).

Mechanism of action

TNBS dissolved in ethanol is required to break the mucosal barrier. TNBS can bind covalently to the E-amino group of lysine and modify cell surface proteins. Colitis may develop when pre-sensitized T lymphocytes lyse hapten-modified autologous cells (54-55). T-lymphocytes will lyse hapten-modified autologous cells only if the animal has been pre-sensitized, whereas macrophages will destroy TNBS-modified autologous cells in the absence of pre-sensitization (56). In addition, TNBS may be metabolized to yield O_2^- and H_2O_2 from the interaction between ascorbate and TNBS (57) indicating that TNBS-induced colitis may partly be mediated by cytotoxic reactive oxygen species generated by the oxidative metabolism of TNBS.

A variety of inflammatory mediators may be involved in TNBS-induced colitis. The predominant arachidonate metabolites found in TNBS colitis are leukotriene B4 (LTB4) and the monhydroxyl fatty acids 5-HETE, 12-HETE and 15-HETE (58). The synthesis of LTB4 increased within 4 h and peaked 24-72 h after the administration of TNBS and this increase is correlated with colonic myeloperoxidase activity (59). Furthermore, it has been shown that a significant level of luminal eicosanoids such as prostaglandin E2 (PGE2), 6-keto $PGF_1\alpha$, TXB2 and LTB4 were increased 3 days after intracolonic instillation of TNBS (59) suggesting that eicosanoids play an important role in the pathogenesis of TNBS-induced colitis. Another potential mechanism of TNBS-induced colitis may be the increased level of platelet-activating factor (PAF). High PAF production was not seen during the time of

maximal neutrophil infiltration (1-4 days after TNBS) but was seen 1-3 weeks after the induction of colitis (60). This finding suggests that PAF is unlikely to play an important role in the acute inflammatory response but may be important in the prolongation of the inflammation in this model.

Types of TNBS-induced colitis models

The TNBS-induced colitis model may be used in 3 different scenarios, (i) in acute TNBS-induced colitis in which the primary phase of the induction of Th1 response, a nonspecific inflammatory response, is analyzed (ii) established TNBS-induced colitis in which the local delayed-type hypersensitivity response is mimicked and a specific response can be analyzed and (iii) chronic TNBS-induced colitis in which repeated local induction of DTH response will lead to fibrotic lesions and a Crohn's disease-like cytokine profile (52). These forms are not well described and documented in current practices therefore it is essential to predefine the objective for using this type of experimental colitis to better understand the pathophysiology of the chosen type of colitis.

5.3 Dinitrobenzene sulfonic acid (DNBS) model of colitis

Features of DNBS-induced colitis

DNBS is another hapten which can be used to induce colonic inflammation. DNBS is less hazardous than TNBS and can be used safely in a well-ventilated room with personnel wearing protective gloves, clothing and goggles. The DNBS model produces acute and chronic inflammation and ulceration in the colon similar to TNBS (61). The feature of colitis in this model is similar to that of the TNBS model with bloody diarrhea and significant loss of body weight evident. Four days after DNBS administration, colon damage was characterized by areas of mucosal necrosis and neutrophil infiltration and the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of caecum, colon and rectum showed presence of mucosal congestion, erosion, and hemorrhagic ulcerations (62). The histopathological features included a transmural necrosis and oedema and diffuse leukocyte cellular infiltrate in the submucosa.

In comparison, rats treated with DNBS have no granulomas whereas about half of the TNBS rats have granulomas (53). In the rat, DNBS causes an overproduction of nitric oxide (NO) due to induction of inducible nitric oxide synthase (iNOS), which contributes to the inflammatory process (63-64). As in the TNBS model, DNBS induces a strong inflammatory response and a significant increase in myeloperoxidase (MPO) activity compared to controls (60). Since TNBS is no longer available in the United States, DNBS can be an alternative compound for inducing experimental models of ulcerative colitis.

Induction of TNBS colitis

Colitis was induced by using a technique of acid-induced colon inflammation as described by Morris et al., (53). In fasted rats lightly anaesthetized with isoflurane, a 3.5 F catheter was inserted into the colon via the anus until the splenicflexure was reached (approximately 8 cm from the anus). 2,4-dinitrobenzenesulphonic acid (DNBS; 25 mg/rat), dissolved in 50% ethanol (total volume, 0.8 ml) was administered as an enema. While other investigator have modified the method that was first described (53), where colitis was induced in lightly anesthetized mice by an intra-rectal injection of 3 mg of DNBS in 100 µl of 50% ethanol, delivered 3 cm into the colon via a polyethylene catheter (65).

Mechanism of action

DNBS and TNBS both bind to proteins, but TNBS has an additional active nitro group and binds more readily at lower concentrations. However, DNBS is more selective and binds only to the ε-amino group of lysine (61).

5.4 Oxazolone-induced colitis

Features of oxazolone-induced colitis

A number of experimental models of colitis have been proposed. However, there are limited colitis models that have a Th2 profile. The oxazolone-induced colitis model is Th2-mediated and has important implications for investigating the pathogenesis and treatment of ulcerative colitis (66-67). The administration of low intrarectal doses of a inducing agent (oxazolone) in ethanol to BALB/c mice every 7 days for 10 weeks showed that in the first 3 weeks of this treatment, the mice lost about 10-15% of their starting weight and exhibited ruffled coats, hunched posture, and restricted movement. During this period 10-15% of animals died. Over the next 3 weeks the surviving mice regained weight and no longer exhibited obvious signs of chronic illness. Repetitive administration of intra-rectal ethanol alone led to a weight loss of up to 5% in the early phase of the disease (68).

Induction of oxazolone colitis

The oxazolone-induced colitis model is established by painting the skin with 0.2 ml 3% oxazolone in 100% ethanol on days 0 and 1 followed by intrarectal administration of 0.15 ml 1% oxazolone in 50% ethanol on day 7 (66). However, another study (51) used carmellose sodium/peanut oil as a non-irritating vehicle in place of ethanol and found that the oxazolone-induced colitis model was still a reproducible animal model of human colonic inflammation. The oxazolone challenge resulted in rapid development of inflammation characterized by diarrhoea, mild ulcerations, hyperemia, infiltration of inflammatory cells, epithelial damage and submucosal edema.

More recently, oxazolone-induced colitis has been established as a chronic model via repeated intrarectal administration of oxazolone in ethanol. This allows the model to be used to define specific features of the inflammatory milieu that favors tumor development (69). Chronic oxazolone-induced colitis begins as severe inflammation with corresponding weight loss, which transforms into chronic inflammation and partial weight recovery. The inflammation is marked by the rapid increase in the production of IL13 in the lamina propria and the appearance of NK T cells, which are both immunologic features of acute oxazolone-induced colitis (69). The authors also concluded that the chronic oxazolone-induced colitis model supports epithelial tumour development induced by administration of a carcinogenic agent, azoxymethane.

Mechanism of action

Similar to the TNBS-induced colitis model, oxazolone, a hapten, induces delayed-type hypersensitivity and contact hypersensitivity reactions to subsequently induce inflammation. Oxazolone-induced colitis has been suggested to be dependent on the presence of IL13 producing invariant NK T cells (70). Thus the oxazolone-induced colitis model is one of the few models suitable for the study of the Th2 dependent immune response in intestinal inflammation.

5.5 Carrageenin-induced colitis

Carrageenan is a high molecular weight sulfated polygalactan, derived from several species of red seaweeds (*Rhodophyceae*) including *Gigartina*, *Chondrus*, and *Eucheuma* (71). The most common forms of carrageenan are lambda (λ), kappa (κ), and iota (ι) (71). Carrageenans are used by the food industry to improve the texture of food products by thickening, stabilizing, or emulsifying dairy products, salad dressings, infant formulas, processed meat, soy milk, and other food products (72-73). Its use has increased markedly during the last half century, and is known to induce inflammation in rheumatological models and in intestinal models of colitis (71).

Features of carrageenan-induced colitis

Early work in animal models has demonstrated that carrageenan may cause gastrointestinal pathology, including ulcerations and tumours of the gastrointestinal tract (71-72). In guinea pigs deprived of ascorbic acid, the oral administration of degraded *E. spinosum* carrageenan induced mild to moderate colitis, while E. cottonii carrageenan consistently induced severe colitis. The severe colitis induced by *E. cottonii* in scorbutic animals markedly affected the mid and distal colon and showed histological changes similar to human ulcerative colitis (74). Delivery of 10% carrageen (degraded carrageenan) for 10 days in the drinking water of CF1 mice induced bloody diarrhoea and pericryptal inflammation, and produced marked dilatation of the cecum and ascending colon (75). Histologically, the mucosa was characterized by distorted crypt architecture, inflammatory infiltration of the lamina propria, and ulceration, conditions which were more pronounced in the proximal colon but were also present in the distal colon.

Induction of carrageenan colitis

Carrageenan causes a reproducible inflammatory reaction and remains a standard chemical for examining acute inflammation and effects of anti-inflammatory drugs. With or without sensitizing the animals with carrageenan, colitis is induced by supplementing the drinking water of 2-10% degraded carrageenan (74-75).

Mechanism of action

Carrageenan has been widely used to induce inflammation in experimental models of colitis in animal models (72-73), that resemble human ulcerative colitis. NFκB is a key determinant of the intestinal epithelial inflammatory cascade and occupies a central role in the transcriptional activation of pro-inflammatory genes (76). Furthermore, Borthakur et al. (71) suggested that activation of NFκB in the intestinal cells following carrageenan exposure is largely attributable to an increase in Bcl10. Bcl10 resides in the cytoplasm which relays receptor mediated signals to activate NFκB (77).

6. Genetic-induced colitis

More recently, various experimental animal model of colitis, especially transgenic mice models with spontaneous colitis (78-79) have been reported and demonstrated that T cells are necessary for involvement and initiation of intestinal inflammation. New genetically engineered animals with spontaneous colitis, such as IL2 and IL10 knockout mice models, are promising tools for further understanding of the etiology of intestinal inflammation.

6.1 IL2 knockout mice

When reared and maintained under conventional specific pathogen-free conditions, IL2 deficient (IL2$^{-/-}$) mice spontaneously develop disorders of the hemopoietic and immune system characterized by anemia, lymphocytic hyperplasia, progressive loss of B cells, and disturbances in bone marrow hemopoietic cells. Animals that survive more than 8-9 weeks of age also develop a chronic, non-granulomatous inflammation of the colonic and caecal submucosa and mucosa. (80).

Features of IL2 deficiency-induced colitis

The histopathology of colitis observed in IL2$^{-/-}$ mice seems to vary depending on the method of induction and the location of animal housing. The original paper (80) described the features of spontaneously developing colitis in IL2$^{-/-}$ mice which included ulceration, crypt abscesses, destruction of the mucosal layer with epithelial dysplasia, but also mononuclear cell infiltration of the mucosa and submucosa. However, the histopathology of immunization-induced colitis and spontaneously-developing colitis in mice reared at the NIH animal facility, seem to resemble human Crohn's disease (transmural inflammation, lymphoid hyperplasia) (81). These findings suggest that the histopathological characteristics in these animals may not only be dependent on genetic but also on environmental factors.

Mechanism of action

IL2$^{-/-}$ mice reared and maintained under gnotobiotic conditions do not develop intestinal lesions (82). Furthermore, colitis that develops in IL2$^{-/-}$ mice under conventional conditions suggests a direct result of an abnormal immune response in the colonic mucosa to intestinal bacterial flora. It is not known specifically how the absence of IL2 accounts for colitis in IL2$^{-/-}$ mice and the role this cytokine plays in homeostatic regulation of mucosal immunity. However, Baumgart et al. (83) suggests that IL2 is required for the generation and the function of a regulatory population of mucosal T cells or is directly involved in preventing the development of inflammatory responses to enteric antigens.

6.2 IL10 knockout mice

Only a small number of spontaneous models of chronic colitis have been employed by researchers to yield detailed information on the penetrance, severity and reproducibility of the gut inflammation (84). The most widely used of the gene-targeted models of spontaneous colitis is the IL10 deficient (IL10$^{-/-}$) mouse model. The IL10$^{-/-}$ model is a well established Th1-mediated model of transmural colitis (85). IL10$^{-/-}$ mice were generated by disrupting the IL10 gene in embryonic stem cells and although the mice were considered to have normal lymphocyte development and antibody response, growth retardation and anemia were observed (86).

Features of IL-10 deficiency-induced colitis

Mice with targeted disruption of the IL10 gene develop spontaneous pancolitis and caecal inflammation by 2-4 months of age (85). Histopathological examination of the colons obtained from mice with active disease show many of the same characteristics as those observed in human inflammatory bowel disease. The initial changes in intestinal inflammation consisted of small, focal infiltrates of inflammatory cells in the lamina propria with minimal or no epithelial hyperplasia (85, 87). Inflammatory infiltrates consisted of a mixture of lymphocytes, plasma cells, and macrophages with smaller numbers of

neutrophils and eosinophils. IL10$^{-/-}$ mice also develop ulcers and crypt abscesses, exhibit epithelial hyperplasia, mucin production is reduced, an increased numbers of mitotic figures were observed and increased expression of major histocompatibility complex class II molecule were also observed in the intestinal epithelial cells (85-87). As mice become older, inflammation involved the submucosa or less frequently became transmural (87).

Mechanism of action

It has been demonstrated that there is a lower number of caecal bacteria observed before colitis (7 weeks of age) in IL10$^{-/-}$ compared to C57Bl/6J mice. This suggests differences in intestinal bacteria that might be associated with the genotype which could contribute to the development of colitis in this mouse model (88).

6.3 CD45RB^{Hi} T cell transfer model of colitis

CD4$^+$ T cells can be separated on the basis of their CD45RB expression into populations expressing high (CD4$^+$ CD45RB^{Hi}) or low (CD4$^+$ CD45RB^{Lo}) levels of this antigen (89). CD4$^+$ CD45RB^{Hi} T cells isolated via fluorescence activated cell sorting from spleens of donor mice transferred to immuno-deficient SCID or RAG1/2$^{-/-}$ recipient mice cause a wasting syndrome with transmural intestinal inflammation primarily in the colon starting 5-10 weeks after cell transfer (90-91).

Features of CD45RB^{Hi} T cell transfer model of colitis

Initial lesions consisted of minimal multifocal or diffuse inflammatory cell infiltrates in the lamina propria.In mice with more severe colitis, changes were diffuse and sometimes transmural (90). Inflammatory infiltrates consisted of macrophages and lymphocytes, accompanied by smaller numbers of neutrophils and eosinophils (90-91). Occasional multinucleated giant cells and ulcers were observed, whereas crypt abscesses were sparse (90-91). Epithelial changes included hyperplasia with lengthening and branching of glands, mucin depletion, increased numbers of mitotic figures, and enhanced levels of major histocompatibility complex class II molecule expression on intestinal epithelial cells (90).

Mechanism of action

Recipient mice repopulated with the CD$^+$CD45RB^{Lo} T cell subset or both populations (CD$^+$CD45RB^{Hi} and CD$^+$CD45RB^{Lo} T cell subsets) do not develop colitis. CD25$^+$FoxP3$^+$ cells within the CD$^+$CD45RB^{Lo} population account for the prevention of colitis since depletion of CD25$^+$ cells from CD45RB^{Lo} cells abrogates their colitis prevention potential (92). Treg cells which produce IL-10 due to co-culture with IL-10, prevent the onset of gut inflammation and antigen-specific immune responses when transferred together with pathogenic CD4$^+$-CD45RB^{Hi} T cells. Furthermore, SCID mice administered both CD45RB^{Hi} T cells and Treg cells together with anti-IL10 receptor antibodies develop colitis (93). These results suggest that the progeny of CD45RB^{Hi} T cells mount a pathogenic Th1-like response in the colon of these immuno-deficient mice.

6.4 Which colitis model to use?

As increasingly more sophisticated experimental colitis models are being described and characterized, researchers have the potential to exploit the unique potential of each model to ask specific questions. No single experimental model of colitis recapitulates all of the pathogenic and clinical features of human ulcerative colitis, however each animal model has

contributed to our understanding of the mechanisms underlying initiation and perpetuation of chronic intestinal inflammation.

7. Current treatments for ulcerative colitis

At present, conventional therapies or pharmaceutical treatments have remained the mainstay of treatment for most patients suffering from ulcerative colitis. However, these treatments are variably effective with significant adverse effects and approximately 25 to 40% of patients will eventually require colectomy (94). The aim of these treatments is to induce and maintain the patient in remission. First line therapy for mild to moderate ulcerative colitis comprise of anti-inflammatory drugs containing 5-aminosalycylic acid (5-ASA), such as oral and rectal mesalamine. Sulfasalazine, the archetype for this class of medications, is cleaved upon reaching the colon releasing mesalamine. Second generation 5-ASA medications include olsalazine and balsalazide (95). Approximately 60 and 80% of patients are adequately treated with these medications and the remainder who exhibit severe ulcerative colitis are treated with a combination of corticosteriods (prednisolone). Immunosuppressives or immunomodulators such as a azathioprine and mercaptopurine that treat severe inflammatory bowel disease and/or administered to patients who have inadequate response to corticosteroids can be beneficial but there is little information about their effectiveness in treating ulcerative colitis and are also associated with risks of infection and malignancy (96). Up to 20% of inflammatory bowel disease sufferers discontinue immunosuppressant therapy because of side effects (97). Biologic drugs that interfere with the inflammatory response such as the anti-TNFα agent infliximab can be effective in inducing remission for ulcerative colitis patients that are refractory to initial treatments.

It has been demonstrated that colonic bacteria may initiate inflammation of inflammatory bowel disease (97-98) and a combination therapy with antibiotics has been shown to offer significant benefit in ulcerative colitis (99-100). Most clinicians use antibiotics as an adjuvant therapy for severe ulcerative colitis despite relatively few trials conducted on their use. Although recent advances have been made in understanding the etiology and pathophysiological mechanisms underlying the pathogenesis of ulcerative colitis, the problem still remains for patients refractory to conventional treatments or not responding and being able to maintain remission effectively with maintenance treatments (101).

Currently, there is no cure for ulcerative colitis and there is increasing evidence that alternative therapies (102-103) may provide some insights into developing a potential successful treatment. The remainder of this chapter will focus on the potential therapeutic interventions which target various aspects of the etiology of ulcerative colitis including the use of pre- and probiotics to manipulate the gut microflora and molecules that mediate the action of inflammatory cells (104). Biological therapies for ulcerative colitis will not be covered in this chapter as this area is reviewed by Rutgeerts et al. (105).

8. Novel therapies for ulcerative colitis

8.1 n-3 fatty acids

Over the years, dietary n-3 fatty acids have gained a reputation in preventing and treating several disorders including cardiovascular diseases, rheumatoid arthritis and Alzheimer's disease by way of anti-inflammatory, antithrombotic, antiarrythmic, hypolipidemic and vasodilatory activities (101, 106-111). It has been shown in human and animal studies that

these n-3 fatty acids have potent immunomodulatory and anti–inflammatory effects by inhibiting the production of inflammatory mediators, eicosanoids, PGE2 and LTB4 and cytokines, TNFα and IL1β (112). It stands to reason that supplemental n-3 fatty acids might therefore be beneficial in treating or preventing relapse in chronic inflammatory diseases such as ulcerative colitis (113).

Animal studies

There have been numerous studies utilising experimentally induced colitis animal models to define the role of dietary n-3 fatty acids in disease prevention and progression. In a severe combined immunodeficient (SCID) mouse model of colitis, Whiting et al. (114) found that dietary n-3 fatty acids reduced clinical colitis and colonic immunopathology by decreasing the synthesis of proinflammatory cytokines, reducing myeloid cell recruitment and activation, and enhancing epithelial barrier function and mucosal wound healing mechanisms. Li et al. (115) demonstrated within the TNBS rat colitis model that rats pretreated with n-3 fatty acids showed significant attenuation of colonic injury and protection. Compromised epithelial barrier in ulcerative colitis by chronic immune cell activation might be explained by the altered expression and distribution of tight junction proteins in tight junction membrane microdomains of the intestinal mucosa, and n-3 fatty acids have been shown to positively affect this altered expression and distribution (115). Many studies have shown adiponectin, a protein hormone produced and secreted primarily by adipocytes and more recently by colonic myofibroblasts to play a beneficial role in ulcerative colitis due to it's anti-inflammatory effect (116-118). Interestingly, Matsunaga et al. (119) who found a decrease in adiponectin expression in DSS-induced colitic mice also found a further decrease in adiponectin expression in colitic mice fed with n-3 fatty acids, which could have contributed to the observed exacerbated colitis for this group. This is in contrast to the beneficial effects other studies have shown regarding dietary n-3 fatty acids and colitis.

Human studies

Fish oil is the best source of n-3 fatty acids. Although numerous studies have focused on oral supplementation in patients with inflammatory bowel disease which ultimately results in the incorporation of n-3 fatty acids into the gut mucosal tissue thereby modifying inflammatory mediators (120-121), the evidence of clinical benefits remains unclear due to conflicting results. A systematic review of the effects of fish oil in human ulcerative colitis by MacLean et al. (113) found significant improvements in clinical scores in three studies at one or more time points relative to the comparative study arm. Studies that were restricted to patients with ulcerative colitis (122-123) reported a statistical improvement in the endoscopic score with fish oil relative to comparative treatment. Together with studies that observed induction of remission (122, 124), prevention of relapse (124-128) and the requirement for immunosuppressive agents (122, 124, 129). MacLean et al. (113) deduced that there were insufficient data to draw any conclusions. However, the observed efficacy of fish oil delivered by enteric coated capsule on reducing steroid requirements did warrant more attention (113).

Another systematic review and meta-analyses by Turner et al. (101) looked at the efficacy and safety of n-3 fatty acid or fish oil therapy in maintaining remission in inflammatory bowel disease. Of the nine studies eligible for inclusion, only three involved ulcerative colitis. There was no difference in the relapse rate between the n-3 fatty acid therapy (fish

oil) and control groups. Pooled analysis showed an increase in diarrhea and symptoms of the upper gastrointestinal tract in the n-3 fatty acid group (fish oil) suggesting troublesome side effects. In short, there was insufficient data to recommend the use of n-3 fatty acids for the maintenance of remission of ulcerative colitis. Given the biologic rationale and the benefit of n-3 fatty acid therapy derived from tissue samples and animal models, it is difficult to explain the lack of clinical benefit in inflammatory bowel disease, although it has been suggested that the dosing regimen may be inadequate or the formulation not optimal (101). Enterically coated n-3 fatty acid that has a timed release of 60 minutes upon ingestion was found to be more beneficial with the lowest adverse events compared with other timed release points and triglyceride compounds (130). In conclusion, further studies are warranted to address appropriate dosing and delivery systems of fish oil for the treatment of ulcerative colitis.

8.2 Plant derived therapies

Other novel therapies that possess anti-oxidant, anti-inflammatory and immuno-modulatory properties have been investigated in experimentally-induced animal colitis models and to some extent in human trials for the treatment of ulcerative colitis. Persistent ulcerative colitis is associated with a 10-fold increased risk of colorectal cancer (131) and therefore limiting chronic colonic inflammation will appear to reduce this risk.

Resveratrol

Resveratrol is a natural polyphenol found in fruits and vegetables and abundantly in grapes and red wine. Sanchez-Fidalgo et al. (132) investigated the protective/preventive effects of dietary resveratrol in the DSS-induced colitis mouse model. There were significant attenuations of clinical signs of colitis such as loss of body weight, diarrhea and rectal bleeding. All mice fed the resveratrol diet survived and finished the treatment while mice fed the standard diet showed a 40% mortality rate. Resveratrol caused significant reductions in TNFα and IL1β and an increase in IL10, an anti-inflammatory cytokine. Expression of prostaglandin E synthase-1 (PGES-1), cyclooxygenase (COX-2) and iNOS, proteins involved in the inflammatory response, were also reduced. Cui et al. (133) investigated the protective/preventive effects as well as the chemopreventive properties of dietary resveratrol in the chronic DSS-induced colitis mouse model. Resveratrol was shown to ameliorate colitis in a dose dependant manner and reduce the tumour incidence by 60%. The number of tumours per animal was also reduced. Resveratrol is tolerated at high doses and a diet rich in this polyphenol could represent a novel approach to treating ulcerative colitis and preventing colon cancer associated with ulcerative colitis.

Andrographis paniculata

Andrographis paniculata, a member of the plant family Acanthaceae, is used extensively in Asian countries, Sweden and Chile for the treatment of various inflammatory and infectious diseases. HMPL-004, an aqueous ethanol herbal extract of Andrographis paniculata has been shown to inhibit TNFα and IL1β and prevent colitis in animal models. A pilot human clinical trial conducted by Tang et al. (134) investigated the efficacy and safety of HMPL-004 in patients with mild to moderate ulcerative colitis. In comparison to a parallel group treated with the standard first line therapy, mesalazine, there were no significant differences observed for clinical remission and disease activity. 13% of patients treated with HMPL-004 and 27% treated with mesalazine had at least one adverse event although the majority of

events were not strongly linked to the study medications. In conclusion, HMPL-004 could be an efficacious alternative to mesalazine for the treatment of ulcerative colitis.

Black raspberries

As well as exhibiting ability to limit the inflammatory response in cell culture (135-137), black raspberries (BRB) have the highest concentration of antioxidant polyphenols compared to other dark berries (138-139). These antioxidants (anthocyanins and ellagic acid) have been shown to scavenge free radicals, increase expression of detoxification enzymes and increase the capacity of the cell to absorb radicals (140-143). A study conducted by Montrose et al. (144), the first to utilise a DSS-induced mouse model of ulcerative colitis to explore the effects of freeze-dried BRB on disease severity, demonstrated the high anti-inflammatory potency of BRB. Dietary BRB markedly reduced colonic injury to the epithelium and tissue levels of TNFα and IL1β were suppressed. Biomarkers of oxidative stress remained unaffected by BRB treatment, however the findings still demonstrated potent anti-inflammatory properties which support a possible therapeutic role for the treatment of ulcerative colitis.

American ginseng

American ginseng (AG), a natural herb, has been shown to improve mental performance and end points associated with conditions such as cardiovascular disease, cancer and diabetes (145-147). In a study by Jin et al. (148), AG extract was mixed in with the chow of DSS-induced colitic mice and given before and after the onset of colitis. Results showed prevention and treatment of colitis with AG along with the downregulation of iNOS and COX-2 and p53 (induced by inflammatory stress). In part, leukocyte activation in colitis causes mucosal and DNA damage which was shown to be inhibited by AG *in vitro* and *in vivo*. A dysfunctional intestinal immune system is a major mechanism by which chronic inflammation occurs in ulcerative colitis and defects in apoptosis of mucosal inflammatory cells is critical in the pathogenesis of ulcerative colitis. Another study conducted by Jin et al. (149), showed that AG extract can drive apoptosis of inflammatory cells through the p53 mechanism *in vitro* which is consistent with dietary AG protecting against DSS-induced colitis in the mouse model.

Ginkgo biloba

Ginkgo biloba extract (EGB) is derived from the green leaves of the Gingko biloba tree and has been used extensively in conditions associated with inflammatory mediators such as acute pancreatitis, central neural system disorders, heart and intestine injury/reperfusion injury (150-152). Zhou et al. (153) investigated the mechanism by which EGB ameliorates inflammation in TNBS-induced colitic rats and its effects on the production of inflammatory mediators. Four weeks of EGB therapy provided protection in ulcerative colitis possibly by radical scavenging and down regulating some of the inflammatory mediators including TNFα, NFκBp65 and IL6. All inflammatory mediators in this study were affected by EGB in a dose dependant manner resulting in the improvement of ulcerative colitis. Another study by Kotakadi et al (154) showed that EGBs have anti-inflammatory properties *in vitro* and prevent and treat colitis in the DSS-induced mouse model. The mechanism underlying the treatment of ulcerative colitis is in part due to the ability of EGB to drive CD4+ effector T cell apoptosis which is fundamental in regulating many chronic inflammatory and autoimmune diseases (155-156).

8.3 Other potential therapies

Crocetin

Crocetin, a carotenoid compound derived from *Crocus sativus L* (saffron) has been used to treat different diseases (157). In the TNBS-induced colitis mouse model, it was revealed that treatment with 50 mg/kg/day intragastrically for 8 days significantly ameliorated diarrhea, inflammation and colonic tissue injury (158). The mechanisms by which crocetin exerted these beneficial effects is through the reduction of neutrophil infiltration and MDA in the inflamed colon. Increased production of NO by iNOS and activation of NFκB, known to play a central role in the early steps of inflammation were also reduced. With further investigation, crocetin could prove to be an alternative therapy or perhaps be used alongside conventional therapies.

Pomegranate

Punica granatum or the pomegranate is used in traditional medicine in China, India, Europe and South Africa. Studies have shown that pomegranate has protective properties against liver fibrosis and ultraviolet-induced pigmentation (159-160). Furthermore, it has antibacterial, anti-inflammatory, anti-diabetic effects and is cardio-protective (161-163). Singh et al. (164) explored the effect of Punica granatum extract and its component, ellagic acid, in the DSS-induced colitis mouse model and found significant attenuation of colonic inflammation. Mast cell degranulation, which releases various inflammatory mediators, including histamine, has been implicated in the pathogenesis of ulcerative colitis and the use of mast cell stabilizers have been documented to attenuate the severity of ulcerative colitis in humans (165). Singh et al (164) found that Punica granatum extract and its ellagic acid component had anti-ulcerative effects comparable to sodium cromoglycate (mast cell stabilser) and sulphasalazine (standard first line treatment for ulcerative colitis).

Helminths

Immune-mediated diseases such as inflammatory bowel disease are becoming more prevalent in highly developed and industrialized countries (166). It is suggested that the adoption of hygienic lifestyles in these countries have contributed to a decline in helminths or parasitic worm infections (166). Epidemiological studies (167-169) have suggested that helminths may provide protection against some immune-mediated diseases and the eradication may in fact promote these diseases. Animal studies have shown helminth protection by promoting regulatory immune responses. In a DNBS-induced mouse colitis model Melon et al. (170) showed that mice infected with the tapeworm, *Hymenolepis diminuta*, increased the production of IL4 and IL10 that protected them from colitis, in contrast to steroid treatment (dexamethasone) which offered little benefit. Khan et al. (171) also showed protection with the nematode, *Trichinella spiralis*, in the TNBS- induced colitis mouse model. However, it was found that helminth infection enhanced disease severity in the oxazolone induced colitis mouse model (172). In a human randomized crossover trial conducted by Summers et al (173), a significant percentage of patients with ulcerative colitis receiving the porcine whipworm, *Trichuris suis*, improved when compared to placebo. As well, the treatment seemed to be safe with no reported side effects. In conclusion, there is potential value for helminth therapy for specific inflammatory bowel disease patients however further studies are needed to fully understand the mechanisms underlying the pathophysiology of ulcerative colitis and the type of helminth therapy required to avoid the possibility of disease aggravation.

8.4 Prebiotics

There is a diverse and large population of micro-organisms naturally living on the mucosal surfaces or in the lumen of the human intestine. The number of resident bacteria increases along the small bowel, with the colon being the most heavily populated region of intestine. The microbiota refers to the particular ecological niche of a host individual in which the community of living micro-organisms is assembled (174). A healthy or balanced microbiota has been considered to be predominantly saccharolytic and comprises of significant numbers of lactobacilli and bifidobacteria (175). A prebiotic can be defined as a non-digestible food ingredient that exerts a beneficial effect on the host through the selective stimulation and metabolism in the intestine, thereby improving host health (176). Inulin and oligofructose are prebiotic carbohydrates that resist digestion by intestinal and pancreatic enzymes in the human gastrointestinal tract and are fermented by bacteria living in the intestinal ecosystem. Prebiotics increase saccharolytic activity within the gut and selectively promote the growth of bifidobacteria when administered in significant amounts (177-178).

Animal models

Videla et al. (179) investigated the effectiveness of inulin, which stimulates intracolonic generation of butyrate and growth of lactic acid bacteria, in the protection against colitis. In a rat model of DSS-induced colitis, oral inulin treatment significantly reduced colonic tissue MPO activity and mucosal release of inflammatory mediators (179). Histologically, oral inulin treatment reduced the extent of damaged mucosa, decreased the severity of crypt disruption and lowered histological damage severity scores compared with controls. Inulin induced an acidic environment from the caecum to the left colon and increased counts of Lactobacilli (179).

Fructooligosaccharides (FOS) increase the growth of lactic acid bacteria and promote butyrate and lactate production, therefore possessing beneficial properties for intestinal inflammation (180). Intracolonic TNBS-induced colitic rats treated with intragastric infusions of FOS resulted in a reduction of pH and inflammation assessed by MPO activity. Furthermore, FOS treatment increased lactate and butyrate concentrations including lactic acid bacteria counts in the caecum (180).

Madsen et al. (181) investigated the role of colonic aerobic luminal bacteria and lactobacillus species in IL10 gene-deficient mice that spontaneously develop colitis. These knockout mice have a decreased level of lactobacillus species in the colon and an increase in adherent and translocated bacteria in the neonatal period. Normalising Lactobacillus levels via oral lactulose therapy reduced colonic mucosal bacteria and prevented colitis (181). Similarly, lactulose treatment has demonstrated protective effects against DSS- and TNBS-models of colitis (182-183). Rumi et al. (182) demonstrated that lactulose therapy ameliorated DSS-induced colitis in a dose-dependent manner and significantly reduced the severity of colonic lesions and decreased MPO activity. Furthermore, Camuesco et al. (183) indicated that lactulose treatment in TNBS-induced colitis exerted a preventive anti-inflammatory effect as evidenced by a significant reduction of MPO activity, a decrease of colonic TNFα and leukotriene B4 production and an inhibition of colonic inducible nitric oxygen synthase expression, which is a result of the inflammatory process (183). Furthermore, this effect was associated with increased levels of lactobacilli and bifidoacteria species in colonic content when compared with untreated colitic rats (183). Overall, the experimental evidence provides significant indications of the anti-inflammatory and beneficial properties of prebiotics in settings of ulcerative colitis.

Clinical studies

Recently, Casellas et al. (184) tested the effect of oligofructose-enriched inulin, which promote the selective growth of saccharolytic bacteria with low inflammatory potential, in patients with active ulcerative colitis. Eligible patients in the randomized, placebo-controlled double blinded pilot trial had been previously in remission with mesalazine as maintenance therapy or no drug, and presented with a relapse of mild to moderate activity (184). Nineteen subjects were treated with mesalazine and randomly allocated to receive either oligofructose-enriched inulin or placebo for two weeks. Patients treated with oligofructose-enriched inulin displayed a reduction of faecal calprotectin, a protein found in granulocytes that resist metabolic degradation (184).

8.5 Probiotics

Probiotics are defined as living, non-pathogenic bacteria which are able to exert beneficial therapeutic or physiologic activities when administered in sufficient numbers (185). Bacteria can be derived from various sources such as cultured food and the normal human microbiota. Lactobacillus or bifidobacterium genera are the most common strains of probiotic bacteria and have also been identified from enterococcus, streptococcus, and lactococcus species, while certain non-pathogenic Escherichia strains are also classified as probiotics (186). Furthermore, genetic engineering of probiotic strains can ensure the release of bioactive compounds16. The beneficial effects of probiotics are highly species and strain specific and therefore the mechanism of action is not well understood. Common mechanisms of action identified in probiotics include improvement of epithelial barrier function, inhibition of pathogenic enteric bacteria and manipulation of host immunoregulation (185).

In vitro models

The effects of probiotics have been investigated using recent comprehensive cell culture experiments which are model systems of inflammation and infection similar to ulcerative colitis. Schlee et al. (187) investigated the ability and mechanism by which different probiotic lactobacillus strains, including *L. Acidophilus PZ1138*, *L. Fermentum PZ-1138*, *E. coli Nissle 1917* and VSL#3 (a combination of 8 bacterial strains), stabilize gut barrier function via induction of the anti-microbial peptide human beta defensin-2 (hBD2) gene. The expression of hBD2 gene by probiotic bacteria was both time- and dosage-dependent, and the promoter activation by probiotics was completely inhibited via deletion of NFκB and activator protein-1 (AP1) binding sites on the hBD-2 promoter (187). Furthermore, hBD-2 induction was also hindered by the inhibition of mitogen-activated protein kinase (MAPK). Overall, Schlee et al. (187) demonstrated that lactobacilli and the VSL#3 bacterial combination strengthened intestinal barrier functions via the up-regulation of hBD-2 through induction of MAPKs and pro-inflammatory pathways including NFκB and AP1. In support of the finding of Schlee et al. (187), *E. coli Nissle 1917* was further demonstrated to strengthen intestinal barrier function using a polarized T84 epithelial monolayer model to monitor barrier disruption by E.coli infection (188). Co-incubation of the enteropathogenic *E. coli* strain with *E. coli Nissle 1917* or addition of *E. coli Nissle 1917* following infection abolished barrier disruption and restored barrier integrity (188). DNA-microarray analysis of T84 cells incubated with the enteropathogenic *E. coli* identified altered expression of over 300 genes, including the distribution of zonla occludens-2 (ZO-2) protein and of distinct

protein kinase C isotypes, all of which are involved in the maintenance of epithelial tight junctions (188).

Furthermore, *E. coli Nissle 1917* has been shown to exert anti-inflammatory effects on human colonic epithelial cells *in vitro* (189). Enzyme-linked immunosorbent assays and real-time quantitative PCR demonstrated that *E. coli Nissle 1917* treatment in vitro suppressed TNFα-induced IL8 transcription and production and inhibited IL8 promoter activity. These properties, in conjunction with the hBD2 results from Schlee et al (187) and T84 epithelial monolayer model results from Zyrek et al. (188) contribute to the reported efficacy in the treatment of inflammatory bowel diseases. Due to the unfortunate idiopathic nature of IBD, pre-treatment with probiotics may be more beneficial for either genetically susceptible individuals or to help IBD sufferers maintain remission.

Animal models

Several murine models of intestinal damage have been utilised to assess the efficacy of probiotics *in vivo* (190-192). Ukena et al. (191) orally administered the probiotic *E. coli Nissle 1917* to BALB/c mice with acute dextran sulphate sodium (DSS)-induced colitis. The probiotic treatment resulted in an upregulation of the tight junction molecule ZO-1 in intestinal epithelial cells at both mRNA and protein levels and reduced intestinal barrier permeability (191). Additionally, infiltration of the colon with leukocytes was ameliorated in E.coli Nissle 1917 inoculated mice (191). Furthermore, Grabig et al (193) demonstrated that *E. coli Nissle 1917* treatment in a wildtype DSS-induced colitis mouse model significantly reduced pro-inflammatory cytokine expression, myeloperoxidase (found in the intracellular granules of neutrophils) activity and disease activity. The inability of *E. coli Nissle 1917* to exert its beneficial effect in the absence of toll-like receptor (TLR)-2 and TLR4 signaling using TLR2 and TLR4 knockout mice indicates that the amelioration of experimentally-indiced colitis in mice was elicited via TLR2- and TLR4-dependent pathways (193). This finding highlights the fact that *E. coli Nissle 1917* may improve the ability of TLRs, which are key components of the innate immune system that trigger antimicrobial host defence responses, to recognise microbial pathogens, improving the host immune response.

Lee et al. (194) demonstrated that oral *L. plantarum HY115* treatment to mice with DSS-induced colitis inhibited colon shortening and MPO production. Furthermore, *L. plantarum HY115* repressed the mRNA expressions of IL1β, TNFα and IFNγ, including colonic IL1 beta and IL6 protein expression and reduced the degradation activities of chondroitin sulphate and hyaluronic acid of intestinal bacteria (194). Similarly, Schultz et al. (195) immune-mediated colitic (induced by IL10 deficiency) mice treated with L. plantarum had decreased levels of mucosal IL12, IFNγ and immunoglobulin G2a (195).

A study by Peran et al. (196) assessed the intestinal anti-inflammatory effects of probiotics with immunomodulatory properties in the TNBS rat model of colitis. *L. casei, L. acidophilus* and bifidobacterium lactis elicited intestinal anti-inflammatory effects, evidenced macroscopically by a decreased colonic weight/length ratio and biochemically, all probiotics restored colonic glutathione levels, depleted due to oxidative stress (196). Interestingly, each probiotic displayed a unique anti-inflammatory profile; bifidobacterium lactis reduced colonic TNFα production, L. casei decreased colonic COX-2 expression and L. acidophilus reduced leukotrine B4 production and MPO activity (196). These findings indicate that probiotics exert their beneficial effects via different mechanisms. Menard et al. (197) inoculated gnotobiotic mice with bifidobacterium longum NCC2705 and nine bifidobacterium strains isolated from infants' faecal flora to investigate the effect of these

probiotics on the Th1/Th2 balance. Immunomodulatory responses including induction of the Th1 and Th2 cytokines, increased ileal IL10, IL4, TNFα and IFNγ secretions and TGFβ1 gene expressions, were observed from only specific strains (197). It was concluded that bifidobacterium's capacity to stimulate immunity is species specific however its influence on the orientation of the immune system is strain specific.

Clinical trials

To date, probiotics have been investigated in several clinical trials as treatments for ulcerative colitis, with conflicting results. However, there have been relatively few large, placebo-controlled, randomised and double-blinded clinical studies to test the efficacy of probiotics in humans (198). Tsuda et al. (199) evaluated the efficacy of the probiotics combination therapy BIO-THREE, comprising of Streptococcus faecalis T-110, Clostridium butyricum TO-A and Bacillus mesentericus TO-A, in patients with mild to moderate distal ulcerative colitis. Patients ingested nine BIO-THREE tablets per day for four weeks. Clinical symptoms and endoscopic findings were evaluated as ulcerative colitis disease activity index and faecal samples were collected to assess the microflora, pre- and post-treatment (199). Remission was observed in nine patients (45%), response in two patients (10%), no response in eight patients (40%) and worsening in one patient (5%) (199). Interestingly, terminal restriction fragment length polymorphism (T-RFLP) analysis indicated that the principal alteration in microflora was an increase in bifidobacteria (199); an unusual finding as no bifidobacteria was administered in the probiotic supplement.

8.6 Zinc

Zinc is ubiquitous in biologic systems and has abundant and varied functions. The zinc atom has the ability to participate in readily exchangeable ligand binding in addition to assuming a number of coordination geometries to provide functional needs to other ligands (200). Zinc has numerous central roles in DNA and RNA metabolism (201). Zinc metalloenzymes and zinc-dependent enzymes have been identified and are involved in nucleic acid metabolism and cellular proliferation, differentiation and growth (202). Zinc also plays a regulatory role in apoptosis (203), with cytoprotective functions that suppress major pathways, leading to programmed cell death.

Animal studies

Zinc administration has been shown to suppress the development of DSS-induced colitis in mice as indicated by decreased clinical disease activity index and histological severity scores (204-206). Ohkawara et al. (205) demonstrated that polaprezinc (N-(3-Aminopropionyl)-L-histidinato zinc), an anti-ulcer drug, suppresses DSS-induced colitis in mice, partly through inhibition of production of pro-inflammatory cytokines, suppression of neutrophils accumulation and cytoprotection by overexpression of heat shock proteins. This is consistent with Iwaya et al (207) whom reported that marginal zinc deficiency exacerbated colitis by modulating the immune response through the impairment of TNFα production and TNFR1 expression, rather than through the impairment of epithelial barrier function. Another potential mechanism of action of zinc in ulcerative colitis has been suggested by Luk et al. (208) by reducing inflammation, inhibiting mast cell degranulation and histamine release. In addition, high dose of zinc has been shown to improve tight-junction permeability (209). A novel zinc compound, Z-103, a chelate compound consisting of zinc ion and L-carnosine, was utilized to assess the protective effect against colonic damage

induced by TNBS in rats (210). The authors demonstrated that treatment with Z-103 reduced he inflammatory responses induced by TNBS, suggesting Z-103 may be as effective against TNBS-induced colitis.

Metallothioneins (MTs) are zinc-binding proteins whose overexpression may lead to sequestration of zinc ions. We have shown that the absence of MT was beneficial in the suppression of colitis in MT knockout (MT$^{-/-}$) mice receiving DSS, suggesting that the presence of MT may have promoted the induction of colitis. Similarly, as indicated by the histological severity scores, MT wildtype mice appeared more susceptible to DSS-induced colitis compared to MT$^{-/-}$ animals (204). Furthermore, Bruewer et al. (211) reported that MT overexpression may represent an important early step in the development of carcinogenesis of ulcerative colitis independent of p53 expression. This should be further investigated in the long term as an independent cancer risk factor in ulcerative colitis.

Human studies

The only double-blind controlled trial of oral zinc sulphate as adjuvant treatment in idiopathic ulcerative colitis or proctitis in relapse was reported by Dronfield et al. (212). In this trial, 51 patients were treated with zinc and the clinical and sigmoidoscopic improvement was similar in the treated and placebo group. Furthermore, it has been shown that zinc administration decreased peripheral blood natural killer cell activity in 13 inflammatory bowel disease patients, with stable disease and mild-moderate disease activity, in a double-blind randomized cross-over trial (213).

9. Conclusions

Animal models of acute and chronic intestinal inflammation are indispensable for our understanding of the pathogenesis of ulcerative colitis and Crohn's disease, even though the etiology of inflammatory bowel disease remains unclear. In conclusion, administration of the above novel therapies have potential benefits in suppressing clinical features, histological pathology scores and inflammatory indicators in colitis in experimental models. There are four types of experimental animal models of colitis; spontaneous colitis models, inducible colitis models with normal immune system, adoptive transfer models in immuno-compromised hosts, and genetically engineered models (knockout and transgenic mice). There is not one single experimental model of colitis that incorporates all the clinical and histopathological characteristics of human inflammatory bowel disease, however, information gained from studies using these different types of colitis models has revealed three fundamental underlying principles. Firstly, chronic intestinal inflammation is mainly mediated by T cells. Secondly, commensal enteric bacteria are required to initiate and achieve intestinal inflammation and finally, the genetic background of the animal is a pivotal factor of disease onset and severity (84). Using these different models of colitis, *in vitro* and *in vivo* studies have shown a variety of novel therapies including, pre- and probiotics, n-3 fatty acids, plant bioactives (resveratrol, black raspberries, ginseng, ginkgo) and helminthes which have potential benefits in suppressing clinical features, histological pathology scores and inflammatory indicators. These novel therapies act on specific mechanisms of action such as intestinal barrier function, mucosal immune function and intestinal microbiota, however, there are no single therapies that have a multifunctional mechanism of action to prevent and treat ulcerative colitis. Newer therapies which use a combination of agents to restore gut homeostasis should be more promising and closer to

achieving long-term remission of ulcerative colitis. Thus, further studies are warranted to determine the mechanism of action by which these agents are able to protect against ulcerative colitis and to explore whether combination therapy could produce synergistic effects.

10. Acknowledgement

11. References

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APPENDIX 4

DIETARY EMU OIL SUPPLEMENTATION SUPPRESSES 5-FLUOROURACIL CHEMOTHERAPY-INDUCED INFLAMMATION, OSTEOCLAST FORMATION, AND BONE LOSS

STATEMENT OF AUTHORSHIP

Dietary emu oil supplementation suppresses 5-fluorouracil chemotherapy-induced inflammation, osteoclast formation and bone loss

American Journal of Physiology: Endocrinology and Metabolism 2012; 302 (11): E1440-9

Rethi Raghu Nadhanan

Performed bone experiments, analysed data, interpreted results, prepared figures and manuscript.

I hereby certify that the statement of contribution is accurate.

Signed   Date 27/11/2012

Suzanne Mashtoub ABIMOSLEH (Candidate)

Conducted all animal trials, contributed to experimental conceptualisation and design.

I hereby certify that the statement of contribution is accurate.

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Yu-Wen Su

Assisted with bone removal and experiments.

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Micheala A. Scherer

Interpreted experimental results.

I hereby certify that the statement of contribution is accurate.

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Gordon S. Howarth

Contributed to experimental conceptualisation and design.

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Cory J. Xian

Contributed to experimental conceptualisation and data interpretation, critical manuscript evaluation.

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