EMU OIL IN COMBINATION WITH HERBAL PREPARATIONS PROVIDES A NOVEL ADJUNCTIVE APPROACH TO COLITIS-ASSOCIATED COLORECTAL CANCER PREVENTION

LAUREN CLAIRE CHARTIER

Discipline of Physiology

Adelaide Medical School

The University of Adelaide

A thesis submitted to The University of Adelaide in fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY

February 2021

CONTENTS

ABSTRACT

Ulcerative colitis (UC) is an incurable and unremitting disease that presents as chronic inflammatory damage in the large intestine. UC is a lifelong condition that is often diagnosed in adolescence. The current pharmaceutical treatments for UC result in undesirable side effects and place patients at an increased risk of developing colorectal cancer (CRC). Emu Oil, derived from the adipose tissue of the Australian Emu, comprises fatty acids and has displayed anti-inflammatory, anti-oxidant and reparative properties in rodent models of gastrointestinal conditions. Notably, mice administered Emu Oil displayed improved clinical indicators of disease and resulted in fewer small colorectal tumours compared with untreated controls in an azoxymethane (AOM)/dextran sulphate sodium (DSS) mouse model. However, the total number of colorectal tumours was unaffected by Emu Oil administration (**Chapter 2**). Experimental studies have indicated that other naturally-sourced compounds (nutraceuticals), including grape seed extract (GSE) and Japanese Kampo medicine, have anti-neoplastic potential. Consequently, such nutraceuticals may be beneficial in colitis-associated CRC (CA-CRC) and amplify the therapeutic potential of Emu Oil.

In Chapter 3, the combination of Emu Oil and GSE was most effective at reducing clinical disease severity scores compared to each treatment alone in AOM/DSS mice. Furthermore, the number of colonic tumours was decreased by Emu Oil, GSE and the combined treatments. Additionally, impaired intestinal permeability was abrogated by Emu Oil and the combination of Emu Oil and GSE; however, GSE was ineffective when administered alone.

This thesis also includes the first study to investigate Saireito, a Japanese Kampo medicine, in pre-clinical CA-CRC. Notably, the combination of Emu Oil and Saireito proved more effective at reducing colonoscopically-assessed inflammation than each treatment alone (**Chapter 4**). Importantly, mice administered a combination of Emu Oil and Saireito displayed greater reductions in the total number of colonic tumours compared to AOM/DSS controls.

In pre-clinical investigations, it is necessary to monitor animal wellbeing and behaviour to ensure welfare and ethical standards are upheld. In this thesis, a number of methods were used, including burrowing (**Chapters 2–4**), clinical disease scores **Chapters 2–6**) and the mouse grimace scale (**Chapter 5**). **Chapter 5** provides details of the first study conducted to compare and correlate retrospective and real-time grimace analyses. Though, the results obtained in this study were not significant. Thus, it was concluded that the clinical disease score was the most reliable method for welfare monitoring in CA-CRC mice. Further, **Chapter 6** aimed to develop a modified AOM/DSS model whereby chemotherapy treatment causes intestinal mucositis, resulting in a more reliable method to test future treatments. However, in this chapter the chemotherapy dose administered was insufficient to induce mucositis coincident with CA-CRC.

Overall, this thesis includes rigorous scientific investigations into alternative and adjunct treatment options relevant to patients with CA-CRC. Additionally, modifications to the AOM/DSS model are discussed and the need to include animal wellbeing techniques in pre-clinical investigations is emphasised. Finally, the therapeutic action of Emu Oil was potentiated when administered with either GSE or Saireito, indicating that combined nutraceuticals could be utilised in CA-CRC management.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

1) **LC Chartier,** GS Howarth, D Trinder and S Mashtoub. Emu Oil and grape seed extract reduce tumour burden and disease parameters in murine colitis-associated colorectal cancer. 2020. *Carcinogenesis*, doi.org/10.1093/carcin/bgaa099 (e-pub ahead of print)

   Copyright: © *2020 Oxford University Press*

2) **LC Chartier,** ML Hebart, GS Howarth, AL Whittaker and S Mashtoub. Affective state determination in a mouse model of colitis-associated colorectal cancer. 2020. *PLoS One*, Vol 15(1): e0228413

   Copyright: © *2020 Chartier et al.*

3) **LC Chartier,** GS Howarth and S Mashtoub. Chemotherapy-induced mucositis development in a murine model of colitis-associated colorectal cancer. 2020. *Scandinavian Journal of Gastroenterology*, Vol 55 (1): pp 47–54

4) **LC Chartier,** GS Howarth and S Mashtoub. Combined Nutraceuticals: A novel approach to colitis-associated colorectal cancer? 2019. *Nutrition and Cancer*, Vol 71 (2): pp 199–206

5) **LC Chartier,** GS Howarth, IC Lawrance, D Trinder, SJ Barker and S Mashtoub. Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. 2018. *Digestive Diseases and Sciences*, Vol 63 (1): pp 134–145

Appendices

6) Z Lotfollahi, S Mashtoub, GS Howarth, **LC Chartier**, L Simson, KY Cheah and SEP Bastian. Comparative effects of mistletoe extracts in combination with 5-Fluorouracil on viability of IEC-6 and Caco-2 intestinal epithelial cells. 2018. *Australian Journal of Herbal and Naturopathic Medicine*, Vol 30(1): pp 174–179

7) CJ Mitchell, GS Howarth, **LC Chartier**, D Trinder, IC Lawrance, LS Huang and S Mashtoub. Orally administered emu oil attenuates disease in a mouse model of Crohn's-like colitis. 2020. *Experimental Biology and Medicine (Maywood)*. (e-pub ahead of print) DOI: 10.1177/1535370220951105

   Copyright: © *2020 by the Society for Experimental Biology and Medicine*

8) SJ Barker, GS Howarth, **LC Chartier**, BL Scherer and S Mashtoub. Mucosal stimulation following oral administration of emu oil represents a process of normal intestinal growth in rats. 2020. *Australian Journal of Herbal and Naturopathic Medicine,* Vol 32(1): pp 15–23

   Copyright: © *NHAA 2020*

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship and a PhD top-up scholarship from AgriFutures Australia.

Lauren Claire Chartier **Date:** 18 February 2021

ACKNOWLEDGEMENTS

First and foremost, I would like to offer a huge heartfelt thanks to my PhD supervisors, Dr Suzanne Mashtoub and Professor Gordon Howarth. Thank you for seeing my potential and for encouraging me to pursue a PhD. You have both taught me many invaluable research and life skills over the years. Gordon, I have enjoyed your endlessly witty e-mails, extensive vocabulary and incredible wealth of knowledge. I would like to thank Suzanne, for being a wonderful mentor and always the kindest friend. Your calm and rational nature was particularly appreciated in moments of office or animal house meltdowns. I thank you both for your wise guidance and for celebrating my achievements along the way. My PhD has been a unique journey that I will always look back on fondly and of which I am incredibly proud.

My sincere thanks to all members of the Gastroenterology Department at the Women's and Children's Hospital for providing such a warm and supportive environment throughout my studies. I am also grateful to the University of Adelaide where I completed my undergraduate degree, Honours Thesis and PhD.

I would like to extend my appreciation to all the funding bodies and organisations that awarded me with travel and other financial scholarships throughout my PhD. I was fortunate enough to receive an Australian Government Research Training Program scholarship and a top-up scholarship from AgriFutures Australia for the tenure of my candidature. Without this support, I would not have been able to continue my education. Additionally, I would like to mention Capstone Editing who contributed to the final touches on my Thesis and who awarded me with the Thesis Editing Grant in 2019. I would also like to thank the 7News Young Achiever Awards SA for nominating me as a Semi-Finalist for the University of Adelaide STEM Award category in 2020.

To all members of the wider Digestive Health Research Group, whose help, advice and companionship has been deeply valued. I would like to give a special mention to Chloe Mitchell

and Rebecca George for always being willing to help with experiments and for being there in the stressful, exhausting and amusing times.

I would like to thank all my incredible friends for their ongoing love and kindness. A special mention to my surf boat crew, the Somerton Allsorts, for brightening every day and for providing the best stress relief.

To the special ladies in my life and the best friends a girl could ask for, Alice, Imogen, Mikaela, Molly and Sarah, I would like to thank them for always being there and for encouraging me to be the best version of myself. I cannot express how much their friendship means to me and how grateful I am to have such inspirational women in my life.

To all my family and especially my beautiful big sister Emily and little brother Mason, I am so lucky to have two siblings to travel through life with. Finally, I dedicate this Thesis to my parents, Kathleen and Simon. Thank you for bringing me into the world, for always being my biggest advocate and for providing me with the love and support to succeed and enjoy all that life has to offer.

PUBLICATIONS ARISING FROM THESIS

LC Chartier, J Fujino, GS Howarth, J Freysdóttir, I Harðardóttir and S Mashtoub. Emu Oil and Saireito in combination reduce tumour development and clinical indicators of disease in a mouse model of colitis-associated colorectal cancer. 2020. *Biomedicine & Pharmacotherapy*, Under Review.

LC Chartier, GS Howarth, D Trinder and S Mashtoub. Emu Oil and grape seed extract reduce tumour burden and disease parameters in murine colitis-associated colorectal cancer. 2020. *Carcinogenesis*, doi.org/10.1093/carcin/bgaa099 (e-pub ahead of print).

LC Chartier, ML Hebart, GS Howarth, AL Whittaker and S Mashtoub. Affective state determination in a mouse model of colitis-associated colorectal cancer. 2020. *PLoS One*, Vol 15(1): e0228413.

LC Chartier, GS Howarth and S Mashtoub. Chemotherapy-induced mucositis development in a murine model of colitis-associated colorectal cancer. 2020. *Scandinavian Journal of Gastroenterology*, Vol 55 (1): pp 47–54.

LC Chartier, GS Howarth and S Mashtoub. Combined Nutraceuticals: A Novel Approach to Colitis-Associated Colorectal Cancer? 2019. *Nutrition and Cancer*, Vol 71 (2): pp 199–206.

LC Chartier, GS Howarth, IC Lawrance, D Trinder, SJ Barker and S Mashtoub. Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. 2018. *Digestive Diseases and Sciences*, Vol 63 (1): pp 134–145.

Z Lotfollahi, S Mashtoub, GS Howarth, **LC Chartier**, L Simson, KY Cheah and SEP Bastian. Comparative effects of mistletoe extracts in combination with 5-Fluorouracil on viability of IEC-6 and Caco-2 intestinal epithelial cells. 2018. *Australian Journal of Herbal and Naturopathic Medicine*, Vol 30(1): pp 174–179.

CJ Mitchell, GS Howarth, **LC Chartier**, D Trinder, IC Lawrance, LS Huang and S Mashtoub. Orally administered emu oil attenuates disease in a mouse model of Crohn's-like colitis. 2020. *Experimental Biology and Medicine (Maywood)*. (e-pub ahead of print) DOI: 10.1177/1535370220951105.

SJ Barker, GS Howarth, **LC Chartier**, BL Scherer and S Mashtoub. Mucosal stimulation following oral administration of emu oil represents a process of normal intestinal growth in rats. 2020. *Australian Journal of Herbal and Naturopathic Medicine*, Vol 32(1): pp 15–23.

KE Maiolo, GS Howarth, **LC Chartier**, D Trinder, IC Lawrance, SJ Barker, BL Scherer and S Mashtoub. Emu Oil reduces proximal colonic crypt hyperplasia in a murine model of colitis-associated colorectal cancer. 2020. *Prostaglandins, Leukotrienes & Essential Fatty Acids*, Under Review.

S Mashtoub, **LC Chartier**, D Trinder, IC Lawrance and GS Howarth. Emu Oil attenuates disease severity and results in fewer large colonic tumours in a mouse model of colitis-associated colorectal cancer. 2020. *Nutrition and Cancer*, Under Review.

TL Kennewell, GS Howarth, **LC Chartier**, J Fujino, CJ Mitchell, AL Whittaker and S Mashtoub. Kampo medicine (Orengedokuto) improves stool consistency in a mouse model of 5-Fluorouracil-induced mucositis. 2020. *Ethnopharmacology*, Under Review.

PUBLICATIONS ARISING FROM CONFERENCES

LC Chartier, GS Howarth, D Trinder and S Mashtoub. Su1815—Grape seed extract and emu oil in combination reduces colitis severity and tumour number in a mouse model of colitis-associated colorectal cancer. 2019. *Gastroenterology*, Vol 156(6): pp S-622–ßS-623.

LC Chartier, KE Maiolo, GS Howarth, IC Lawrance, D Trinder, SJ Barker, BL Scherer, CJ Mitchell, S Mashtoub. Emu Oil improves clinical indicators of disease and reduces proximal colonic crypt hyperplasia in a murine model of colitis-associated colorectal cancer. 2018. *Gastroenterology*, Vol 154(6) (Supp 1): Abstract Mo1994: S–875.

LC Chartier, GS Howarth, IC Lawrance, D Trinder, SJ Barker, S Mashtoub. Emu oil improves bodyweight in a mouse model of colitis-associated colorectal cancer, whilst not impacting healthy controls. 2017. *J Nutr Interned Metab,* Vol 8: p 94.

LC Chartier, GS Howarth, IC Lawrance, D Trinder, SJ Barker, S Mashtoub. Emu oil improves clinical indicators of disease in a mouse model of colitis-associated colorectal cancer. 2017. *J Gastro Hepatol*, Vol 32 (Supp 2):56–56.

CONFERENCE PRESENTATIONS AND AWARDS

Poster presentation titled 'Emu Oil improves clinical indicators of disease and reduces proximal colonic crypt hyperplasia in a murine model of colitis-associated colorectal cancer' at Digestive Diseases Week, Washington DC, USA, 2–5 June 2018

Oral presentation titled 'Emu Oil improves clinical indicators of disease and reduces proximal colonic crypt hyperplasia in a murine model of colitis-associated colorectal cancer' at the Australian Society for Medical Research Meeting, Adelaide, South Australia, 4 June 2018

Awarded runner-up in the Best Abstract Award

Poster presentation titled 'Orally administered emu oil reduced disease severity in a murine model of colitis-associated colorectal cancer' at Multinational Association of Supportive Care in Cancer, Vienna, Austria, 28–30 June 2018

Supported by a Walter and Dorothy Duncan Trust Travel Award and the Hans-Jürgen and Marianne Ohff Research Travel Grant

Poster presentation titled 'Proximal colonic crypt hyperplasia is reduced by emu oil in a murine model of colitis-associated colorectal cancer' at Nutrition Society of Australia Annual Meeting, Canberra, Australian Capital Territory, 27–30 November 2018

Supported by a Nutrition Society of Australia Student Travel Award and Adelaide Medical School Research Travel Award

Poster presentation titled 'Grape seed extract and emu oil in combination reduces colitis severity and tumour number in a mouse model of colitis-associated colorectal cancer' at Digestive Diseases Week, San Diego, USA, 18–21 May 2019

Supported by the Cancer Council Beat Cancer Project Travel Grant

Oral presentation titled ‘Grape seed extract and emu oil in combination reduces colitis severity and tumour number in a mouse model of colitis-associated colorectal cancer’ at the Australian Society for Medical Research Meeting, Adelaide, South Australia, 5 June 2019

Poster presentation titled ‘Emu Oil and Saireito (Japanese Kampo Medicine) reduce tumour development and clinical indicators of disease in a mouse model of colitis-associated colorectal cancer’ at the Biennial International Society for the Study of Fatty Acids and Lipids Congress, Qingdao, China, 14–17 June 2020 (conference did not proceed)

Supported by a Healthy Development Adelaide Travel Grant

THESIS STRUCTURE

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

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

CHAPTER 1

COMBINED NUTRACEUTICALS: A NOVEL APPROACH TO COLITIS-ASSOCIATED COLORECTAL CANCER?

CONTEXT STATEMENT

The research described in this chapter has been published in *Nutrition and Cancer*.

> *LC Chartier, GS Howarth and S Mashtoub. Combined Nutraceuticals: A Novel Approach to Colitis-Associated Colorectal Cancer? 2019. Nutrition and Cancer, Vol 71 (2): pp 199–206.*

Ulcerative colitis (UC) is an incurable and lifelong inflammatory bowel disease. Patients suffering from this disease are likely to develop colitis-associated colorectal cancer (CA-CRC). **Chapter 1** provides an overview of the characteristics and current treatments of UC and CA-CRC and highlights the need for novel therapies. This chapter also identifies the attraction towards naturally-sourced therapies for gastrointestinal conditions and reviews the current literature on Emu Oil, grape seed extract and Japanese Kampo medicine. Finally, **Chapter 1** also discusses the rationale for combining the selected nutraceuticals to strengthen efficacy as investigated in **Chapters 3** and **4**. In addition to the nutraceuticals discussed in this review, mistletoe extract has also been identified as a potential anti-cancer agent for gastrointestinal conditions as described in **Appendix 1**.

STATEMENT OF AUTHORSHIP

Title of Paper: Combined Nutraceuticals: A Novel Approach to Colitis-Associated Colorectal Cancer?

Publication Status: Published

Publication Details: Nutrition and Cancer, 2019, Vol 71, pages 199-206

Principal Author

Name of Principal Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Conceptualisation, full review of the literature, writing, formatting and submission of the manuscript.		
Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	31/8/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, revision and editing of the manuscript.		
Signature		Date	17/9/20

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, revision and editing of the manuscript.		
Signature		Date	17/9/20

NUTRITION AND CANCER
2019, VOL. 71, NO. 2, 199–206
https://doi.org/10.1080/01635581.2019.1578391

Taylor & Francis
Taylor & Francis Group

Combined Nutraceuticals: A Novel Approach to Colitis-Associated Colorectal Cancer?

Lauren C. Chartier[a,b], Gordon S. Howarth[a,b,c], and Suzanne Mashtoub[a,b]

[a]Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia; [b]Department of Gastroenterology, Women's and Children's Hospital, North Adelaide, South Australia; [c]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia

ABSTRACT

Ulcerative colitis is an unremitting and lifelong inflammatory bowel disease that is increasing in prevalence worldwide. Patients display various clinical symptoms such as abdominal pain, diarrhea and fatigue. The etiology of ulcerative colitis remains unknown and the current pharmaceutical treatments are variably effective and not curative, highlighting the need for improved therapeutic approaches. Furthermore, patients with ulcerative colitis are at an increased risk of developing colorectal cancer. Some naturally sourced agents, named nutraceuticals, have been identified to possess anti-inflammatory and antioxidant properties. Of particular interest is Emu Oil, grape seed extract and Japanese Kampo medicine. Previously, Emu Oil has protected and repaired intestinal damage in models of gastrointestinal diseases including colitis and colitis-associated colorectal cancer. Additionally, grape seed extract possesses anticancer properties *in vitro*. Moreover, Kampo medicine, composed of herbal ingredients, is widely used in Japan for the treatment of various medical conditions and has demonstrated efficacy in targeting cancer cells *in vitro*. Nutraceuticals in combination have not yet been widely investigated in a setting of colitis-associated colorectal cancer. Investigation into the efficacy of Emu Oil combined with other nutraceuticals, including grape seed extract and Kampo medicine, is warranted as they may provide a novel approach to conventional colitis and colorectal cancer management.

ARTICLE HISTORY
Received 7 September 2018
Accepted 10 January 2019

Introduction

Inflammatory bowel disease (IBD) is the collective term for a group of lifelong and debilitating disorders that are emerging in global prevalence. IBD encompasses two conditions: Crohn's disease and ulcerative colitis (UC). Crohn's disease is characterized by discontinuous transmural inflammation which can affect any region of the gastrointestinal (GI) tract. Alternatively, UC presents as continuous inflammation and ulceration of the GI mucosa, and is confined to the large intestine (1). Western countries have the highest prevalence of IBD and particularly areas in Northern Europe and North America are most affected; however, IBD prevalence is increasing worldwide (2).

There are two categories of inflammatory damage: acute and chronic. Acute inflammation occurs rapidly, is of short duration and involves the migration of neutrophils to the injury site (3). An acute response aims to kill bacterial and viral infections by facilitating wound repair, whereas chronic inflammation is a sustained response that can manifest histologically to result in tissue necrosis. Furthermore, chronic inflammation has the potential to develop to degenerative diseases such as rheumatoid arthritis, heart disease, IBD, and cancer (3).

Ulcerative Colitis

Patients diagnosed with UC present with GI and extra-intestinal symptoms including bloody or loose stools, weight loss, fever and fatigue. The disease course occurs in cycles of idiopathic 'disease flares' followed by periods of remission (4). Current treatments for UC are variably effective and typically not curative. The categories of pharmaceuticals prescribed include antibiotics, corticosteroids, aminosalicylates, and tumor necrosis factor-α (TNF-α) blockers. Many

CONTACT Suzanne Mashtoub suzanne.mashtoub@adelaide.edu.au Gastroenterology Department, Women's and Children's Hospital, 72 King William Road, North Adelaide 5006, South Australia.

of these drugs are immunosuppressants, consequently increasing the risk of secondary infections (5). Additionally, surgery can be required to remove and resect severely inflamed sections of the colon. However, these treatments can be invasive and result in complications that reduce patient quality of life. UC is generally diagnosed in patients in their youth or early adult life; therefore patients must manage their condition and treatment over many decades.

The etiology of UC remains unknown; however, lifestyle and environmental factors have been identified as being important in the pathogenesis of the condition rather than ethnic, racial or regional differences (6). The potential pathological cascade for the development of IBD involves an underlying genetic susceptibility, followed by a population change of commensal and pathogenic bacteria within the GI tract as a result of intestinal insult. This disturbance to gut microbiota populations, named 'dysbiosis', is common in IBD patients (7). When prolonged, dysbiosis can result in transient breaks of the mucosal barrier, thus contributing to chronic inflammation and IBD pathogenesis (4,6).

Intestinal microflora may be a main contributing factor in the development of intestinal lesions in IBD patients as lesions are predominantly found in areas of high bacterial concentration (8). Studies have concluded that germ-free conditions significantly decrease the severity and development of IBD in genetically susceptible hosts. Wild-type HLA-B27 transgenic rats spontaneously develop chronic colitis; however, when kept in a germ-free environment no colitis or disease developed (9).

In 2005, a Nobel Prize was awarded for identifying *Helicobacter pylori* to be the causative organism of gastritis and peptic ulcers (10). However, *H. pylori* and its association with UC is controversial. A study of 49 UC patients found a 57% positivity for *H. pylori* in the gastric mucosa (11) and Yu et al. (2015) (12) concluded that non-*H. pylori* enterohepatic Helicobacter species (EHS) are likely involved in the etiology of UC. EHS can colonize lower in the GI tract and are discussed as having a primary relationship with the causative action or risk of IBD development. Furthermore, following fluorescence *in situ* hybridization analyses, non-pylori Helicobacter species were prevalent in 79% of adult UC patients, compared with 23% of the adult control population. Similarly non-pylori Helicobacter species were present in 87% of pediatric UC patients, compared with 40% of pediatric controls (13,14). These results highlight the potential association between non-pylori Helicobacter species and UC pathogenesis.

The limited effectiveness of currently available therapies for UC patients is problematic, as poorly managed UC can often progress to colorectal cancer (CRC) (15). Hartnett and Egan (2012) (16) concluded that UC patients have a 2- to 3-fold greater lifetime risk of developing CRC due to the multifaceted contributing factors and ineffective treatment. Moreover, chronic inflammation is known to be associated with approximately one-fifth of all cancers, highlighting the link between inflammation and tumorigenesis (4). Therefore, there is a need to develop novel effective treatment strategies for UC to prevent the subsequent development of cancer.

Colorectal Cancer

CRC can develop spontaneously or non-spontaneously due to underlying inflammatory damage. Spontaneous CRC develops when normal colonic enterocytes are transformed to malignant cells due to an accumulation of genetic and epigenetic events. There are three main molecular pathways involved in sporadic tumorigenesis: chromosomal instability (CIN), microsatellite instability, and the serrated pathway (16–18). CIN is the dominating genetic pathway for spontaneous CRC development and it occurs in approximately 85% of CRC cases (17). However, the chronic inflammatory environment experienced by UC patients results in decreased T_{reg} cells and an increase in Th17 levels, additional to elevated expression of interleukins (IL-17a, IL-1β, and IL-6) (19), DNA damage, and tissue destruction. These factors combined with poor long-term management of UC, results in the development of cancer (16). The cancer risk increases with a greater amount of surface area affected by colitis, and patients with colitis-associated CRC are typically younger in age than spontaneous CRC patients (17).

Colitis-associated CRC pathogenesis differs from spontaneous CRC as inflammation alters the timing and frequency of malignancy, although affecting the same molecular pathways. Dysplasia in colitis-associated CRC can be polypoid, flat, localized, or diffused, making surveillance more challenging. Clinical and histological symptoms are often quiescent in UC patients with carcinomata arising without a typical inflammation–dysplasia–carcinoma progression (17). In most cases, neoplastic lesions arise in areas of the colon greatly affected by inflammation; however, genomic alterations can also occur without disturbing morphology of the colon, thus making colitis-associated CRC complicated to diagnose at early stages.

Mutations of *APC* occur later in development and are less common in colitis-associated carcinogenesis than sporadic cancers. However, *p53* deletion is evident in 50–85% of colitis-associated CRC cases (17).

It is well-established that inflammation plays a role in all stages of tumor development. Additional to the carcinogenic pathways involved, inflammation-associated genes such as, cyclooxygenase-2 (COX-2), are increased in the inflamed colonic mucosa and neoplastic tissue, contributing to pathogenesis. The presence of cytokines and reactive nitrogen and oxygen species (RNS and ROS) is increased resulting in DNA changes, mutations, and genomic instability of the colonic epithelium. IBD causes an increased turnover of epithelial cells, including mitosis and apoptosis, and although this turnover rate may be attributed to cancer, it is not a definitive cause of CRC. Consequently, tumor development is promoted through proliferation of these altered cells and the inflammatory environment inhibits apoptosis and promotes cell proliferation causing tumors to increase in size. Additional genetic changes from the toxic environment cause the carcinoma to metastasize from the primary site of the large intestine (16).

Pro- and Anti-Inflammatory Cytokines

Increased levels of the pro-inflammatory cytokine TNF-α are positively correlated with colitis-associated CRC development (20). TNF-α has a crucial role in tumor initiation by stimulating existing DNA damage to cause further mutations and promoting the presence of RNS and ROS. Additionally, TNF-α acts as a tumor promoter by altering basal cell proliferation and apoptosis levels. Infliximab, a humanized monoclonal antibody against TNF-α, is a currently prescribed treatment for IBD. Infliximab has been extensively investigated in pre-clinical models and was able to reduce the development of colonic tumors in a mouse model of chronic UC (21). This result reinforces the importance of TNF-α in the progression of UC to colitis-associated CRC.

Nuclear factor-κB (NF-κB) is an oncogenic transcription factor that is activated by pro-inflammatory cytokines (16). The NF-κB pathway is activated in colonic epithelial cells in patients with IBD and in cases of CRC. Additionally, the STAT3 pathway plays a crucial role in tumorigenesis. IL-6 (pro-inflammatory) activates STAT3 to increase inflammation to the point of constitutively active STAT3 expression, which is common in many cancers (16). The role of STAT3 in tumorigenesis was investigated in a mouse model by Grivennikov et al. (22). The authors concluded that IL-6 activated STAT3 and consequently led to tumor development. However, when STAT3 was inhibited in the intestinal epithelial cells, colitis-associated CRC was also inhibited (22). Therefore, therapies that target the STAT3 pathway may be crucial in preventing colitis-associated tumor development.

Colorectal Cancer Therapy

Despite recent advances in CRC diagnosis and therapy, the cure rate remains poor. Chemotherapy and radiation treatment provide modest survival depending on the stage of cancer progression (23). 5-Fluorouracil (5-FU) has been the principal anticancer drug used in CRC for decades. 5-FU is an antimetabolite that blocks DNA synthesis by inhibiting the enzymes topoisomerase II and thymidylate synthase (23). 5-FU can be incorporated into RNA causing mitotic inhibition, disruption of cell–cell and cell–substratum interactions and impaired epithelial integrity (24). Although patients may respond differently to chemotherapy, approximately 80% that undergo 5-FU treatment subsequently develop inflammation of the oral and/or intestinal mucosa, resulting in a condition known as mucositis (25). Mucositis is a debilitating side-effect of cancer therapy associated with painful ulceration of the GI tract. It may be present from mouth to anus and can severely impact quality of life (26). Patients with chronic mucositis may decide to terminate cancer therapy to control the secondary condition, and this decision can be detrimental. Recent investigations aiming to develop new cytotoxic agents have seen pharmaceuticals including raltitrexed, oxaliplatin, and irinotecan introduced for CRC therapy (23). However, like 5-FU, these agents have been associated with adverse side-effects which may result in the cessation of treatment.

As the prevalence of cancer increases and pharmaceuticals are unable to provide complication-free treatment, we require therapeutic approaches and management that allow patients to live with cancer. This approach is needed to increase the body's natural healing, tumor immunity and to attenuate cancer progression (27). Furthermore, recent investigations have indicated a number of naturally sourced products, named nutraceuticals (plant and animal-derived extracts) to be therapeutic in inflammatory disorders and display anticancer properties with scarce side-effects. This article discusses some nutraceuticals of interest and the evidence available to suggest the potential efficacy in conjunction with conventional

treatment for colitis and colitis-associated cancer. These nutraceuticals elicit a protective effect on the intestine and consequently may limit the undesirable symptoms of inflammatory damage and provide alternatives to currently available therapies.

Nutraceuticals

Emu Oil

Animal-derived oils have previously been investigated for their use in various inflammatory conditions. Oils derived from ratites (flightless birds) including the ostrich (*Struthio camelus*), rhea (*Rhea americana*), cassowary (*Casuarius*), and the Australian emu (*Dromaius novaehollandiae*) are of interest. Native Aboriginal people traditionally used Emu Oil topically to alleviate pain and promote wound healing due to the anti-inflammatory, skin-permeation, and antioxidant properties it possesses (28). Emu Oil is derived from subcutaneous and retroperitoneal Emu adipose tissue and comprises 98.8 and 98.0%, fatty acids respectively, Table 1. Sourced from Mashtoub (2017) (29) details the fatty acid composition of Emu Oil compared to other ratite oils and an olive oil control. The fatty acid fraction comprises oleic acid (omega-9; 42%), linoleic acid (omega-6; 21%), palmitic acid (21%), and lower levels of α-linolenic acid (omega-3; 1%) (28).

Antioxidant Properties of Emu Oil

A study conducted by Bennett et al. (30) investigated the antioxidant properties of Emu Oil in comparison with other avian oils such as ostrich and rhea oil. The authors used the 2,2-diphenyl-1-picryl hydracyl (DPPH) radical assay and concluded that Emu Oil elicited a greater effect on reducing levels of thiobarbituric acid-reactive substance (a marker of oxidative stress) than the other oils tested (30), indicating that Emu Oil may offer greater protection than alternative ratite oils against oxidative damage. Additionally, this effect may consequently contribute to CRC inhibition. Furthermore, Bennett et al. (30) proposed that the ratio of unsaturated to saturated fatty acids is likely responsible for cellular protection against oxidative stress. In a study conducted by Mashtoub et al. (31), the free radical scavenging activity (RSA) and the primary oxidation status of ratite oils was investigated. The oils tested varied in farm location, rendering, and storage procedures. The authors concluded that Emu Oil represented the greatest overall RSA compared to ostrich and rhea oil, thus identifying the antioxidant potential of Emu Oil.

Table 1. Composition of emu oil (29).

Major FA composition of emu, 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

MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Anti-Inflammatory Properties of Emu Oil

A number of studies have investigated the anti-inflammatory properties of Emu Oil following both topical and oral application. Yoganathan et al. (2003) (32) induced auricular inflammation in CD-1 mice by applying 5 µl of croton oil and treating topically with fish, flaxseed, olive or Emu Oil. Topical application of Emu Oil resulted in a decrease of pro-inflammatory cytokines (TNF-α, IL-1α), which are known to be directly involved in the development of IBD. Moreover, the anti-inflammatory effect of Emu Oil was more pronounced than that of the other oils tested, which contain higher levels of the anti-inflammatory fatty acids; oleic and α-linolenic acid (32). Consequently, this study indicated that the unique benefits of Emu Oil may be attributed to components in the nontriglyceride fraction as well as the fatty acid composition.

Additional to topical application, orally administered Emu Oil has displayed anti-inflammatory and reparative properties in a number of pre-clinical models of GI disorders (33). These include chemotherapy-induced mucositis (34), nonsteroidal anti-inflammatory drug (NSAID)-induced enteropathy (35), UC (36), and colitis-associated CRC (37,38). In a rat model of NSAID (indomethacin)-induced enteropathy, daily administration of Emu Oil resulted in significantly decreased myeloperoxidase (MPO) activity in the jejunum and ileum, thereby reducing acute intestinal inflammation compared to NSAID-treated controls (35). Furthermore, Mashtoub et al. (34) concluded that Emu Oil improved the integrity of the intestinal mucosa following mucositis-induced damage in rats. Emu Oil has also been shown to be efficacious in reducing inflammation in animal models of dextran sulfate sodium (DSS)-induced UC. In DSS-treated rats, daily oral-administration of Emu Oil resulted in significantly decreased severity of histologically assessed proximal and distal colonic damage (36). Emu Oil (low dose: 0.5 ml) significantly lengthened colonic crypts in DSS-treated rats compared to UC

controls. Emu Oil (high dose: 1.0 ml) also increased crypt depth by 26% (36). The authors discussed that reparative properties of Emu Oil may be due to an increase in crypt cell proliferation, a decrease in the rate of crypt cell apoptosis, or a combination of the two mechanisms (36). Most recently, studies by Mashtoub et al. (37) and Chartier et al. (38) investigated the anti-inflammatory and potential neoplastic-preventative action of Emu Oil in mouse models of colitis-associated CRC. When Emu Oil was administered thrice weekly for 12 weeks, mice with colitis-associated CRC and treated with Emu Oil improved bodyweight and resulted in fewer large colorectal tumors than CRC controls (37). Furthermore, in a 9-week colitis-associated CRC study mice administered Emu Oil (low dose: 0.08 ml) resulted in fewer small colorectal tumors compared to CRC controls (38). Although Emu Oil-administration resulted in significant reductions in disease severity, Emu Oil did not reduce the total number of tumors compared to CRC controls in either of these studies. Therefore, further investigation into the tumor inhibiting effect of Emu Oil in colitis-associated cancer and its efficacy when combined with other naturally sourced compounds to enhance its efficacy is warranted in colitis-associated CRC models.

Grape Seed Extract

Grape seed extract is a by-product of the wine and grape-juice industries. It contains high levels of phytochemicals, such as flavonoids, and has historically been used to treat digestive problems (39). Procyanidins in grape seed extract are potent antioxidants and free-radical scavengers that possess anti-inflammatory, antibacterial, antiviral, and anti-neoplastic potential (39). Proanthocyanidins in grape seed extract bind to the apical surface of the gut mucosa without being absorbed across the intestine, thus allowing them to be bioavailable within the gut following ingestion (39).

Previous *in vitro* investigations have displayed the anti-neoplastic potential of grape seed extract and *in vivo* models have highlighted its anti-inflammatory and antioxidant properties. In a study conducted by Cheah et al. (40), grape seed extract was fractionized from mature and immature Cabernet Sauvignon seeds and applied to colon cancer (Caco-2) cells for viability assessment. All procyanidin fractions of grape seed extract analyzed reduced cell viability of Caco-2 cells and enhanced the potency of the chemotherapeutic 5-FU, demonstrating its anti-neoplastic properties (40). Moreover, in a rat model of DSS-induced colitis, grape seed extract decreased intestinal villus height and mucosal thickness of colitis animals towards normal values and decreased damage to the intestinal architecture. Importantly, in this study, administration of grape seed extract did not impact any metabolic parameters or have GI side effects in healthy animals, demonstrating its safety for oral-application (39). These studies highlight the anti-neoplastic potential of grape seed extract and if combined with Emu Oil, may elicit a desired effect on tumor-inhibition in a model of colitis-associated CRC.

Kampo Medicine

Kampo medicine is a traditional Japanese herbal medicine derived from ancient China (41), and contains combinations of 3–30 plant-based ingredients. Many Kampo formulae are covered by health insurance in Japan, therefore patients often receive Western and Kampo treatment simultaneously. Kampo is believed to enhance immunity, improve pre- and post-operative conditions and alleviate adverse drug reactions in GI conditions and cancers (42). In 2012, a review of 900 physicians from core cancer hospitals in Japan found 92% of the physicians surveyed actively prescribed Kampo and 74% of these were prescribed to cancer patients (43). Interestingly, only 23% of physicians expected high efficacy in tumor suppression; however, patients believed that Kampo suppressed tumor growth and exhibited fewer side-effects than Western medicine (43,44). This discrepancy warrants further investigation into the efficacy of Kampo in pre-clinical models of disease and cancer to ease expectations between physician and patient.

Daikenchuto [*ginger root*, *Japanese green pepper (Zanthoxylum)*, and *ginseng radix root*] is a Kampo medicine that has conventionally been used to improve intestinal function (42,45,46). Additionally, it has been suggested that Kampo medicines may target tumor growth promotion rather than initiation. This was detailed in a study where Daikenchuto limited the size of tumors as well as decreasing the overall quantity of tumors (45). Recently, Nagata et al. investigated the anti-cancer effects of Daikenchuto and its components through cell culture and *in vivo* analyses. The Kampo medicine was tested in breast, esophageal, gastric, and colon cancer cell lines. Daikenchuto inhibited tumor growth in all cell lines compared to controls and to a similar extent of the known chemotherapeutic, 5-FU (42). Furthermore, Daikenchuto pro-longed

the survival time of mice with DSS-induced colitis and decreased levels of inflammatory cytokines IL-1β and TNF-α (47). Together, these findings suggest that Daikenchuto may be efficacious in reducing tumor growth and survival in colitis-associated CRC. The Kampo medicine, Juzentaihoto (*Astragalus Root*, *Cinnamon bark*, *Rehmannia Root*, *Peony Root*, *Cridium Rhizome*, *Atractylodes Lancea Rhizone*, *Japanese Angelica Root*, *Ginseng*, *Poria Sclerotium*, and *Glucyrrhiza*) may also be of interest for colitis-associated CRC therapy. Juzentaihoto is commonly prescribed post-operatively to promote and restore physical strength and alleviate traditional cancer-treatment side-effects (27). Juzentaihoto was shown to prevent cancer recurrence and suppress metastasis by activating macrophages and increasing natural killer cells (27). These characteristics of Juzentaihoto suggest that it may be beneficial as an adjunct-treatment for colitis-associated CRC patients to limit the side-effects of conventional treatment.

Other Nutraceuticals

Other naturally sourced products may also be effective in a setting of colitis-associated CRC. Lyprinol^{TM}, an extract derived from the New Zealand green-lipped mussel (48), was identified to be therapeutic in reducing small intestinal damage and acute inflammation when combined with Emu Oil (48). Moreover, D'Argenio et al. (49) administered apple polyphenols extract to rats induced with colitis. It was concluded that the apple extract reduced the severity of the condition and returned levels of biomarkers including COX-2 and TNF-α to baseline levels. Finally, evidence suggests that supplementing fruits and vegetables, dietary fiber, and probiotic bacteria may also be therapeutic in reducing inflammation and symptoms of IBD (50).

Combination Treatment

Scientific literature has discussed Emu Oil as an effective anti-inflammatory treatment when applied topically and orally in various conditions. When Emu Oil was administered in a model of colitis-associated CRC chronic colitis was significantly reduced; however, Emu Oil did not significantly impact the total number of colorectal tumors (37,38). Nutraceuticals including grape seed extract and Kampo are documented to possess anti-neoplastic potential following *in vitro* and *in vivo* investigations. Combining the anti-inflammatory action of Emu Oil with a substance that displays anti-neoplastic properties may elicit a more potent tumor-inhibiting effect in colitis-associated CRC.

Conclusion

UC is an unremitting, chronic, and lifelong condition that is increasing in prevalence worldwide. The link between inflammation and carcinogenesis is well-established and CRC remains the second most prominent cancer in Australia. Current treatments for UC and CRC have debilitating side-effects, and more effective therapies without these manifestations are required. Therapies that inhibit cancer promotion and progression may elicit an effect on cancer development that can potentially be used as a therapy long after cancer has developed or gone undetected. Naturally sourced compounds are gaining popularity as treatments for conditions of the GI tract including UC and colitis-associated CRC, as they are affordable, renewable and have limited or no side-effects. By investigating combination treatments of these compounds, a potent natural therapy may be developed.

Disclosure Statement

No potential conflict of interest was reported by the authors.

References

1. Head KA and Jurenka JS: Inflammatory bowel disease Part 1: ulcerative colitis-pathophysiology and conventional and alternative treatment options. *Altern Med Rev* 8, 247–283, 2003.
2. Loftus EV: Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* 126, 1504–1517, 2004.
3. Iwalewa EO, McGaw LJ, Naidoo V, and Eloff JN: Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. *Afr J Biotechnol* 6, 2868–2885, 2007.
4. Westbrook AM, Szakmary A, and Schiestl RH: Mechanisms of intestinal inflammation and development of associated cancers: lessons learned from mouse models. *Mutat Res Rev Mutat Res* 705, 40–59, 2010.
5. Ananthakrishnan AN and Binion DG: Treatment of ulcerative colitis in the elderly. *Dig Dis Sci* 27, 327–334, 2009.
6. Hansen R, Thomson JM, El-Omar EM, and Hold G: The role of infection in the aetiology of inflammatory bowel disease. *J Gastroenterol* 45, 266–276, 2010.
7. Martinez-Medina M and Garcia-Gil LJ: *Escherichia coli* in chronic inflammatory bowel diseases: an update on adherent invasive *Escherichia coli*

pathogenicity. *World J Gastrointest Pathophysiol* 5, 213–227, 2014.
8. Khajah MA: The potential role of fecal microbiota transplantation in the treatment of inflammatory bowel disease. *Scand J Gastroenterol* 52, 1172–1184, 2017.
9. Lidar M, Langevitz P, and Shoenfeld Y: The role of infection in inflammatory bowel disease: initiation, exacerbation and protection. *Isr Med Assoc J* 11, 558–563, 2009.
10. Mégraud F: A humble bacterium sweeps this year's Nobel Prize. *Cell* 123, 975–976, 2005.
11. Caner S, Altinbaş A, Yeşıl Y, Yavuz B, Barış Y, et al.: The relation between *Helicobacter pylori* and ulcerative colitis. *Turk J Med Sci* 44, 820–823, 2014.
12. Yu Q, Zhang S, Li L, Xiong L, Chao K, et al.: Enterohepatic Helicobacter species as a potential causative factor in inflammatory bowel disease: A meta-analysis. *Medicine* 94, e1773, 2015.
13. Thomson JM, Hold G, Berry SH, El-Sakka NE, Mowat NA, et al.: Variable detection of entero-hepatic Helicobacter species in colonic mucosal pinch biopsies by different molecular techniques. *Gastroenterology* 134, A-655, 2008.
14. Hansen R, El-Sakka NE, Berry SH, Thomson JM, Bisset WM, et al.: Are non-pylori Helicobacter organisms associated with paediatric ulcerative colitis? Retrospective observational study. *Gastroenterology* 136, A-671, 2009.
15. Kinugasa T and Akagi Y: Status of colitis-associated cancer in ulcerative colitis. *World J Gastrointest Oncol* 8, 351–357, 2016.
16. Hartnett L and Egan LJ: Inflammation, DNA methylation and colitis-associated cancer. *Carcinogenesis* 33, 723–731, 2012.
17. Itzkowitz SH and Yio X: Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 287, G7–G17, 2004.
18. Mundade R, Imperiale TF, Prabhu L, Loehrer PJ, and Lu T: Genetic pathways, prevention, and treatment of sporadic colorectal cancer. *Oncoscience* 1, 400–406, 2014.
19. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, and Barry S: Foxp3^{+} regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. *J Clin Immunol* 30, 80–89, 2010.
20. Zheng H, Lu Z, Wang R, Chen N, and Zheng P: Establishing the colitis-associated cancer progression mouse models. *Int J Immunopathol Pharmacol* 29, 759–763, 2016.
21. Kim YJ, Hong KS, Chung JW, Kim JH, and Hahm KB: Prevention of colitis-associated carcinogenesis with infliximab. *Cancer Prev Res* 3, 1312–1333, 2010.
22. Grivennikov S, Karin E, Terzic J, Mucida D, Yu G-Y, et al.: IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* 15, 103–113, 2009.
23. Jansman F, Sleijfer D, Graaf J, Coenen J, and Brouwers J: Management of chemotherapy-induced adverse effects in the treatment of colorectal cancer. *Drug Saf* 24, 353–367, 2001.
24. Denham JW and Hauer-Jensen M: The radiotherapeutic injury-a complex 'wound'. *Radiother Oncol* 63, 129–145, 2002.
25. Chang C-T, Ho T-Y, Lin H, Liang J-A, Huang H-C, et al.: 5-Fluorouracil induced intestinal mucositis via Nuclear Factor-κB activation by transcriptomatic analysis an in vivo bioluminescence imaging. *PLoS ONE* 7, e31808, 2012.
26. Duncan M and Grant G: Oral and intestinal mucositis - causes and possible treatments. *Aliment Pharmacol Ther* 18, 853–874, 2003.
27. Yamakawa J-I, Moyoo Y, Moriya J, Ogawa M, Uenishi H, et al.: Role of Kampo medicine in integrative cancer therapy. *Evid Based Complementary Alternat Med* 2013, 570848, 2013.
28. Abimosleh SM, Tran CD, and Howarth GS: Emu oil: a novel therapeutic for disorders of the gastrointestinal tract? *J Gastroenterol Hepatol* 27, 857–861, 2012.
29. Mashtoub S: Potential therapeutic applications for emu oil. *Lipid Technol* 29, 28–31, 2017.
30. Bennett DC, Code WE, Godin DV, and Cheng KM: Comparison of the antioxidant properties of emu oil with other avian oils. *Aust J Exp Agric* 48, 1345–1350, 2008.
31. Mashtoub S, Bennett DC, Tran CD, and Howarth GS: Processing and storage of ratite oils affects primary oxidation status and radical scavenging ability. *Anim Prod Sci* 55, 1332–2014, 2015.
32. Yoganathan S, Nicolosi R, Wilson T, Handelman G, Scollin P, et al.: Antagonism of croton oil inflammation by topical emu oil in CD-1 mice. *Lipids* 38, 603–607, 2003.
33. Mashtoub S, Cheah KY, Lymn KA, and Howarth GS: Intestinal homeostasis is restored in mice following a period of intestinal growth induced by orally-administered emu oil. *Exp Biol Med* 243, 945–952, 2018.
34. Mashtoub S, Tran CD, and Howarth GS: Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. *Exp Biol Med* 238, 1305–1317, 2013.
35. Abimosleh SM, Tran CD, and Howarth GS: Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. *Evid Based Complement Alternat Med* 2013, 429706, 2013.
36. Abimosleh SM, Lindsay RJ, Butler RN, Cummins AG, and Howarth GS: Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. *Dig Dis Sci* 57, 887–896, 2012.
37. Mashtoub S, Ghaemi R, Lawrance I, Trinder D, and Howarth GS: Tu1632 Emu oil attenuates disease severity in a mouse model of colitis and inflammation-associated colorectal cancer. *Gastroenterology* 150, S1154, 2016.
38. Chartier LC, Howarth GS, Lawrance IC, Trinder D, Barker SJ, et al.: Emu oil improves clinical indicators of disease in a mouse model of colitis-associated colorectal cancer. *Dig Dis Sci* 63, 135–145, 2018.
39. Cheah KY, Bastian SEP, Acott TMV, Abimosleh SM, Lymn KA, et al.: Grape seed extract reduces the severity of selected disease markers in the proximal colon

of dextran sulphate sodium-induced colitis in rats. *Dig Dis Sci* 58, 970–977, 2013.
40. Cheah KY, Howarth GS, Bindon KA, Kennedy JA, and Bastian SEP: Low molecular weight procyanidins from grape seeds enhance the impact of 5-Fluorouracil chemotherapy on Caco-2 human colon cancer cells. *PLos ONE* 9, e98921, 2014.
41. Motoo Y, Seki T, and Tsutani K: Traditional Japanese medicine, Kampo: its history and current status. *Chin J Integr Med* 17, 85–87, 2011.
42. Nagata T, Toume K, Long LX, Hirano K, Watanabe T, et al. .: Anticancer effect of a Kampo preparation Daikenchuto. *J Nat Med* 70, 627–633, 2016.
43. Ito A, Munakata K, Imazu Y, and Watanabe K: First Nationwide attitude survey of Japanese physicians on the use of Traditional Japanese Medicine (Kampo) in cancer treatment. *Evid Based Complement Alternat Med* 2012, 957082, 2012.
44. Takeda T, Yamaguchi T, and Yaegashi N: Perceptions and attitudes of Japanese gynecologic cancer patients to Kampo (Japanese herbal) medicines. *Int J Clin Oncol* 17, 143–149, 2012.
45. Hasebe T, Matsukawa J, Ringus D, Miyoshi J, Hart J, et al.: Daikenchuto (TU-100) suppresses tumor development in the azoxymethane and APCmin/+ mouse models of experimental colon cancer. *Phytother Res* 31, 90–99, 2017.
46. Nagano T, Itoh H, and Takeyama M: Effect of Daikenchu-to on levels of 3 brain-gut peptides (motilin, gastrin and somatostatin) in human plasma. *Biol Pharm Bull* 22, 1131–1133, 1999.
47. Matsunaga T, Hashimoto S, Yamamoto N, Kawasato R, Shirasawa T, et al.: Protective effect of Daikenchuto on dextran sulfate sodium-induced colitis in mice. *Gastroenterol Res Pract* 2017, 1298263, 2017.
48. Mashtoub S, Lampton L, Eden G, Cheah KY, Lymn KA, et al.: Emu oil combined with Lyprinol^{TM} reduces small intestinal damage in a rat model of chemotherapy-induced mucositis. *Nutr Cancer* 68, 1171–1180, 2016.
49. D'Argenio G, Mazzone G, Tuccillo C, Ribecco MT, Graziani G, et al.: Apple polyphenols extract (APE) improves colon damage in a rat model of colitis. *Dig Liver Dis* 44, 555–562, 2012.
50. Mijan MA and Lim BO: Diets, functional foods, and nutraceuticals as alternative therapies for inflammatory bowel disease: present status and future trends. *World J Gastroenterol* 24, 2673–2685, 2018.

CHAPTER 2

EMU OIL IMPROVES CLINICAL INDICATORS OF DISEASE IN A MOUSE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

CONTEXT STATEMENT

The research described in this chapter has been published in *Digestive Diseases and Sciences*.

> *LC Chartier, GS Howarth, IC Lawrance, D Trinder, SJ Barker and S Mashtoub. Emu Oil Improves Clinical Indicators of Disease in a Mouse Model Of Colitis-Associated Colorectal Cancer. 2018. Digestive Diseases and Sciences, Vol 63 (1): pp 134–145.*

Previous scientific literature has highlighted the anti-inflammatory potential of Emu Oil when applied topically (Yoganathan et al. 2003). Additionally, previous animal studies have investigated orally-administered Emu Oil in rodent models of gastrointestinal diseases, including dextran sulphate sodium (DSS)-induced ulcerative colitis (UC) (Abimosleh et al. 2012) and Crohn's disease (**Appendix 2**). Both these studies concluded that orally-administered Emu Oil reduced disease severity and repaired the damaged intestine compared to disease control animals.

Chapter 2 describes the first study to investigate orally-administered Emu Oil in a 9-week azoxymethane (AOM)/DSS mouse model of colitis-associated colorectal cancer (CA-CRC). This publication forms the foundation of this Doctor of Philosophy project and provides suitable background information to subsequently investigate Emu Oil combined with other naturally-sourced compounds in CA-CRC, as introduced in **Chapter 1** and described in **Chapters 3** and **4**. Previously, the mechanistic action of Emu Oil was investigated immunohistochemically in normal tissue using the Ki-67 stain for proliferation (**Appendix 3**). Tissue collected from the current study (**Chapter 2**), was assessed immunohistochemically for Ki-67 (**Appendix 4**), whereby Emu Oil attenuated aberrant cell proliferation in AOM/DSS mice. Furthermore, the AOM/DSS model of CA-CRC was used in the studies described in **Chapters 5**, **6** and **Appendix 5.**

STATEMENT OF AUTHORSHIP

Title of Paper: Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer

Publication Status: Published

Publication Details: Digestive Diseases and Sciences, 2018, Volume 63, pages 135-145

Principal Author

Name of Principal Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Completed animal trials, sample and data collection, data analyses and interpretation. Prepared manuscript including writing, formatting and submission.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	9/9/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, revision and editing of the manuscript.		
Signature		Date	17/9/20

Name of Co-Author	Ian C Lawrance		
Contribution to the Paper	Conceptualisation and methodology development.		
Signature		Date	31 Aug 2020

Name of Co-Author	Debbie Trinder		
Contribution to the Paper	Conceptualisation and methodology development.		
Signature		Date	1st Sept 2020

Name of Co-Author	Scott J Barker		
Contribution to the Paper	Assistance in experimental trial and data collection.		
Signature		Date	05 Sep 2020

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, supervision of analyses, data interpretation, revision and editing of the manuscript.		
Signature		Date	17/9/20

Digestive Diseases and Sciences (2018) 63:135–145
https://doi.org/10.1007/s10620-017-4876-4

ORIGINAL ARTICLE

Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer

Lauren C. Chartier[1,2] · Gordon S. Howarth[1,2,3] · Ian C. Lawrance[4,5] · Debbie Trinder[4,6] · Scott J. Barker[1,2] · Suzanne Mashtoub[1,2,4]

Received: 4 July 2017 / Accepted: 1 December 2017 / Published online: 6 December 2017

Abstract

Background/Aims Ulcerative colitis is a remitting and relapsing inflammatory bowel disorder. Current treatments are limited, and if poorly controlled, colitis may progress to colorectal cancer. Previously, Emu Oil protected the intestine in experimental models of gut damage. We aimed to determine whether Emu Oil could reduce the severity of chronic colitis and prevent the onset of neoplasia in a mouse model of colitis-associated colorectal cancer.

Methods Female C57BL/6 mice were injected (day 0) with azoxymethane, followed by ad libitum access to three dextran sulfate sodium/water cycles (7 days of dextran sulfate sodium and 14 days of water). Mice ($n = 9$/group) were orally administered either water or Emu Oil (low dose 80 µL or high dose 160 µL), thrice weekly for 9 weeks. Bodyweight and disease activity index were measured daily. Colitis progression was monitored by colonoscopy on days 20, 41 and 62. At killing, tumor number and size were recorded.

Results Azoxymethane/dextran sulfate sodium induced significant bodyweight loss (maximum 24%) which was attenuated by Emu Oil treatment (low dose days 9, 10, 14: maximum 7%; high dose days 7–15, 30–36: maximum 11%; $p < 0.05$). Emu Oil reduced disease activity index of azoxymethane/dextran sulfate sodium mice at most time points (maximum 20%; $p < 0.05$). Additionally, Emu Oil reduced colonoscopically assessed colitis severity (days 20 and 62) compared to disease controls ($p < 0.05$). Finally, in azoxymethane/dextran sulfate sodium mice, low-dose Emu Oil resulted in fewer small colonic tumors ($p < 0.05$) compared to controls.

Conclusions Emu Oil improved clinical indicators and reduced severity of colitis-associated colorectal cancer, suggesting therapeutic potential in colitis management.

Keywords Emu Oil · Colitis · Colorectal cancer · Inflammatory bowel disease

✉ Suzanne Mashtoub
suzanne.mashtoub@uwa.edu.au

Lauren C. Chartier
lauren.chartier@adelaide.edu.au

Gordon S. Howarth
gordon.howarth@adelaide.edu.au

Ian C. Lawrance
ian.lawrance@uwa.edu.au

Debbie Trinder
debbie.trinder@uwa.edu.au

Scott J. Barker
scott.barker@student.adelaide.edu.au

[1] Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, SA, Australia

[2] Gastroenterology Department, Women's and Children's Hospital, 72 King William Road, North Adelaide, SA 5006, Australia

[3] School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA, Australia

[4] School of Medicine, The University of Western Australia, Fiona Stanley Hospital, Murdoch, WA, Australia

[5] Centre for Inflammatory Bowel Diseases, Saint John of God Hospital, Subiaco, WA, Australia

[6] Harry Perkins Institute of Medical Research, Nedlands, WA, Australia

Introduction

The inflammatory bowel diseases (IBD), encompassing Crohn's disease and ulcerative colitis (UC), are chronic inflammatory conditions of the gastrointestinal tract that present in young people [1]. More than 75,000 Australians are living with IBD, a figure expected to increase to > 100,000 by 2022 [2]. UC is characterized by confluent inflammation of the colonic mucosa presenting in periods of cyclic relapse and remission [3]. The exact cause of UC is not well understood, and treatment options are variably effective with drugs including steroids, thiopurines, and aminosalicylates commonly prescribed [3]. A significant risk to patients with long-standing UC is the well-established link between inflammation and tumorigenesis [1]. Herrinton et al. [4] observed UC patients throughout 14.5 years and concluded that the risk of developing colorectal cancer (CRC) was 60% higher than that of the general population. Therefore, an effective treatment for chronic colitis may inhibit the onset of CRC.

Cell growth in a chronic inflammatory environment of the large bowel may lead to the development of CRC [5]. An inflamed colonic epithelium accompanied by an influx of pro-inflammatory cytokines (interleukin-6 [IL-6], tumor necrosis factor-alpha [TNF-α], and interferon-gamma [IFNγ]) upregulate transcription factors STAT3 and NFκB. Furthermore, mutation or deletion of transcription factors *p53* and *Apc* causes rapid dysplasia, invasion, and uncontrolled growth of colonic tumors [5].

Dextran sulfate sodium (DSS)/water cycling is used to induce chronic colitis in preclinical animal models [6, 7, 11]. A single injection of the carcinogen azoxymethane (AOM) in combination with ad libitum DSS cycles accelerates the progression of colitis-associated CRC. AOM induces colonic tumors that share histopathological characteristics of human CRC, including mutations in *Kras* and β-catenin [6].

Emu Oil (EO) is derived from the subcutaneous and retroperitoneal adipose tissue of the native Australian Emu (*Dromaius novaehollandiae*). Tissue is rendered and undergoes a series of liquefied fat filtrations to yield a purified product [7]. Native Australian Aboriginal people have traditionally used EO topically on rashes, burns, and skin to alleviate pain, facilitate wound healing, and treat inflamed joints [8]. EO comprises 98.8% (subcutaneous) and 98.0% (retroperitoneal) fatty acids, including oleic acid (omega-9; 42%), linoleic acid (omega-6; 21%), palmitic acid (21%), and lower levels of α-linolenic acid (omega-3; 1%) [8]. The mode of EO action is still not well understood. However, it has been suggested that the anti-inflammatory properties may be due to the interactions and ratios of the omega fatty acids, in combination with antioxidant components (carotenoids and flavones) within the 2% non-triglyceride fraction [8].

Oral administration of EO has displayed anti-inflammatory and reparative properties in a number of preclinical models, including chemotherapy-induced mucositis [9], nonsteroidal anti-inflammatory drug-induced enteropathy [10], and ulcerative colitis [7]. Previously, Mashtoub et al. [11] investigated the impact of orally administered EO on tumor development in a 12-week colitis-associated CRC study. From this study, it was concluded that high-dose EO (160 μL) resulted in fewer large colorectal tumors [11]. However, EO did not reduce the total number of tumors compared to CRC controls. Therefore, an additional study where the mice were euthanized at an earlier time point was warranted to determine whether EO could delay the onset of neoplasia.

It was hypothesized that orally administered EO would decrease the severity of chronic colitis, thereby delaying the onset of colorectal tumors in mice. The aims of the study were to determine whether (1) EO could reduce the severity of chronic colitis assessed by clinical indicators and histology and (2) EO could delay the onset of colorectal tumors and hence result in fewer tumors in an AOM/DSS mouse model of colitis-associated CRC.

Methods

Emu Oil

Commercially available EO was purchased from Emu Tracks (Marleston, Adelaide, South Australia, batch no. 0214660). EO was aliquoted into 5-ml tubes (for storage) and kept in darkness at 4°. The composition of Emu Oil was analyzed by gas chromatography at the Waite Lipid Analysis Service—FOODPlus Research Centre (Urrbrae, South Australia; Table 1).

Animal Studies

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, The University of Adelaide and The University of Western Australia. Female C57BL/6 mice (at 8 weeks) were sourced from Animal Resource Centre (Perth, Western Australia) and group-housed at room temperature with a light/dark cycle of 14:10 h. Mice were fed standard mouse chow and provided with drinking water for the duration of the trial.

Table 1 Fatty acid composition of the Emu Oil used in the current study

Analyte	Common name	Total lipids (%)
Total saturates		34.4
14:00	Myristic acid	0.3
16:00	Palmitic acid	24.1
18:00	Stearic acid	9.8
20:00	Arachidonic acid	0.1
Total monos		55.7
16:1n-7 *Omega 7*	Palmitoleic acid	4
18:1n-9 *Omega 9*	Oleic acid	48.8
18:1n-7 *Omega 7*	Vaccenic acid	2.6
20:1n-9 *Omega 9*	Gondoic acid	0.3
Total *Omega 9*		49.1
Total *Omega 7*		6.6
Total *Omega 3*		0.4
18:3n-3	α Linolenic acid	0.4
Total *Omega 6*		9.5
18:2n-6	Linoleic acid	9.3
20:2n-6	Eicosadienoic acid	0.1
20:4n-6	Arachidonic acid	0.1

Monos Monounsaturated fatty acids

Experimental Trial

Female C57BL/6 mice (n = 45) were randomly assigned to five groups (n = 9/group): (1) saline + water + water, (2) saline + water + EO (160 µL), (3) AOM + DSS + water, (4) AOM + DSS + EO (80 µL), and (5) AOM + DSS + EO (160 µL). Water or EO was administered via oral-gastric gavage thrice weekly for the duration of the trial.

On day 0, all mice were i.p. injected with saline or AOM (7.4 mg/kg bodyweight; Sigma-Aldrich, Castle Hill NSW). Groups 3–5 underwent three DSS/water cycles, whereby one cycle consisted of 7 days of DSS (MP Biomedicals LLC, Santa Ana California, USA) solution (2%w/v; 2 g/100 ml distilled water), followed by 2 weeks of plain water in drinking bottles. Groups 1 and 2 were provided with plain water throughout the 9-week trial.

Bodyweight and Disease Activity Index

Bodyweight was recorded daily. Disease activity index (DAI) scores were calculated daily from weight loss, general condition, stool consistency, and rectal bleeding using an increasing severity scale of 0–3 for each parameter as described by Abimosleh et al. [7].

Behavioral Studies

Burrowing studies were conducted to assess welfare and indicate levels of stress and pain under pathological conditions [12]. All mice were acclimatized in darkness for one and a half hours before commencing burrowing. Remaining in darkness, mice were placed in individual cages with a pre-weighed (400 g) burrow containing pebbles (Kitty litter, Black and Gold, Adelaide, Australia) for 1 h. The burrow was then re-weighed and recorded. Mice were acclimatized to the burrowing apparatus by allowing two practice tests in pairs. Individual burrowing was measured on day − 1 for baseline recordings. Burrowing studies were conducted at the end of each DSS week (days 6, 27, and 48) and at the end of each cycle (days 19, 40, and 62).

Colonoscopy

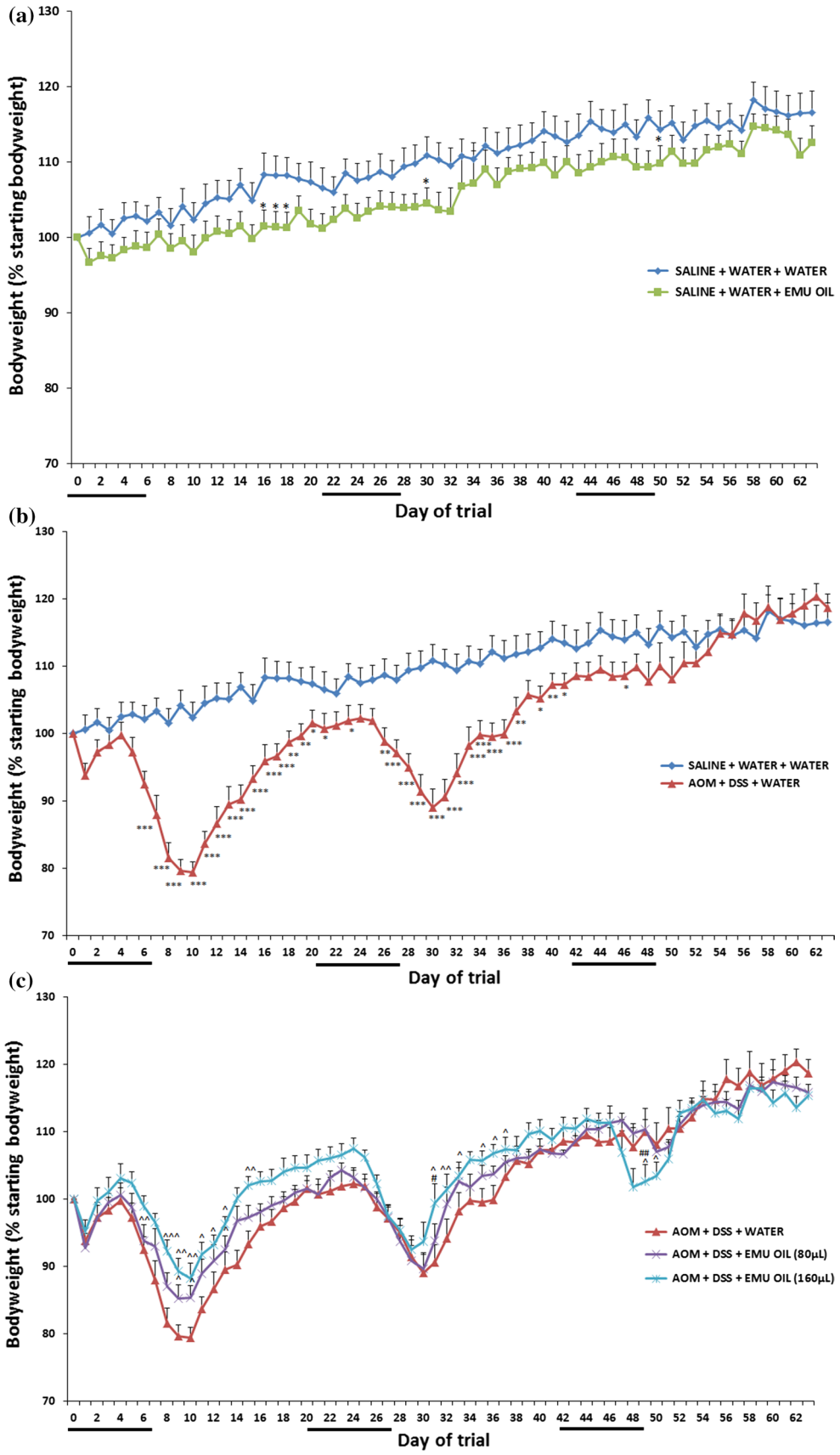
Colitis progression and tumor development were assessed using a high-resolution Karl Storz colonoscope (1.9 mm outer diameter; Tuttlingen, Germany) at the end of each DSS/water cycle (days 20, 41, and 62). Mice were anesthetized using isoflurane for the duration of the procedure and closely monitored. Colitis severity was measured from recorded videos in a blinded fashion using five parameters described by Becker et al. [13], including thickening of the colon, vasculature pattern, presence of fibrin, granularity of mucosal surface, and stool consistency. Each parameter was scored in a blinded fashion from 0 to 3 with increasing severity and totaled.

Tissue Collection

On day 63, mice were killed via CO_2 asphyxiation. Mouse colon was removed, opened longitudinally, and photographed. Small intestine and colon were weighed, measured, and excised into jejunum, ileum, duodenum, proximal colon, and distal colon. Sections (2 cm) were then placed into cassettes (fixed in formalin) for histology. Additionally, the adjacent 2 cm of tissue was collected in yellow-cap tubes (placed in liquid nitrogen) for biochemical analysis. Visceral organs including heart, liver, spleen, thymus, lung, and kidneys were removed and weighed.

Tumor Analysis

Photographs of longitudinally opened colons were analyzed in a blinded fashion using Olympus Soft Imaging Solutions GmbH computer software analySIS version 5.2 (Tokyo, Japan). The number and size of tumors were measured, whereby < 2 mm in diameter was categorized as "small," 2–3 mm as "medium," and "large" tumors were those with > 3 mm diameter.


(a)
Bodyweight (% starting bodyweight)
Day of trial
SALINE + WATER + WATER
SALINE + WATER + EMU OIL
(b)
Bodyweight (% starting bodyweight)
Day of trial
SALINE + WATER + WATER
AOM + DSS + WATER
(c)
Bodyweight (% starting bodyweight)
Day of trial
AOM + DSS + WATER
AOM + DSS + EMU OIL (80µL)
AOM + DSS + EMU OIL (160µL)

◂**Fig. 1** Daily bodyweight change of mice ($n = 9$/group). **a** Normal and EO controls, **b** normal and disease controls, and **c** comparisons across disease groups (solid line DSS week). Data are expressed as mean (% change of starting bodyweight) ± SEM. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ compared to saline + water + water, ^^^$p < 0.001$; ^^$p < 0.01$; ^$p < 0.05$ compared to AOM + DSS + water, ##$p < 0.01$; #$p < 0.05$ compared to AOM + DSS + EO (80 µL)

Crypt Depth

Intestinal sections were routinely processed, embedded in paraffin wax, sectioned (4 µm), and stained with hematoxylin and eosin. Samples were analyzed using a light microscope (Olympus Corporation, Tokyo, Japan) and Olympus Soft Imaging Solutions GmbH software analySIS version 5.2 (Tokyo, Japan). Crypt depth measurements of the proximal and distal colon were obtained from 40 well-orientated crypts per tissue section per mouse.

Myeloperoxidase Assay

Myeloperoxidase (MPO) assays were performed to detect neutrophil infiltration using techniques previously described [10]. Tissue was homogenized and centrifuged at 13,000*g* for 12 min, supernatant was discarded, and a detergent solution of 0.5% hexadecyltrimethyl ammonium bromide buffer (Sigma Chemicals, Sydney, Australia) was used to re-suspend the tissue homogenate. After vortexing and centrifuging for 2 min each, water background and test samples were aliquoted (50 µL) into duplicate wells of a microtiter 96-well plate. A reaction solution containing 4.2 mg of O-dianisidine dihydrochloride reagent, 12.5 µL H_2O_2, 2.5 mL potassium phosphate buffer [pH 6.0], 22.5 mL distilled water was added to each well (200 µL). Absorbance was measured at 450 nm at 1-min intervals for 15 min with a spectrophotometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore).

Statistical Analysis

Statistical analysis was completed using SPSS, version 17 for Windows (SPSS Inc. Chicago, Illinois, USA). Data were tested for normality using a Shapiro–Wilk test. Bodyweight, DAI, burrowing activity, and colitis grading were analyzed by repeated measures ANOVA with least significance difference (LSD) to compare among and within a group. Cull data, crypt depth, MPO, and tumor size were analyzed using ANOVA with Tukey's post hoc test. Data are displayed as means with their standard errors. $p < 0.05$ were considered statistically significant.

Results

Clinical Indicators

In normal animals, EO reduced bodyweight (days 16–18, 30, 49) compared to controls (Fig. 1a; maximum 7%; $p < 0.05$) with no impact on other days. AOM/DSS caused significant bodyweight loss on days 1, 6–41, 44, 45, 49, 50, and 62 compared to normal controls (Fig. 1b; maximum 24%; $p < 0.05$). Importantly, in AOM/DSS mice, both doses of EO attenuated bodyweight loss compared to disease controls (Fig. 1c; low dose days 9, 10, 14—maximum 7%; high dose days 7–15, 30–36—maximum 11%; $p < 0.05$). Furthermore, mice treated with low-dose EO presented greater bodyweight compared to mice receiving the high dose on days 48 and 49 (maximum 7%; $p < 0.05$). In normal animals, treatment with EO did not increase DAI parameters compared to controls (Fig. 2; $p > 0.05$). AOM/DSS increased DAI compared to normal throughout the study (days 1, 6–18 and 20–63; maximum 41%; $p < 0.05$). EO treatment in AOM/DSS animals significantly reduced DAI compared to disease controls at most time points (Fig. 2; low dose maximum 20%; high dose maximum 18%; $p < 0.05$). Additionally, high-dose EO reduced DAI compared to low-dose EO on days 15, 23–25, 28–29, 48–50, and 63 (maximum 13%; $p < 0.05$).

Burrowing Activity

In normal animals, EO decreased burrowing on day 48 compared to normal controls (Fig. 3; $p < 0.05$), with no significant impact on other days. Additionally, impaired burrowing activity was displayed by AOM/DSS controls on days 48 and 61 ($p < 0.05$). Importantly, both doses of EO increased burrowing activity of AOM/DSS mice on day 61 compared to AOM/DSS controls (Fig. 3; $p < 0.05$).

Visceral Organ Weights

Weights of the liver (maximum: 76%) and spleen (maximum: 22%) were increased following AOM/DSS compared to normal controls ($p < 0.001$), with no impact observed following EO-treatment ($p > 0.05$).

Gastrointestinal Lengths and Weights

Colon shortening was observed in AOM/DSS controls compared to normal ($p < 0.05$), with no significant impact following EO treatment. Colon (40%) and small intestine (34%) weights were increased following AOM/DSS compared to

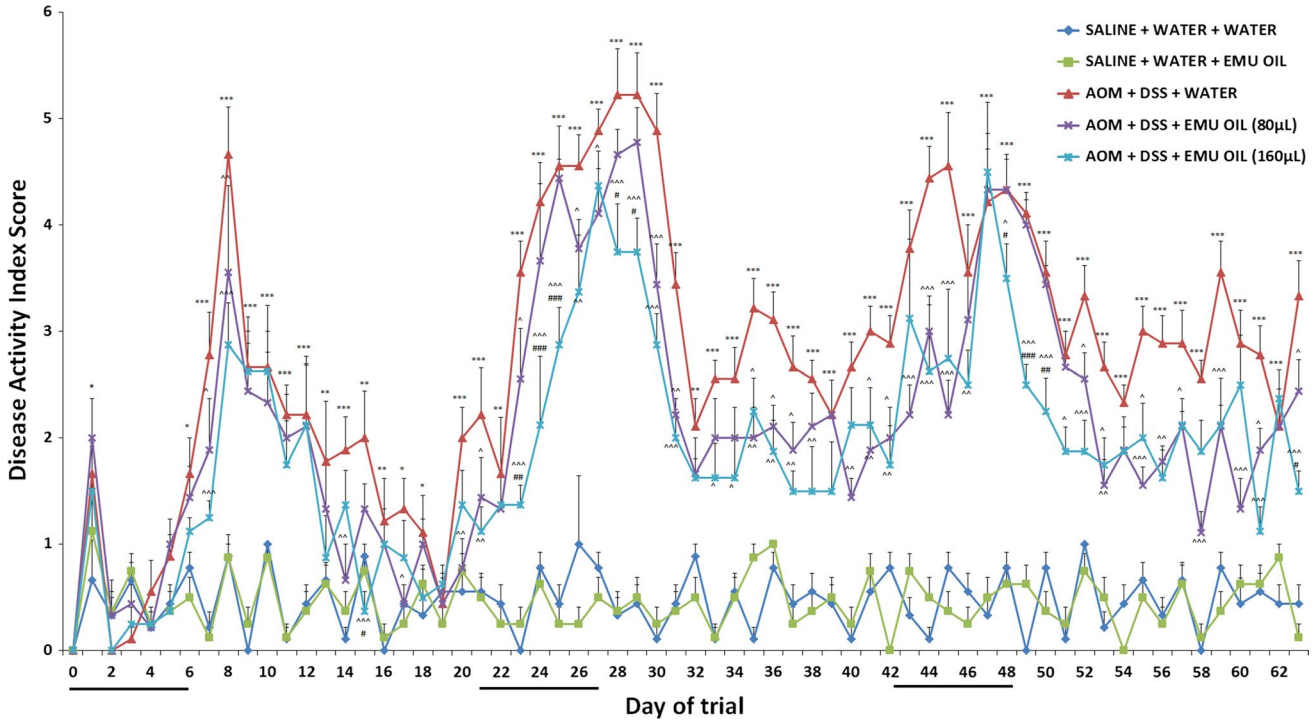


Fig. 2 Daily disease activity index (DAI) score (n = 9/group). Data are expressed as mean DAI score ± SEM (solid line DSS week). ***p < 0.001; **p < 0.01; *p < 0.05 compared to saline + water + water, ^^^p < 0.001; ^^p < 0.01; ^p < 0.05 compared to AOM + DSS + water, ###p < 0.001; ##p < 0.01; #p < 0.05 compared to AOM + DSS + EO (80 µL)

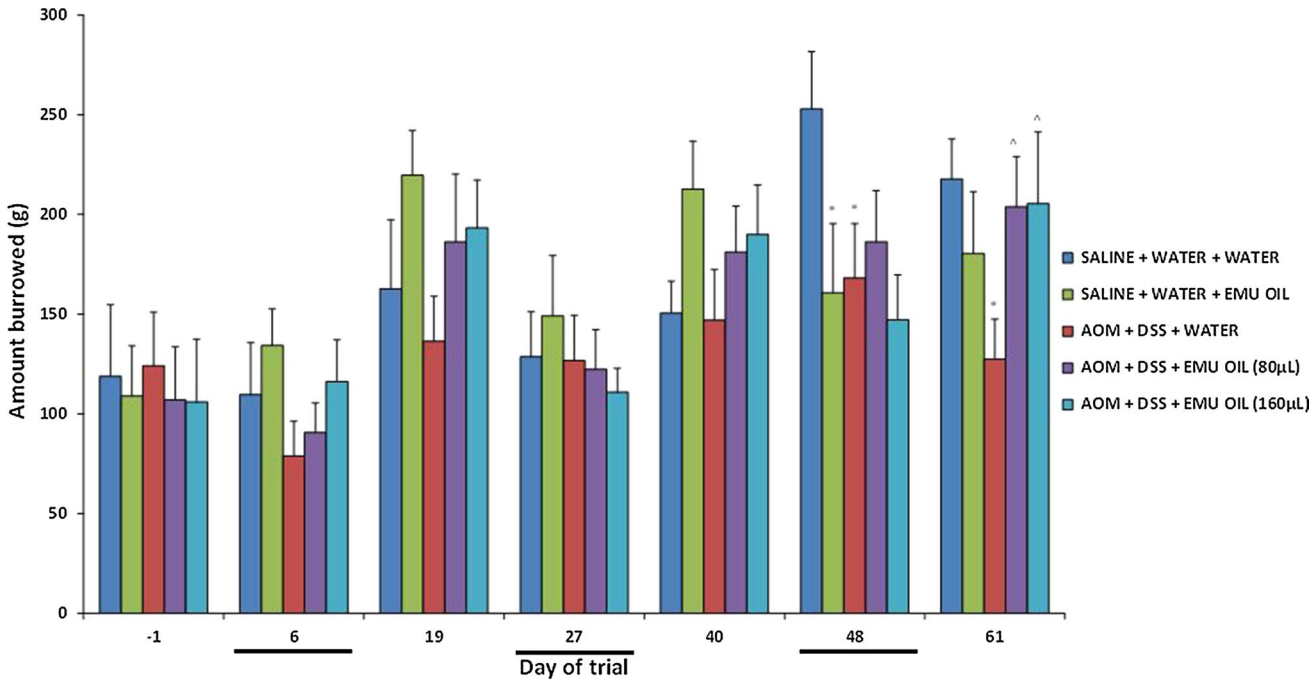


Fig. 3 Burrowing activity (n = 9/group). Data are expressed as mean amount burrowed (g) ± SEM (solid line DSS week). *p < 0.05 compared to saline + water + water, ^p < 0.05 compared to AOM + DSS + water

normal controls ($p < 0.01$), with no impact elicited by EO ($p > 0.05$).

Crypt Depth

Long-term EO treatment in normal animals did not impact crypt depth of the proximal or distal colon compared to controls (Fig. 4a, b; $p > 0.05$). AOM/DSS increased colonic crypt depth in both regions compared to normal controls (proximal; $p < 0.01$ and distal; $p < 0.001$). Additionally, EO did not affect crypt depth in AOM/DSS mice ($p > 0.05$).

Myeloperoxidase Activity

Colonic MPO levels remained unaffected by AOM/DSS, EO treatment and by EO in normal animals (data not shown; $p > 0.05$).

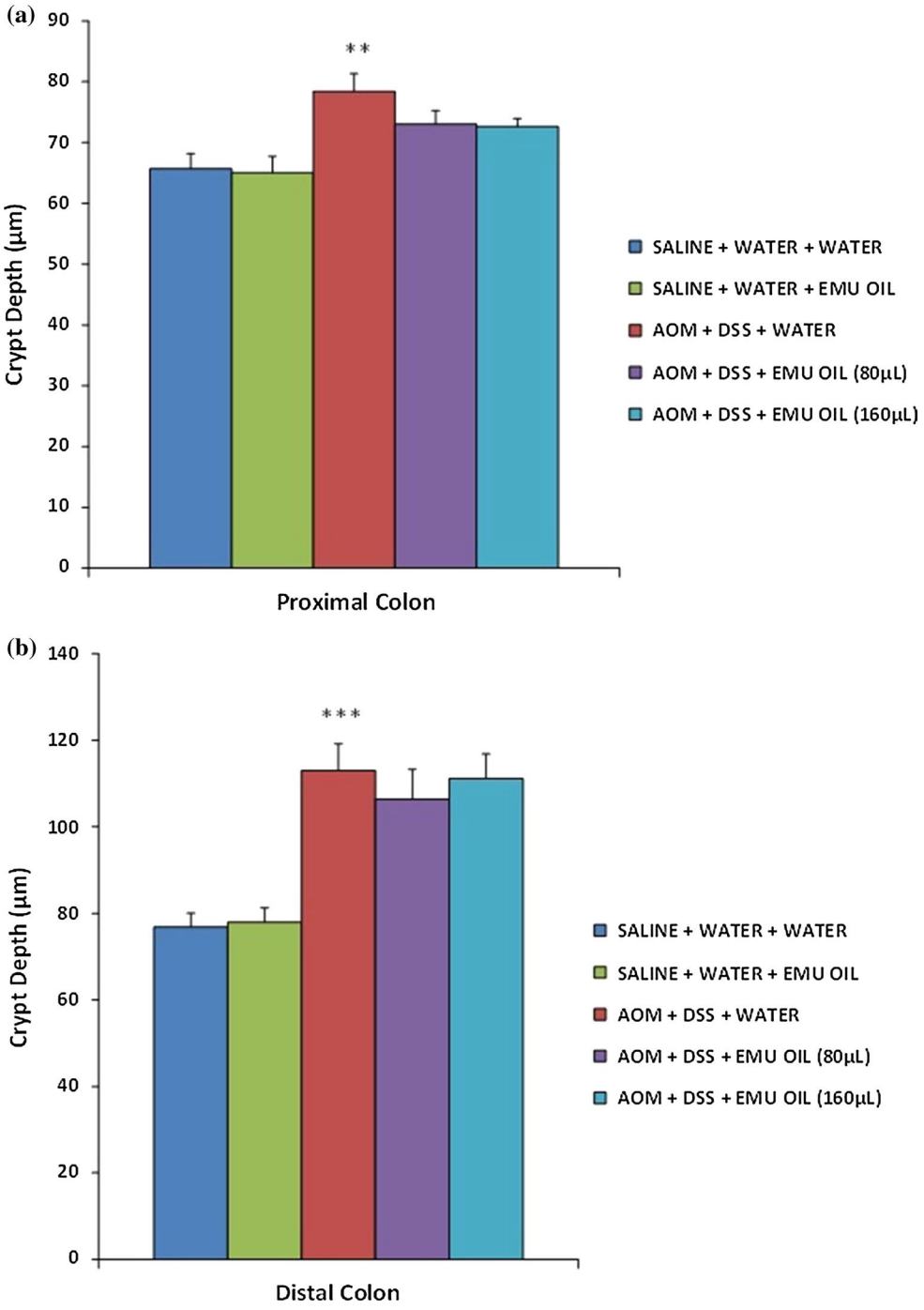


Fig. 4 Crypt depth of the **a** proximal colon and **b** distal colon ($n = 9$/group). Data are expressed as mean crypt depth (µm) ± SEM. ***$p < 0.001$; **$p < 0.01$ compared to saline + water + water

Colitis

Normal and EO controls did not develop colitis throughout the trial (Fig. 5). AOM/DSS induced colitis in mice and was most severe in disease control animals at each time point ($p < 0.001$). Low- and high-dose EO reduced disease severity compared to AOM/DSS alone on days 20 ($p < 0.001$) and 62 (low dose; $p < 0.01$ and high dose; $p < 0.05$). Additionally, high-dose EO reduced colitis compared to low-dose EO on day 20 ($p < 0.001$). Furthermore, on day 41 high-dose EO reduced colitis severity compared to both AOM/DSS controls and low-dose EO ($p < 0.05$).

Tumor Development

Colonoscopy confirmed tumor development by day 41. On day 63, tumor number and size were analyzed in a blinded fashion from photographs of the mouse colons. Importantly, no tumors were present in normal animals treated with EO. AOM/DSS caused few tumors to develop in the proximal colon (AOM/DSS control one small tumor and one large tumor; low-dose EO one small tumor; high-dose EO one small tumor). Tumors developed predominantly in the distal colon (descending and sigmoid colon) following AOM/DSS treatment. Disease control animals displayed the highest number of tumors (2.5 ± 0.5; $p < 0.001$). Neither dose of EO reduced the total number of tumors in the distal colon compared to disease controls at killing (low-dose EO 1.5 ± 0.5; high-dose EO 2.5 ± 0.6; $p > 0.05$).

When arranged into sizes, low-dose EO reduced the number of small tumors (< 2 mm) in the distal colon ($p < 0.05$), while not impacting the number of medium (2–3 mm) or large tumors (> 3 mm). Additionally, high-dose EO treatment in AOM/DSS mice did not significantly impact tumor sizes (Fig. 6).

Discussion

In the current study, low-dose EO-treated animals displayed fewer small tumors, despite both doses of EO decreasing colitis severity. However, these results are encouraging as fewer small tumors after 9 weeks likely corresponded to fewer large tumors at the 12-week time point, as observed by Mashtoub and colleagues [11].

Although the mechanism of EO action remains uncertain, its anti-inflammatory and antioxidant properties have been documented in the literature. In a rat model indomethacin-induced enteropathy, MPO activity was assessed in jejunal and ileal tissue. MPO was significantly decreased following daily EO treatment compared to olive oil or water controls, thereby reducing acute intestinal inflammation [10]. Additionally, Mashtoub et al. [14] investigated the free radical scavenging activity (RSA) and the primary oxidation status of ratite oils, whereby EO represented the greatest overall RSA compared to Ostrich and Rhea oil [14]. Moreover, Bennett et al. [15] compared the antioxidant properties of EO with other avian oils such as Ostrich and Rhea oil via the

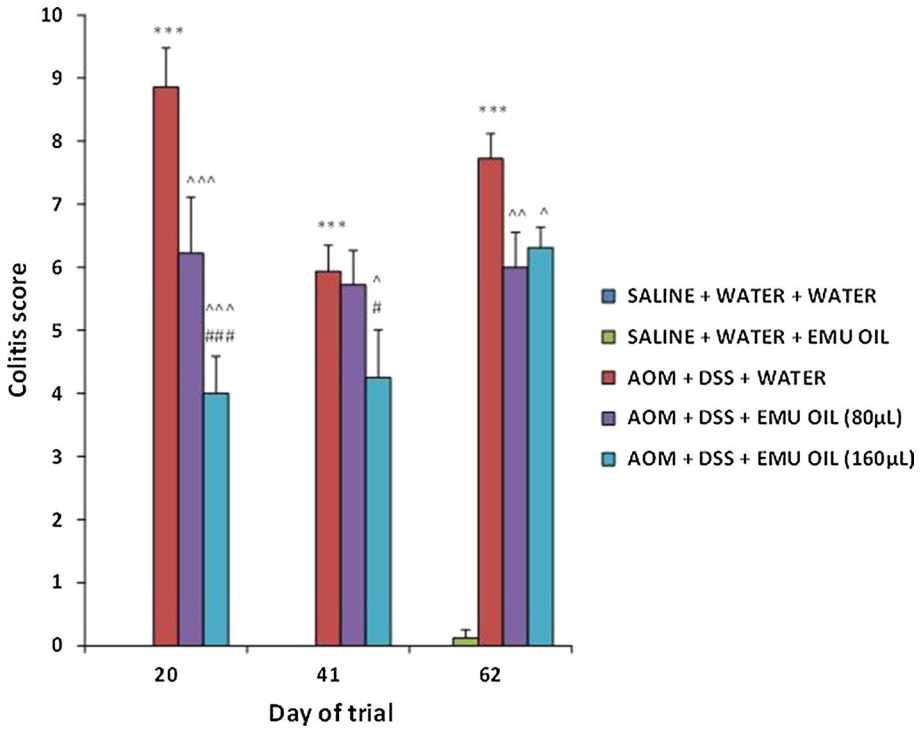


Fig. 5 Colitis severity score calculated from colonoscope videos ($n = 9$/group). Data are expressed as mean (colitis score) ± SEM. ***$p < 0.001$ compared to saline + water + water, ^^^$p < 0.001$; ^^$p < 0.01$; ^$p < 0.05$ compared to AOM + DSS + water, ###$p < 0.001$ compared to AOM + DSS + EO (80 µL)

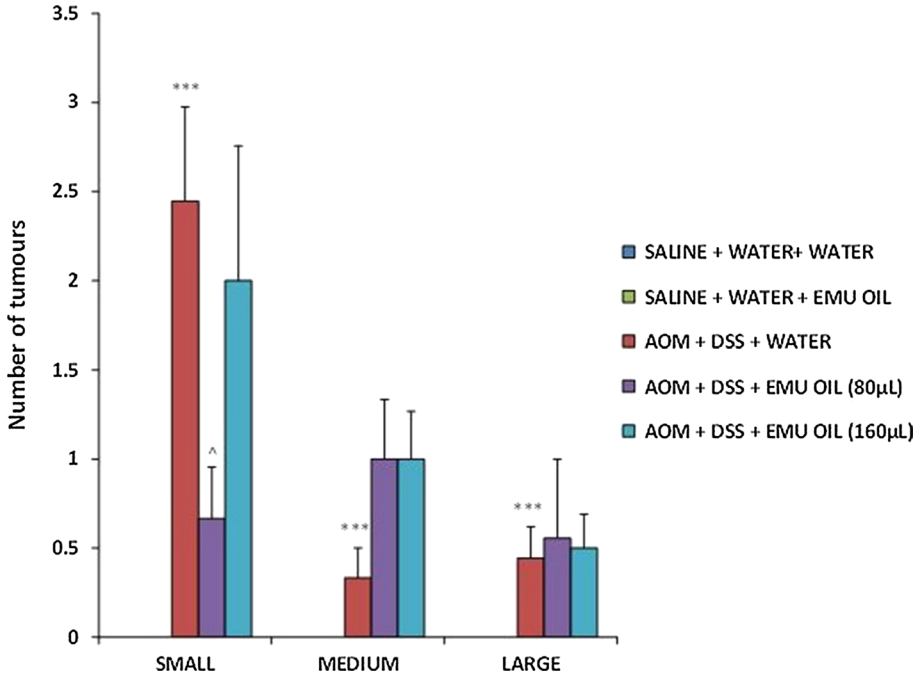


Fig. 6 Distal colonic tumors arranged into sizes ($n = 9$/group). Tumors were categorized depending on their diameters; small (< 2 mm), medium (2–3 mm), and large (> 3 mm). Data are expressed as mean number of tumors ± SEM. ***$p < 0.001$ compared to saline + water + water, ^$p < 0.05$ compared to AOM + DSS + water

2,2-diphenyl-1-picryl hydracyl radical assay. EO elicited a greater effect on reducing levels of thiobarbituric acid-reactive substance than the other oils tested [15]. Hence, EO may offer greater protection than alternative ratite oils against oxidative damage, which may promote the development of CRC. Furthermore, the authors proposed that the ratio of unsaturated to saturated fatty acids is likely responsible for cellular protection against oxidative stress [15].

With appropriate characterization of colonoscope scoring and grading of tumors, tumor size and progression could be quantified and therefore may conclude whether tumor progression was inhibited by EO. Nonetheless, high-dose EO did not affect tumor number or size compared to CRC controls. Although an unexpected result, this suggested that the effect of EO on tumor size and progression was unlikely associated with a dose-dependent response. Zheng and colleagues [16] investigated the expression of several therapeutic targets in the AOM/DSS model. The authors found that colonic mRNA and protein expression of tumor suppressors *Apc* and *p53* were largely downregulated by AOM/DSS. Additionally, TNF-α in the blood of mice was increased in colitis-associated CRC compared to controls. These results correlated with worsened survival due to tumor progression [16]. Yoganathan et al. [17] induced auricular inflammation in CD-1 mice by applying 5 µL of croton oil. Mice were then treated with topical-application of fish, flaxseed, olive or EO. Only EO application resulted in a decrease in levels of both the pro-inflammatory cytokines TNF-α and IL-1α, an effect that was more pronounced in comparison with the other oils tested [17]. Consequently, future studies would benefit from assessing the colonic expression of *Apc, p53* and pro-inflammatory cytokines TNF-α and IL-1α in the blood and tissue, in order to determine whether EO has an impact on these markers.

Previously, Sanchez-Fidalgo and colleagues [18] concluded that nutraceuticals, such as extra-virgin olive oil and dietary enriched sunflower oil, protected mice from marked bodyweight loss and clinically assessed disease severity in a model of DSS-induced colitis. Furthermore, orally administered processed Aloe Vera gel attenuated bodyweight loss and prevented AOM/DSS colon carcinogenesis in mice [19]. In the current study, mice treated with AOM/DSS alone displayed significant bodyweight loss and increased clinical indicators of disease following DSS treatment compared to normal controls. These results are consistent with the previous work by Cooper et al. [20] and Parang et al. [21]. Carlsson et al. [22] showed that weight loss in this model may be attributed to increased intestinal permeability promoted by DSS. In this study, EO attenuated bodyweight loss in AOM/DSS mice, suggesting a protective effect on intestinal permeability. Moreover, bodyweight of normal animals was unaffected by EO, suggesting that bodyweight attenuation may have been due to a protected intestinal barrier rather than an increase in dietary lipids.

Bidirectional communication between the gastrointestinal tract and the brain, namely the gut-brain-axis, is well established and demonstrates that inflammation and microbiota in the gut affect physiology and emotions and can alter behavior [12, 23]. In the current study, burrowing activity was reduced in AOM/DSS mice on days 48 and 61, indicating impaired behavior, likely anxiety or depression. Emge et al. [23] concluded similar anxiety-like behavior

using a light/dark box test in mice with DSS-induced colitis. In the current study, EO treatment improved burrowing activity of AOM/DSS mice when disease was most severe. These results could suggest that EO may have altered the gut microbiota, consequently affecting glial cells in the brain and improving behavior. However, qPCR on cecal content is necessary to understand the potential influence of EO on microflora populations.

In the current study, shortening of the colon and an increase in colon weight in AOM/DSS animals was likely due to the presence of edema and bowel wall thickening as a compensatory response in order to increase surface area of the DSS-challenged bowel [19]. AOM/DSS control mice also displayed an increase in mean platelet volume compared to normal. Observational studies have identified mean platelet volume to be a predictor of inflammatory activity and UC [24]. However, MPO, indicative of acute inflammation, remained unchanged, despite a reduction in MPO following EO treatment in previous acute studies of intestinal disease [9, 10]. Acute experimental colitis initiates a T-helper (Th1) response, while a combination of responses incorporating Th1 and Th2 pathways results from chronic models of colitis [25]. Therefore, in this chronic model it would be beneficial to further analyze pro-inflammatory cytokines including IL-6, IL-8, IL-13, and IL-18 in comparison with MPO.

Finally, this study is the first to investigate the safety of long-term EO administration in normal animals, whereby EO did not alter bodyweight or wellbeing. Despite high volumes of EO, liver weights were unaffected, suggesting that EO did not cause liver toxicity in normal animals. Importantly, EO administration in healthy animals did not cause changes in intestinal architecture (crypt lengthening), supporting evidence that EO does not contribute to uncontrolled stimulation or proliferation of the gastrointestinal tract. Thus, as EO is readily available for purchase in Australia, these results support the safety of EO for long-term use in normal individuals.

In summary, EO attenuated bodyweight loss and improved clinically assessed disease severity in colitis-associated CRC. However, EO did not reduce the total number of colorectal tumors. Nevertheless, future studies including altered dosage, frequency, and timing of EO administration are required to conclude the antineoplastic activity of EO. Furthermore, additional studies investigating intra-rectal administration of EO may elicit a more localized effect on preventing tumor development.

Acknowledgments S.M. is the recipient of a National Health and Medical Research Council Early Career (Peter Doherty) Biomedical Postdoctoral Fellowship. The authors would like to acknowledge Dr. Alexandra Whittaker for burrowing input and Dr. Tamsin Lannagan for colonoscopy expertise.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

References

1. Kinugasa T, Akagi Y. Status of colitis-associated cancer in ulcerative colitis. *World J Gastrointest Oncol*. 2016;8:351–357.
2. PricewaterhouseCoopers Austalia (PwC) Improving Inflammatory Bowel Disease care across Australia (March 2013).
3. Head KA, Jurenka JS. Inflammatory bowel disease part 1: ulcerative colitis–pathophysiology and conventional and alternative treatment options. *Altern Med Rev*. 2003;8:247–283.
4. Herrinton LJ, Liu L, Levin TR, Allison JE, Lewis JD, Velayos F. Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1998 to 2010. *Gastroenterology*. 2012;143:382–389.
5. Rogler G. Chronic ulcerative colitis and colorectal cancer. *Cancer Lett*. 2014;345:235–241.
6. De Robertis M, Massi E, Poeta ML, et al. The AOM/DSS murine model for the study of colon carcinogenesis: from pathways to diagnosis and therapy studies. *J Carcinog*. 2011;10:9.
7. Abimosleh SM, Lindsay RJ, Butler RN, Cummins AG, Howarth GS. Emu Oil increases colonic crypt depth in a rat model of ulcerative colitis. *Dig Dis Sci*. 2012;57:887–896. https://doi.org/10.1007/s10620-011-1979-1.
8. Abimosleh SM, Tran CD, Howarth GS. Emu Oil: a novel therapeutic for disorders of the gastrointestinal tract? *J Gastroenterol Hepatol*. 2012;27:857–861.
9. Mashtoub S, Tran CD, Howarth GS. Emu Oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. *Exp Biol Med*. 2013;238:1305–1317.
10. Abimosleh SM, Tran CD, Howarth GS. Emu Oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. *Evid Based Complement Altern Med*. 2013;2013:429706.
11. Mashtoub S, Ghaemi R, Lawrance IC, Trinder D, Howarth GS. Emu Oil attenuates disease severity in mouse models of colitis and inflammation-associated colorectal cancer. *Gastroenterology*. 2016;150:1154.
12. Jirkof P, Leucht K, Cesarovic N, et al. Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice. *Lab Anim*. 2013;47:274–283.
13. Becker C, Fantini MC, Wirtz S, et al. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut*. 2005;54:950–954.
14. Mashtoub S, Bennett DC, Tran CD, Howarth GS. Processing and storage of ratite oils affects primary oxidation status and radical scavenging ability. *Anim Prod Sci*. 2014;55:1332–1337.
15. Bennett DC, Code WE, Godin DV, Cheng KM. Comparison of the antioxidant properties of emu oil with other avian oils. *Aust J Exp Agric*. 2008;48:1345–1350.
16. Zheng H, Lu Z, Wang R, Chen N, Zheng P. Establishing the colitis-associated cancer progression mouse models. *Int J Immunopathol Pharmacol*. 2016;29:759–763.
17. Yoganathan S, Nicolosi R, Wilson T, et al. Antagonism of croton oil inflammation by topical emu oil in CD-1 mice. *Lipids*. 2003;38:603–607.
18. Sánchez-Fidalgo S, Cárdeno A, Sánchez-Hidalgo M, et al. Dietary unsaponifiable fraction from extra virgin olive oil supplementation

attenuates acute ulcerative colitis in mice. *Eur J Pharm Sci*. 2013;48:572–581.
19. Im SA, Kim JW, Kim HS, et al. Prevention of azoxymethane/dextran sodium sulfate-induced mouse colon carcinogenesis by processed *Aloe vera* gel. *Int Immunopharmacol*. 2016;40:428–435.
20. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Investig*. 1993;69:238–249.
21. Parang B, Barrett CW, Williams CS. AOM/DSS model of colitis-associated cancer. *Methods Mol Biol*. 2016;1422:297–307.
22. Carlsson AH, Yakymenko O, Olivier I, et al. Faecalibacterium prausnitzii supernatant improves intestinal barrier function in mice DSS colitis. *Scand J Gastroenterol*. 2013;48:1136–1144.
23. Emge JR, Huynh K, Miller EN, et al. Modulation of the microbiota-gut-brain axis by probiotics in a murine model of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol*. 2016;310:G989–G998.
24. Gasparyan AY, Ayvazyan L, Mikhailidis DP, Kitas GD. Mean platelet volume: a link between thrombosis and inflammation? *Curr Pharm Des*. 2011;17:47–58.
25. Valatas V, Vakas M, Kolios G. The value of experimental models of colitis in predicting efficacy of biological therapies for inflammatory bowel diseases. *Am J Physiol Gastrointest Liver Physiol*. 2013;305:G763–G785.

CHAPTER 3

EMU OIL AND GRAPE SEED EXTRACT REDUCE TUMOUR BURDEN AND DISEASE PARAMETERS IN MURINE COLITIS-ASSOCIATED COLORECTAL CANCER

CONEXT STATEMENT

The research described in this chapter has been published in *Carcinogenesis*.

> *LC Chartier, GS Howarth, D Trinder and S Mashtoub. Emu Oil and grape seed extract reduce tumour burden and disease parameters in murine colitis-associated colorectal cancer. 2020. Carcinogenesis, doi.org/10.1093/carcin/bgaa099 (e-pub ahead of print).*

In the study described in **Chapter 2**, Emu Oil improved clinical indicators of disease in mice and resulted in fewer small colorectal tumours than disease controls. As introduced in **Chapter 1,** grape seed extract (GSE) is a naturally-sourced compound that comprises polyphenols and other anti-oxidants. In cellular-based studies and rat models of chemotherapy-induced mucositis and dextran sulphate sodium (DSS)-induced colitis; GSE has reduced intestinal inflammation, damage and improved intestinal architecture compared to untreated controls (Cheah et al. 2009, Cheah et al. 2014, Cheah et al. 2013). **Chapter 3** describes the first study to investigate GSE in a model of colitis-associated colorectal cancer. In this study, GSE was also combined with Emu Oil to increase its efficacy in the azoxymethane/DSS model.

STATEMENT OF AUTHORSHIP

Title of Paper: Emu Oil and Grape Seed Extract Reduce Tumour Burden and Disease Parameters in Murine Colitis-Associated Colorectal Cancer

Publication Status: Published

Publication Details: Carcinogenesis, 2020, doi:10.1093/carcin/bgaa099 (online ahead of print)

Principal Author

Name of Principal Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Completed animal trials, sample and data collection, data analyses and interpretation. Prepared manuscript including writing, formatting and submission.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	8/10/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodological development, revision and editing of the manuscript.		
Signature		Date	9/10/20

Name of Co-Author	Debbie Trinder		
Contribution to the Paper	Conceptualisation and methodology development.		
Signature		Date	16th Sept 2020

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodological development, supervision of analyses, data interpretation, revision and editing of the manuscript.		
Signature		Date	8/10/20

Carcinogenesis, 2020, 1–8

doi:10.1093/carcin/bgaa099
Advance Access publication September 17, 2020
Original Article

ORIGINAL ARTICLE

Emu oil and grape seed extract reduce tumour burden and disease parameters in murine colitis-associated colorectal cancer

Lauren C. Chartier[1,2], Gordon S. Howarth[1,2,3], Debbie Trinder[4,5] and Suzanne Mashtoub[1,2,4,*]

[1]Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, 5005, Australia, [2]Department of Gastroenterology, Women's and Children's Hospital, North Adelaide, South Australia, 5006, Australia, [3]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy campus, Roseworthy, South Australia, 5371, Australia, [4]Medical School, The University of Western Australia, Fiona Stanley Hospital, Murdoch, Western Australia, 6150, Australia and [5]Harry Perkins Institute of Medical Research, Murdoch, Western Australia, 6150, Australia

*To whom correspondence should be addressed: Tel: +61 08 8161 6991; Email: suzanne.mashtoub@adelaide.edu.au

Abstract

Ulcerative colitis is an incurable condition whereby patients are at an increased risk of developing colorectal cancer (CRC). We aimed to investigate the combination of Emu oil (EO) and grape seed extract (GSE) in an azoxymethane (AOM)/dextran sulphate sodium (DSS) model of colitis-associated CRC (CA-CRC). C57BL/6 mice (n = 10/group) were injected i.p. with saline or AOM (7.4 mg/kg) and underwent three DSS/water cycles. Mice were orally-gavaged thrice weekly with water (80 µl), EO (80 µl), GSE (80 µl; 400 mg/kg) or combined EO/GSE (160 µl). Mice were euthanized on day 63. AOM/DSS induced significant bodyweight loss (max –21%) and increased disease activity index (DAI) (max +83%) throughout the trial ($P < 0.05$). EO (max –53%), GSE (max –51%) and EO/GSE (max –71%) reduced DAI scores in AOM/DSS mice in all DSS cycles ($P < 0.05$). EO/GSE-treatment in AOM/DSS mice resulted in further DAI reduction compared with EO (max –62%) and GSE (max –71%) alone ($P < 0.05$). AOM/DSS mice presented with severe colonoscopically-assessed colitis at all time-points, which was reduced by EO, GSE and EO/GSE ($P < 0.05$). EO, GSE and EO/GSE reduced the number of colonic tumours compared with AOM/DSS controls ($P < 0.05$). Myeloperoxidase (acute inflammation) and fluorescein isothiocyanate-dextran levels (intestinal permeability) were increased in AOM/DSS controls ($P < 0.05$). EO (–58%) and EO/GSE (–77%) reduced fluorescein isothiocyanate-dextran compared with AOM/DSS controls ($P < 0.05$), with no effect on myeloperoxidase. Histologically-assessed severity scores were increased in the distal colon of AOM/DSS mice compared with saline ($P < 0.05$), with no effect observed following treatment. The combination of EO and GSE improved clinical indicators and reduced colonic tumours in AOM/DSS treated mice, suggesting potential in CA-CRC management.

Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease characterised by continuous ulceration and damage of the large bowel (1,2). Patients present with gastrointestinal and extra-intestinal symptoms which include, but are not limited to; abdominal pain, fever, fatigue, urgency, weight loss, and bloody/loose stools (2). Furthermore, UC is a lifelong unremitting disorder most commonly diagnosed early in life with peak onset occurring around 30 years of age (2). Currently, pharmaceuticals (aminosalicylates, steroids and immunosuppressants) are prescribed to manage symptoms; however, these drugs are not curative (2,3). Additional to drug intervention, surgical procedures (colectomy) are required in ~15% of UC patients (4).

Received: March 29, 2020; **Revised:** August 3, 2020; **Accepted:** September 16, 2020

Abbreviations

AOM	azoxymethane
CA-CRC	colitis-associated CRC
CRC	colorectal cancer
DAI	disease activity index
DSS	dextran sulphate sodium
EO	Emu oil
GSE	grape seed extract
MPO	myeloperoxidase
UC	ulcerative colitis

The highest incidences of UC are documented in western countries; however, its prevalence is increasing world-wide (1,5,6). The pathogenesis of UC remains unknown, although disease is thought to arise from a multitude of factors including genetic susceptibility, a compromised immune system and other environmental factors (1,2,7). A history of smoking has been linked to UC diagnosis, whereas breast-feeding has been reported to decrease UC risk (8,9). As UC is an incurable condition, management is critical over many decades, and consequently, UC patients are at significant risk of developing colorectal cancer (CRC) (10–12). Globally, CRC ranks third compared with all cancers in terms of incidence, and second in mortality (13). Furthermore, Europe, Australia and New Zealand have the largest number of diagnoses, and like UC, environmental factors such as obesity, chronic inflammation and malnutrition are known to increase CRC risk (13). In addition, chemotherapy-treatment for CRC often results in significant gastrointestinal toxicities including nausea, weight loss and mucositis (14). These debilitating side-effects further impair patient quality of life. Consequently, the development of novel alternative treatments for CRC capable of reducing the side-effects of conventional therapies, is crucial. Although colitis-associated CRC (CA-CRC) accounts for only 2% of all CRC related mortalities, it is the cause of death for ~15% of all inflammatory bowel disease patients (15).

Recently, plant and animal-derived products, termed nutraceuticals, have gained interest for their potential to benefit inflammatory and gastrointestinal conditions (16). In particular, fruits and vegetables, containing polyphenols are reported to exert health benefits, mainly by positively increasing diversity and abundance of gut microbiota (17). For example, a low dose of curcumin, a polyphenol derived from the spice Turmeric, was found to maintain remission in UC patients (18). Furthermore, 60% of inflammatory bowel disease patients reported that a poor and processed diet played a major role in triggering disease relapse (19). More recently, anti-inflammatory and reparative properties have been attributed to the nutraceuticals Emu oil (EO) and grape seed extract (GSE) (16).

EO, derived from the adipose tissue of the Native Australian Emu (*Dromaius Novahollindae*), has previously been reported to display anti-inflammatory and reparative properties in preclinical models of chemotherapy-induced mucositis (20,21), NSAID-induced enteropathy (22), UC (23,24) and CA-CRC (25–27). Chartier *et al.* (2018) concluded that orally-administered EO improved clinical indicators and reduced the number of small colonic tumours in an azoxymethane (AOM)/ dextran sulphate sodium (DSS) mouse model of CA-CRC, although overall tumour number was not significantly affected (25). Therefore, combining EO with other antioxidant or anticancer nutraceuticals could potentiate the capacity to decrease colonic tumours in this model of CA-CRC.

GSE, a naturally-sourced by-product of wine-making, comprises anthocyanidins, B-type proanthocyanidins, hydroxycinnamic acid and flavanols (28). These compounds have displayed antioxidant, anti-inflammatory, anti-bacterial and anti-neoplastic potential *in vitro* and *in vivo* (29). Cheah *et al.* (2014) applied procyanidin fractions of GSE to colon cancer (Caco-2) cell lines and determined that GSE reduced cell viability and enhanced the potency of the common chemotherapeutic drug, 5-Fluorouracil (30). In rodent models of chemotherapy-induced mucositis (31,32) and DSS-induced colitis (29), orally-administered GSE reduced intestinal inflammation, decreased severity scores of damage and improved intestinal architecture compared with un-treated animals, indicating its efficacy in gastrointestinal disorders. However, GSE has not yet been investigated in a preclinical setting of CA-CRC. It was hypothesized that EO and GSE, alone and in combination, would decrease inflammation and clinical indicators of disease while concurrently inhibiting colorectal tumour development in an AOM/DSS mouse model of CA-CRC.

Materials and methods

Animal studies

All animal studies were conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committees of the Children, Youth and Women's Health Service and The University of Adelaide. Female C57BL/6 mice (C57BL/6JArc, n = 80) at 8 weeks of age were sourced from Animal Resource Centre (Perth, Western Australia) and group-housed at room temperature with a light/dark cycle of 14:10 h. Mice were fed standard mouse chow and provided with drinking water for the duration of the trial.

Experimental trial

Female C57BL/6 mice (n = 10/group) were randomly assigned to eight treatment groups (n = 10/group); (1) saline + water + water, (2) saline + water + GSE, (3) saline + water + EO, (4) saline + water + combination of EO and GSE (EO/GSE), (5) AOM + DSS + water, (6) AOM + DSS + GSE, (7) AOM + DSS + EO and (8) AOM + DSS + EO/GSE. All mice were orally-administered (gavage) with either water (80 µl), EO (80 µl; Emu Tracks, Marleston, South Australia, Australia; Supplementary Table 1, available at *Carcinogenesis* Online.), GSE (80 µl; 400 mg/kg; Tarac Technologies, South Australia, Australia) or a combination of EO/GSE (160 µl), thrice weekly for the duration of the trial. On day 0, mice received a single injection (i.p.) of saline or AOM (carcinogen; 7.4 mg/kg; Sigma–Aldrich, Castle Hill, New South Wales, Australia), and then underwent three DSS/water cycles comprised of 7 days DSS (2%w/v, 2g/100 ml distilled water; MP Biomedicals, Santa Ana, CA) followed by 14 days plain water (ad libitum). Healthy control animals (groups 1–4) received plain water for the duration of the 9-week experimental period. All animals were euthanized on day 63 via CO_2 asphyxiation followed by cervical dislocation. Blood was collected (groups 1 and 5–8) via cardiac puncture for intestinal permeability analyses. Heart, liver, lung, thymus, spleen, kidney and stomach were removed from all mice and weighed, and intestinal lengths and weights were measured. Colon (2 cm) was collected for biochemical analyses. Remaining sections of the proximal and distal colon were collected for histological analyses.

Daily measurements

Mice were weighed and observed daily for the duration of the trial. Disease activity index (DAI) was calculated by scoring four parameters from 0–3 (increasing in severity), and then summed to provide a total DAI score for each day (33). The parameters include bodyweight loss, general condition/ appearance, stool consistency and rectal bleeding.

Colonoscopy

At the end of each DSS/water cycle (days 20, 41 and 62), a high-resolution colonoscope (Karl Storz, 1.9 mm outer diameter, Tuttlingen, Germany) was used to assess colitis progression and tumour development. Mice were placed on a heating pad and anesthetized using Isoflurane inhalant (AbbVie Pty Ltd, Mascot, New South Wales, Australia) for the duration

of the colonoscopy procedure. Immediately after, mice were placed in a recovery cage on a heating pad and closely monitored before returning to their home cage. Colonoscopy videos were later scored in a blinded fashion as described by Becker *et al.* (2005) (34). Thickening of the colon, vasculature pattern, fibrin, granularity of mucosa and stool consistency were each scored from 0 to 3 (increasing in severity) and summed for a final colitis severity score. The number of colonic tumours at each time-point was also counted from colonoscopy videos in a blinded fashion.

Burrowing analyses

During the experimental period mouse behaviour was determined by their burrowing ability as a measure of daily living and general wellbeing. A modified protocol (35) was implemented to measure burrowing ability on day 1 (baseline), at the end of each DSS week (days 5, 26 and 47) and at the end of each DSS/water cycle (days 19, 40 and 61). At each of these time-points, mice were acclimatised in the dark for at least 1 h prior to commencing burrowing analyses. Remaining in the dark, mice were then placed in individual cages with a pre-weighed burrow attached (400 g; kitty litter 'pebbles'; Black and Gold, Australian Asia/Pacific Wholesalers Pty Ltd) and left for 1 h. Burrows were then re-weighed, recorded and the weight difference taken to represent the amount burrowed.

Myeloperoxidase assay

Sections of the mid-colon were biochemically analysed for myeloperoxidase (MPO) levels as an indicator of acute inflammation. As described by Abimosleh *et al.* (2013) (22), thawed, homogenised samples were centrifuged at 13 000*g* (4°C) for 12 min and supernatant was discarded. Tissue homogenate was then re-suspended in 200 µl of 0.5% hexadecyltrimethyl ammonium bromide buffer, a detergent (Sigma). After vortexing for 2 min, samples were again centrifuged at 13 000*g* for 2 min. Background, negative and positive control samples (50 µl) and the supernatants of each test sample were then aliquoted into duplicate wells of a microtitre 96-well plate. Following the addition of a reaction solution (200 µl to each well; 4.2 mg of O-dianisidine dihydrochloride reagent, 12.5 µl H_2O_2, 2.5 ml potassium phosphate buffer [pH 6.0], 22.5 mL distilled water) the change in absorbance was measured at 450 nm at 1 min interval for 15 min with a spectrophotometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore) (22). Data were expressed as MPO units per gram of tissue.

FITC-dextran assay

A fluorescein isothiocyanate (FITC)-dextran assay was performed to assess intestinal permeability. Mice from saline + water + water (1), AOM + DSS + water (5), AOM + DSS + GSE (6), AOM + DSS + EO (7) and AOM + DSS + EO/GSE (8) groups were orally-administered (gavage) with a 500 mg/kg dose of FITC-dextran (mol wt 4000, 75 mg/ml; Sigma), 3 h prior to get killed. Blood was collected via cardiac puncture at time of getting killed. Samples were centrifuged (11 000*g* at 23°C) for 12 min and serum collected. Serum samples were diluted 1:3 with 0.2 M PBS and FITC-dextran was quantified using a BioTek Synergy Mx Microplate Reader (BioTek, Winooski, Vermont) and Gen5 version 2.00.18 software relative to a standard curve (0.001–100 µg/ml).

Histological analysis

Sections of the proximal and distal colon were routinely processed and embedded in paraffin wax following collection. Sections (4 µm) were then stained with haematoxylin and eosin (H and E). Damage severity was assessed in a blinded fashion using an Olympus BH-2 light microscope (Olympus Corporation, Tokyo, Japan) as described previously (36). Six parameters including goblet cell reduction, crypt and crypt cell disruption, polymorphonuclear infiltration and thickening/oedema of the submucosa and muscularis externa were scored from 0 to 3 with increasing severity for four cross sections of colonic tissue per mouse. Median scores for each parameter were then calculated and summed to determine a final severity score per mouse per colonic section.

Statistical analysis

Statistical analysis was completed using SPSS, version 25 for Windows (SPSS Chicago, IL). Data were tested for normality using a Shapiro–Wilk test. Bodyweight, DAI, burrowing activity, colonoscopically-assessed colitis score and tumour number were analysed by repeated measures ANOVA with least significance difference to compare among and within a group. Organ data, FITC-dextran, histologically-assessed severity scores and MPO activity were analysed using a one-way ANOVA, with a Tukey's *post hoc* test. For all analyses, $P < 0.05$ was considered statistically significant. All data figures were created using Microsoft Excel (version 15).

Results

Bodyweight

In normal mice, EO alone did not affect bodyweight loss. However, administration of GSE and the combination of EO/GSE decreased bodyweight on days 7, 48 and 22–24, 29–31, 33–39, 41–63 respectively ($P < 0.05$; Figure 1). AOM/DSS induced significant bodyweight loss throughout the trial (days 6–39, 47–59 and 61) compared with saline controls ($P < 0.05$; Figure 1). In AOM/DSS animals, individual administration of EO and GSE did not affect bodyweight ($P > 0.05$; Figure 1). Combined EO/GSE decreased bodyweight on day 60 compared with AOM/DSS controls ($P < 0.05$). Additionally, EO/GSE in combination further reduced bodyweight compared with administration of EO and GSE alone on days 29, 30, 32, 52, 54, 59, 60 and days 60–62, respectively ($P < 0.05$; Figure 1).

Disease activity index

In normal mice, GSE and combined EO/GSE-administration increased DAI scores, attributed to weight loss, compared with saline controls on days 5, 43, 48 and 13, 43, 48, 52, respectively ($P < 0.05$; Figure 2). Whereas EO alone did not impact DAI scores on any days in normal mice compared with saline controls ($P > 0.05$; Figure 2). AOM/DSS control mice presented with the highest DAI score throughout the trial and was significantly increased compared with saline controls (days 2, 3 and 5–63; $P < 0.05$; Figure 2). EO (days 7, 8, 11, 14, 15, 17, 21, 24–26, 35, 36, 39, 41, 42, 45, 46, 48, 53, 55, 57 and 59–63) and GSE (days 8–10, 14, 15, 17, 24, 34, 35, 39, 43, 53–57, 59–63) alone decreased DAI scores in AOM/DSS mice compared with AOM/DSS controls ($P < 0.05$; Figure 2). Furthermore, combined EO/GSE decreased DAI scores on days 6, 8, 11, 12, 13 15,23-25, 34–37, 39, 42, 43, 46, 48, 51, 53, 54–57 and 60–63 compared with AOM/DSS controls ($P < 0.05$; Figure 2). Importantly, the combination of EO/GSE further decreased DAI scores in AOM/DSS mice compared with EO (days 8, 40, 44 and 46) and GSE (days 11–12, 18, 25, 32, 34, 36, 37, 42, 51 and 63) alone ($P < 0.05$; Figure 2).

Burrowing activity

In normal mice, there was no significance determined in the burrowing ability of saline controls compared with nutraceutical-treated animals ($P > 0.05$). Furthermore, AOM/DSS controls did not exhibit impaired burrowing ability during the trial. However, EO-administration (197 ± 41 g) significantly increased burrowing activity of AOM/DSS mice on day 61 (+79%; $P < 0.05$) compared with AOM/DSS controls (110 ± 19 g), with no significant effect, evident by any treatment at the other time-points.

Colonoscopically-assessed colitis severity

At all three time-points, AOM/DSS controls presented with significantly increased colitis-severity scores than saline controls ($P < 0.05$; Figure 3). In AOM/DSS mice, EO and GSE alone resulted in significantly decreased colitis severity on day 62 compared with AOM/DSS controls ($P < 0.05$; Figure 3). Furthermore, the combination of EO/GSE significantly reduced colitis severity scores on days 41 and 62 compared with untreated AOM/DSS

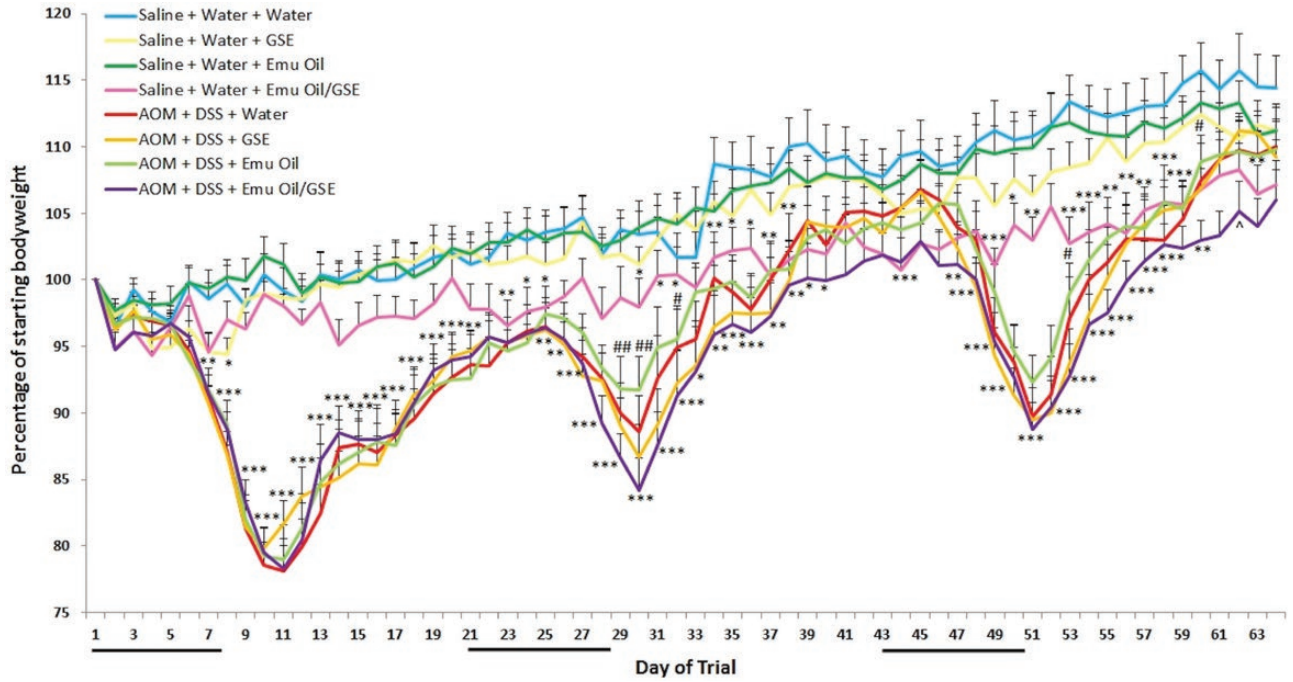


Figure 1. Daily bodyweight change of mice. Data are expressed as mean (% starting bodyweight) ± SEM (n = 10/group). Black line on the x-axis represents a DSS week. *P < 0.05 **P < 0.01, ***P < 0.001 compared with Saline + Water + Water; *P < 0.05 compared with AOM + DSS + Water; **P < 0.01, *P < 0.05 compared with AOM + DSS + EO/GSE on the same day.

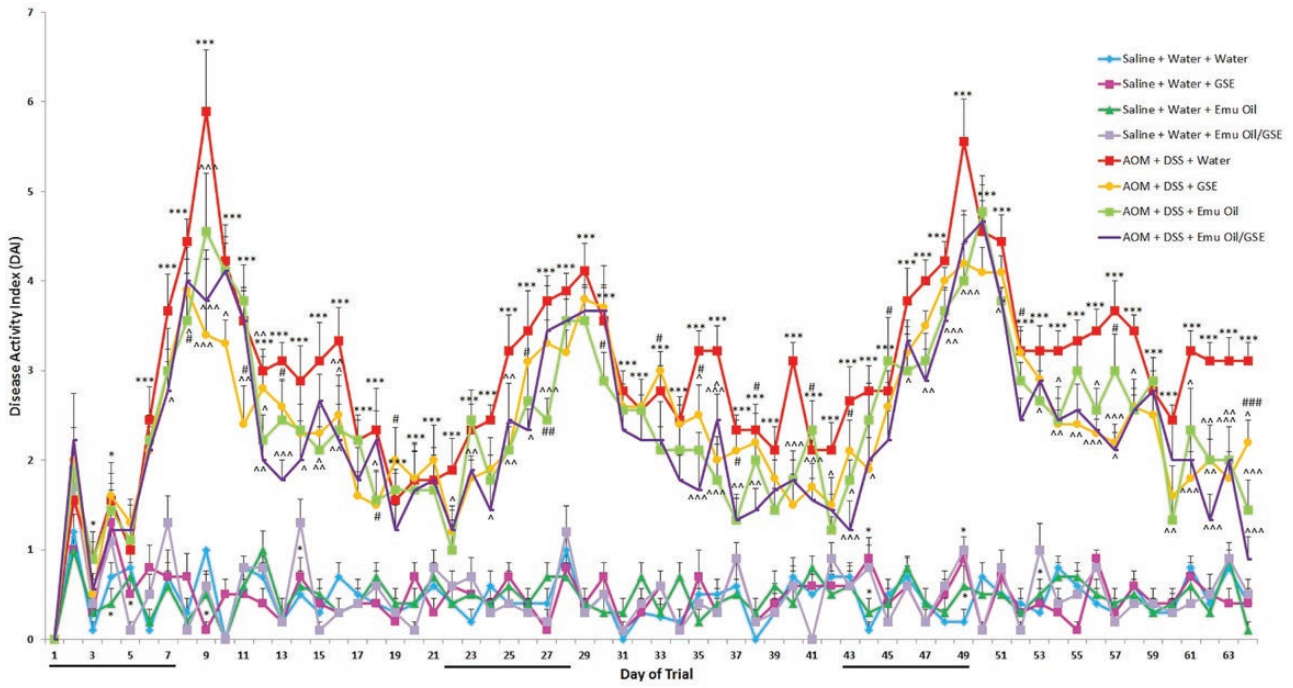


Figure 2. Daily DAI score. Data are expressed as mean ± SEM (n = 10/group). Black line on the x-axis represents a DSS week. ***P<0.001, **P < 0.01, *P < 0.05 compared to Saline + Water + Water; ^^^P < 0.001, ^^P < 0.01, ^P < 0.05 compared to AOM + DSS + Water; ###P < 0.001, ##P < 0.01, # P < 0.05 compared compared with AOM + DSS + EO/GSE on the same day.

mice (P < 0.05; Figure 4). However, the combination of EO/GSE did not further reduce colitis severity compared with EO and GSE alone (P > 0.05; Figure 3).

Colonoscopically-assessed tumour development

Saline controls and nutraceutical-treated saline animals did not develop colonic tumours during the 9-week experimental period (Supplementary Figure 1). However, colorectal tumours were achieved in 100% of mice administered with AOM/DSS. AOM/DSS control mice presented with the greatest number of colonic tumours at all three time-points (days 20, 41 and 62; P < 0.05; Figure 4). EO (days 20 and 62) and GSE (day 20) alone significantly reduced colonic tumour number compared with AOM/DSS controls (P < 0.05; Figure 4). Furthermore, EO/GSE in combination

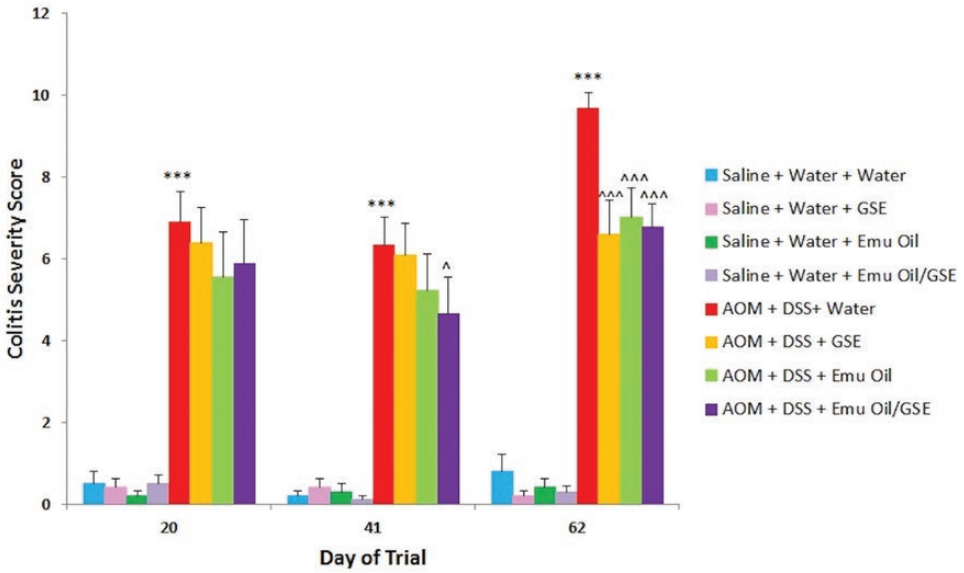


Figure 3. Colonoscopically-assessed colitis severity. Data are expressed as mean (colitis severity score) ± SEM (n = 10/group). ***$P < 0.001$ compared with Saline + Water + Water; ^^^$P < 0.001$, ^$P < 0.05$ compared with AOM + DSS + Water on the same day.

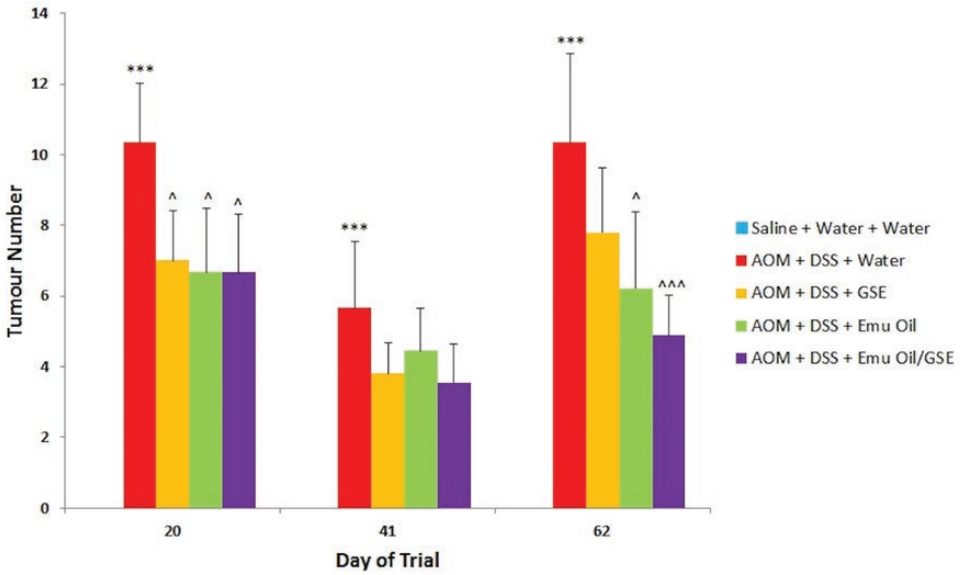


Figure 4. Colonoscopically-assessed tumour number. Data are expressed as mean (tumour number) ± SEM (n = 10/group). ***$P < 0.001$ compared with Saline + Water +Water; ^^^$P < 0.001$, ^$P < 0.05$ compared with AOM + DSS + Water on the same day.

resulted in fewer colonic tumours on days 20 and 62 compared with AOM/DSS controls ($P < 0.05$; Figure 4). However, in AOM/DSS mice, there was no further reduction in tumour numbers in the combined EO/GSE-treated mice compared with EO and GSE alone ($P > 0.05$; Figure 4).

Mouse organ weights and lengths

The weight of the colon ($166 \times 10^{-2} \pm 10$ g), liver ($552 \times 10^{-2} \pm 25$ g) and spleen ($86 \times 10^{-2} \pm 10$ g) were significantly increased in AOM/DSS controls compared with saline controls, with no impact observed in other treatment groups ($P < 0.05$; Supplementary Tables 2–3, available at *Carcinogenesis* Online). There was no difference in the weights of the duodenum, small intestine, heart, thymus, lung, kidneys or stomach amongst experimental groups. Furthermore, there were no significant differences in intestinal lengths (small and large) within all treatment groups ($P > 0.05$; Supplementary Tables 2–3, available at *Carcinogenesis* Online).

Acute inflammation (MPO)

In normal mice, MPO levels were unaffected by EO, GSE or EO/GSE ($P > 0.05$; Supplementary Figure 2). AOM/DSS controls (117 ± 23) had significantly increased MPO per gram of tissue compared with saline controls (13 ± 2; $P < 0.05$; Supplementary Figure 2). However, EO, GSE or the combination of EO/GSE did not affect MPO levels in AOM/DSS mice compared with AOM/DSS controls ($P > 0.05$; Supplementary Figure 2).

Intestinal permeability (FITC-dextran)

AOM/DSS significantly increased intestinal permeability, indicated by an increased serum FITC-dextran level, compared with

saline controls ($P < 0.05$; Figure 5). Additionally, in AOM/DSS mice, EO and the combination of EO/GSE, significantly decreased serum FITC-dextran levels compared with AOM/DSS controls ($P < 0.05$; Figure 5). However, GSE-administration alone did not impact FITC-dextran levels ($P > 0.05$).

Histologically-assessed severity scores

In the proximal colon, histologically-assessed severity scores remained unchanged across all experimental groups ($P > 0.05$; Table 1). However, in the distal colon, AOM/DSS controls presented with significantly increased damage severity scores compared with saline controls ($P < 0.05$; Table 1; Supplementary Figure 3, available at *Carcinogenesis* Online), with no effect observed following administration of EO, GSE or the combination of EO/GSE ($P > 0.05$).

Discussion

In the current study, administration of EO, GSE and the combination of EO and GSE did not result in the manifestation of any aberrant colonic features in normal healthy control mice. The combination of EO and GSE slightly increased DAI scores in normal animals at selected time-points; an effect that was attributed to some minor bodyweight loss. However, this was not consistently observed across the experimental period and was probably a result of mice receiving a larger gavage volume compared with the EO and GSE alone treatment groups, thus eating less mouse chow. This effect was also observed in a similar study by Chartier *et al.* (25). The 9-week AOM/DSS model used in the current study successfully induced characteristics of CA-CRC, consistent with previous investigations (25,37–41). These markers included significant bodyweight loss, increased DAI scores, colonoscopically-assessed colitis-severity, tumour development and liver and spleen weights. Furthermore, intestinal permeability was elevated in addition to histological parameters of disease severity in the colon.

Thrice weekly oral-administration of EO, GSE and a combination of the two resulted in a marked decrease in clinical indicators of disease in mice with CA-CRC. Although bodyweight alone was not largely impacted by administration of EO or GSE, DAI scores were significantly reduced by these agents in CA-CRC mice at most time-points. This result was attributed to a protective effect against the other parameters of the DAI score which included general condition/appearance, stool consistency and rectal bleeding. In a recent study, *Ziziphus jujube* (ZJ), a fruit containing bioactive compounds including flavonoids and polysaccharides was administered to AOM/DSS mice (42). The authors concluded that *Ziziphus jujube* significantly decreased faecal blood, diarrhoea and overall DAI, similar to the results observed by the combination of EO and GSE. In the current study, burrowing behaviour, as a measure of affective state and animal welfare, was unaffected by nutraceutical-administration in AOM/DSS mice. However, Chartier *et al.* (37) recently concluded that DAI was the most reliable measure of affective state for the AOM/DSS model, which is supported by the significant DAI results obtained in the current study. Moreover, MPO, a measure of acute inflammation was elevated in AOM/DSS controls though remained unchanged in nutraceutical-administered mice. This effect was also observed by Chartier *et al.* in 2018, and as the AOM/DSS model induces chronic inflammation, it is probably that a more complex immune response incorporating T-helper 1 (Th1) and Th2 pathways would have occurred by the end of the 9-week experimental period when tissue was collected (25). Therefore, analysis of pro-inflammatory cytokines including, but not limited to, IL-6, IL-8, IL-12 and IL-18 should be incorporated in future studies to compare with the obtained MPO data.

The murine AOM/DSS model of CA-CRC results in tumour development exacerbated by chronic inflammation (41). This inflammatory environment alters the diversity and abundance of the gut microbiota, and is linked to increased intestinal permeability (43–45). The large intestine (colon and rectum) is home to large populations of microbes and bacteria, accounting for 70% of all bacteria in the human body (46) and thus disruption to these populations can have significant implications on overall intestinal homeostasis. A compromised barrier function results in translocation of commensal microbes that can activate pro-inflammatory transcription factors such as NF-κB, and cytokines, including IL-6 and TNF-α (15,47). In the current study,

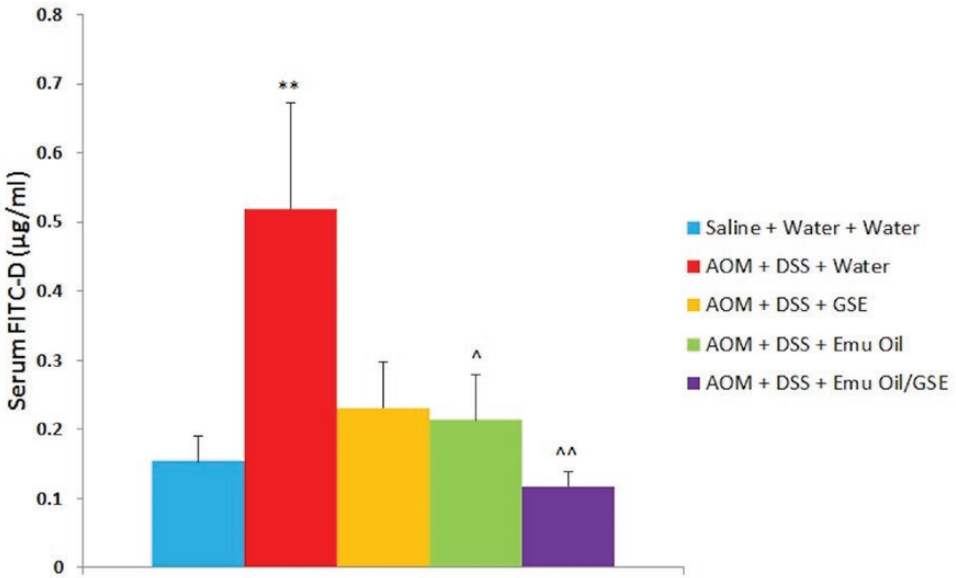


Figure 5. Intestinal permeability assessed by FITC-dextran levels. Data are expressed as mean serum FITC-dextran (µg/ml) ± SEM ($n = 10$/group). **$P < 0.01$ compared with Saline + Water + Water; ^^$P < 0.01$, ^$P < 0.05$ compared with AOM + DSS + Water.

Table 1. Histological severity scores of colonic tissue

Group	Proximal colon	Distal colon
Saline + Water + Water	2.35 ± 0.5	2.65 ± 0.5
Saline + Water + GSE	1.94 ± 0.4	2.00 ± 0.3
Saline + Water + EO	1.70 ± 0.4	1.75 ± 0.4
Saline + Water + EO/GSE	2.45 ± 0.4	1.20 ± 0.2
AOM + DSS + Water	4.44 ± 0.6	7.90 ± 0.7***
AOM + DSS + GSE	4.10 ± 0.6	6.90 ± 1.1
AOM + DSS + EO	4.10 ± 0.9	6.94 ± 0.9
AOM + DSS + EO/GSE	3.20 ± 0.7	5.50 ± 1.1

***$P < 0.001$ compared with Saline + Water + Water.

serum collected from AOM/DSS controls exhibited significantly elevated levels of FITC-dextran, indicating an increase in intestinal permeability, accompanied by increased liver and spleen weights compared with saline control mice. This was an expected result as the gut-liver axis links the two organs, resulting in similar immunological qualities (15). Although neither EO nor GSE affected visceral organ weights, intestinal permeability was restored to saline control levels in AOM/DSS mice treated with EO and the combination of EO and GSE. This protective effect on intestinal barrier function could represent an interaction with the gut microbiota, which should be defined by future 16S rRNA sequencing of faecal/caecal samples. Furthermore, assessing the impact of EO and GSE on mucin secreting goblet cells may also highlight their protective effect on the intestinal barrier.

Colonoscopic analysis revealed that EO, GSE and the combination of EO and GSE were most effective at reducing colitis severity in the latter stages of the experimental period. Interestingly, the combination of EO and GSE reduced colitis severity at the mid- and final time-points, whereas EO and GSE alone only reduced severity at the final time-point. In a previous study using the AOM/DSS model, EO reduced colonoscopically-assessed colitis severity at all three time-points; however, this was primarily delivered at a larger volume of 160 µl, compared with the 80 µl used in the current study (25). Colonic tumours were evident from day 20 of the current AOM/DSS trial, whereas mice treated with EO, GSE and the combination of EO and GSE presented with fewer tumours than CA-CRC control mice. In the mid-stage of the trial (day 41), the number of colonic tumours was fewer than that observed on day 20. This was probably because the tumours counted on day 20 were small slightly raised polyps on the mucosal surface of the colon. By day 41, these small polyps had coalesced to form larger protruding tumours and thus the overall number of colonic tumours decreased. At the end of the study, EO and the EO/GSE combination significantly reduced the number of colonic tumours, while GSE alone had no effect on day 62. Although this is the first study to test whole GSE in an *in vivo* CA-CRC model, previously, Derry *et al.* (2013) and (2014) investigated the efficacy of GSE in AOM-induced colon tumourigenesis (48,49). It was concluded that dietary-GSE reduced tumour multiplicity and overall tumour size compared with untreated mice, and furthermore, GSE also inhibited CRC metastasis to the lungs compared with untreated AOM-treated mice (48,49). Considering previous research suggesting the anti-neoplastic potential of GSE in cell lines (30–32), it was interesting that GSE alone did not elicit a more profound effect on tumour burden in AOM/DSS mice. In future studies, increasing the dose of whole GSE or by refining the compound to deliver specific grape procyanidin extracts may increase the tumour-inhibiting action of GSE when administered alone (30,31,50). Reddivari *et al.* (2016) tested dietary supplementation of the grape bioactive compound, resveratrol -GSE in a mouse model of AOM-induced carcinogenesis and concluded that resveratrol-GSE reduced tumorigenesis by 50% compared with CRC controls, an effect similar to sulindac, a non-steroidal anti-inflammatory drug (51). Additionally, cytoplasmic and nuclear protein levels of ß-catenin were decreased by resveratrol-GSE, supressing downstream Wnt/ß-catenin signals including c-Myc and cyclin D1 that drive cell proliferation in CRC (51). This suppressive effect of GSE on Wnt/ß-catenin signalling may have acted to accelerate the anti-neoplastic potential of EO when combined with decrease tumour burden at the last time-point in the current study. *In vitro* studies have suggested that GSE inhibits cell proliferation and enhances apoptosis in colon cancer cell lines (52,53), therefore, incorporating immunohistochemical staining for apoptosis/proliferation in future studies would conclude if such mechanisms are occurring in the AOM/DSS mouse model. Moreover, to further understand tumour development in this AOM/DSS model, it would be beneficial to obtain tumour biopsies during colonoscopy procedures for profiling and microscopic analysis of neoplastic tissue.

In conclusion, EO and GSE were both effective at reducing DAI scores, colitis severity, the number of colonoscopically-assessed tumours and intestinal permeability in this AOM/DSS model of CA-CRC. In the current study, the greatest protection against CA-CRC was exerted by the combination of GSE and EO, whereas EO tended to improve disease parameters to a greater extent than GSE at the dose tested. Future studies should include increasing the dose of GSE to potentiate its effect in CA-CRC mice. Additionally, further investigation into the effects of EO and GSE on the intestinal microbiota would assist in understanding the mechanisms underlying their protective action in CA-CRC.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

Funding

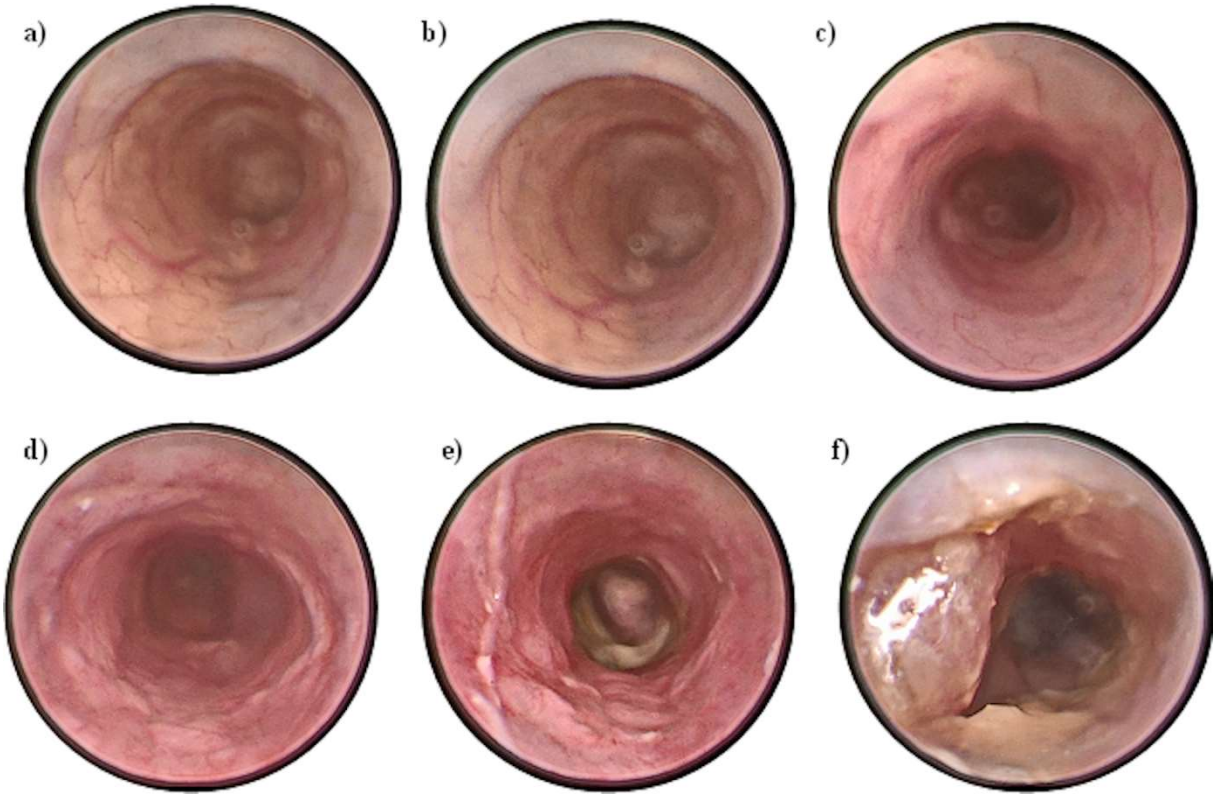
LCC is supported by an Australian Government Research Training Program Scholarship; and receives a top-up scholarship from AgriFutures Australia. DT is funded by a National Health and Medical Research Council (NHMRC) Senior Research Fellowship (1020437).
Conflict of Interest Statement: None declared.

References

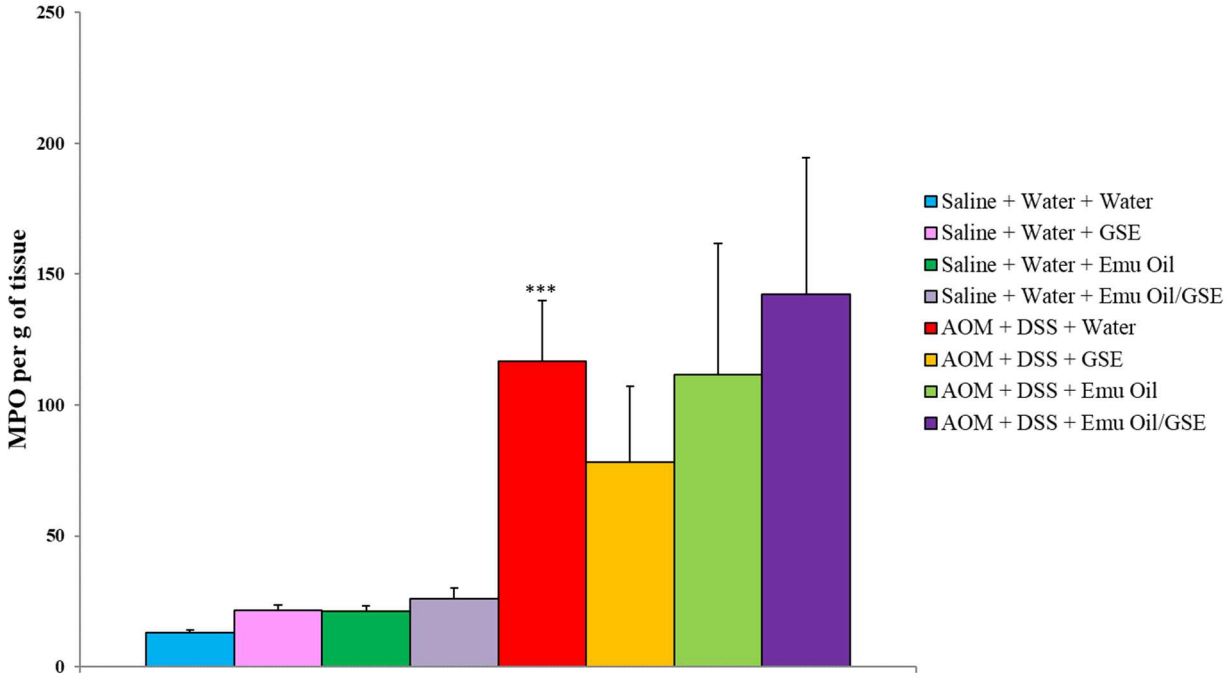
1. Bernstein, C.N. *et al.* (2006) The epidemiology of inflammatory bowel disease in Canada: a population-based study. *Am. J. Gastroenterol.*, 101, 1559–1568.
2. Ungaro, R. *et al.* (2017) Ulcerative colitis. *Lancet*, 389, 1756–1770.
3. Antonelli, E. *et al.* (2018) Novel oral-targeted therapies for mucosal healing in ulcerative colitis. *World J. Gastroenterol.*, 24, 5322–5330.
4. Magro, F. *et al.* (2012) Review of the disease course among adult ulcerative colitis population-based longitudinal cohorts. *Inflamm. Bowel Dis.*, 18, 573–583.
5. Molodecky, N.A. *et al.* (2012) Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterol.*, 142, 46–54.e42; quiz e30.
6. Shivananda, S. *et al.* (1996) Incidence of inflammatory bowel disease across Europe: is there a difference between north and south? Results of the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Gut*, 39, 690–697.
7. Hendrickson, B.A. *et al.* (2002) Clinical aspects and pathophysiology of inflammatory bowel disease. *Clin. Microbiol. Rev.*, 15, 79–94.

8. Klement, E. *et al.* (2004) Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis. *Am. J. Clin. Nutr.*, 80, 1342–1352.
9. Mahid, S.S. *et al.* (2006) Smoking and inflammatory bowel disease: a meta-analysis. *Mayo Clin. Proc.*, 81, 1462–1471.
10. Biancone, L. *et al.* (2020) Cancer risk in inflammatory bowel disease: A 6-year prospective multicenter nested case-control IG-IBD study. *Inflamm Bowel Dis*, 26, 450–459.
11. van Hogezand, R.A. *et al.* (2002) Malignancies in inflammatory bowel disease: fact or fiction? *Scand. J. Gastroenterol. Suppl.*, 236, 48–53.
12. Brenner, H. *et al.* (2014) Colorectal cancer. *Lancet*, 383, 1490–1502.
13. Bray, F. *et al.* (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.*, 68, 394–424.
14. O'Brien, M.E. *et al.* (2006) Mortality within 30 days of chemotherapy: a clinical governance benchmarking issue for oncology patients. *Br. J. Cancer*, 95, 1632–1636.
15. Shalapour, S. *et al.* (2020) Cruel to be kind: epithelial, microbial, and immune cell interactions in gastrointestinal cancers. *Annu. Rev. Immunol.*, 38, 649–671.
16. Chartier, L.C. *et al.* (2019) Combined nutraceuticals: a novel approach to colitis-associated colorectal cancer? *Nutr. Cancer*, 71, 199–206.
17. Liu, W. *et al.* (2017) Grape seed proanthocyanidin extract ameliorates inflammation and adiposity by modulating gut microbiota in high-fat diet mice. *Mol Nutr Food Res*, 61, 1–14. doi:10.1002/mnfr.201601082.
18. Hanai, H. *et al.* (2006) Curcumin maintenance therapy for ulcerative colitis: randomized, multicenter, double-blind, placebo-controlled trial. *Clin. Gastroenterol. Hepatol.*, 4, 1502–1506.
19. Casanova, M.J. *et al.* (2017) Prevalence of malnutrition and nutritional characteristics of patients with inflammatory bowel disease. *J. Crohns. Colitis*, 11, 1430–1439.
20. Mashtoub, S. *et al.* (2016) Emu oil combined with Lyprinol™ reduces small intestinal damage in a rat model of chemotherapy-induced mucositis. *Nutr. Cancer*, 68, 1171–1180.
21. Mashtoub, S. *et al.* (2013) Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. *Exp. Biol. Med. (Maywood).*, 238, 1305–1317.
22. Abimosleh, S.M. *et al.* (2013) Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. *Evid. Based. Complement. Alternat. Med.*, 2013, 429706.
23. Abimosleh, S.M. *et al.* (2012) Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. *Dig. Dis. Sci.*, 57, 887–896.
24. Safaeian, R. *et al.* (2019) Emu oil reduces disease severity in a mouse model of chronic ulcerative colitis. *Scand. J. Gastroenterol.*, 54, 273–280.
25. Chartier, L.C. *et al.* (2018) Emu oil improves clinical indicators of disease in a mouse model of colitis-associated colorectal cancer. *Dig. Dis. Sci.*, 63, 135–145.
26. Mashtoub, S. *et al.* (2017) Emu oil attenuates disease severity and results in fewer large colonic tumours in a mouse model of colitis-associated colorectal cancer. *Gastroenterol.*, 152, S737–S737.
27. Mashtoub, S., *et al.* (2016) Emu oil attenuates disease severity in models of colitis and colitis-associated colorectal cancer. *J. Gastroenterol. Hepatol.*, 31, 125.
28. Roopchand, D.E. *et al.* (2013) Concord grape pomace polyphenols complexed to soy protein isolate are stable and hypoglycemic in diabetic mice. *J. Agric. Food Chem.*, 61, 11428–11433.
29. Cheah, K.Y. *et al.* (2013) Grape seed extract reduces the severity of selected disease markers in the proximal colon of dextran sulphate sodium-induced colitis in rats. *Dig. Dis. Sci.*, 58, 970–977.
30. Cheah, K.Y. *et al.* (2014) Low molecular weight procyanidins from grape seeds enhance the impact of 5-Fluorouracil chemotherapy on Caco-2 human colon cancer cells. *PLoS One*, 9, e98921.
31. Cheah, K.Y. *et al.* (2014) Grape seed extract dose-responsively decreases disease severity in a rat model of mucositis; concomitantly enhancing chemotherapeutic effectiveness in colon cancer cells. *PLoS One*, 9, e85184.
32. Cheah, K.Y. *et al.* (2009) Grape seed extract protects IEC-6 cells from chemotherapy-induced cytotoxicity and improves parameters of small intestinal mucositis in rats with experimentally-induced mucositis. *Cancer Biol. Ther.*, 8, 382–390.
33. Howarth, G.S. *et al.* (2000) Predisposition to colonic dysplasia is unaffected by continuous administration of insulin-like growth factor-I for 20 weeks in a rat model of chronic inflammatory bowel disease. *Growth Factors*, 18, 119–133.
34. Becker, C. *et al.* (2005) In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut*, 54, 950–954.
35. Deacon, R.M. (2006) Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. *Nat. Protoc.*, 1, 118–121.
36. Yazbeck, R. *et al.* (2008) Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice. *Front. Biosci.*, 13, 6850–6858.
37. Chartier, L.C. *et al.* (2020) Affective state determination in a mouse model of colitis-associated colorectal cancer. *PLoS One*, 15, e0228413.
38. Chartier, L.C. *et al.* (2020) Chemotherapy-induced mucositis development in a murine model of colitis-associated colorectal cancer. *Scand. J. Gastroenterol.*, 55, 47–54.
39. Mashtoub, S. *et al.* (2015) Emu oil promotes bodyweight gain in a mouse model of inflammation-associated colorectal cancer. *J..Gastroenterol. Hepatol.*, 30, 80.
40. De Robertis, M. *et al.* (2011) The AOM/DSS murine model for the study of colon carcinogenesis: from pathways to diagnosis and therapy studies. *J. Carcinog.*, 10, 9.
41. Parang, B. *et al.* (2016) AOM/DSS model of colitis-associated cancer. *Methods Mol. Biol.*, 1422, 297–307.
42. Periasamy, S. *et al.* (2020) Dietary Ziziphus jujuba fruit attenuates colitis-associated tumorigenesis: a pivotal role of the NF-κB/IL-6/JAK1/STAT3 Pathway. *Nutr. Cancer*, 72, 120–132.
43. Lucas, C. *et al.* (2017) Microbiota, inflammation and colorectal cancer. *Int J Mol Sci*, 18, 1310. doi:10.3390/ijms18061310.
44. Prosberg, M. *et al.* (2016) The association between the gut microbiota and the inflammatory bowel disease activity: a systematic review and meta-analysis. *Scand. J. Gastroenterol.*, 51, 1407–1415.
45. Rizzetto, L. *et al.* (2018) Connecting the immune system, systemic chronic inflammation and the gut microbiome: The role of sex. *J. Autoimmun.*, 92, 12–34.
46. Hillman, E.T. *et al.* (2017) Microbial ecology along the gastrointestinal tract. *Microbes Environ.*, 32, 300–313.
47. Zheng, H. *et al.* (2016) Establishing the colitis-associated cancer progression mouse models. *Int. J. Immunopathol. Pharmacol.*, 29, 759–763.
48. Derry, M.M. *et al.* (2014) Characterization of azoxymethane-induced colon tumor metastasis to lung in a mouse model relevant to human sporadic colorectal cancer and evaluation of grape seed extract efficacy. *Exp. Toxicol. Pathol.*, 66, 235–242.
49. Derry, M.M. *et al.* (2013) Grape seed extract efficacy against azoxymethane-induced colon tumorigenesis in A/J mice: interlinking miRNA with cytokine signaling and inflammation. *Cancer Prev. Res. (Phila).*, 6, 625–633.
50. Pérez-Ortiz, J.M. *et al.* (2019) Antiproliferative and cytotoxic effects of grape pomace and grape seed extracts on colorectal cancer cell lines. *Food Sci. Nutr.*, 7, 2948–2957.
51. Reddivari, L. *et al.* (2016) Grape compounds suppress colon cancer stem cells *in vitro* and in a rodent model of colon carcinogenesis. *BMC Complement. Altern. Med.*, 16, 278.
52. Velmurugan, B. *et al.* (2010) Dietary-feeding of grape seed extract prevents azoxymethane-induced colonic aberrant crypt foci formation in fischer 344 rats. *Mol. Carcinog.*, 49, 641–652.
53. Dinicola, S. *et al.* (2012) Antiproliferative and apoptotic effects triggered by Grape Seed Extract (GSE) versus epigallocatechin and procyanidins on colon cancer cell lines. *Int. J. Mol. Sci.*, 13, 651–664.

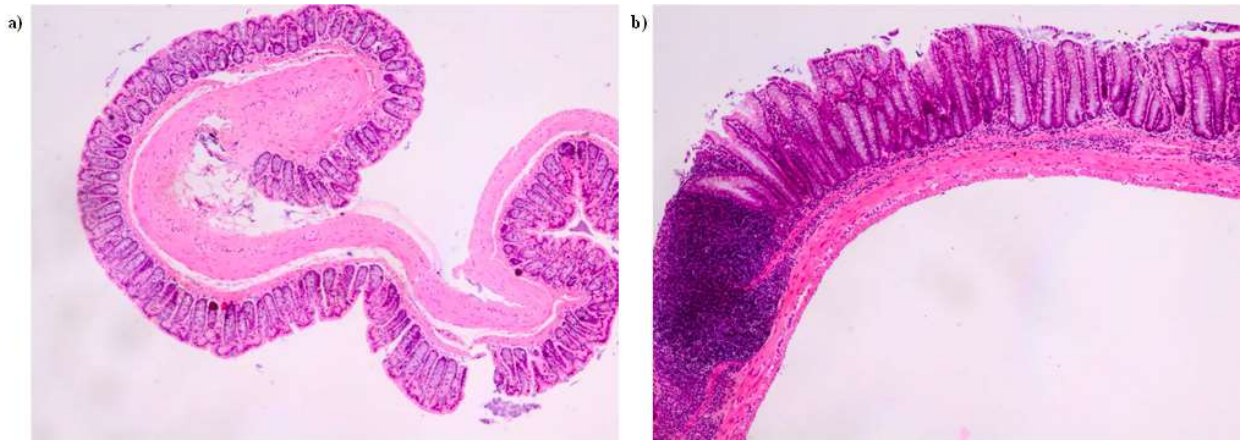
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Table 1. Fatty acid composition of the Emu Oil used in the current experiment (Emu Tracks; batch #11170128)

Analyte	Common Name	Total Lipids (%)
Total Saturates		49.1
14:00	Myristic acid	6.2
16:00	Palmitic acid	21.2
18:00	Stearic acid	11.8
20:00	Arachidonic acid	0.2
Total Monos		36.7
16:1n-7 Omega 7	Palmitoleic acid	2.8
18:1n-9 Omega 9	Oleic acid	32.2
18:1n-7 Omega 7	Vaccenic acid	2.6
20:1n-9 Omega 9	Gondoic acid	0.5
Total Omega 9		32.9
Total Omega 7		3.8
Total Omega 3		0.9
18:3n-3	α-Linolenic acid	0.7
Total Omega 6		12.9
18:2n-6	Linoleic acid	14.3
20:2n-6	Eicosadienoic acid	1.1
20:4n-6	Arachidonic acid	1.2

Supplementary Table 2. Visceral Organ Weights (% relative to BW, all weights are*10^{-2})

	Saline + Water + Water	Saline + Water + GSE	Saline + Water + Emu Oil	Saline + Water + Emu Oil/GSE	AOM + DSS + Water	AOM + DSS + GSE	AOM + DSS + Emu Oil	AOM + DSS + Emu Oil/GSE
Heart	55 ± 2	54 ± 3	51 ± 2	51 ± 2	51 ± 2	55 ± 2	54 ± 3	53 ± 2
Liver	444 ± 13	406 ± 21	443 ± 15	429 ± 13	552 ± 25*	520 ± 28	531 ± 25	558 ± 21
Spleen	32 ± 2	36 ± 2	32 ± 2	38 ± 2	86 ± 10***	68 ± 7	77 ± 10	65 ± 10
Thymus	18 ± 1	26 ± 3	19 ± 2	18 ± 2	16 ± 2	18 ± 3	15 ± 1	18 ± 3
Lung	66 ± 3	77 ± 4	73 ± 4	75 ± 3	73 ± 2	74 ± 3	76 ± 7	74 ± 3
L Kidney	54 ± 2	53 ± 2	50 ± 3	52 ± 2	56 ± 2	55 ± 2	52 ± 2	53 ± 2
R Kidney	52 ± 2	55 ± 2	53 ± 1	55 ± 2	57 ± 2	58 ± 1	60 ± 2	56 ± 2
Stomach	75 ± 5	72 ± 3	71 ± 3	69 ± 2	66 ± 2	70 ± 4	73 ± 4	70 ± 4

***$p<0.001$, *$p<0.05$ compared to Saline + Water + Water

Supplementary Table 3. (a) Intestinal Weights (% relative to BW, all weights are $*10^{-2}$) and (b) Lengths (cm)

3(a)

	Saline + Water + Water	Saline + Water + GSE	Saline + Water + Emu Oil	Saline + Water + Emu Oil/GSE	AOM + DSS + Water	AOM + DSS + GSE	AOM + DSS + Emu Oil	AOM + DSS + Emu Oil/GSE
Colon	92 ± 3	85 ± 2	91 ± 3	83 ± 3	166 ± 10***	146 ± 7	153 ± 7	150 ± 12
Duodenum	44 ± 3	48 ± 3	48 ± 4	49 ± 3	49 ± 3	47 ± 3	44 ± 3	46 ± 3
Small Intestine	336 ± 10	310 ± 7	340 ± 9	322 ± 10	344 ± 11	327 ± 9	346 ± 9	318 ± 8

3(b)

	Saline + Water + Water	Saline + Water + GSE	Saline + Water + Emu Oil	Saline + Water + Emu Oil/GSE	AOM + DSS + Water	AOM + DSS + GSE	AOM + DSS + Emu Oil	AOM + DSS + Emu Oil/GSE
Colon	7.5 ± 0.3	7.5 ± 0.3	7.7 ± 0.2	7.3 ± 0.2	6.8 ± 0.2	6.1 ± 0.4	6.9 ± 0.2	6.7 ± 0.3
Duodenum	3.5 ± 0.1	3.4 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.5 ± 0.3	3.7 ± 0.1	4.0 ± 0.2	3.6 ± 0.1
Small Intestine	28.4 ± 0.5	26.8 ± 0.6	29.1 ± 0.5	27.5 ± 0.4	29.8 ± 0.7	29.5 ± 0.5	30.4 ± 0.8	28.3 ± 0.8

***$p<0.001$ compared to Saline + Water + Water

CHAPTER 4

EMU OIL AND SAIREITO IN COMBINATION REDUCE TUMOUR DEVELOPMENT AND CLINICAL INDICATORS OF DISEASE IN A MOUSE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

CONTEXT STATEMENT

The research in this chapter is under review by *Biomedicine & Pharmacotherapy* and has been formatted according to the journal requirements.

In the previous chapter, grape seed extract in combination with Emu Oil (EO) offered the greatest protection against colitis-associated colorectal cancer (CA-CRC). This was evidenced by a decrease in disease severity scores and colorectal tumour number and the restoration of intestinal permeability. **Chapter 1** also presented Japanese Kampo medicines as potential therapies for ulcerative colitis and CA-CRC. Kampo medicine has been used and prescribed in Japan alongside Western therapies for centuries and has demonstrated efficacy in pre-clinical models of dextran sulphate sodium (DSS)-induced colitis and colorectal cancer (Yamakawa et al. 2013, Nagata et al. 2016, Matsunaga et al. 2017). Recently, Orengodokuto (Kampo medicine) was shown to decrease diarrhoea and limited side effects of chemotherapy in a mouse model of 5-Fluorourcail-induced mucositis (**Appendix 6**).

Saireito, which comprises 12 herbal ingredients, has not yet been investigated in a setting of CA-CRC. **Chapter 4** describes the first study to investigate the effects of Saireito when administered alone and in combination with Emu Oil in the azoxymethane (AOM)/DSS model. This study was the result of an international collaboration with Assistant Professor Junko Fujino from Dokkyo Medical University, Tokyo, Japan. Furthermore, this chapter details cellular-based cytokine analyses for interleukin-12p40 and interleukin-10 that were conducted during a two week placement with Professors Jóna Freysdóttir and Ingibjörg Harðardóttir at the Landspitali University Hospital in Reykjavik, Iceland in June 2019.

STATEMENT OF AUTHORSHIP

Title of Paper: Emu Oil and Saireito in combination reduce tumour development and clinical indicators of disease in a mouse model of colitis-associated colorectal cancer

Publication Status: Under Review

Publication Details: Biomedicine & Pharmacotherapy

Principal Author

Name of Principal Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Completed cellular and animal trials, sample and data collection, data analyses and interpretation. Prepared manuscript including writing, formatting and submission.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	20|10|20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Junko Fujino		
Contribution to the Paper	Intellectual development.		
Signature		Date	23/9/2020

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodological development, revision and editing of the manuscript.		
Signature		Date	9/10/20

Name of Co-Author	Jóna Freysdóttir		
Contribution to the Paper	Supervision of *in vitro* analyses and interpretation data.		
Signature		Date	28-Sept-2020

Name of Co-Author	Ingibjörg Harðardóttir		
Contribution to the Paper	Supervision of *in vitro* analyses and interpretation data.		
Signature		Date	28 sept 20

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodological development, supervision of analyses, data interpretation, revision and editing of the manuscript.		
Signature		Date	8/10/20

EMU OIL AND SAIREITO IN COMBINATION REDUCE TUMOUR DEVELOPMENT AND CLINICAL INDICATORS OF DISEASE IN A MOUSE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

Lauren C Chartier[a, b], Junko Fujino[c], Gordon S Howarth[a, b, d], Jona Freysdottir[e], Ingibjorg Hardardottir[e] and Suzanne Mashtoub[a, b, f*]

a. Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, Australia
b. Gastroenterology Department, Women's and Children's Hospital, North Adelaide, South Australia, Australia
c. Department of Paediatric Surgery, Saitama Medical Centre, Dokkyo Medical University, Saitama, Japan
d. School of Animal & Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia, Australia
e. Faculty of Medicine, Biomedical Centre, University of Iceland and Landspitali-the National University Hospital of Iceland, Reykjavik, Iceland
f. School of Medicine, The University of Western Australia, Murdoch, Western Australia, Australia

***Corresponding Author**

Dr Suzanne Mashtoub

Gastroenterology Department, Women's and Children's Hospital

72 King William Road, North Adelaide, South Australia 5006

Email: suzanne.mashtoub@adelaide.edu.au

Author Email Addresses: lauren.chartier@adelaide.edu.au; fujunko@dokkyomed.ac.jp; gordon.howarth@adelaide.edu.au; jonaf@landspitali.is; ih@hi.is

ABSTRACT

Background: Emu Oil (EO) previously demonstrated therapeutic potential in a mouse model of colitis-associated CRC (CA-CRC). Saireito, a traditional Japanese medicine, has not been investigated in CA-CRC. **Aim:** To determine whether EO and Saireito could be therapeutic in an azoxymethane (AOM)/dextran sulphate sodium (DSS) model of CA-CRC. **Methods:** Female C57BL/6 mice were assigned to groups (n=10/group); 1) saline control, 2) saline+Saireito, 3) saline+EO, 4) saline+EO/Saireito, 5) AOM/DSS control, 6) AOM/DSS+Saireito, 7) AOM/DSS+EO and 8) AOM/DSS+EO/Saireito. Mice were intraperitoneally injected with saline or AOM (7.4mg/kg) on day 0 and underwent three DSS/water cycles (2%w/v DSS for 7 days, 14 days water). Mice were orally-gavaged with either water (80µL), Saireito (80µL), EO (80µL) or EO/Saireito (160µL; 80µL EO + 80µL Saireito) thrice weekly. Daily bodyweight and disease activity index (DAI) were recorded and colonoscopies performed on days 20, 41 and 62. Mice were euthanized on day 63. $p<0.05$ was considered statistically significant. **Results:** AOM/DSS induced significant bodyweight loss throughout the trial (max -36%), which was attenuated by Saireito (max +7%), EO (max +5%) and EO/Saireito (max +14%; $p<0.05$). AOM/DSS increased DAI compared to saline controls ($p<0.05$), which was reduced by Saireito, EO and EO/Saireito ($p<0.05$). All treatments reduced colonoscopically-assessed colitis severity (days 20 and 41; $p<0.05$). EO/Saireito further decreased colitis severity compared to Saireito and EO alone (day 20; $p<0.05$). Finally, EO and EO/Saireito resulted in fewer colonic tumours compared to AOM/DSS controls ($p<0.05$). **Conclusion:** Combined EO and Saireito reduced disease and tumour development in AOM/DSS mice, suggesting therapeutic potential in CA-CRC.

Keywords: Emu Oil, Kampo medicine, Nutraceuticals, ulcerative colitis, colorectal cancer, mouse model

1.1 INTRODUCTION

Ulcerative colitis (UC) is a debilitating and lifelong inflammatory bowel disease (IBD) that presents as continuous damage and inflammation of the large intestine [1]. IBD pathogenesis is complex and not fully understood, however, a combination of immunological dysregulation, environmental, diet, lifestyle and psychological wellbeing can contribute to disease onset [1, 2]. Furthermore, 8-14% of UC patients have a family history of IBD, highlighting a genetic predisposition. UC has historically been most common in Western societies, although prevalence is now increasing in Asian and Latin American countries [2]. There is currently no cure for UC and patients must resort to anti-inflammatory and immunomodulatory drugs and potent steroids to manage symptoms and maintain remission [2]. Unfortunately, 15% of UC patients will require surgery to remove segments of the colon, or sometimes the entire colon, approximately 20 years following diagnosis [2]. Furthermore, cancer is the major cause of mortality for IBD patients, with UC patients being at an increased risk of developing colitis-associated colorectal cancer (CA-CRC) due to uncontrolled and long-term chronic inflammation [3, 4].

Recently, a disruption of the epithelial barrier and intestinal microbial biodiversity has been a focus of UC and CA-CRC research. Treatments are now centring on restoring intestinal integrity, impeding migration of immune cells and controlling microbial populations via transplants from healthy donors [2, 5, 6]. Naturally-sourced compounds derived from animals or plants have also gained interest as adjunct therapies for gastrointestinal conditions [7-10].

Emu Oil, derived from the subcutaneous and retroperitoneal adipose tissue of the native Australian Emu (*Dromaius Novaehollandiae*) has been investigated as an orally-administered anti-inflammatory agent in pre-clinical settings of gastrointestinal disorders including UC, Crohn's disease, intestinal mucositis and CA-CRC [11-21]. Traditionally, Emu Oil has been used topically by Indigenous Australian people to alleviate pain and promote wound healing [12]. Although the mechanisms are still not fully understood, the anti-inflammatory and anti-oxidant properties of Emu Oil are attributed to the fatty acid composition and the

uncharacterised 2% non-triglyceride fraction of the oil [7, 12, 22]. Previously in a study of CA-CRC in mice, Emu Oil application resulted in decreased clinical indicators of disease and reduced small colonic tumours compared to disease controls over a 9-week experimental period [14]. However, the overall tumour number was unaffected by Emu Oil treatment. Combining Emu Oil with other naturally-sourced compounds could therefore be one means to increase its potency and elicit a more pronounced effect on tumour development in pre-clinical CA-CRC.

Kampo medicines are Traditional Japanese Herbal formulations that comprise various ratios of plant-based constituents. Kampo is widely accepted in Japan with 148 Kampo extractions being covered by national health insurance and approximately 85% of practitioners regularly prescribing these traditional medicines [23]. Kampo is used in conjunction with Western medicines to enhance immunity and relieve adverse drug reactions for a plethora of conditions including gastrointestinal diseases and cancer [24]. Specifically, Daikenchuto and Juzentaihoto have been widely investigated in pre-clinical settings of UC and CRC, whereby these Kampo formulations inhibited tumour growth, decreased levels of inflammatory cytokines (TNF-α and IL-1β) and increased the abundance of natural killer cells [24-26]. Saireito, a combination of two Kampo medicines (Shosaikoto and Goreisan) is composed of 12 herbs; *Bupleuri radix*, *Pinelliae tuber, Alismatis rhizoma, Scutellariae radix, Ginseng radix, Poria, Polyporus, Astractylodis lanceae rhizoma, Zizyphi fructus, Glycyrrhizae radix, Cinnamomi cortex* and *Zingiberis rhizome.* Saireito has displayed anti-inflammatory and immunomodulatory effects in clinical and experimental investigations of UC, chronic hepatitis and rheumatoid arthritis [27-29]. However, Saireito is yet to be investigated in the setting of CA-CRC.

The aim of the current study was to investigate whether Emu Oil and Saireito in combination could reduce the severity of chronic colitis and inhibit the subsequent development of colorectal tumours in an azoxymethane (AOM)/ dextran sulphate sodium (DSS) mouse model of CA-CRC. Furthermore, the anti-inflammatory action of Emu Oil and Kampo medicines is well understood; however, the mechanism of action and effect on inflammatory cytokines has not yet been defined, prompting further analyses *in vitro*.

1.2 MATERIALS AND METHODS

1.2.1 Dendritic cell maturation and ELISA

The potential anti-inflammatory effect of Emu Oil and/or Saireito was determined using dendritic cell (DC) model as described previously (Hardardottir, Olafsdottir et al. 2015). In short, $CD14^+$ monocytes were isolated from peripheral blood mononuclear cells that were obtained from buffy coat from healthy blood donors (Blood Bank, Landspitali - the National University Hospital of Iceland, Reykjavik, Iceland) using CD14 Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The $CD14^+$ monocytes were cultured in RPMI medium supplemented with 10% foetal calf serum (FCS) (both from Gibco, Thermo Fisher Scientific, Paisley, UK) and 12.5 ng/ml interleukin (IL)-4 and 25 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; both from R&D Systems, Bio-techne, Abingdon, UK) for differentiation into immature dendritic cells (imDC). The 48-well tissue culture plate was incubated at 37ºC with 5% CO_2 and 100% humidity for seven days. On day seven, the imDCs were harvested and activated into matured dendritic cells (mDC) by culturing them in 48-well culture plates for 2 days in RPMI medium supplemented with 10% FCS and 10 ng/ml IL-1β, 50 ng/ml tumour necrosis factor (TNF)-α (both from R&D Systems, Bio-techne, Abingdon, UK), and 500 ng/ml lipopolysaccharides (LPS) (Sigma-Aldrich, trading as Merck, Darmstadt, Germany). At the same time 10 µl of 100 µg/ml Saireito, sonicated Emu Oil and combined Emu Oil/Saireito were added to three wells each (final concentration 2 µg/ml) and three wells left without addition. mDCs were harvested and centrifuged (300g for 10 minutes at 4ºC) and supernatant collected and stored at -80°C until used [30].

The maturation of DCs was determined by measuring the concentration of the pro-inflammatory cytokine IL-12p40 and anti-inflammatory cytokine IL-10 in the cell supernatants using DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. Results are presented as secretion index (SI), indicating the concentration of the cytokine in supernatant from cultured DCs cultured with Saireito, sonicated Emu Oil and combined Emu Oil/Saireito,

divided by the concentration of the cytokine in supernatant from DCs cultured without any addition.

1.2.2 Experimental Timeline

Animal studies were conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committees of The University of Adelaide and Children, Youth and Women's Health Service (AE 1079/3/21). Female C57BL/6 mice (C57BL/6JArc, n=80) were sourced from the Animal Resource Centre (Perth, Western Australia, Australia) at 8 weeks of age and were group-housed in standard open-top cages (polypropylene; 470mm x 175mm x 120mm; Crestware Industries) at room temperature with a light/dark cycle of 14:10 hours. Mice were fed standard mouse chow (meat-free mouse diet; Specialty Feeds, Glen Forrest, Western Australia, Australia) and were provided with enrichment items including shredded paper, polycarbonate 'houses' and cardboard toilet paper rolls for the duration of the trial. Plain drinking water was also provided *ad libitum* throughout the trial, except when precluded by group allocation.

Mice (n=10/group) were randomly allocated to eight treatment groups (displayed as intraperitoneal (i.p.) injection + *ad libitum* + oral gavage); 1) Saline + Water + Water, 2) Saline + Water + Saireito, 3) Saline + Water + Emu Oil, 4) Saline + Water + combined Emu Oil and Saireito (Emu Oil/Saireito), 5) AOM + DSS + Water, 6) AOM + DSS + Saireito, 7) AOM + DSS + Emu Oil and 8) AOM + DSS + Emu Oil/Saireito. On day 0, all mice were injected (i.p.) with either saline or the carcinogen AOM (7.4mg/kg; Sigma-Aldrich, Castle Hill New South Wales, Australia) and underwent three DSS/water cycles, each comprising of seven days DSS (2%w/v; 2g/100ml distilled water; MP Biomedicals LLC, Santa Ana California, USA) followed by 14 days of plain water recovery. Starting immediately, all mice were administered thrice weekly via oral gavage with 80µL of either water, Saireito (1g/kg; Tsumura & Co., Tokyo, Japan), Emu Oil (100%; Emu Tracks, Marleston, South Australia, Australia) or a combination of Emu Oil and Saireito (160µL; 80µL Emu Oil + 80µL Saireito) for the duration of the trial. All animals were euthanized on day 63 via CO_2 asphyxiation and cervical dislocation. Blood

was collected via cardiac puncture from groups 1 and 5-8 for intestinal permeability analyses. Visceral organs (heart, liver, lungs, spleen, thymus, kidney, stomach and caecum) were weighed and gastrointestinal organs (small and large intestine) measured and weighed. Sections of the proximal and distal colon were also collected for histological analysis.

1.2.3 Daily measurements

Bodyweight and disease activity index (DAI) were measured daily during routine morning monitoring over the experimental period. DAI was calculated from bodyweight loss, general condition, stool consistency and rectal bleeding as described [11]. Each parameter was scored from 0-3 with increasing severity and then totalled to calculate the DAI score for each mouse on each day, with a maximum obtainable score of 12.

1.2.4 Colonoscopy

At the end of each three-week DSS/water cycle (days 20, 41 and 62), disease progression was monitored via colonoscopy using a high resolution colonoscope (Karl Storz, 1.9mm outer diameter, Tuttlingen, Germany). Mice were individually placed on a heating pad and anesthetised using isoflurane inhalant (AbbVie Pty Ltd, Mascot New South Wales, Australia) for the duration of the colonoscopy procedure. Immediately following the procedure, mice were transferred to a recovery cage on a heating pad and closely and continuously monitored until their behaviour returned to normal. Mice were then returned to their home cage. Videos obtained from the colonoscopy procedures were subsequently scored in a blinded fashion for colitis severity and tumour development as described by Becker et al. (2005). Colitis severity was calculated from five parameters (scored 0-3 with increasing severity) including, thickening of the colon, vasculature pattern, fibrin, granularity of the mucosal surface and stool consistency and summed for a total colitis severity score, with a maximum attainable score of 15 [31]. Colonic tumours were also counted from colonoscopy videos for each mouse at each time-point.

1.2.5 Burrowing analyses

Burrowing behaviour was analysed during the experimental period as a measure of wellbeing and affective state [32]. At baseline (day -1) and at the end of each DSS/water cycle (days 19, 40 and 61), nearing their dark cycle, mice were placed in the dark for at least one hour to acclimatize. In the dark, mice were then placed in individual cages containing an attached burrow with 400g of pre-weighed pebbles (400g; kitty litter 'pebbles'; Black and Gold, Australian Asia/Pacific Wholesalers Pty Ltd, Australia). Mice were then left to burrow for an hour. After this time, mice were removed from the burrowing cages and burrows were re-weighed to determine the amount burrowed for each mouse at each time-point.

1.2.6 Nesting behaviour

Nest building is a normal behaviour that is carried out by mice in their home cage. Mice were provided with shredded paper and tissues in their home cage and nesting was analysed twice per week (every 3-4 days), 24 hours after home cages were changed and cleaned. Scores of 0 or 1 were determined for each treatment group at each time-point, where 0 indicated no nest was built and 1 represented positive nesting behaviour [33, 34].

1.2.7 Intestinal permeability (FITC-dextran assay)

Three hours prior to sacrifice, mice from groups 1 and 5-8, were orally-gavaged with a 500mg/kg dose of fluorescein isothiocyanate (FITC)-dextran (mol wt 4000, 75mg/ml; Sigma, Castle Hill, New South Wales, Australia). Blood was then collected at kill via cardiac puncture. Samples were centrifuged (11,000g at 23°C) for 12 minutes and serum collected. Serum samples were diluted 1:3 with 0.2M PBS and FITC-dextran was quantified using a BioTek Synergy Mx Microplate Reader (BioTek, Winooski, Vermont, USA) and Gen5 version 2.00.18 software relative to a standard curve (0.001-100µg/mL).

1.2.8 Tumour photographs

At time of kill, colons were removed and opened longitudinally to visualise tumours. Photographs of longitudinally-opened mouse colons (Canon 5D Mark IV with 17-40mm lens) were analysed in a blinded fashion using Olympus Soft Imaging Solutions GmbH computer

software analysis version 5.2 (Tokyo, Japan). The number of tumours was determined and categorised into sizes as described previously [14, 17]. Colonic tumours with a diameter <2mm were determined as 'small', 2-3mm as 'medium' and 'large' tumours were those with a diameter >3mm.

1.2.9 Histological analysis

Sections of the proximal and distal colon were routinely processed and embedded in paraffin wax following collection. Sections (4µm) were then stained with haematoxylin and eosin (H&E) and mounted on plain glass microscope slides. Histologically-assessed damage severity was assessed in a blinded-fashion using an Olympus BH-2 light microscope (Olympus Corporation, Tokyo, Japan) as previously described [35]. Six parameters including goblet cell reduction, crypt and crypt cell disruption, polymorphonuclear infiltration and thickening/oedema of the submucosa and muscularis externa were scored from 0-3 with increasing severity for four cross sections of colonic tissue per mouse. Median scores for each parameter were then calculated and summed to determine a final severity score per mouse per colonic section, with a maximum attainable score of 18.

1.2.10 Statistical analyses

Statistical analyses were completed using SPSS, version 25 for Windows (SPSS Inc. Chicago, Illinois, USA). Data were tested for normality using a Shapiro–Wilk test. Bodyweight, DAI, burrowing activity, colonoscopically-assessed colitis score and tumour number were analysed by repeated measures ANOVA with least significance difference (LSD) to compare among and within a group. ELISA results were analysed by two samples t-test (assuming equal variance) at a 95% confidence interval. Nesting behaviour was analysed with a non-parametric Kruskal-Wallis test. FITC-dextran data were logarithmically transformed and analysed using linear regression. Organ data and histologically-assessed severity scores were analysed using a one-way ANOVA with a Tukey's *post hoc* test. For all analyses, $p<0.05$ was considered statistically significant.

1.3 RESULTS

1.3.1 Cytokine secretion by dendritic cells *in vitro*

Culturing dendritic cells in the presence of Emu Oil or in the presence of the combination of Emu Oil and Saireito decreased their secretion of IL-12p40 compared to that when they were cultured in the absence of the nutraceuticals ($p<0.05$; Figure 1). However, culturing dendritic cells in the presence of Saireito alone did not significantly impact their IL-12p40 secretion compared to the negative control. None of the treatments affected dendritic cell secretion of IL-10 (Figure 1).

1.3.2 Bodyweight

In normal mice, Saireito (day 63) and Emu Oil (day 18) slightly increased bodyweight compared to saline controls, with no effect exhibited by the treatments on other days ($p<0.05$; Figure 2a). AOM/DSS induced significant bodyweight loss on most days of the experimental trial compared to saline controls (days 4-16, 25-33, 46-56 and 61; $p<0.05$; Figure 2b). Saireito-administration increased bodyweight of AOM/DSS mice on day 50 and slightly decreased bodyweight on days 41 and 43 compared to AOM/DSS controls ($p<0.05$; Figure 2c). Additionally, Emu Oil increased bodyweight on days 15, 57, 58, 62 and 63 of the trial compared to AOM/DSS controls ($p<0.05$; Figure 2c). However, Emu Oil slightly decreased bodyweight of AOM/DSS mice on days 41-43 compared to AOM/DSS controls ($p<0.05$; Figure 2c). The combination of Emu Oil/Saireito attenuated bodyweight loss in AOM/DSS mice on days 6-12, 29-32 and 47-51 compared to AOM/DSS controls ($p<0.05$; Figure 2c). Finally, the combination of Emu Oil and Saireito further attenuated bodyweight loss of AOM/DSS mice compared to Saireito along (days 6-11, 29-32, 34, 37, 38, 43, 47 and 48) and Emu Oil alone (days 7-18, 20-21, 25-27, 30-35, 38, 46-58 and 60-63; $p<0.05$; Figure 2c).

1.3.3 DAI

In normal mice, administration of Saireito (day 48), Emu Oil (day 57) and Emu Oil/Saireito (day 6) slightly increased DAI scores compared to saline controls ($p<0.05$; Figure 3); however, this result was not consistently observed throughout the nine-week trial. As expected,

AOM/DSS controls displayed significantly increased DAI scores compared to saline controls at most time-points (days 4-19, 21-33, 35-36 and 38-63; $p<0.05$; Figure 3). Saireito (days 6, 9, 12, 13, 15, 17, 19, 20, 22, 24, 25, 27, 29-32, 39, 40, 42, 44-46, 50-56, 59-61 and 63) and Emu Oil (days 6, 9, 12, 16, 17, 19, 21, 22, 25, 27-29, 31, 32, 39, 40, 44-47, 51, 53, 54, 56 and 59-63) significantly decreased DAI scores of AOM/DSS mice, compared to AOM/DSS controls ($p<0.05$; Figure 3). Moreover, the combination of Emu Oil/Saireito in AOM/DSS mice decreased DAI scores in comparison with AOM/DSS controls on days 5-32, 34, 36-40, 42-57 and 59-63 ($p<0.05$; Figure 3). Finally, the Emu Oil/Saireito combination further decreased DAI scores compared to Saireito alone (days 4, 6-10, 24-26, 28, 32, 34, 43 and 46-48) and Emu Oil alone (7-10, 15, 18, 23-26, 30, 38, 40, 42, 45-48, 50 and 55; $p<0.05$; Figure 3).

1.3.4 Colonoscopically-assessed parameters

AOM/DSS mice presented with severe colitis as determined by colonoscopy on days 20, 41 and 62 ($p<0.05$; Figure 4a). Saireito, Emu Oil and the combination of Emu Oil/Saireito significantly reduced colitis severity scores on days 20 and 4, compared to AOM/DSS controls, with no effect exhibited by treatments on day 62 ($p<0.05$; Figure 4a). Furthermore, Emu Oil/Saireito in combination further decreased colitis severity scores in comparison with both Saireito and Emu Oil alone on day 20 ($p<0.05$; Figure 4a).

Colonic tumours were evident in all AOM/DSS mice. AOM/DSS controls presented with the highest number of colonic tumours at all time-points of the trial ($p<0.05$; Figure 4b). Emu Oil-administration significantly decreased the number of tumours on day 20 compared to AOM/DSS controls ($p<0.05$; Figure 4b). Saireito alone did not significantly impact tumour development at any time-point; however, the combination of Emu Oil/Saireito significantly reduced tumour burden on day 41 of the trial ($p<0.05$; Figure 4b), with no effect observed on other days. Finally, Emu Oil/Saireito further decreased tumour number compared to Saireito alone on days 20 and 62 ($p<0.05$; Figure 4b); with no further effect compared to Emu Oil alone ($p>0.05$).

1.3.5 Burrowing

In normal mice, Saireito, Emu Oil or combined Emu Oil/Saireito did not significantly impact burrowing ability on the days analysed ($p>0.05$). AOM/DSS impaired burrowing activity on days 19 and 40 compared to saline controls ($p<0.05$; Figure 5), with no effect exhibited on day 61. Administration of Saireito alone significantly increased burrowing activity of AOM/DSS mice on day 19 compared to AOM/DSS controls ($p<0.05$; Figure 5). Emu Oil and the combination of Emu Oil/Saireito did not affect burrowing behaviour of AOM/DSS mice during the experimental trial.

1.3.6 Nesting behaviour

In normal mice, positive nesting behaviour was evident in all treatment groups (1±0). Furthermore, AOM/DSS did not affect nesting ability as all mice successfully building nests (1±0). Administration of Saireito and Emu Oil, alone and in combination, had no effect on nest building compared to AOM/DSS controls (1±0; $p>0.05$).

1.3.7 Intestinal permeability (FITC-dextran)

FITC-dextran concentrations (ug/ml) were unchanged in AOM/DSS mice compared to normal controls ($p>0.05$; Figure 6). Moreover, Saireito, Emu Oil and combined Emu Oil/Saireito did not impact intestinal permeability in comparison with AOM/DSS controls ($p>0.05$; Figure 6).

1.3.8 Intestinal and visceral organ measurements

In normal animals, weights of the visceral organs and intestinal lengths/weights remained unaffected by administration of Emu Oil, Saireito and the combination of Emu Oil/Saireito ($p>0.05$; Table 1). AOM/DSS mice presented with significantly increased colonic weights compared to saline controls ($p<0.05$; Table 1), with no effect exhibited by Emu Oil, Saireito or the combined treatment ($p>0.05$). Furthermore, colonic shortening was evident in AOM/DSS controls in comparison with saline controls ($p<0.05$; Table 1). Finally, weights of the liver and spleen were increased in AOM/DSS control mice compared to saline controls ($p<0.05$; Table 1), with no effect exhibited by Emu Oil, Saireito or the combination of Emu Oil/ Saireito ($p>0.05$). All other visceral organ weights remained unaffected in AOM/DSS mice (Table 1).

1.3.9 Gross tumour morphology

Mice treated with AOM/DSS presented with significant distal colonic tumours (Figure 7). At kill (day 63) Emu Oil and Saireito-treatment alone did not significantly impact tumour sizes or overall tumour number ($p>0.05$; Figure 8). The numbers of large tumours in Emu Oil, Saireito and the combination of Emu Oil/Saireito treatment groups were less than in AOM/DSS controls; however, this did not achieve statistical significance. Finally, the combination of Emu Oil/Saireito significantly reduced the number of small colonic tumours (diameter <2mm) and the overall number of tumours compared to AOM/DSS controls ($p<0.05$; Figure 8).

1.3.10 Histologically-assessed disease severity

In the proximal colon, there were no significant differences in histologically-assessed severity scores across experimental groups ($p>0.05$; Figure 9). However, AOM/DSS controls presented with significant damage and increased histological severity scores in the distal colon compared with normal controls ($p<0.05$; Figure 9). Neither Emu Oil nor Saireito-treatment impacted histologically-assessed severity scores in the distal colon ($p>0.05$).

1.4 DISCUSSION

In the current study, the combination of Emu Oil and the herbal formula Saireito decreased disease severity in mice with experimentally-induced CA-CRC. Clinical indicators including bodyweight attenuation, DAI, colonoscopically-assessed inflammation and tumour burden were improved by the combined treatment to a greater extent than Emu Oil or Saireito alone. Importantly, in normal mice, administration of Emu Oil/Saireito or each nutraceutical alone, did not result in any adverse side-effects during the nine-weeks of treatment. Minor weight gain and a slightly increased DAI score was evident in normal control mice treated with Emu Oil and Saireito alone; however, this was only observed on a few days throughout the 63-day study and was therefore deemed to be of negligible significance.

Colonoscopy procedures are crucial in the diagnosis and monitoring of humans with IBD and CRC; however, only in the past twenty years has it been executable in pre-clinical mouse

models [36, 37]. Additional to mimicking both human monitoring and diagnosis techniques, the colonoscopy procedure is advantageous during long-term mouse trials as it allows researchers to obtain greater amounts of data during *in vivo* studies with a singular end-point. Furthermore, colonoscopies may also identify when pathogenesis is progressing rapidly or severely, early enough for interventions to be implemented without impacting animal welfare. In the current study, and previous AOM/DSS investigations [14, 38-40], colonoscopies were performed at three time-points at the end of each DSS/water cycle. The nutraceuticals administered in the current study were protective against colitis, with the combination of Emu Oil and Saireito offering greater protection compared to the treatments alone. Interestingly, these protective effects were only observed at the first two colonoscopy time-points (days 20 and 41), with no effect determined on the final procedure (day 62). However, this was not an unexpected finding as the parameters defining colitis severity score assess tissue void of tumours, which inevitably becomes progressively sparse as CRC development is more pronounced [31]. At the final colonoscopy time-point, tumours are often very large and occupy a large circumference of the video diameter, and in severe cases, are almost unpassable. Nonetheless, colonoscopically-assessed colorectal tumours were significantly decreased by Emu Oil and the combination of Emu Oil and Saireito, with the combination resulting in the greatest reduction in tumour number. Notably, these results were reflected in tumour assessment at time of euthanasia, whereby the combination of Emu Oil and Saireito decreased the number of small colonic tumours and overall tumour development.

Although Emu Oil has previously been investigated in CA-CRC [14], the present study is the first to investigate the Japanese Kampo formula, Saireito, in the AOM/DSS model. Gastrointestinal tumours are the most common cause of cancer-related death in Japan and Kampo formulae, including Juzentaihoto, Daikenchuto, Hocheuekkito and Shosaikoto, have been investigated in randomized clinical trials of such cancers [41-43]. Kato et al. (2015) investigated the efficacy of Saireito in 5-Flourouracil (5-FU)-induced mucositis in comparison with Daikenchuto [44]. Similar to that observed in the current study, Saireito-administration

significantly protected against bodyweight loss and diarrhoea compared to 5-FU controls [44]. Furthermore, twice-daily administration of Saireito dose-dependently (100-1000mg/kg) improved histologically-assessed morphological changes, including villus shortening and crypt disruption that were induced by 5-FU [44]. Daikenchuto was also effective at improving these histological parameters but to a lesser effect than Saireito. However, in the current study, there were no significant impacts of Saireito or Emu Oil on histologically-assessed disease severity, although villus height and crypt disruption were not investigated as individual parameters. 5-FU also caused an upregulation of TNF-alpha and IL-1beta mRNA that was attenuated by Saireito at a dose of 1000mg/kg [44]. Furthermore, in the Kato study, tumour growth following implantation was reduced by 5-FU without a specific effect of Saireito on tumourigenesis. Importantly, Saireito significantly decreased diarrhoea and bodyweight loss caused by the chemotherapeutic agent [44], an effect that was also observed in the present study.

The Chinese herbal medicine *Panax notoginseng* and American ginseng (*Panax quinquefolius* L.) have previously increased the anti-tumour effects of chemotherapy and been investigated in AOM/DSS induced CA-CRC [45, 46]. American ginseng decreased colitis severity, DAI and histological severity during the acute pre-neoplastic stage of the study, and later supressed tumour multiplicity [45]. Wang et al. (2016) highlighted that tumour suppression was linked to attenuation of the inflammatory-associated cytokines, IL-1α, IL-1β and IL-6. IL-6 is proposed to be involved in the pathogenesis of IBD and CRC by direct promotion of tumour cell proliferation (*in vitro* and *in vivo)* and survival through activation of the STAT3 pathway, thus creating a tumour-promoting microenvironment [47, 48]. This is largely supported by a study whereby IL-6 knockout mice presented with significantly reduced tumour growth in a STAT3 dependent manner [49]. Additionally, IL-12 and lack of IL-10 have been implicated in the pathogenesis of colitis and specifically, IL-10 deficient mice can develop UC and CRC just weeks after birth [50]. In the current study, the effects of Emu Oil and Saireito on secretion of IL-12p40 and IL-10 from mDCs were analysed *in vitro*. Cells treated with Emu Oil and the combination of Emu Oil and Saireito exhibited potent anti-inflammatory potential by reducing

the secretion of the pro-inflammatory cytokine IL-12p40; an effect that was not observed following treatment of Saireito alone. Moreover, the anti-inflammatory cytokine IL-10 was unaffected by nutraceutical treatment. Future investigations should determine the effects of the selected nutraceuticals on IL-12, IL-10 and IL-6 in intestinal tissue collected from CA-CRC animal studies. Furthermore, novel therapies could target IL-6 as a primary mediator for inflammation-associated carcinogenesis.

Techniques that monitor pain, stress and daily-living behaviour are important in laboratory animal studies in order to evaluate how disease or treatments are impacting overall animal well-being [51]. Recently, DAI, facial grimace and burrowing were assessed and compared in CA-CRC mice [52]. It was concluded, that DAI was the most reliable method of welfare evaluation in this model of chronic gastrointestinal disease [52]. In the present study, burrowing and nesting techniques were utilised, whereby there was a significant improvement in burrowing activity of AOM/DSS mice treated with Saireito on day 19 of the trial. However, nesting behaviour was not impacted in AOM/DSS mice compared to normal controls or by nutraceutical treatment. Although the present study scored nesting using the simplest scoring method of a zero for no nest and a one for a successful nest built, future studies in the AOM/DSS model could evaluate nesting in a more complex format using methods described by Deacon et al. (2003) and Paumier et al. (2013) [53, 54]. Moreover, hoarding and judgement bias techniques may also be of interest in the AOM/DSS model [53, 55].

In conclusion, the combination of Emu Oil and Saireito represents a promising adjuvant treatment to conventional CA-CRC treatments. This combination improved clinical indicators of CA-CRC while reduced numbers of colonic tumours. However, further investigation into the effects of these nutraceuticals on inflammatory-associated cytokines, such as IL-6, linked to the pathogenesis of CA-CRC is crucial to understand the mechanism by which Emu Oil and Saireito exert their beneficial action in combination.

REFERENCES

[1] R. Ungaro, S. Mehandru, P.B. Allen, L. Peyrin-Biroulet, J.F. Colombel. Ulcerative colitis. Lancet. 389 (2017) 1756-1770.

[2] M. Eisenstein. Ulcerative colitis: towards remission. Nature. 563 (2018) S33.

[3] L. Biancone, A. Armuzzi, M.L. Scribano, F. Castiglione, R. D'Inca, A. Orlando, C. Papi, M. Daperno, M. Vecchi, G. Riegler, W. Fries, P. Alvisi, G. Meucci, F. Mocciaro, F. Rogai, S. Festa, L. Guidi, A. Testa, L. Spina, S. Renna, A. Viola, M. Patturelli, R. Di Mitri, I. Frankovic, E. Calabrese, C. Petruzziello, E. De Cristofaro, G. Sena, A. Ruffa, B. Neri, A. Rossi, D. Italian Group for the Study of Inflammatory Bowel. Cancer Risk in Inflammatory Bowel Disease: A 6-Year Prospective Multicenter Nested Case-Control IG-IBD Study. Inflamm Bowel Dis. (2019).

[4] L. Herszenyi, L. Barabas, P. Miheller, Z. Tulassay. Colorectal cancer in patients with inflammatory bowel disease: the true impact of the risk. Dig Dis. 33 (2015) 52-57.

[5] T. Klag, E.F. Stange, J. Wehkamp. Defective antibacterial barrier in inflammatory bowel disease. Dig Dis. 31 (2013) 310-316.

[6] A.D. Kostic, R.J. Xavier, D. Gevers. The microbiome in inflammatory bowel disease: current status and the future ahead. Gastroenterology. 146 (2014) 1489-1499.

[7] L.C. Chartier, G.S. Howarth, S. Mashtoub. Combined Nutraceuticals: A Novel Approach to Colitis-Associated Colorectal Cancer? Nutr Cancer. (2019) 1-8.

[8] T.S. Ahn, D.G. Kim, N.R. Hong, H.S. Park, H. Kim, K.T. Ha, J.H. Jeon, I. So, B.J. Kim. Effects of Schisandra chinensis extract on gastrointestinal motility in mice. Journal of ethnopharmacology. 169 (2015) 163-169.

[9] K.K. Auyeung, Q.B. Han, J.K. Ko. Astragalus membranaceus: A Review of its Protection Against Inflammation and Gastrointestinal Cancers. Am J Chin Med. 44 (2016) 1-22.

[10] F.C. Blum, J. Singh, D.S. Merrell. In vitro activity of neem (Azadirachta indica) oil extract against Helicobacter pylori. Journal of ethnopharmacology. 232 (2019) 236-243.

[11] S.M. Abimosleh, R.J. Lindsay, R.N. Butler, A.G. Cummins, G.S. Howarth. Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. Dig Dis Sci. 57 (2012) 887-896.

[12] S.M. Abimosleh, C.D. Tran, G.S. Howarth. Emu Oil: a novel therapeutic for disorders of the gastrointestinal tract? J Gastroenterol Hepatol. 27 (2012) 857-861.

[13] S.M. Abimosleh, C.D. Tran, G.S. Howarth. Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. Evid Based Complement Alternat Med. 2013 (2013) 10.

[14] L.C. Chartier, G.S. Howarth, I.C. Lawrance, D. Trinder, S.J. Barker, S. Mashtoub. Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. Dig Dis Sci. 63 (2018) 135-145.

[15] L.C. Chartier, K.E. Maiolo, G.S. Howarth, I. Lawrance, D. Trinder, S.J. Barker, B. Scherer, C.J. Mitchell, S. Mashtoub. Emu Oil Improves Clinical Indicators of Disease and Reduces Proximal Colonic Crypt Hyperplasia in a Murine Model of Colitis-Associated Colorectal Cancer. Gastroenterology. 154 (2018) S875-S875.

[16] S. Mashtoub, R. Ghaemi, I. Lawrance, D. Trinder, G.S. Howarth. Emu Oil attenuates Disease Severity in Mouse Models of Colitis and Inflammation-Associated Colorectal Cancer. Gastroenterology. 150 (2016) S1154-S1154.

[17] S. Mashtoub, G.S. Howarth, D. Trinder, I. Lawrance. Emu Oil Attenuates Disease Severity and Results in Fewer Large Colonic Tumours in a Mouse Model of Colitis-Associated Colorectal Cancer. Gastroenterology. 152 (2017) S737-S737.

[18] S. Mashtoub, L.S. Lampton, G.L. Eden, K.Y. Cheah, K.A. Lymn, J.E. Bajic, G.S. Howarth. Emu Oil Combined with Lyprinol (TM) Reduces Small Intestinal Damage in a Rat Model of Chemotherapy-Induced Mucositis. Nutrition and Cancer-an International Journal. 68 (2016) 1171-1180.

[19] S. Mashtoub, R.J. Lindsay, K.A. Lymn, T.W.V. Acott, R. Yazbeck, A.G. Cumins, R.N. Butler, G.S. Howarth. EMU oil increases crypt depth but only minimally affects other indicators of colonic integrity in a rat model of colitis. J Gastroen Hepatol. 24 (2009) A243-A244.

[20] S. Mashtoub, C.D. Tran, G.S. Howarth. Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. Exp Biol Med (Maywood). 238 (2013) 1305-1317.

[21] R.J. Lindsay, M.S. Geier, R. Yazbeck, R.N. Butler, G.S. Howarth. Orally administered emu oil decreases acute inflammation and alters selected small intestinal parameters in a rat model of mucositis. British Journal of Nutrition. 104 (2010) 513-519.

[22] S. Yoganathan, R. Nicolosi, T. Wilson, G. Handelman, P. Scollin, R. Tao, P. Binford, F. Orthoefer. Antagonism of croton oil inflammation by topical emu oil in CD-1 mice. Lipids. 38 (2003) 603-607.

[23] Y. Motoo, T. Seki, K. Tsutani. Traditional Japanese medicine, Kampo: its history and current status. Chin J Integr Med. 17 (2011) 85-87.

[24] T. Nagata, K. Toume, L.X. Long, K. Hirano, T. Watanabe, S. Sekine, T. Okumura, K. Komatsu, K. Tsukada. Anticancer effect of a Kampo preparation Daikenchuto. J Nat Med. 70 (2016) 627-633.

[25] T. Matsunaga, S. Hashimoto, N. Yamamoto, R. Kawasato, T. Shirasawa, A. Goto, K. Fujisawa, T. Takami, T. Okamoto, J. Nishikawa, I. Sakaida. Protective Effect of Daikenchuto on Dextran Sulfate Sodium-Induced Colitis in Mice. Gastroenterol Res Pract. 2017 (2017) 1298263.

[26] J. Yamakawa, Y. Motoo, J. Moriya, M. Ogawa, H. Uenishi, S. Akazawa, T. Sasagawa, M. Nishio, J. Kobayashi. Role of Kampo medicine in integrative cancer therapy. Evid Based Complement Alternat Med. 2013 (2013) 570848.

[27] M.J. Borigini, M.J. Egger, H.J. Williams, H.E. Paulus, J.R. Ward. TJ-114 (Sairei-To), an Herbal Medicine in Rheumatoid Arthritis. J Clin Rheumatol. 2 (1996) 309-316.

[28] H. Miki, K. Tokuhara, M. Oishi, R. Nakatake, Y. Tanaka, M. Kaibori, M. Nishizawa, T. Okumura, M. Kon. Japanese Kampo Saireito Has a Liver-Protective Effect Through the

Inhibition of Inducible Nitric Oxide Synthase Induction in Primary Cultured Rat Hepatocytes. JPEN J Parenter Enteral Nutr. 40 (2016) 1033-1041.

[29] T. Watanabe, T. Yamamoto, M. Yoshida, K. Fujiwara, N. Kageyama-Yahara, H. Kuramoto, Y. Shimada, M. Kadowaki. The traditional herbal medicine saireito exerts its inhibitory effect on murine oxazolone-induced colitis via the induction of Th1-polarized immune responses in the mucosal immune system of the colon. Int Arch Allergy Immunol. 151 (2010) 98-106.

[30] I. Hardardottir, E.S. Olafsdottir, J. Freysdottir. Dendritic cells matured in the presence of the lycopodium alkaloid annotine direct T cell responses toward a Th2/Treg phenotype. Phytomedicine : international journal of phytotherapy and phytopharmacology. 22 (2015) 277-282.

[31] C. Becker, M.C. Fantini, S. Wirtz, A. Nikolaev, R. Kiesslich, H.A. Lehr, P.R. Galle, M.F. Neurath. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. Gut. 54 (2005) 950-954.

[32] P. Jirkof, K. Leucht, N. Cesarovic, M. Caj, F. Nicholls, G. Rogler, M. Arras, M. Hausmann. Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice. Lab Anim. 47 (2013) 274-283.

[33] T.M. Ballard, M. Pauly-Evers, G.A. Higgins, A.M. Ouagazzal, V. Mutel, E. Borroni, J.A. Kemp, H. Bluethmann, J.N. Kew. Severe impairment of NMDA receptor function in mice carrying targeted point mutations in the glycine binding site results in drug-resistant nonhabituating hyperactivity. J Neurosci. 22 (2002) 6713-6723.

[34] C.A. Browne, G. Clarke, T.G. Dinan, J.F. Cryan. An effective dietary method for chronic tryptophan depletion in two mouse strains illuminates a role for 5-HT in nesting behaviour. Neuropharmacology. 62 (2012) 1903-1915.

[35] R. Yazbeck, G.S. Howarth, M.S. Geier, H.U. Demuth, C.A. Abbott. Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice. Front Biosci. 13 (2008) 6850-6858.

[36] C. Becker, M.C. Fantini, M.F. Neurath. High resolution colonoscopy in live mice. Nat Protoc. 1 (2006) 2900-2904.

[37] E.H. Huang, J.J. Carter, R.L. Whelan, Y.H. Liu, J.O. Rosenberg, H. Rotterdam, A.M. Schmidt, D.M. Stern, K.A. Forde. Colonoscopy in mice. Surg Endosc. 16 (2002) 22-24.

[38] E. Lippert, P. Ruemmele, F. Obermeier, S. Goelder, C. Kunst, G. Rogler, N. Dunger, H. Messmann, A. Hartmann, E. Endlicher. Anthocyanins Prevent Colorectal Cancer Development in a Mouse Model. Digestion. 95 (2017) 275-280.

[39] N. Seiwert, J. Fahrer, G. Nagel, J. Frank, D. Behnam, B. Kaina. Curcumin Administered as Micellar Solution Suppresses Intestinal Inflammation and Colorectal Carcinogenesis. Nutr Cancer. (2020) 1-8.

[40] S.P. Sharp, R.A. Malizia, T. Walrath, S.S. D'Souza, C.J. Booth, B.J. Kartchner, E.C. Lee, S.C. Stain, W. O'Connor, Jr. DNA damage response genes mark the early transition from colitis to neoplasia in colitis-associated colon cancer. Gene. 677 (2018) 299-307.

[41] J.J. Gao, P.P. Song, F.H. Qi, N. Kokudo, X.J. Qu, W. Tang. Evidence-based research on traditional Japanese medicine, Kampo, in treatment of gastrointestinal cancer in Japan. Drug Discov Ther. 6 (2012) 1-8.

[42] F. Ikegami, M. Sumino, Y. Fujii, T. Akiba, T. Satoh. Pharmacology and toxicology of Bupleurum root-containing Kampo medicines in clinical use. Hum Exp Toxicol. 25 (2006) 481-494.

[43] K. Yoshikawa, M. Shimada, M. Nishioka, N. Kurita, T. Iwata, S. Morimoto, T. Miyatani, M. Komatsu, H. Kashihara, C. Mikami. The effects of the Kampo medicine (Japanese herbal medicine) "Daikenchuto" on the surgical inflammatory response following laparoscopic colorectal resection. Surg Today. 42 (2012) 646-651.

[44] S. Kato, S. Hayashi, Y. Kitahara, K. Nagasawa, H. Aono, J. Shibata, D. Utsumi, K. Amagase, M. Kadowaki. Saireito (TJ-114), a Japanese traditional herbal medicine, reduces 5-fluorouracil-induced intestinal mucositis in mice by inhibiting cytokine-mediated apoptosis in intestinal crypt cells. PLoS One. 10 (2015) e0116213.

[45] C.Z. Wang, C. Yu, X.D. Wen, L. Chen, C.F. Zhang, T. Calway, Y. Qiu, Y. Wang, Z. Zhang, S. Anderson, Y. Wang, W. Jia, C.S. Yuan. American Ginseng Attenuates Colitis-Associated Colon Carcinogenesis in Mice: Impact on Gut Microbiota and Metabolomics. Cancer Prev Res (Phila). 9 (2016) 803-811.

[46] X.D. Wen, C.Z. Wang, C. Yu, L. Zhao, Z. Zhang, A. Matin, Y. Wang, P. Li, S.Y. Xiao, W. Du, T.C. He, C.S. Yuan. Panax notoginseng attenuates experimental colitis in the azoxymethane/dextran sulfate sodium mouse model. Phytother Res. 28 (2014) 892-898.

[47] M.J. Waldner, M.F. Neurath. Master regulator of intestinal disease: IL-6 in chronic inflammation and cancer development. Semin Immunol. 26 (2014) 75-79.

[48] H. Lahm, D. Petral-Malec, A. Yilmaz-Ceyhan, J.R. Fischer, M. Lorenzoni, J.C. Givel, N. Odartchenko. Growth stimulation of a human colorectal carcinoma cell line by interleukin-1 and -6 and antagonistic effects of transforming growth factor beta 1. Eur J Cancer. 28A (1992) 1894-1899.

[49] S. Grivennikov, E. Karin, J. Terzic, D. Mucida, G.Y. Yu, S. Vallabhapurapu, J. Scheller, S. Rose-John, H. Cheroutre, L. Eckmann, M. Karin. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell. 15 (2009) 103-113.

[50] M.J. Waldner, M.F. Neurath. Cytokines in colitis associated cancer: potential drug targets? Inflamm Allergy Drug Targets. 7 (2008) 187-194.

[51] P. Jirkof. Burrowing and nest building behavior as indicators of well-being in mice. J Neurosci Methods. 234 (2014) 139-146.

[52] L.C. Chartier, M.L. Hebart, G.S. Howarth, A.L. Whittaker, S. Mashtoub. Affective state determination in a mouse model of colitis-associated colorectal cancer. PLoS One. 15 (2020) e0228413.

[53] R.M. Deacon, C. Penny, J.N. Rawlins. Effects of medial prefrontal cortex cytotoxic lesions in mice. Behav Brain Res. 139 (2003) 139-155.

[54] K.L. Paumier, S.J. Sukoff Rizzo, Z. Berger, Y. Chen, C. Gonzales, E. Kaftan, L. Li, S. Lotarski, M. Monaghan, W. Shen, P. Stolyar, D. Vasilyev, M. Zaleska, D.H. W, J. Dunlop. Behavioral characterization of A53T mice reveals early and late stage deficits related to Parkinson's disease. PLoS One. 8 (2013) e70274.

[55] R.P. George, T.H. Barker, K.A. Lymn, D.A. Bigatton, G.S. Howarth, A.L. Whittaker. A Judgement Bias Test to Assess Affective State and Potential Therapeutics in a Rat Model of Chemotherapy-Induced Mucositis. Sci Rep. 8 (2018) 8193.

ACKNOWLEDGEMENTS

LCC is supported by an Australian Government Research Training Program Scholarship and receives a top-up scholarship from AgriFutures Australia.

DISCLOSURE OF INTERESTS

The authors declare that there are no conflicts of interest.

FIGURE LEGENDS

Figure 1. Secretion of IL-12p40 and IL-10 by mature dendritic cells. Dendritic cells were matured with IL-1β, TNF-α and LPS for 24 h in the absence (negative) or presence of Saireito, Emu Oil or the combination of Emu Oil and Saireito at the concentration of 2μg/ml. The concentrations of IL-12p40 and IL-10 in the supernatants were determined by ELISA. The data are presented as SI, i.e. the concentration of each cytokine in the supernatant of cells in the presence of the nutraceutical divided by the concentration of the cytokine in the supernatant of cells in the absence of the nutraceutical. Data are expressed as mean ± SEM (n=2 donors). **p<0.01, *p<0.05 compared to negative.

Figure 2. Daily bodyweight change of (a) Saline mice, (b) Saline and AOM/DSS controls and (c) AOM/DSS mice. Data are expressed as mean (% starting bodyweight) ± SEM (n=10/group). ***p<0.001, **p<0.01, *p<0.05 compared to Saline + Water + Water;^^^p<0.001, ^^p<0.01, ^p<0.05 compared to AOM + DSS + Water; ###p<0.001, ##p<0.01, #p<0.05 compared to AOM + DSS + Saireito; $$$p<0.001, $$p<0.01, $p<0.05 compared to AOM + DSS + Emu Oil on the same day.

Figure 3. Daily Disease Activity Index (DAI) score. Data are expressed as mean ± SEM (n =10/group). ***p<0.001, **p<0.01, *p<0.05 compared to Saline + Water + Water;^^^p<0.001, ^^p<0.01, ^p<0.05 compared to AOM + DSS + Water; ###p<0.001, ##p<0.01, #p<0.05 compared to AOM + DSS + Saireito; $$$p<0.001, $$p<0.01, $p<0.05 compared to AOM + DSS + Emu Oil on the same day.

Figure 4. Colonoscopically-assessed (a) colitis severity and (b) tumour development. Data are expressed as mean (colitis severity score or tumour number) ± SEM (n=10/group). ***p<0.001, compared to Saline + Water + Water; ^^^p<0.001, ^^p<0.01, ^p<0.05 compared to AOM + DSS + Water; ###p<0.001, ##p<0.01, #p<0.05 compared to AOM + DSS + Saireito; $$$p<0.001 compared to AOM + DSS + Emu Oil on the same day.

Figure 5. Burrowing ability of mice. Data are expressed as mean (amount burrowed) ± SEM (n=10/group). ***p<0.001, *p<0.05 compared to Saline + Water + Water; ^^p<0.01 compared to AOM + DSS + Water on the same day.

Figure 6. Intestinal permeability assessed by FITC-dextran levels. Data are expressed as mean serum FITC-dextran (μg/ml) ± SEM (n=10/group).

Figure 7. Photos of longitudinally-opened mouse colons taken at time of euthanasia.

Figure 8. Tumour number obtained from longitudinally-opened colons. Data are expressed as mean (tumour number) ± SEM (n=10/group). Colonic tumours with a diameter <2mm were determined as 'small', 2-3mm as 'medium' and a diameter of >3mm was 'large'. ***p<0.001, **p<0.01, *p<0.05 compared to Saline + Water + Water; ^p<0.05 compared to AOM + DSS + Water; #p<0.05 compared to AOM + DSS + Saireito.

Figure 9. Histologically-assessed disease severity scores of the proximal and distal colon. Data are expressed as mean (severity score) ± SEM (n=10/group). ***p<0.001 compared to Saline + Water + Water.

Table 1. Intestinal and visceral organ measurements

	Saline + Water + Water	Saline + Water + Saireito	Saline + Water + EO	Saline + Water + EO/Saireito	AOM + DSS + Water	AOM + DSS + Saireito	AOM + DSS + EO	AOM + DSS + EO/ Saireito
	Visceral Organ Weights (% relative to bodyweight and *10^{-2})							
Heart	0.50 ± 0.01	0.50 ± 0.02	0.52 ± 0.03	0.52 ± 0.02	0.50 ± 0.03	0.53 ± 0.03	0.56 ± 0.02	0.54 ± 0.02
Liver	4.62 ± 0.12	4.52 ± 0.14	4.64 ± 0.16	4.31 ± 0.15	5.34 ± 0.16**	5.18 ± 0.17	5.76 ± 0.33	5.57 ± 0.11
Spleen	0.35 ± 0.01	0.38 ± 0.01	0.40 ± 0.01	0.37 ± 0.13	0.86 ± 0.07***	0.77 ± 0.07	0.80 ± 0.06	0.82 ± 0.08
Thymus	0.21 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.19 ± 0.02	0.18 ± 0.03	0.24 ± 0.03	0.23 ± 0.03	0.24 ± 0.01
Lung	0.68 ± 0.04	0.75 ± 0.03	0.73 ± 0.02	0.75 ± 0.02	0.68 ± 0.02	0.72 ± 0.02	0.73 ± 0.04	0.71 ± 0.01
L Kidney	0.48 ± 0.01	0.49 ± 0.01	0.48 ± 0.02	0.47 ± 0.02	0.49 ± 0.02	0.52 ± 0.01	0.55 ± 0.01	0.55 ± 0.02
R Kidney	0.51 ± 0.02	0.51 ± 0.02	0.55 ± 0.02	0.50 ± 0.01	0.55 ± 0.01	0.54 ± 0.01	0.53 ± 0.04	0.58 ± 0.03
Stomach	0.79 ± 0.03	0.78 ± 0.03	0.84 ± 0.05	0.83 ± 0.04	0.71 ± 0.03	0.73 ± 0.01	0.69 ± 0.06	0.76 ± 0.02

	Saline + Water + Water	Saline + Water + Saireito	Saline + Water + EO	Saline + Water + EO/Saireito	AOM + DSS + Water	AOM + DSS + Saireito	AOM + DSS + EO	AOM + DSS + EO/ Saireito
Intestinal Weights (% relative to bodyweight and *10^{-2})								
Colon	1.03 ± 0.03	0.89 ± 0.03	0.94 ± 0.04	0.88 ± 0.04	1.71 ± 0.18***	1.88 ± 0.17	1.82 ± 0.38	1.81 ± 0.22
Duodenum	0.51 ± 0.04	0.53 ± 0.02	0.53 ± 0.02	0.54 ± 0.03	0.51 ± 0.04	0.55 ± 0.03	0.46 ± 0.03	0.54 ± 0.00
Small Intestine	3.55 ± 0.14	4.79 ± 1.47	3.52 ± 0.06	3.42 ± 0.12	3.61 ± 0.16	3.64 ± 0.12	3.38 ± 0.20	3.76 ± 0.11
Intestinal Lengths (cm)								
Colon (cm)	8.01 ± 0.15	7.16 ± 0.30	7.58 ± 0.28	7.2 ± 0.33	5.55 ± 0.24***	6.95 ± 0.23	6.33 ± 0.17	6.25 ± 0.43
Duodenum (cm)	3.96 ± 0.07	3.47 ± 0.11	3.51 ± 0.10	3.84 ± 0.20	4.05 ± 0.17	3.98 ± 0.12	3.8 ± 0.15	3.7 ± 0.20
Small Intestine (cm)	29.2 ± 0.53	28.7 ± 0.45	28.8 ± 0.36	27.1 ± 0.61	30.25 ± 1.11	29.33 ± 0.33	29.67 ± 1.45	30.75 ± 0.48

Figure 1.

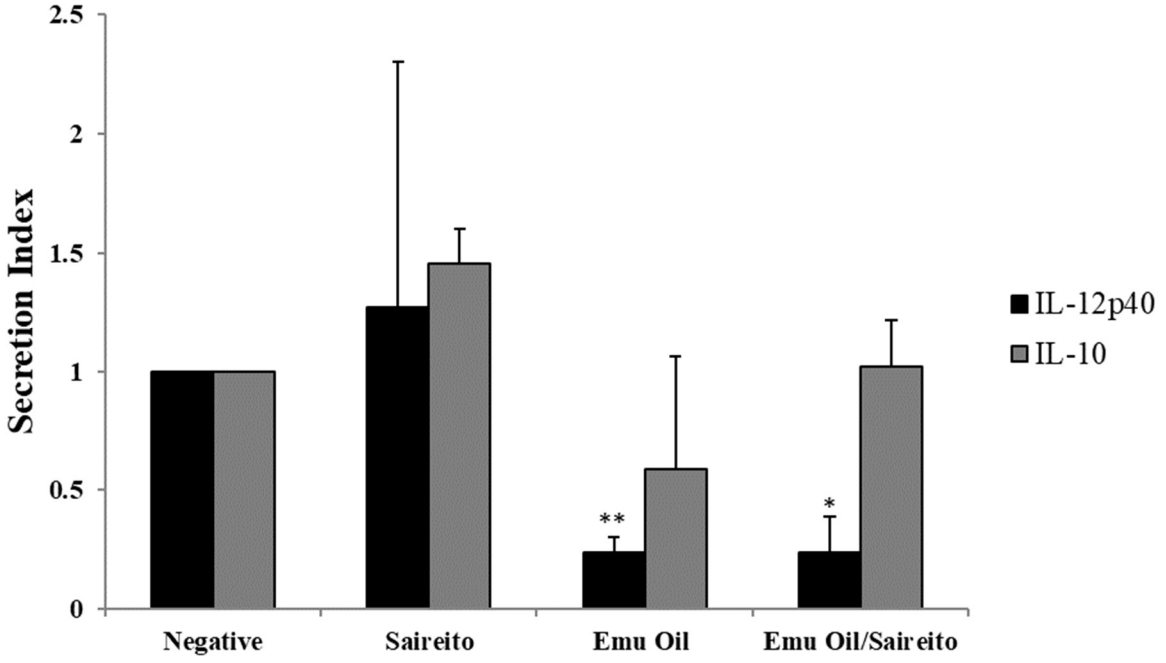

Secretion Index
2.5
2
1.5
1
0.5
0
**
*
Negative
Saireito
Emu Oil
Emu Oil/Saireito
IL-12p40
IL-10

Figure 2a.

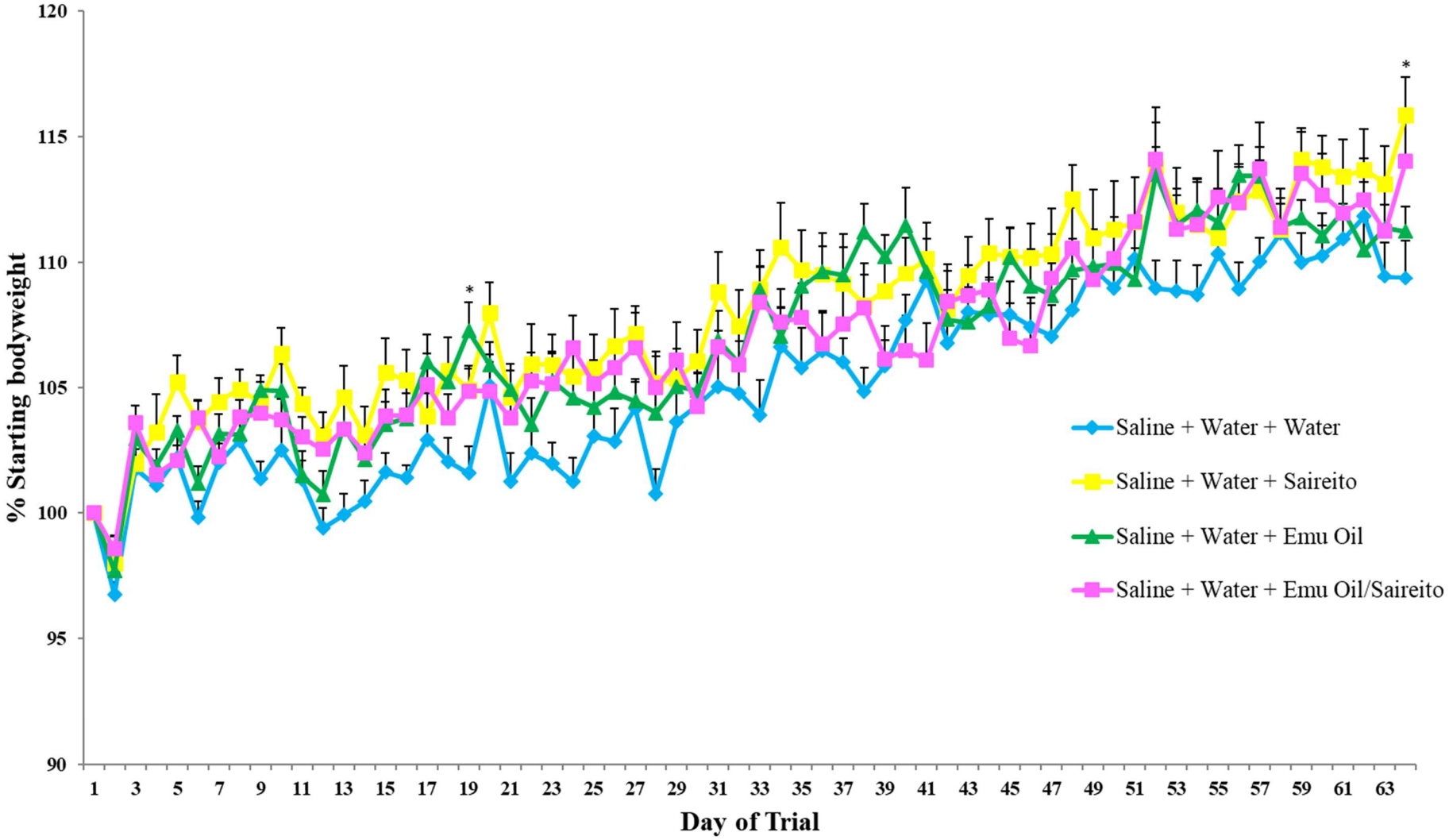

120
115
110
105
100
95
90
% Starting bodyweight
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63
Day of Trial
*
*
Saline + Water + Water
Saline + Water + Saireito
Saline + Water + Emu Oil
Saline + Water + Emu Oil/Saireito

Figure 2b.

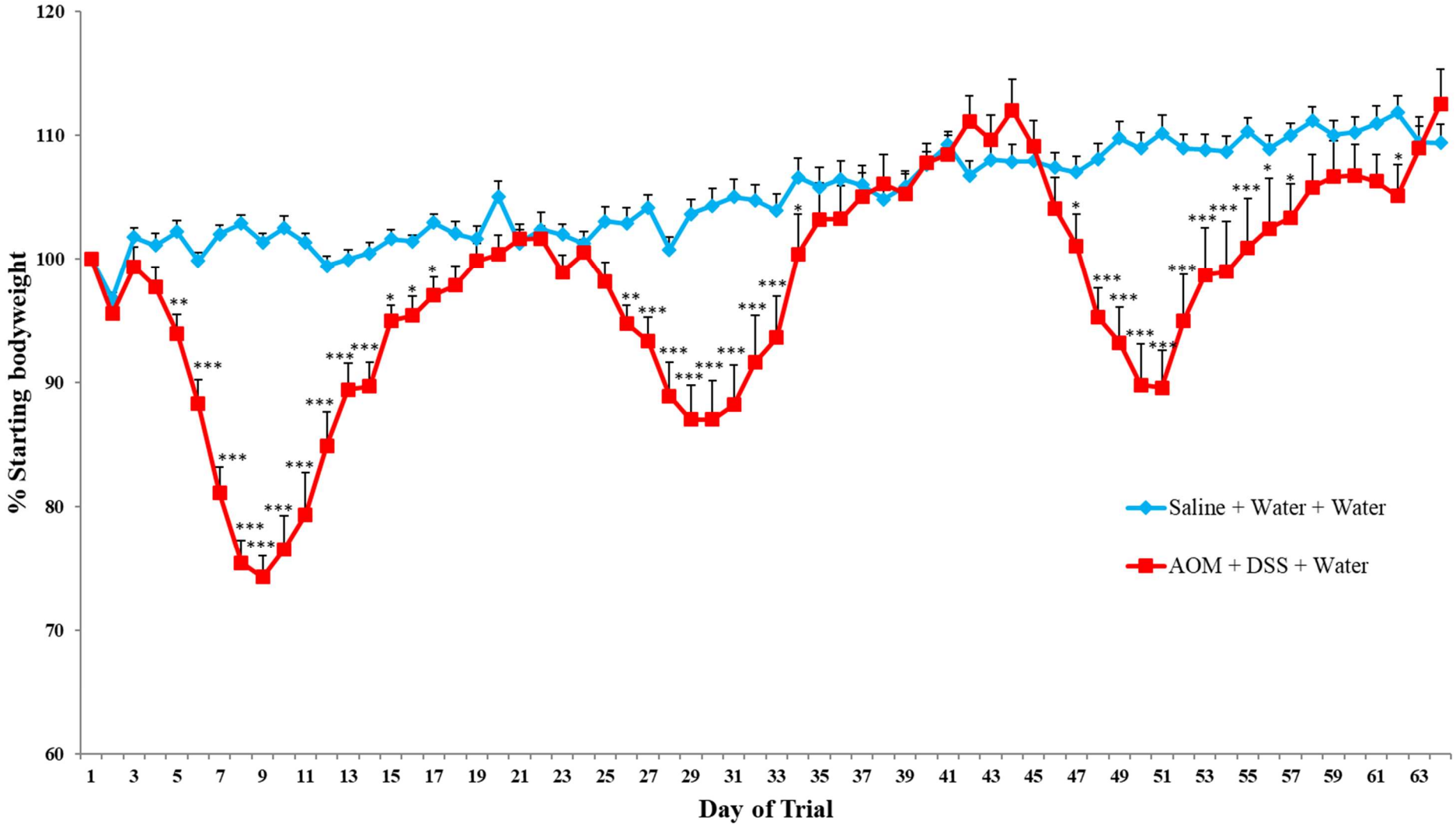

% Starting bodyweight
Day of Trial
Saline + Water + Water
AOM + DSS + Water

Figure 2c.

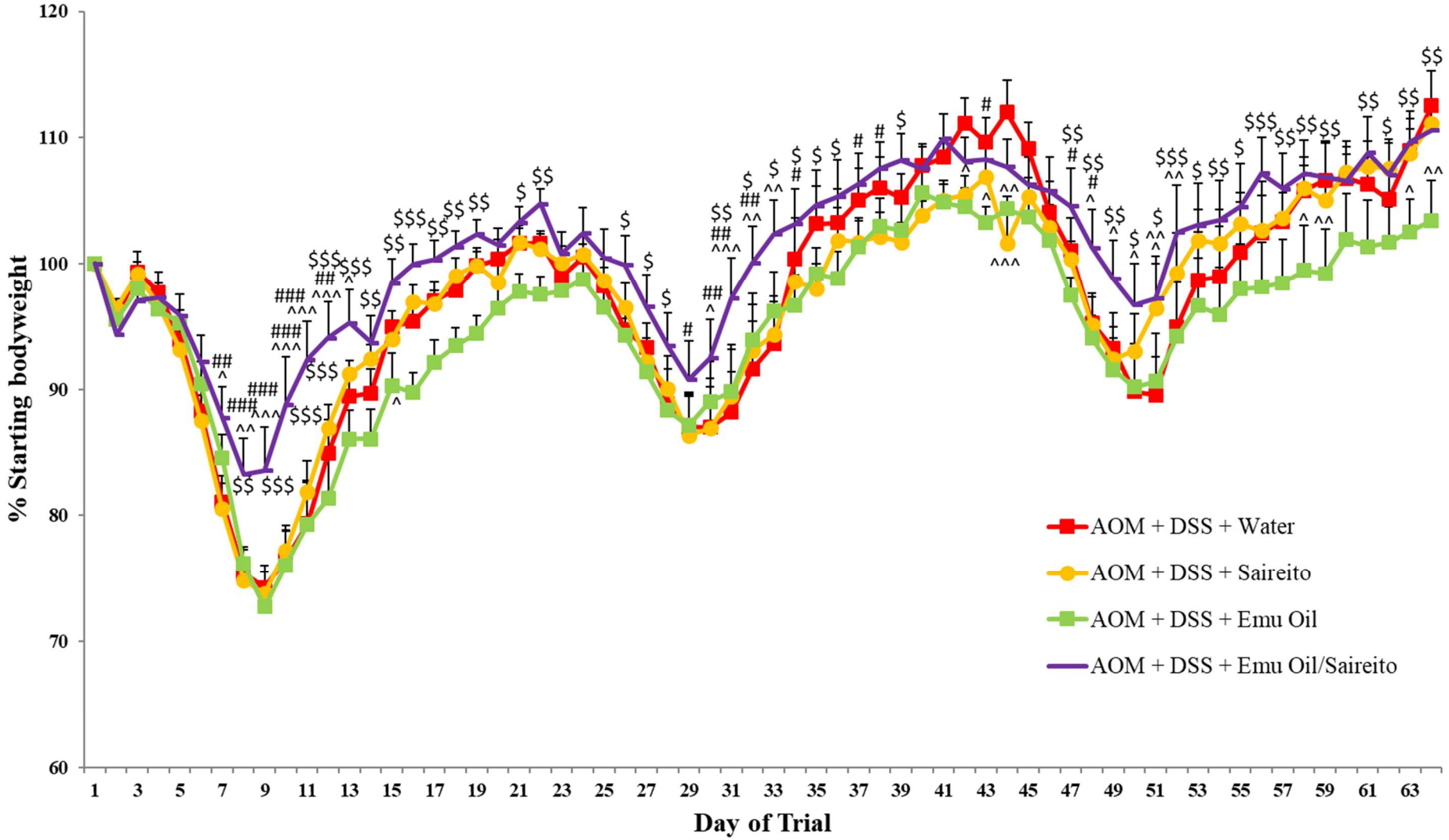

% Starting bodyweight
Day of Trial
AOM + DSS + Water
AOM + DSS + Saireito
AOM + DSS + Emu Oil
AOM + DSS + Emu Oil/Saireito

Figure 3.

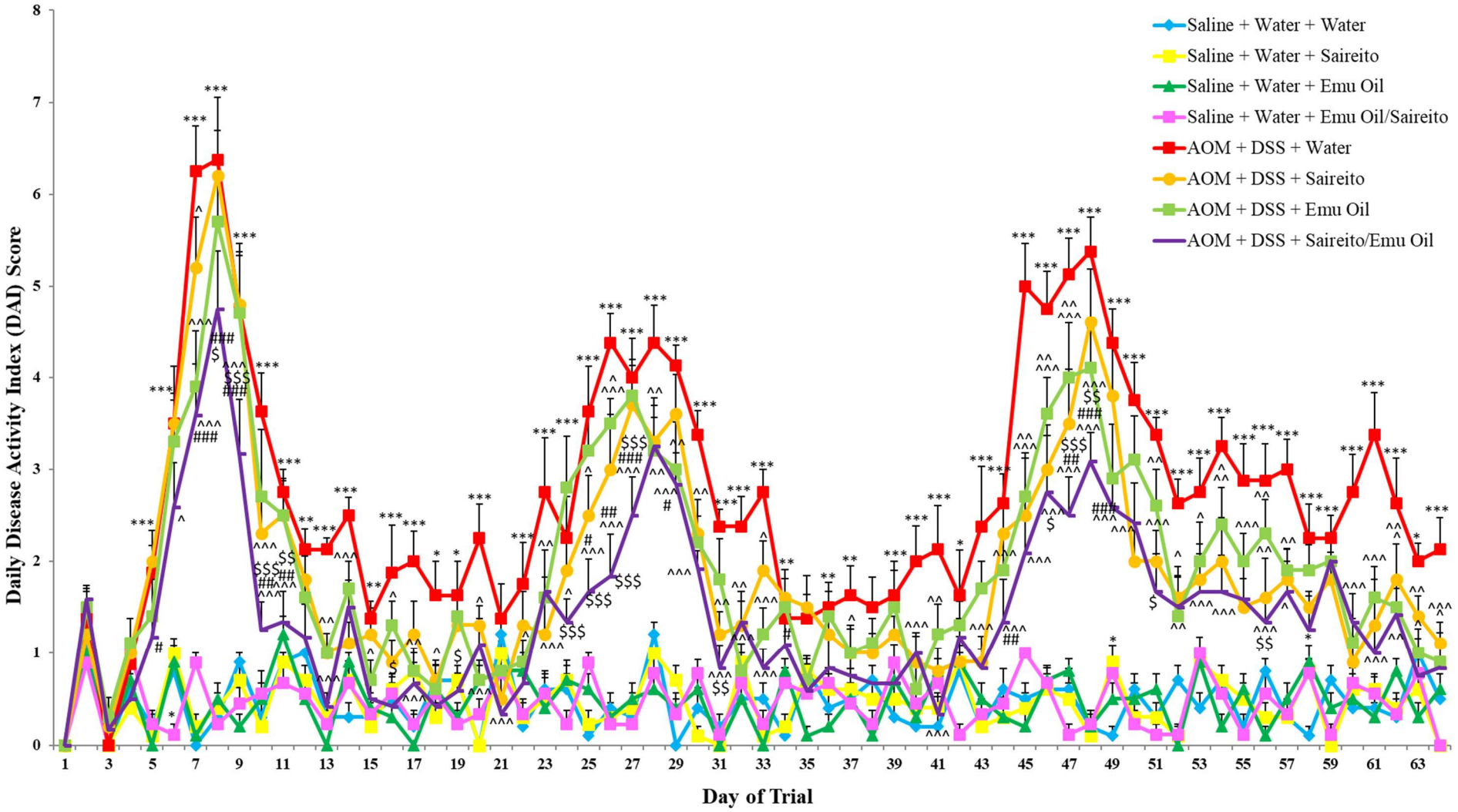

Saline + Water + Water
Saline + Water + Saireito
Saline + Water + Emu Oil
Saline + Water + Emu Oil/Saireito
AOM + DSS + Water
AOM + DSS + Saireito
AOM + DSS + Emu Oil
AOM + DSS + Saireito/Emu Oil
Daily Disease Activity Index (DAI) Score
Day of Trial
0
1
2
3
4
5
6
7
8
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
49
51
53
55
57
59
61
63

Figure 4a.

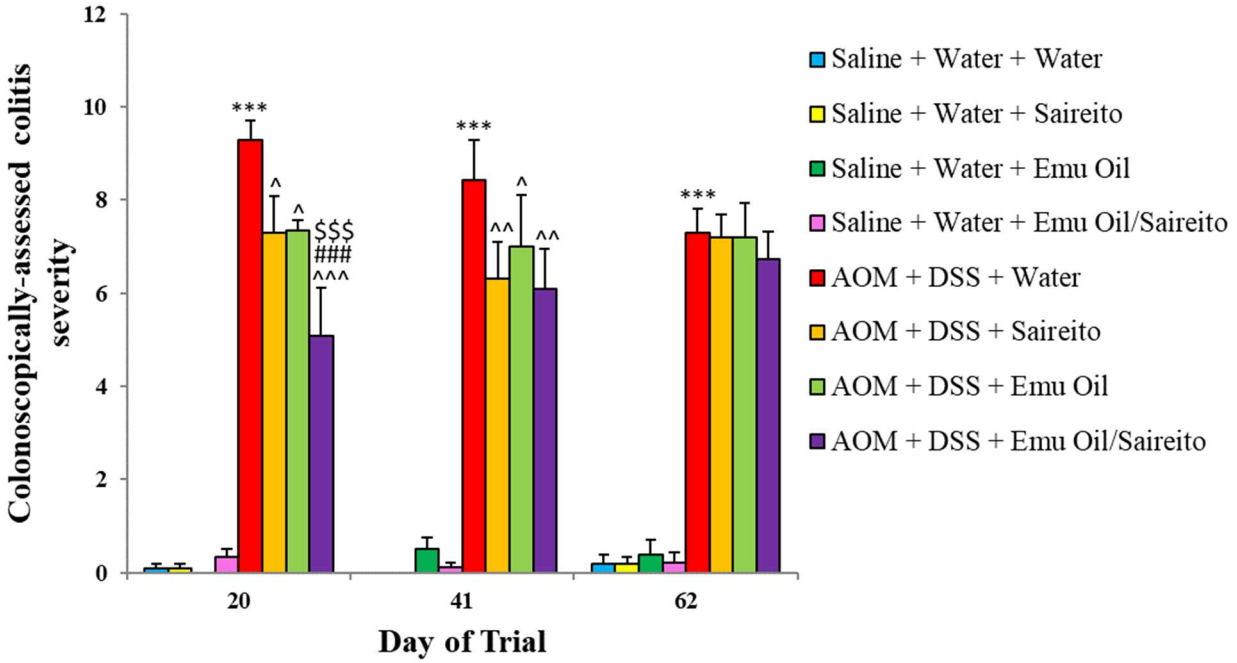


Figure 4b.

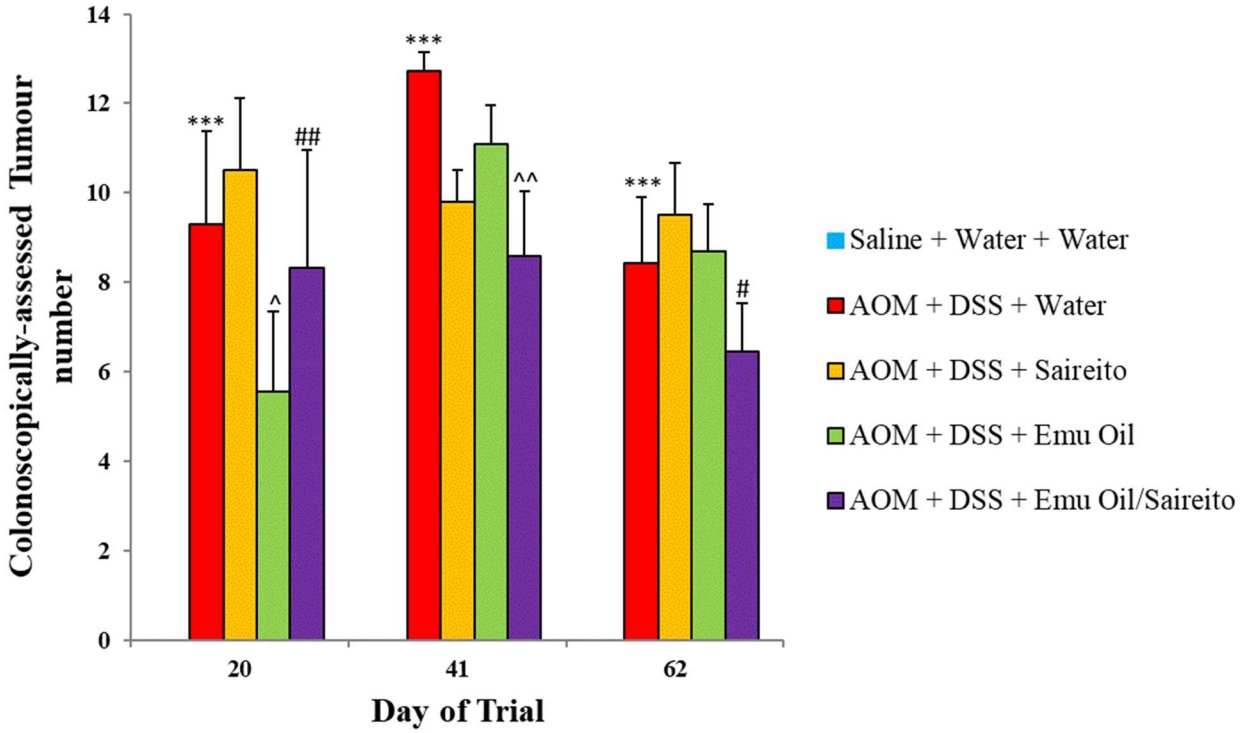


Figure 5.

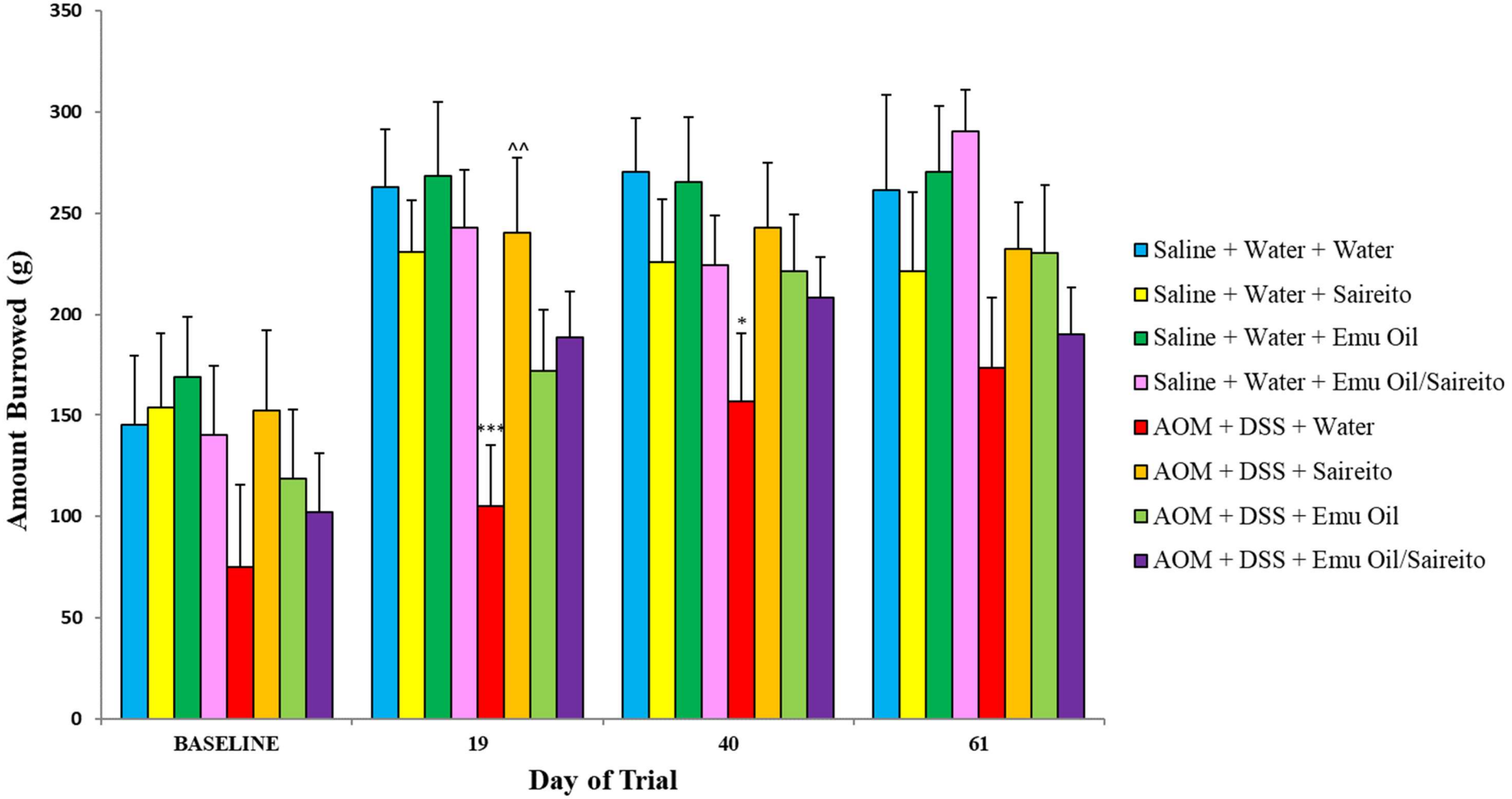

Amount Burrowed (g)
350
300
250
200
150
100
50
0
BASELINE
19
40
61
Day of Trial
***
^^
*
Saline + Water + Water
Saline + Water + Saireito
Saline + Water + Emu Oil
Saline + Water + Emu Oil/Saireito
AOM + DSS + Water
AOM + DSS + Saireito
AOM + DSS + Emu Oil
AOM + DSS + Emu Oil/Saireito

Figure 6.

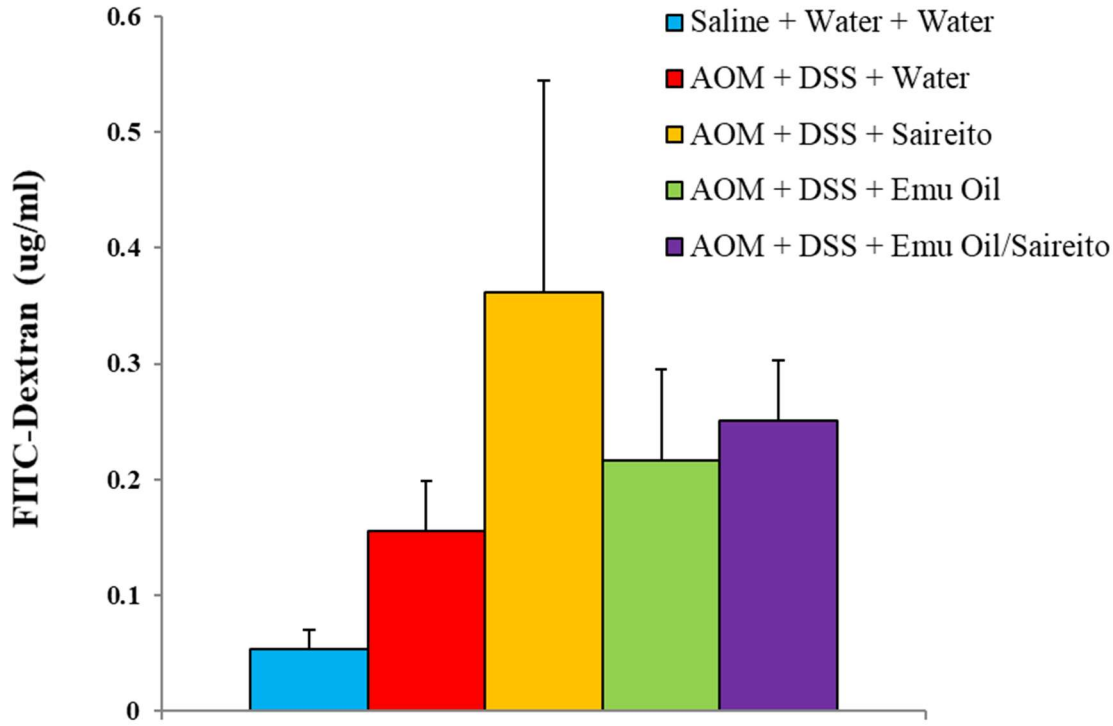

FITC-Dextran (ug/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline + Water + Water
AOM + DSS + Water
AOM + DSS + Saireito
AOM + DSS + Emu Oil
AOM + DSS + Emu Oil/Saireito

Figure 7.

i. **Saline + Water + Water**

ii. **Saline + Water + Saireito**

iii. **Saline + Water + EO**

iv. **Saline + Water + EO/Saireito**

v. **AOM + DSS + Water**

vi. **AOM + DSS + Saireito**

vii. **AOM + DSS + EO**

viii. **AOM + DSS + EO/Saireito**

Figure 8.

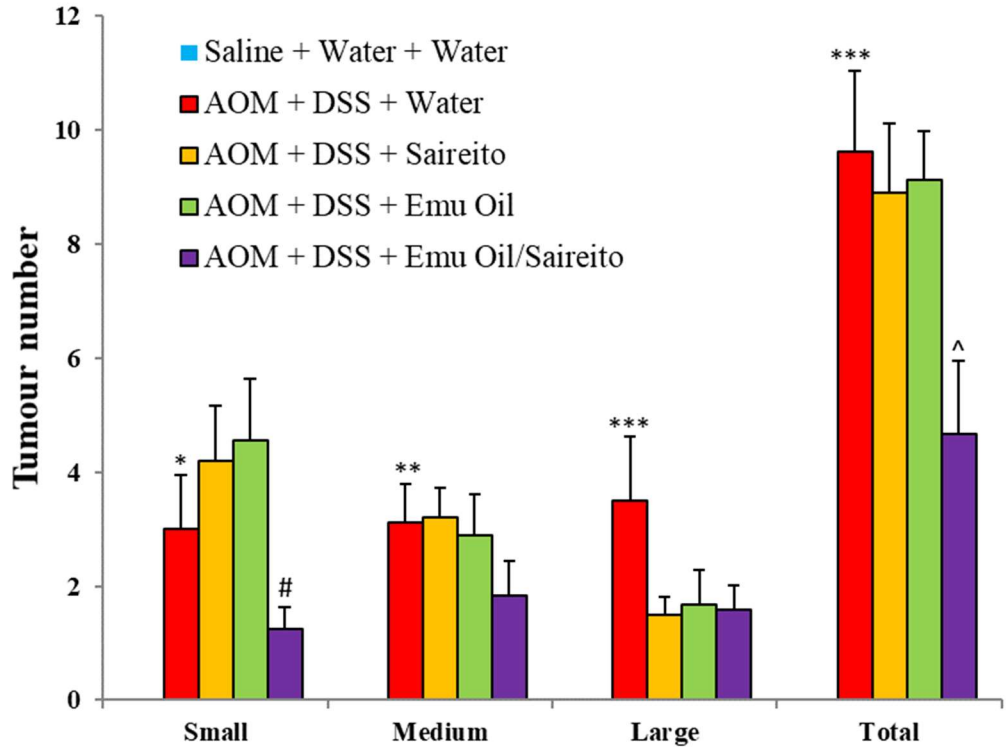
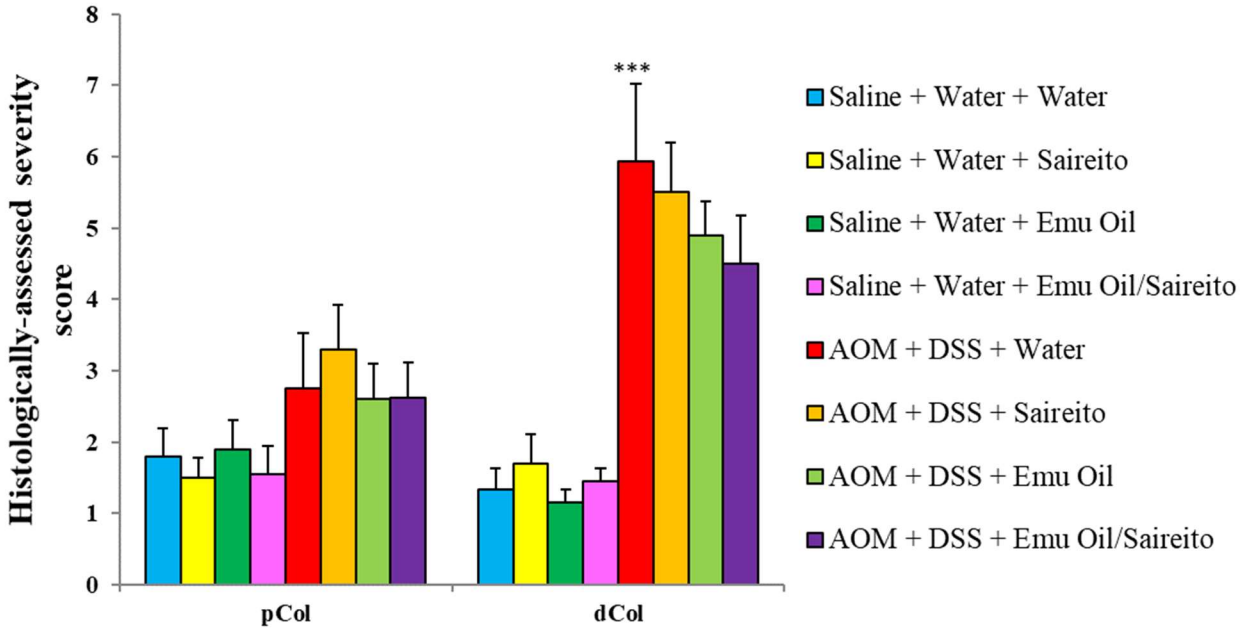


Figure 9.



CHAPTER 5

AFFECTIVE STATE DETERMINATION IN A MOUSE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

CONTEXT STATEMENT

The research described in this chapter has been published in *PLoS One*.

> *LC Chartier, ML Hebart, GS Howarth, AL Whittaker and S Mashtoub. Affective state determination in a mouse model of colitis-associated colorectal cancer. 2020. PLoS One, Vol 15(1): e0228413.*

In **Chapters 2, 3 and 4**, the studies described primarily monitored the welfare of experimental mice through burrowing behaviour analyses and disease severity scores in the azoxymethane (AOM)/dextran sulphate sodium (DSS) mouse model. However, a number of alternative techniques can be used to monitor mouse-specific welfare and affective state behaviour, including the mouse grimace scale. **Chapter 5** investigates and compares a number of species-specific behaviour techniques to measure animal welfare in the AOM/DSS mouse model of colitis-associated colorectal cancer (CA-CRC). Specifically, **Chapter 5** describes the first study to use the grimace scale in a pre-clinical model of chronic gastrointestinal disease and compare real-time and retrospective grimace scoring methods. This study also explores the effects of analgesic (buprenorphine) administration on behavioural and experimental outcomes in the AOM/DSS model. The study described in **Chapter 5** represents an important advancement for AOM/DSS investigations, as it highlights methods to monitor animal welfare that can be used in future experimental trials. Additionally, the outcomes highlight that disease activity index scoring is the most reliable method for monitoring mouse welfare in a pre-clinical setting of CA-CRC. Finally, buprenorphine may potentially be utilised in future studies for pain relief, as the results of the study showed that its administration did not result in side effects or affect experimental outcomes.

STATEMENT OF AUTHORSHIP

Title of Paper: Affective State Determination in a Mouse Model of Colitis-Associated Colorectal Cancer

Publication Status: Published

Publication Details: PLoS ONE, 2020, Vol 15, e0228413

Principal Author

Name of Principal Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Completed animal trials, sample and data collection, data analyses and interpretation. Prepared manuscript including writing, formatting and submission.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	17/9/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Michelle L Hebart		
Contribution to the Paper	Statistical analysis of data.		
Signature		Date	9/92020

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, revision and editing of the manuscript.		
Signature		Date	17/9/20

Name of Co-Author	Alexandra L Whittaker		
Contribution to the Paper	Conceptualisation, methodology development, supervision of analyses and data collection, data interpretation, revision and editing of the manuscript.		
Signature		Date	16/9/2020

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, supervision of analyses, data interpretation, revision and editing of the manuscript.		
Signature		Date	17/9/20

PLOS ONE

RESEARCH ARTICLE

Affective state determination in a mouse model of colitis-associated colorectal cancer

Lauren C. Chartier[1,2], **Michelle L. Hebart**[3], **Gordon S. Howarth**[2,3], **Alexandra L. Whittaker**[3☯], **Suzanne Mashtoub**[1,2,4☯*]

1 Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, Australia, **2** Department of Gastroenterology, Women's and Children's Hospital, North Adelaide, South Australia, Australia, **3** School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy Campus, Roseworthy, South Australia, Australia, **4** School of Medicine, The University of Western Australia, Murdoch, Western Australia, Australia

☯ These authors contributed equally to this work.
* suzanne.mashtoub@adelaide.edu.au

OPEN ACCESS

Citation: Chartier LC, Hebart ML, Howarth GS, Whittaker AL, Mashtoub S (2020) Affective state determination in a mouse model of colitis-associated colorectal cancer. PLoS ONE 15(1): e0228413. https://doi.org/10.1371/journal.pone.0228413

Editor: Mathilde Body-Malapel, University of Lille, FRANCE

Received: August 12, 2019

Accepted: January 14, 2020

Published: January 27, 2020

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pone.0228413

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Abstract

Behavioural indicators of affective state, including burrowing, clinical scores and the Mouse Grimace Score have not yet been validated in mouse models of chronic gastrointestinal disease. Additionally, a comparison of these methods has not been characterised. This study aimed to determine which behavioural assessment was the optimal indicator of disease, evidenced by correlation with clinically-assessed measures, in an azoxymethane (AOM)/dextran sulphate sodium (DSS) mouse model of colitis-associated colorectal cancer. C57BL/6 mice were allocated to four groups (n = 10/group); 1) saline control, 2) saline+buprenorphine, 3) AOM+DSS+water, 4) AOM+DSS+buprenorphine. Mice were gavaged thrice weekly with water or buprenorphine (0.5mg/kg; 80μL) for 9 weeks. Disease activity index (DAI) was measured daily; burrowing and grimace analyses occurred on days -1, 5, 19, 26, 40, 47 and 61. Colonoscopies were performed on days 20, 41 and 62. All animals were euthanized on day 63. Burrowing activity and retrospective grimace analyses were unaffected ($P>0.05$), whilst DAI was significantly increased ($P<0.05$) in mice with colitis-associated colorectal cancer compared to normal controls. In addition, DAI was positively correlated with colonoscopically-assessed severity and tumour number ($P<0.05$). We conclude that traditional measures of DAI or clinical scoring provide the most reliable assessment of wellbeing in mice with colitis-associated colorectal cancer.

Introduction

Pain, as defined by the Oxford dictionary, refers to a 'highly unpleasant physical sensation caused by illness or injury'. In biomedical research, rodents are the most widely used species and it is estimated that globally approximately 4.6 million will experience procedure-related pain [1]. Prevention and alleviation of pain through accurate pain assessment and appropriate analgesic use should be a priority for researchers working with laboratory animals [2]. However, assessment of pain is challenging in all animal species, and is particularly problematic in rodent-prey species that mask pain as part of a survival mechanism [3].

Funding: LCC received partial funding from The Australian Veterinary Association for the current study (Animal Welfare Trust Grant; www.ava.com.au). LCC is also supported by an Australian Government Research Training Program Stipend and a PhD Top-Up Scholarship from AgriFutures Australia (www.agrifutures.com.au). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There was no additional external funding received for this study.

Competing interests: The authors have declared that no competing interests exist.

Although directly measuring pain in animals is near impossible, it can be presumed that animals are in pain when they display pain-like behaviours [4]. Such behaviours include reduced ambulation, agitation and increased grooming of an affected area [4]. A number of techniques have been established to measure pain-like behaviour in animal models. The first included stimulus-evoked measures such as the Von Frey, Randall-Selitto and Hargreaves techniques. These methods are now used less widely due to a growing concern over clinical translatability [4], since these methods are regarded as not measuring the affective pain response. In response to this concern, scientists developed a range of behavioural assessment methods proposed to measure the affective or emotional component of the pain response. A method that has received much attention is the characterisation of facial expression.

The first standardised system for facial expression scoring in rodents, 'The Mouse Grimace Scale' (MGS) was developed by Langford et al. (2010). The MGS scores five facial features or 'action units' from 0–2 (not present to severe). These features are: orbital tightening, nose bulge, cheek bulge, ear position and whisker change. A higher MGS score is indicative of increased levels of pain [5]. Whilst this system represents a considerable advancement in pain assessment of rodents, validation studies have typically involved retrospective assessment through analysis of stored video behavioural data in models of acute pain. Consequently, refinement possibilities are limited, since humane endpoints and analgesic provision are not able to be immediately implemented. Therefore, an alternative live-scoring method should be considered to allow 'cage-side' analysis, whereby interventions can be applied by researchers to rapidly improve animal welfare as needed. Leung et al. (2016) determined that a real-time grimace scoring method was reliable in rats [6]. Miller and Leach (2016) investigated the validity of baseline grimace scoring in various cohorts, strains and sexes of mice [7]; however, the effectiveness of real-time scoring in mice with chronic disease is yet to be determined. Furthermore, there have been relatively few investigations into the validity of MGS in mice expected to be experiencing *chronic* visceral pain, as opposed to acute pain, initiated via a non-surgical insult.

In addition to pain, animals may also experience distress or sickness leading to a negative affective state and potentially compromising their welfare. Negative affective state has traditionally been assessed in biomedical research using general clinical scoring, for example the Morton and Griffiths (1985) schema. This scheme describes appearance, food/water intake, behaviour, digestive and cardiovascular signs on a scale of normal to severe for rodents, guinea pigs, rabbits, cats and dogs [8]. This method remains the predominant method for laboratory rodent welfare assessment globally, as prescribed by animal ethics committees and regulatory documents. More recently, deterioration in activities of daily living (ADL) has been proposed to indicate decreased welfare in mice [9]. The most common measurable ADL in mice are burrowing, nesting and hoarding. These techniques are inexpensive, simple to run and also provide environmental enrichment for laboratory mice.

The current study sought to address these deficiencies in knowledge by determining the effectiveness of a range of measures of pain and well-being in a pre-clinical setting of colitis-associated colorectal cancer, using the azoxymethane (AOM)/dextran sulphate sodium (DSS) mouse model. Methods examined were the MGS, burrowing activity and clinical scoring and we aimed to determine which method was the most reliable in this pre-clinical model of colitis-associated colorectal cancer. Buprenorphine, a long-lasting opioid analgesic (up to 8 hours), has few effects on the immune system and has displayed efficacy in reducing the acute and chronic pain experience of mice and rats [10–12]. Therefore, buprenorphine was administered to validate the tests, especially the pain-specific MGS, to determine if pain was a contributing factor in behavioural response. The current study represents the first validation of a live-scoring method of the MGS, compared to the traditional retrospective scoring, in a mouse

model of chronic disease. Finally, this study aimed to determine the most reliable behavioural assessment technique (MGS, clinical scoring or burrowing) for indication of disease and its progression in experimentally-induced colitis-associated colorectal cancer, as evidenced by correlation with clinically-assessed disease measures in mice.

Materials and methods

Animal studies

All animal studies were conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committee of the Children, Youth and Women's Health Service (AE1095/7/21). This study was conducted as part of another study evaluating naturally-sourced therapies in colitis-associated colorectal cancer with control groups being utilised in the current study (AE1079/3/21). Female C57BL/6 mice (C57BL/6JArc, n = 40; average weight 18.36g) at 8 weeks of age were sourced from a SPF production facility, the Animal Resource Centre (ARC; Perth, Western Australia) and group-housed in standard open-top cages (polypropylene; 470mm x 175mm x 120mm; Crestware Industries) with pelleted paper bedding materials (>99% recycled paper product; Fibrecycle PtyLtd, Helensvale, QLD, Australia). The ARC undertakes a quarterly health screening, covering various bacterial, viral and parasitic organisms, all of which the obtained colony screened negative for. Only female mice were used to remain consistent with data obtained from previous studies [13, 14]. Environment was regulated at 21-24˚C with 42–44% humidity and a light/dark cycle of 14:10 h. Mice were fed standard mouse chow (meat free mouse diet; Specialty Feeds, Glen Forrest, Western Australia) and provided with enrichment items including shredded paper, polycarbonate 'houses' and cardboard toilet paper rolls for the duration of the trial. All mice received *ad libitum* access to plain drinking water during the experimental period (except where group allocation precluded it).

Experimental design

Female C57BL/6 mice (n = 10/group) were randomly assigned to four treatment groups (n = 10/group); 1) saline + water + water, 2) saline + water + buprenorphine, 3) AOM + DSS + water and 4) AOM + DSS + buprenorphine. Mice were stratified to groups based on baseline body weight. Group size (n = 10/group) was calculated using Clin.Calc for mouse grimace scale outcomes from Rosen et al. (2017) [15]. This calculation assumed a power of 80%, and indicated that a minimum sample size of n = 9/group was necessary for statistical power. All mice were administered (oral-gastric gavage) 80μL of either water or buprenorphine (0.5mg/kg; Reckitt Benckiser Healthcare, Hull, U.K) thrice weekly for the duration of the trial. Buprenorphine was administered via oral gavage as control animals (groups 1 and 3) utilised in another study were gavaged with water and thus exposed to the same procedural distress. On day 0, mice received a single intraperitoneal injection of saline or AOM (7.4mg/kg; average injection volume 0.14ml; 27G needle;Sigma-Aldrich, Castle Hill NSW, Australia), and then underwent three DSS/water cycles comprised of 7 days DSS (*ad libitum*; 2%w/v, 2g/100ml distilled water; MP Biomedicals LLC, Santa Ana California, USA) followed by 14 days plain water *(ad libitum)*. Negative control animals (groups 1 & 2) received plain water in their drinking bottles for the duration of the 9-week experimental period. All animals were euthanised on day 63 via CO_2 asphyxiation followed by cervical dislocation (experimental timeline; Fig 1).

Disease activity index

DAI was calculated daily (at 8am, prior to buprenorphine administration) from general clinical signs including bodyweight loss, general condition, stool consistency and rectal bleeding

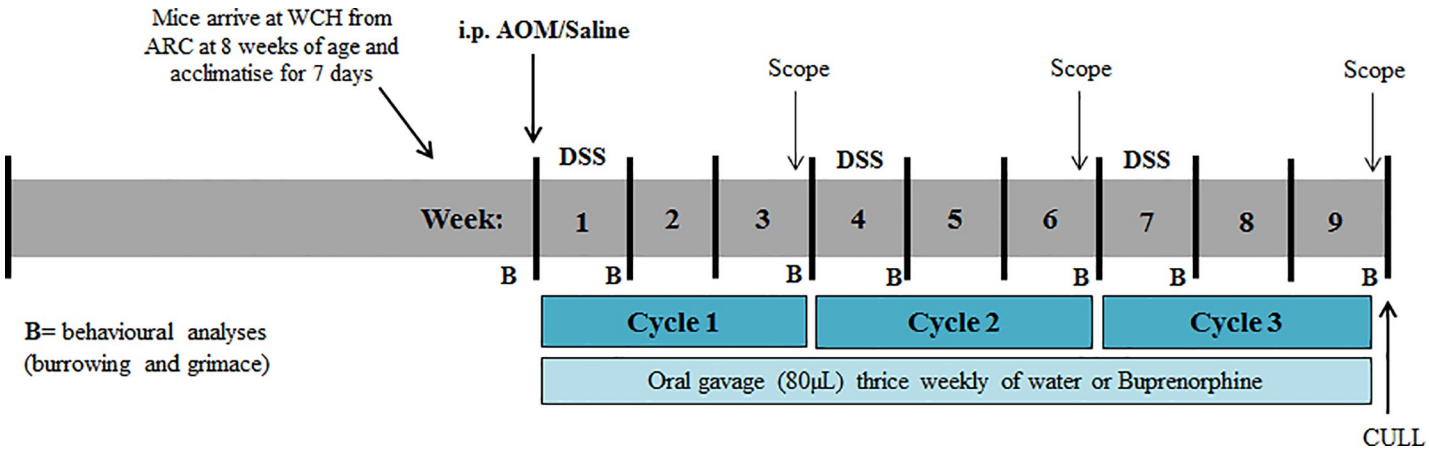


Fig 1. Experimental timeline. Animals were injected (i.p.) on day 0 and underwent three dextran sulphate sodium (DSS)/water cycles, comprising one week 2% DSS followed by 2 weeks of plain water. Animals were gavaged thrice weekly with water or buprenorphine. All mice were euthanised after 9-weeks via CO_2 asphyxiation, followed by cervical dislocation.

https://doi.org/10.1371/journal.pone.0228413.g001

during the experimental period. General condition included features such as ruffled coat and grooming, hunching, alertness and abdominal twitching. Each parameter was scored from 0–3 with increasing severity and totalled to give a final DAI value, with a maximum possible score being 12 [16]. As DAI was a part of daily monitoring and welfare measurements, the researchers were not blinded to treatment groups when obtaining DAI scores.

Colonoscopy

Colonoscopies using a high-resolution Karl Storz colonoscope (1.9mm outer diameter, Tuttlingen, Germany) were performed at the end of each DSS/water cycle (days 20, 41 and 62) to assess colitis progression and tumour development. Mice were anaesthetised using isoflurane inhalant (AbbVie Inc, Illinois, USA) in oxygen via mask for the duration of the procedure, and closely monitored on a heating pad during and immediately following the procedure. From anaesthetic induction to recovery, the colonoscopy procedure lasted approximately 10 minutes. Colitis severity was measured from recorded videos in a blinded fashion using five parameters described by Becker et al. (2005). These parameters include; thickening of the colon, vasculature pattern, presence of fibrin, granularity of mucosal surface, and stool consistency. Each parameter was scored from 0 to 3 with increasing severity and totalled, with the maximum possible severity score being 15 [17]. Additionally, colonic tumours were also counted from videos in a blinded fashion.

Burrowing analyses

Burrowing analyses were conducted as a measure of affective state or activities of daily living using a modified protocol described by Deacon [18]. At 6pm, one hour after commencement of the dark cycle and 8 hours after buprenorphine administration (from 6pm), mice were placed in individual cages with a pre-weighed (400g kitty litter 'pebbles'; Black and Gold, Australian Asia/Pacific Wholesalers Pty Ltd) burrow attached (modified plastic Coca-Cola bottle; 6.9cm diameter, 16cm long) and left for an hour. After this time, the burrows were re-weighed and the weight difference taken to represent the amount burrowed. Burrowing analyses occurred on day -1 (baseline), at the end of each DSS week for a severe disease measure (days 5, 26 and 47) and at the end of each DSS/water cycle to assess recovery (days 19, 40 and 61).

Mouse grimace scale

The affective experience of pain was assessed using the Mouse Grimace Scale (MGS; [5] at baseline, the end of each DSS week and end of a DSS/water cycle (days -1, 5, 19, 26, 40, 47 and 61). Real-time [6] and retrospective [5] MGS scoring methods were conducted in the morning (following buprenorphine administration; approx. 9am-12pm) at all indicated time-points. Five facial features (orbital tightening, nose bulge, cheek bulge, ear position and whisker change) were scored by a treatment-blinded grimace experienced observer from 0–2 (not present to severe), with a maximum possible MGS score being 10.

Real-time MGS

Animals were removed from their home cage and placed individually in a clear plastic cage for scoring by a treatment-blinded experienced observer. The mouse remained in the scoring cage for a five minute period, where the observer assigned a score for each facial feature every 15 seconds. The animal was then returned to its home cage. A median score was calculated for each parameter per 15 second time-point and then an average was obtained of the medians per 90 second period. A final mean was then calculated from each 90 second period to produce a final grimace score for each mouse.

Retrospective MGS

Over the same time period as the real-time method, video recording of the clear cage was performed using two video cameras placed on perpendicular cage sides (Panasonic HC-V180, Osaka, Japan). Still images of the mice were extracted from video footage, cropped to show the face alone and placed into a pre-designed excel spreadsheet by an investigator blinded to treatment allocation. A random number generator was used to select three images for scoring of each mouse at each time-point. These images were then scored by a treatment-blinded scorer using the methods described by Langford et al. 2010. Scores for each parameter were totalled to give a score per photo, and then the three photo scores were averaged to give a final reportable score for each mouse per time-point [5].

Statistical analysis

Statistical analysis was completed using SPSS, version 25 for Windows (SPSS Inc. Chicago, Illinois, USA). Data were tested for normality using a Shapiro–Wilk test. DAI, burrowing activity, colitis score, and tumour number were analysed by repeated measures ANOVA with least significance difference (LSD) to compare among and within a group. MGS data were analysed non-parametrically using a Friedman test to assess temporal differences within groups and a Kruskal-Wallis with a Mann-Whitney *post-hoc* test to compare differences between groups within time-points. To determine any correlations between behavioural outputs and the measured clinical parameters, a non-parametric spearman-rho test was applied. $P<0.05$ was considered statistically significant.

Results

Disease activity index

In normal animals, buprenorphine administration did not impact DAI scores during the experimental period compared to saline controls ($P>0.05$; Fig 2). AOM/DSS significantly increased DAI scores on days 2, 3 and 5–63 compared to untreated saline controls ($P<0.05$). In AOM/DSS mice, buprenorphine administration increased DAI scores on days 16, 18–21, 25, 41 and 59 compared to AOM/DSS controls ($P<0.05$). Furthermore, buprenorphine

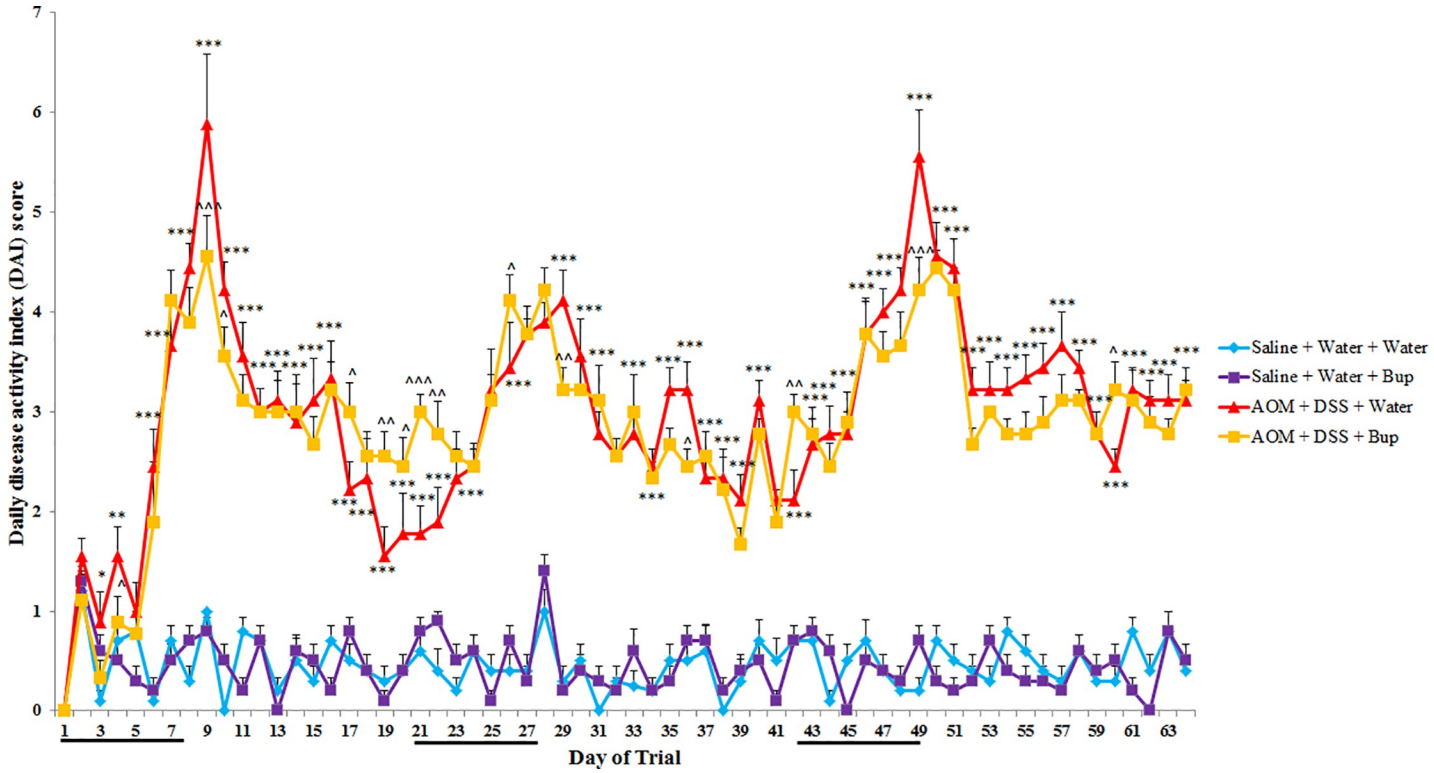


Fig 2. Daily disease activity index (DAI) score (n = 10/group). Data were analysed using a repeated measures ANOVA with least significance difference (LSD) and are expressed as mean DAI score ± SEM (black line on the x axis represents a dextran sulphate sodium; DSS week). ***p<0.001, **p<0.01, *p<0.05 compared to Saline + Water + Water, ^^^p<0.001, ^^p<0.01, ^p<0.05 compared to AOM + DSS + Water.

https://doi.org/10.1371/journal.pone.0228413.g002

administration decreased DAI scores on days 3, 8, 9, 28, 35 and 48 in AOM/DSS animals compared to AOM/DSS alone ($P<0.05$).

Colitis severity and tumour number

Buprenorphine administration did not impact colitis progression in saline control animals throughout the experimental trial ($P>0.05$; Fig 3). AOM/DSS significantly increased colonoscopically-assessed colitis severity compared to saline controls at all three time-points (days 20, 41 and 62; $P<0.05$). Mice administered buprenorphine and treated with AOM/DSS presented with increased colitis severity on day 20 and decreased colitis severity scores on day 62 compared to AOM/DSS controls ($P<0.05$).

Saline control animals and those treated with buprenorphine did not develop colorectal tumours during the experimental period. AOM/DSS resulted in increased tumour number compared to saline controls ($P<0.05$; Fig 4). Additionally, in AOM/DSS mice, buprenorphine did not significantly impact tumour development compared with AOM/DSS controls ($P>0.05$).

Burrowing

Buprenorphine administration significantly increased burrowing activity in normal mice compared to saline controls on days 19, 26 and 40 ($P<0.05$; Fig 5). AOM/DSS did not significantly affect burrowing compared to saline controls at any time-point.

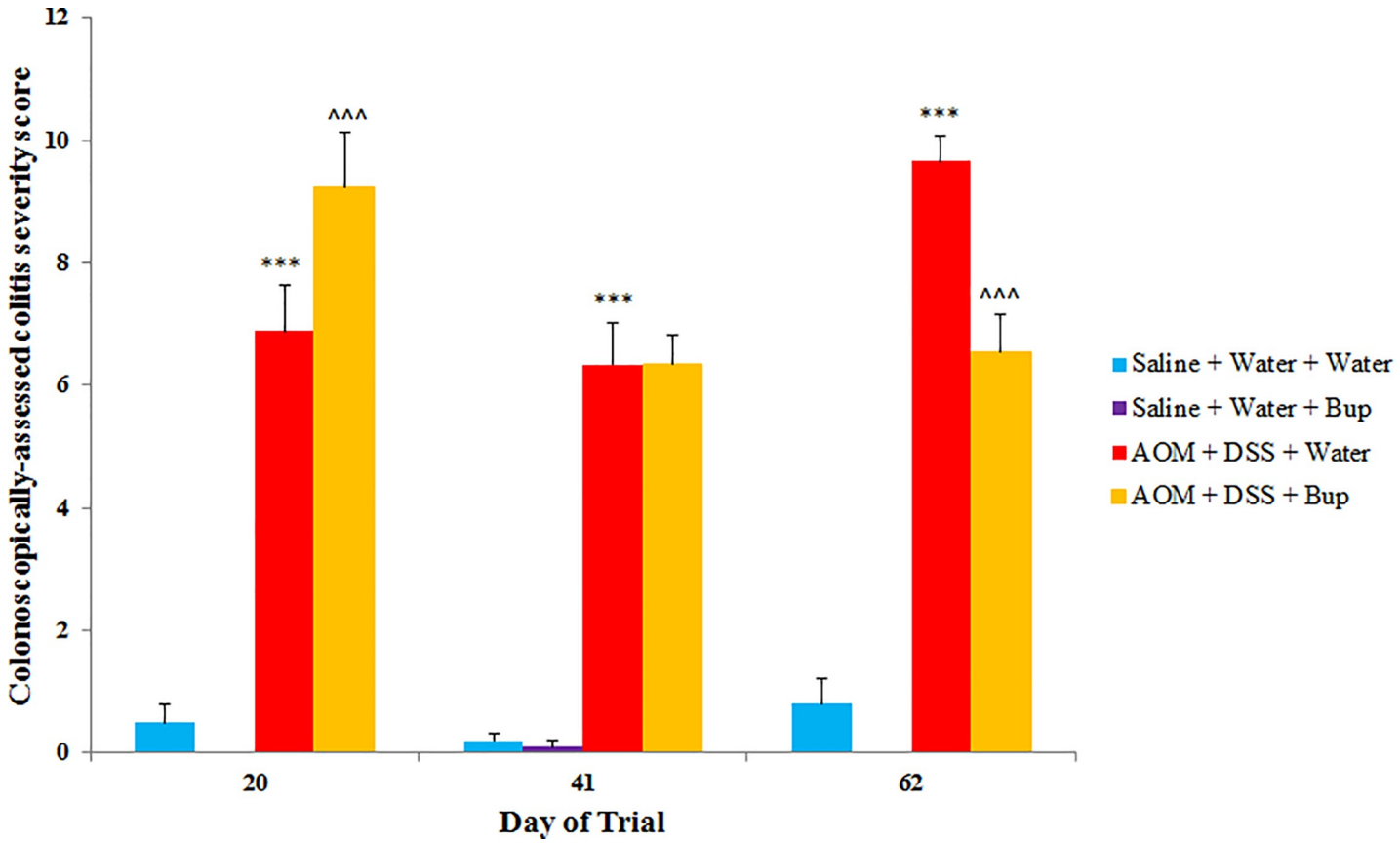


Fig 3. Colonoscopically-assessed colitis severity (n = 10/group). Data were analysed using a repeated measures ANOVA with least significance difference (LSD) and are expressed as mean colitis severity score ± SEM. Colitis-severity is calculated from stool consistency, mucosal thickness, granularity of the mucosal surface, fibrin and vasculature pattern (each scored from 0–3 and summed). $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ compared to Saline + Water + Water, $^{\wedge\wedge\wedge}p<0.001$, $^{\wedge\wedge}p<0.01$, $^{\wedge}p<0.05$ compared to AOM + DSS + Water.

https://doi.org/10.1371/journal.pone.0228413.g003

Mouse grimace scale

Buprenorphine administration in normal mice resulted in no significant differences in real-time grimace scores at any time-point when compared to saline controls (Table 1; $P>0.05$). On day 19, AOM/DSS controls had higher real-time grimace scores compared to saline controls (Table 1; $P<0.05$), with no other significant differences on other days. AOM/DSS controls presented with significantly higher real-time grimace scores on day 19 compared to baseline ($P<0.05$). Buprenorphine administration in AOM/DSS mice resulted in significantly higher real-time grimace scores on day 19 when compared to baseline; and on day 47 compared to day 40 ($P<0.05$). Finally, buprenorphine administration in AOM//DSS mice significantly reduced real-time grimace scores on day 40 compared to day 19 ($P<0.05$). Scoring of retrospective grimace frames resulted in no significant differences within or across groups ($P>0.05$).

Correlations

Real-time grimace scores were positively correlated with colitis severity and tumour number on day 19 (Table 2; $P<0.05$). Burrowing was negatively correlated with colitis severity and tumour number at all three time-points (days 19, 40 and 61; $P<0.05$). Furthermore, DAI was

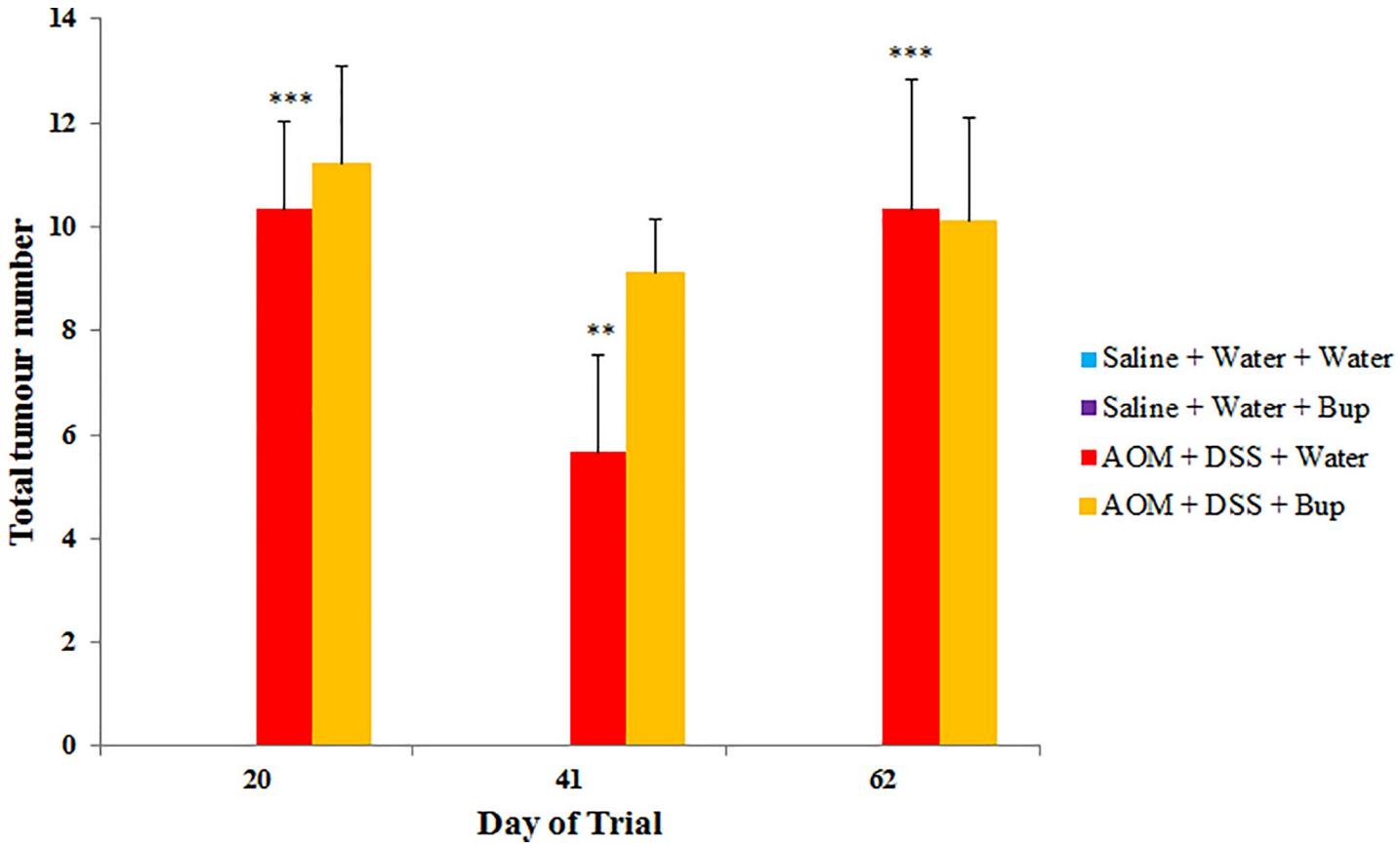


Fig 4. Total tumour number measured from colonoscopy (n = 10/group). Data were analysed using a repeated measures ANOVA with least significance difference (LSD) and are expressed as mean total tumour number ± SEM. ***p<0.001, **p<0.01, *p<0.05 compared to Saline + Water + Water, ^^^p<0.001, ^^p<0.01, ^p<0.05 compared to AOM + DSS + Water.

https://doi.org/10.1371/journal.pone.0228413.g004

positively correlated with colitis severity score and tumour number at all three time-points (days 19, 40 and 61; $P<0.05$).

Discussion

AOM/DSS administration successfully induced colitis-associated colorectal cancer in mice, as evidenced by colonoscopically-assessed severity, tumour development and increased colon weights. However, the disease state was not reliably translated in the results of the two affective state measurement techniques utilised, namely burrowing and MGS. Clinical scores of disease such as DAI used in the current study, include scoring of non-specific mouse illness signs such as bodyweight loss, coat appearance, activity and stool consistency. Our findings suggest that the DAI is in fact the most reliable determinant of the clinical picture presented in these mice, and humane endpoint implementation in this model should continue to be based on this scoring scheme.

Analgesic administration did not affect normal animals; however, interestingly, buprenorphine increased the clinical DAI score of mice with colitis-associated colorectal cancer on selected days, attributed to bodyweight loss. This result was likely to be primarily due to inappetence, possibly brought about by nausea, and consequent bodyweight loss as a side-effect of analgesic intervention [1, 19]. Nonetheless, this effect was not observed consistently throughout the experimental period. Overall, results were unable to conclude a significant

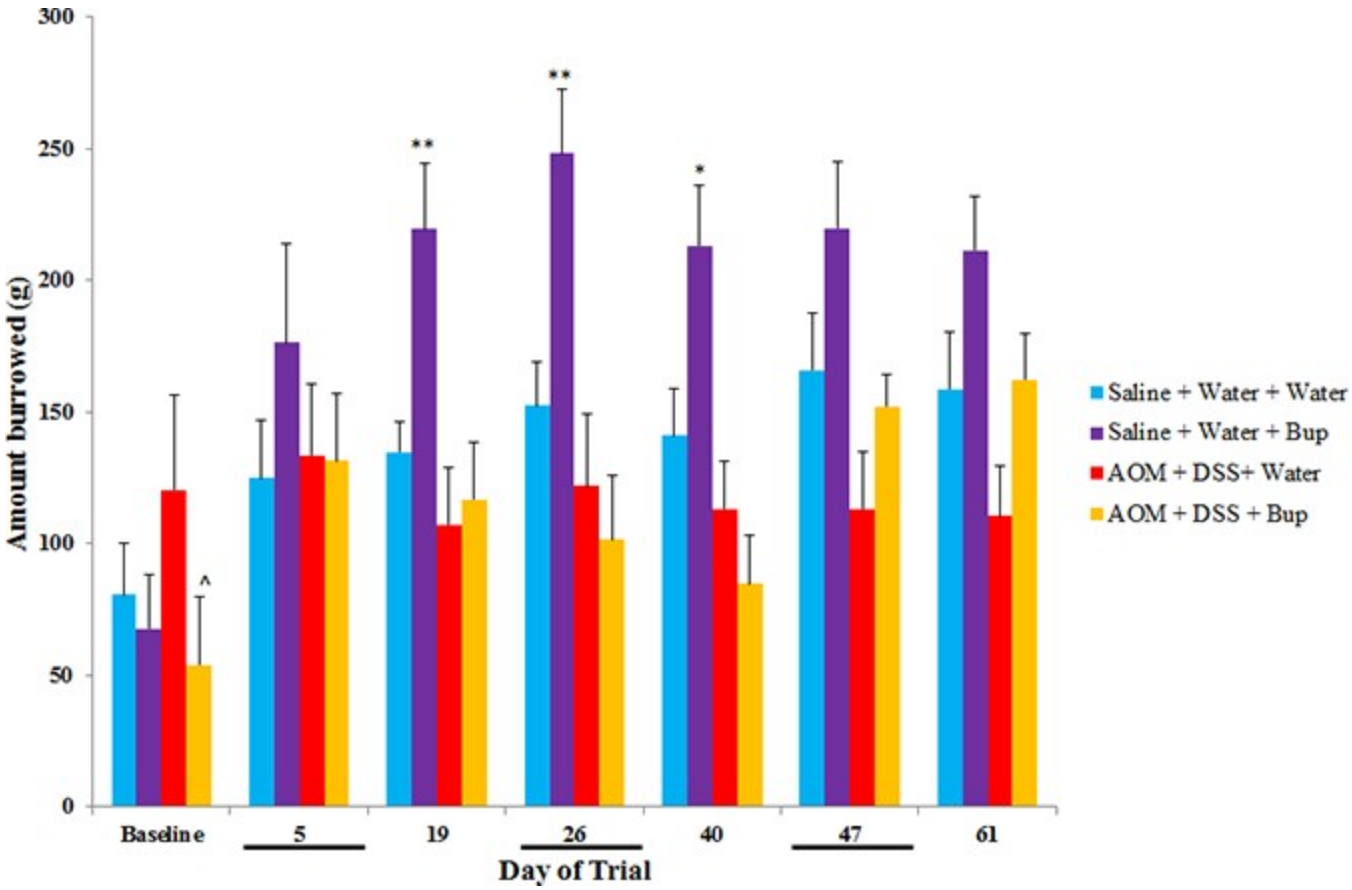


Fig 5. Burrowing activity (n = 10/group). Data were analysed using a repeated measures ANOVA with least significance difference (LSD) and are expressed as mean amount burrowed ± SEM (black line on the x axis represents a dextran sulphate sodium; DSS week). ***p<0.001, **p<0.01, *p<0.05 compared to Saline + Water + Water, ^^^p<0.001, ^^p<0.01, ^p<0.05 compared to AOM + DSS + Water.

https://doi.org/10.1371/journal.pone.0228413.g005

impact of opioid analgesic (buprenorphine) intervention on pain reduction in the measures used at selected time-points, as highlighted by the minimal differences in grimace scores, burrowing behaviour and DAI in disease mice. This implied either that: 1) These animals were

Table 1. Real-time and retrospective MGS scores (n = 10/group).

	Saline + Water + Water		Saline + Water + Bup		AOM + DSS + Water		AOM + DSS + Bup	
	Retrospective	Real-time	Retrospective	Real-time	Retrospective	Real-time	Retrospective	Real-time
Baseline	0 ± 0	0 ± 0	0.133 ± 0.07	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Day 5	0.167 ± 0.06	0 ± 0	0.233 ± 0.11	0.056 ± 0.04	0.296 ± 0.12	0 ± 0	0.185 ± 0.10	0 ± 0
Day 19	0.067 ± 0.04	0 ± 0	0.167 ± 0.13	0 ± 0	0.185 ± 0.08	0.198 ± 0.08**#	0.444 ± 0.22	0.111 ± 0.05#^
Day 26	0.267 ± 0.10	0 ± 0	0 ± 0	0 ± 0	0.222 ± 0.10	0.062 ± 0.03	0.333 ± 0.18	0.049 ± 0.05
Day 40	0.167 ± 0.09	0 ± 0	0.100 ± 0.05	0 ± 0	0.111 ± 0.08	0 ± 0	0.037 ± 0.04	0 ± 0
Day 47	0.100 ± 0.07	0 ± 0	0.133 ± 0.07	0 ± 0	0.259 ± 0.19	0.012 ± 0.012	0.333 ± 0.14	0.086 ± 0.06^
Day 61	0.267 ± 0.12	0 ± 0	0.133 ± 0.05	0 ± 0	0.259 ± 0.11	0 ± 0	0.148 ± 0.11	0 ± 0

Data were analysed non-parametrically using a Friedman test and a Kruskal-Wallis with a Mann-Whitney *post-hoc* test and are expressed as mean MGS score ± SEM.

**p<0.01 compared to Saline + Water + Water at same time-point

#p<0.05 compared to baseline within a group

^p<0.05 compared to day 40 within a group.

https://doi.org/10.1371/journal.pone.0228413.t001

Table 2. Correlations between data sets of behavioural and clinical indicators on days 19, 40 and 61.

		Colitis Severity day 19	Tumour Number day 19	Colitis Severity day 40	Tumour Number day 40	Colitis Severity day 61	Tumour Number day 61
Real-time Grimace	**Correlation Coefficient**	0.517	0.668	n.e	n.e	n.e	n.e
	Significance (2-tailed)	0.001***	0.000***	n.e	n.e	n.e	n.e
Photo Grimace	**Correlation Coefficient**	0.240	0.289	-0.147	-0.194	0.080	-0.027
	Significance (2-tailed)	0.146	0.078	0.379	0.244	0.633	0.872
Burrowing	**Correlation Coefficient**	-0.478	-0.405	-0.393	-0.408	-0.538	-0.266
	Significance (2-tailed)	0.002**	0.012*	0.015*	0.011*	0.000***	0.106
DAI	**Correlation Coefficient**	0.689	0.764	0.751	0.650	0.863	0.838
	Significance (2-tailed)	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***

Data were analysed using a non-parametric spearman-rho test.

***$p<0.001$

**$p<0.01$ and

*$p<0.05$.

Note–no correlation coefficients could be derived between real-time grimace data and other measures on day 40 and 61 due to number of zero scores (n.e.–not estimable due to no variation in real-time grimace scores [all scores were 0]).

https://doi.org/10.1371/journal.pone.0228413.t002

not experiencing pain, 2) The tests utilised were not sensitive enough to detect the type of pain experienced, or that 3) buprenorphine was ineffective in the face at the chosen time-points in mice with colitis-associated colorectal cancer.

Non-facial indicators of pain such as abdominal twitching, hunching, writhing, and belly press were identified in mice throughout the experimental period. Although these characteristics are not considered in facial grimace scores, they have been identified as validated pain associated behaviours that commonly occur following laboratory procedures [20, 21]. This highlights a key point in comparing real-time with retrospective measures especially when using personnel experienced with mice as real-time observers. Experienced observers are likely to subconsciously note general condition, and other pain-like behaviours such as hunching, writhing, belly press and immobility which may influence their scoring. These indicators are unable to be scored with a head-only photo image. Therefore, it would be advisable to use naïve observers for real-time grimace scoring in future studies.

The MGS action units have been validated in acute or moderate pain which lasts from minutes to hours. It has been reported that these action units are unable to be identified in mice days or weeks after a procedure, injury or surgical insult [5]. This is plausible since a fitness advantage would be gained by not communicating evidence of injury to predators via expression of the 'pain face' [22]. Consequently, the time-points selected in the current chronic study may have been too long after procedures to be able to identify pain present in the face. Furthermore, the MGS scores obtained were generally low (maximum 0.4 ± 0.2; live and retrospective analysis), implying a lack of sensitivity which may have precluded the finding of an analgesic effect. Similarly, in a study of rats with the gastrointestinal condition mucositis, Whittaker et al. (2016) reported that other behavioural measures utilised in the mucositis study including writhing, twitching, back-arching and sociability, to be more indicative of a pain status in the

disease rats than facial grimace [23]. Moreover, the low MGS results in the current study may have indicated that the mice were not experiencing pain, it could also highlight the evolutionary characteristic of mice hiding pain in their face to deter predators [4].

Animal ethics committees often recommend analgesic implementation in studies when animals are induced with disease, therefore, it is crucial to understand that analgesic intervention will not affect experimental design. In the current study, a minimal effect on colitis severity and DAI was observed in buprenorphine treated animals; however, these results were not consistently represented throughout the experimental period and may have been due to the timing of DAI scoring in respect to buprenorphine-administration. Furthermore, buprenorphine was orally-administered to mice thrice weekly for 63 days and it is possible that during this time mice established a tolerance to the analgesia. Dum and Herz (1981) concluded that rats subcutaneously injected with buprenorphine twice daily developed a tolerance after just five days [24]. Additionally, in a study of DBA/2 mice with SL2 lymphoma, there was no interaction between dietary-administered buprenorphine and time, indicating that a drug tolerance may have been established during the 20 day period [25]. Furthermore, Van Loo and authors (1997) concluded that there were no clear indicators that buprenorphine impacted the pain experienced by mice with tumours, concluding that it was an undesirable analgesia in a lymphoma tumour model [25]. Hence, these data cannot confirm an action of buprenorphine in reducing pain based on the MGS scores obtained, nor any improvement in wellbeing based on DAI score or burrowing behaviour. However, this needs to be considered in light of the difficulty in teasing apart beneficial, versus side effects using the DAI, and the differences obtained in baseline burrowing score. Moreover, buprenorphine does not modify experimental outcomes which is an important finding when considering analgesic use in gastrointestinal animal models.

In normal mice, burrowing activity was increased in buprenorphine–treated groups on days 19, 26 and 40. This hyper-excitability is supported by Cowan et al. (1977), whereby the authors documented that buprenorphine-administration increased activity (walking and hopping) in non-painful mice [12]. In another study, resting behaviours were decreased in buprenorphine-treated cancerous mice compared to controls [25]. Moreover, increased levels of activity are suggested to be a side-effect of buprenorphine administration in rodents [26]. In the current study, AOM/DSS control animals displayed a higher baseline (day -1) burrowing ability compared to AOM/DSS administered together with buprenorphine, which may have impacted the burrowing results obtained at the other time-points. In future studies, it would be beneficial to allocate treatment groups based on burrowing activity and adjust these to ensure that all experimental groups display similar burrowing abilities at baseline. Furthermore, there was no significant difference in burrowing activity between colitis-associated colorectal cancer and normal control mice in the current study, suggesting that burrowing is not an effective behavioural measure in the AOM/DSS model. Interestingly, DSS-administration alone has been reported to significantly impact burrowing behaviour in mice with acute [27] and chronic [28] colitis.

Conclusions

Although the MGS scores obtained through real-time and retrospective analyses were unable to be validated in regards to pain alleviation in this chronic study, we were able to conclude that real-time grimace scores and daily clinical scores were correlated with increased colitis severity and tumour number across treatment groups. However, retrospective grimace scores were not correlated with other data sets. This indicated that real-time grimace may be a more accurate technique to complement other measures of disease in animal studies. However, as

previously discussed, there are limitations with the use of this method as a practical tool. Importantly, burrowing activity was negatively correlated with colitis severity and tumour number, indicating that as chronic disease develops, mouse behaviour will decrease as wellbeing is impacted. Given the lack of statistically-significant differences between groups we cannot recommend this measure in the colitis-associated colorectal cancer model. We conclude that the traditional disease activity index, or clinical score, presents the most comprehensive welfare assessment tool in the colitis-associated colorectal cancer mouse model, having a degree of sensitivity and comprising of both objective and subjective measures to constitute a final score.

In the current study, use of the MGS was unable to identify pain in the mouse model of colitis-associated colorectal cancer. Furthermore, the live-scoring MGS method was unable to be validated in this model of chronic gastrointestinal disease. Nonetheless, this study is the first to use the MGS in a chronic model of colorectal pain and it is the first to discuss the correlation between live and retrospective scoring methods with other study measurements. Further investigation of the MGS in this model is necessary to validate its reliability in chronic disease Consideration should be given to use of other methods for measuring ADL or affective state, for example nest making or judgement biasing in models of chronic disease. Moreover, DAI or clinical scores may be the most reliable method for affective state assessment in mouse models of chronic gastrointestinal diseases.

Supporting information

S1 Dataset. Disease activity index data files.
(XLSX)

S2 Dataset. Colonoscopically-Assessed colitis severity and tumour scores data files.
(XLSX)

S3 Dataset. Burrowing data files.
(XLSX)

S4 Dataset. Live mouse grimace scale data files.
(XLSX)

S5 Dataset. Retrospective mouse grimace scale data files.
(XLSX)

S6 Dataset. Correlation output data.
(DOCX)

Acknowledgments

The authors would like to acknowledge Rebecca George and Chloe Mitchell for assisting with real-time grimace scoring.

Author Contributions

Conceptualization: Gordon S. Howarth, Alexandra L. Whittaker, Suzanne Mashtoub.

Data curation: Lauren C. Chartier, Michelle L. Hebart.

Formal analysis: Lauren C. Chartier, Michelle L. Hebart.

Funding acquisition: Lauren C. Chartier.

Investigation: Lauren C. Chartier.

Methodology: Lauren C. Chartier, Suzanne Mashtoub.

Project administration: Lauren C. Chartier.

Resources: Lauren C. Chartier.

Supervision: Gordon S. Howarth, Alexandra L. Whittaker, Suzanne Mashtoub.

Validation: Alexandra L. Whittaker.

Visualization: Lauren C. Chartier.

Writing – original draft: Lauren C. Chartier.

Writing – review & editing: Lauren C. Chartier, Gordon S. Howarth, Alexandra L. Whittaker, Suzanne Mashtoub.

References

1. Flecknell P. Rodent analgesia: Assessment and therapeutics. Vet J. 2018; 232:70–7. https://doi.org/10.1016/j.tvjl.2017.12.017 PMID: 29428096
2. Hager C, Biernot S, Buettner M, Glage S, Keubler LM, Held N, et al. The Sheep Grimace Scale as an indicator of post-operative distress and pain in laboratory sheep. PLoS One. 2017; 12(4):e0175839. https://doi.org/10.1371/journal.pone.0175839 PMID: 28422994
3. Stasiak KL, Maul D, French E, Hellyer PW, VandeWoude S. Species-specific assessment of pain in laboratory animals. Contemp Top Lab Anim Sci. 2003; 42(4):13–20. PMID: 12906396
4. Deuis JR, Dvorakova LS, Vetter I. Methods Used to Evaluate Pain Behaviors in Rodents. Front Mol Neurosci. 2017; 10:284. https://doi.org/10.3389/fnmol.2017.00284 PMID: 28932184
5. Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, et al. Coding of facial expressions of pain in the laboratory mouse. Nat Methods. 2010; 7(6):447–9. https://doi.org/10.1038/nmeth.1455 PMID: 20453868
6. Leung V, Zhang E, Pang DS. Real-time application of the Rat Grimace Scale as a welfare refinement in laboratory rats. Sci Rep. 2016; 6:31667. https://doi.org/10.1038/srep31667 PMID: 27530823
7. Miller AL, Leach MC. The effect of handling method on the mouse grimace scale in two strains of laboratory mice. Lab Anim. 2016; 50(4):305–7. https://doi.org/10.1177/0023677215622144 PMID: 26657061
8. Morton DB, Griffiths PH. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. Vet Rec. 1985; 116(16):431–6. https://doi.org/10.1136/vr.116.16.431 PMID: 3923690
9. Deacon R. Assessing burrowing, nest construction, and hoarding in mice. J Vis Exp. 2012(59):e2607. https://doi.org/10.3791/2607 PMID: 22258546
10. Filibeck U, Castellano C, Oliverio A. Development of morphine-induced changes of activity in the mouse. Brain Res. 1981; 254(4):602–5. https://doi.org/10.1016/0165-3806(81)90029-8 PMID: 6272950
11. Kayser V, Besson JM, Guilbaud G. Effects of the analgesic agent tramadol in normal and arthritic rats: comparison with the effects of different opioids, including tolerance and cross-tolerance to morphine. Eur J Pharmacol. 1991; 195(1):37–45. https://doi.org/10.1016/0014-2999(91)90379-5 PMID: 2065712
12. Cowan A, Lewis JW, Macfarlane IR. Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. Br J Pharmacol. 1977; 60(4):537–45. https://doi.org/10.1111/j.1476-5381.1977.tb07532.x PMID: 409448
13. Chartier LC, Howarth GS, Lawrance IC, Trinder D, Barker SJ, Mashtoub S. Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. Dig Dis Sci. 2018; 63(1):135–45. https://doi.org/10.1007/s10620-017-4876-4 PMID: 29214422
14. Mashtoub S, Tran CD, Howarth GS. Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. Exp Biol Med (Maywood). 2013; 238(11):1305–17.
15. Rosen SF, Ham B, Drouin S, Boachie N, Chabot-Dore AJ, Austin JS, et al. T-Cell Mediation of Pregnancy Analgesia Affecting Chronic Pain in Mice. J Neurosci. 2017; 37(41):9819–27. https://doi.org/10.1523/JNEUROSCI.2053-17.2017 PMID: 28877966

16. Abimosleh SM, Lindsay RJ, Butler RN, Cummins AG, Howarth GS. Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. Dig Dis Sci. 2012; 57(4):887–96. https://doi.org/10.1007/s10620-011-1979-1 PMID: 22147247

17. Becker C, Fantini MC, Wirtz S, Nikolaev A, Kiesslich R, Lehr HA, et al. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. Gut. 2005; 54(7):950–4. https://doi.org/10.1136/gut.2004.061283 PMID: 15951540

18. Deacon RM. Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. Nat Protoc. 2006; 1(1):118–21. https://doi.org/10.1038/nprot.2006.19 PMID: 17406222

19. Mitchell D, Krusemark ML, Hafner D. Pica: a species relevant behavioral assay of motion sickness in the rat. Physiol Behav. 1977; 18(1):125–30. https://doi.org/10.1016/0031-9384(77)90103-2 PMID: 561970

20. Wright-Williams S, Flecknell PA, Roughan JV. Comparative effects of vasectomy surgery and buprenorphine treatment on faecal corticosterone concentrations and behaviour assessed by manual and automated analysis methods in C57 and C3H mice. PLoS One. 2013; 8(9):e75948. https://doi.org/10.1371/journal.pone.0075948 PMID: 24098748

21. Miller AL, Leach MC. The Mouse Grimace Scale: A Clinically Useful Tool? PLoS One. 2015; 10(9): e0136000. https://doi.org/10.1371/journal.pone.0136000 PMID: 26406227

22. Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, et al. The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol Pain. 2011; 7:55. https://doi.org/10.1186/1744-8069-7-55 PMID: 21801409

23. Whittaker AL, Leach MC, Preston FL, Lymn KA, Howarth GS. Effects of acute chemotherapy-induced mucositis on spontaneous behaviour and the grimace scale in laboratory rats. Lab Anim. 2016; 50 (2):108–18. https://doi.org/10.1177/0023677215595554 PMID: 26162377

24. Dum JE, Herz A. In vivo receptor binding of the opiate partial agonist, buprenorphine, correlated with its agonistic and antagonistic actions. Br J Pharmacol. 1981; 74(3):627–33. https://doi.org/10.1111/j.1476-5381.1981.tb10473.x PMID: 6271322

25. van Loo PL, Everse LA, Bernsen MR, Baumans V, Hellebrekers LJ, Kruitwagen CL, et al. Analgesics in mice used in cancer research: reduction of discomfort? Lab Anim. 1997; 31(4):318–25. https://doi.org/10.1258/002367797780596211 PMID: 9350703

26. Liles JH, Flecknell PA. The effects of buprenorphine, nalbuphine and butorphanol alone or following halothane anaesthesia on food and water consumption and locomotor movement in rats. Lab Anim. 1992; 26(3):180–9. https://doi.org/10.1258/002367792780740558 PMID: 1501431

27. Jirkof P, Leucht K, Cesarovic N, Caj M, Nicholls F, Rogler G, et al. Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice. Lab Anim. 2013; 47(4):274–83. https://doi.org/10.1177/0023677213493409 PMID: 23828853

28. Safaeian R, Howarth GS, Lawrance IC, Trinder D, Mashtoub S. Emu Oil reduces disease severity in a mouse model of chronic ulcerative colitis. Scand J Gastroenterol. 2019:1–8.

CHAPTER 6

CHEMOTHERAPY-INDUCED MUCOSITIS DEVELOPMENT IN A MURINE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

CONTEXT STATEMENT

This chapter has been published in the *Scandinavian Journal of Gastroenterology*.

> *LC Chartier, GS Howarth and S Mashtoub. Chemotherapy-induced mucositis development in a murine model of colitis-associated colorectal cancer. 2020. Scandinavian Journal of Gastroenterology, Vol 55 (1): pp 47–54.*

The promising results concluded in **Chapters 2, 3** and **4** suggest that selected nutraceuticals may be therapeutic for colitis-associated colorectal (CA-CRC) cancer patients. The azoxymethane/dextran sulphate sodium (AOM/DSS) model closely mimics the human pathogenesis of CA-CRC; however, it was thought that it would be beneficial to develop a model that includes the application of conventional treatments (e.g., chemotherapy) to better understand how novel compounds interact with such therapies. Further, by including chemotherapy administration in the AOM/DSS model, it is possible to investigate the effects of test compounds on intestinal mucositis; a common debilitating side effect of cancer treatment. **Chapter 6** investigates the development of a mouse model of CA-CRC alongside the induction of chemotherapy 5-Fluorouracil-induced mucositis. This new model should enable future investigations of potential therapies to accurately analyse the interaction of compounds with the disease profile in mice.

STATEMENT OF AUTHORSHIP

Title of Paper: Chemotherapy-Induced Mucositis Development in a Murine Model of Colitis-Associated Colorectal Cancer

Publication Status: Published

Publication Details: Scandinavian Journal of Gastroenterology, 2020, Vol 55, pages 47-54

Principal Author

Name of Principal Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Completed animal trials, sample and data collection, data analyses and interpretation. Prepared manuscript including writing, formatting and submission.		
Overall percentage (%)	65%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	31/8/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, revision and editing of the manuscript.		
Signature		Date	17/9/20

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, supervision of analyses, data interpretation, revision and editing of the manuscript.		
Signature		Date	17/9/20

SCANDINAVIAN JOURNAL OF GASTROENTEROLOGY
2020, VOL. 55, NO. 1, 47–54
https://doi.org/10.1080/00365521.2019.1699601

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Chemotherapy-induced mucositis development in a murine model of colitis-associated colorectal cancer

Lauren C. Chartier[a,b], Gordon S. Howarth[a,b,c] and Suzanne Mashtoub[a,b,d]

[a]Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, Australia; [b]Gastroenterology Department, Women's and Children's Hospital, North Adelaide, Australia; [c]School of Animal & Veterinary Sciences, The University of Adelaide, Roseworthy, Australia; [d]School of Medicine, The University of Western Australia, Murdoch, Australia

ABSTRACT

Objectives: Ulcerative colitis is an incurable inflammatory bowel disease that increases the risk of colorectal cancer (CRC). 5-Fluorouracil (5-FU) is the predominant chemotherapy for CRC patients; however, undesirable side-effects, including mucositis, are common. This study utilised 5-FU-treatment in a model of colitis-associated CRC to develop a pre-clinical setting of intestinal mucositis coincident with manifestation of CRC.

Materials/methods: On day 0, female C57BL/6 mice ($n = 10$/group); (1) saline control, (2) AOM/DSS control, or (3) AOM/DSS + 5-FU were injected with saline or AOM (i.p; 7.4 mg/kg). Groups 2 and 3 underwent cycles of seven days 2%w/v DSS followed by 14 days plain water. After three cycles, 5-FU was administered weekly (i.p; 75 mg/kg) to group 3 for five weeks. Clinical indicators were measured daily and colonoscopy performed at four time-points. Mice were euthanized at 13 weeks (day 91). Intestinal sections were collected for histological and biochemical analyses. $p < .05$ was considered significant.

Results: AOM/DSS resulted in bodyweight loss, increased disease activity index, colitis-severity and tumour number compared to saline controls ($p < .05$). 5-FU-treatment in AOM/DSS mice decreased bodyweight and disease activity index at selected time-points compared to AOM/DSS controls ($p < .05$). 5-FU did not impact colitis-severity or overall tumour burden; although, resulted in fewer small tumours compared to AOM/DSS controls (<2mm; $p < .05$). AOM/DSS increased histological severity scores in intestinal sections ($p < .05$), however, 5-FU-treatment did not further increase histologically-assessed disease severity ($p > .05$).

Conclusion: Weekly 5-FU administration at a dose of 75 mg/kg was insufficient to reduce overall tumour burden or induce intestinal mucositis in the AOM/DSS mouse model.

ARTICLE HISTORY
Received 25 October 2019
Revised 11 November 2019
Accepted 26 November 2019

KEYWORDS
5-FU; chemotherapy; colitis-associated colorectal cancer; cancer; IBD; mucositis; mouse model

Introduction

The inflammatory bowel diseases (IBDs), ulcerative colitis (UC) and Crohn's disease (CD), are life-long, incurable conditions that are increasing in frequency with high morbidity [1]. While UC and CD are both characterised by chronic inflammation resulting in ulceration, haemorrhage, fibrosis and perforation, UC damage is confined to the colon, while CD presents are transmural damage in the small and/or large intestine. Another consequence of unrelenting colonic inflammation is the development of colorectal cancer (CRC). Indeed, an increased risk of CRC is well recognised in both UC and CD [2–4]. CRC is ranked as third for incidence and second in regards to mortality compared to other cancers worldwide [5]. CRC is most common in Western societies, and Australia and New Zealand have one of the highest incidence rates [5]. The link between colonic inflammation and carcinogenesis is well established [3,4,6,7], whereby oxidative stress in IBD results in prolonged generation of cellular oxidants and reactive oxygen species (ROS) which can damage cells and promote mutagenic processes [3].

The chemotherapeutic agent 5-Fluorouracil (5-FU) is used widely for the treatment of various cancers including breast, head and neck cancers, and it is the predominant chemotherapy drug for CRC patients [8,9]. 5-FU is an antimetabolite drug that disrupts essential biosynthetic processes and rapidly becomes incorporated into DNA/RNA, inhibiting their action [10]. Approximately 80% of 5-FU is metabolised in the liver by the enzyme dihydropyrimidine dehydrogenase [11]. Current treatment regimens for CRC involve daily administration of 5-FU for four consecutive days while patients are closely monitored. However, due to the non-selective nature of 5-FU action, it is commonly associated with debilitating gastrointestinal toxicities [12]. These non-specific toxicities commonly include fatigue, appetite loss, nausea, diarrhoea and intestinal mucositis [13]. As a result, patient quality of life can be significantly impacted and it is estimated that

CONTACT Suzanne Mashtoub suzanne.mashtoub@adelaide.edu.au Gastroenterology Department, Women's and Children's Hospital, 72 King William Road, North Adelaide 5006, Australia

Supplemental data for this article can be accessed here.

7.5% of cancer patients receiving chemotherapy will die as a result of chemotherapy toxicity rather than cancer [12].

Intestinal mucositis is characterised by ulceration and deterioration of mucosal membranes and can occur anywhere along the gastrointestinal tract from mouth to anus. Surprisingly, approximately 40–60% of patients undergoing chemotherapy treatment will be diagnosed with mucositis [14–16]. Mucositis develops due to the inability of 5-FU to discriminate between normal and neoplastic cells [17,18]. The Dark Agouti rat mammary adenocarcinoma (DAMA) model has been developed to investigate the effects of chemotherapies and chemo-protectants on tumour burden and associated gut-toxicities [19,20]. However, this model results in the development of breast cancer, following subcutaneous injection of cancer cells, and therefore has limitations as it does not mimic an inflammation-associated cancer phenotype.

Recently, a mouse model mimicking human colitis-associated CRC has been developed to investigate novel test compounds with anti-neoplastic potential [6]. A single injection of the carcinogen azoxymethane (AOM) in combination with cycles of dextran sulphate sodium (DSS) and water accelerates the development of colorectal tumours [6]. AOM induces carcinogenesis in rodents that share histopathological characteristics of human CRC, including mutations in *Kras* and β-catenin [6]. Furthermore, the AOM/DSS model in mice results in colonic tumours developing alongside chronic colonic inflammation. In the current study, chemotherapy (5-FU) was administered in the AOM/DSS mouse model to mimic the clinical setting of intestinal mucositis development on a background of colitis-associated CRC. Mimicking these conditions in concert is crucial as a pre-clinical model system for the investigation of new agents with anti-mucositis or anti-neoplastic potential. It was therefore hypothesised that 5-FU-treatment would inhibit tumour development in a mouse model of colitis-associated CRC, while simultaneously resulting in the development of intestinal mucositis.

Materials and methods

Animal trial

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 University of Adelaide and the Children, Youth and Women's Health Service. Female C57BL/6 mice at 8 weeks of age were sourced from the Animal Resource Centre (Perth, Western Australia) and group-housed at room temperature with a light:dark cycle of 14:10 h. Animals were acclimatised for one week before trial commencement. Mice were fed standard mouse chow and provided with drinking water for the duration of the trial.

On Day 0, mice were randomly assigned to three treatment groups ($n = 10$/group); 1) saline control, 2) AOM/DSS control, or 3) AOM/DSS + 5-FU. All mice were then intraperitoneally (i.p.) injected with saline (group 1: InterPharma, Manly, NSW, Australia) or the carcinogen AOM (groups 2 and 3: 7.4 mg/kg; Lot number: SLBN5975V; Sigma-Aldrich, Castle Hill, NSW, Australia). Groups 2 and 3 underwent three DSS/water cycles, consisting of *ad libitum* access to a DSS solution (2%w/v; 2 g/100 ml distilled water; Lot number: Q1408; MP Biomedicals LLC, Santa Ana, California, USA) for seven days, followed by 14 days of plain water in drinking bottles. Mice in Group 1 were provided with plain water throughout the 13-week trial. 5-FU (Mayne Pharma Pty Ltd, Mulgrave, Victoria, Australia; 50 mg/ml; Batch number C032600AB) was administered *via* i.p. injection (75 mg/kg) once per week during the final five weeks of the trial for Group 3. All mice were euthanized *via* CO_2 asphyxiation followed by cervical dislocation after the 13-week experimental period (day 91).

Trial analyses

Daily measurements

Body weight was recorded daily for all mice. A disease activity index (DAI) which scored body weight loss, rectal bleeding, stool consistency, and overall general condition of the animal was utilised to monitor the progression and resolution of disease. Each parameter was scored 0–3 (increasing in severity), and then summed to achieve an overall daily DAI, as described previously [21].

Colonoscopy

Colitis progression and tumour development were assessed using a high-resolution Karl Storz colonoscope (1.9 mm outer diameter; Tuttlingen, Germany) at the end of each DSS/water cycle (days 20, 41 and 62) and immediately prior to cull (day 90). Mice were anaesthetized using isofluorane inhalant and placed on a heating mat for the duration of the procedure. Once the colonoscopy was completed, mice were transferred to a recovery cage on a heating mat for close monitoring. Colitis severity was determined using five parameters described by Becker et al. [22]. These include thickening of the colon, vasculature pattern, presence of fibrin, granularity of mucosal surface and stool consistency. Each parameter was scored in a double-blinded fashion from 0-3 with increasing severity and totalled. Tumours observed from colonoscopy videos were counted and scored based upon their size, as described by [22].

Post-mortem analyses

Tissue collection

On day 91, all mice were sacrificed *via* CO_2 asphyxiation and whole blood was collected *via* cardiac puncture. Colons were excised, opened longitudinally and photographed. Weights and lengths of gastrointestinal (duodenum, small intestine and colon) organs and visceral (heart, liver, spleen, thymus, lung and kidneys) organ weights were recorded. Segments (2 cm) of the ileum, jejunum, colon (proximal and distal) were removed and placed in 10% buffered formalin for histological analyses. Additionally, 2 cm segments were collected from the mid-colon, snap-frozen in liquid nitrogen and stored at −80 °C for biochemical analysis.

Tumour analyses

High-resolution photographs of longitudinally-cut colons were analysed in a blinded manner using Olympus Soft Imaging Solutions GmbH computer software analySIS version 5.2 (Tokyo, Japan). Colonic tumours were counted, measured and categorised into size based upon their diameter (<2 mm 'small', 2–3 mm 'medium', >3 mm 'large').

Complete blood analyses

Comprehensive blood profiling was completed on whole blood collected at sacrifice (Cell-dyn® 3700, Abbott Diagnostics Division, USA) at the Veterinary Diagnostic Laboratory, The University of Adelaide. Blood analytes were quantified, including; red blood cells, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, red cell distribution width, platelet count, mean platelet volume, neutrophils, lymphocytes, monocytes, eosinophils and basophils.

Histological analyses

Small and large intestinal samples were routinely processed and embedded in paraffin wax. Sections (4 μm) were then stained with haematoxylin and eosin (H&E). Measurements of jejunal and ileal villus height/crypt depth and proximal and distal colonic crypt depth were determined for 40 well-orientated crypts/villi per tissue section per mouse and a mean value was then obtained as described by Howarth et al. [23]. All analyses were performed in a blinded fashion, using an Olympus BH-2 light microscope (Olympus Corporation, Tokyo, Japan) and image analysis software, Olympus analysis (version 5.2; Tokyo, Japan). Damage severity was assessed in sections of the jejunum, ileum, proximal and distal colon using a semi-quantitative analysis based on parameters including villus fusion/stunting, crypt area, enterocyte, crypt and crypt cell disruption, reduction in goblet cell numbers, lymphocytic and polymorphonuclear infiltration, and thickening/oedema of the submucosa and muscularis externa.

Biochemical analysis

Myeloperoxidase (MPO) levels in the colon were determined as an indicator of neutrophil infiltration, and hence, acute inflammation, using techniques described by Abimosleh et al. [24]. Thawed, homogenised samples were centrifuged at 13,000 g (4 °C) for 12 min, after which the supernatant was discarded, and the tissue homogenate was re-suspended in 200 μL of 0.5% hexadecyltrimethyl ammonium bromide buffer, a detergent (Sigma Chemicals, Castle Hill, NSW, Australia). After vortexing for 2 min, samples were again centrifuged at 13,000 g for 2 min. Background, negative and positive control samples (50 μL) and the supernatants of each test sample were then aliquoted into duplicate wells of a microtitre 96-well plate. Following the addition of a reaction solution (200 μL to each well; 4.2 mg of O-dianisidine dihydrochloride reagent, 12.5 μL H_2O_2, 2.5 ml potassium phosphate buffer [pH 6.0], 22.5 mL distilled water) the change in absorbance was measured at 450 nm at 1 min intervals for 15 min with a spectrophotometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore). Data were expressed as MPO units per gram of tissue.

Statistical analyses

Statistical comparisons were performed using SPSS version 22.0 for Windows (SPSS Inc. Chicago, IL, USA). Data were tested for normality using a Shapiro-Wilk test. All data were expressed as mean ± standard error of the mean (SEM). Organ data, tumour analyses, complete blood analyses, histologically-assessed parameters and myeloperoxidase activity were analysed using a one-way ANOVA, with a Tukey's *post hoc* test. Bodyweight, disease activity index and colonoscopically-assessed parameters were analysed using a linear mixed-effects model with mouse treated as a random factor and post hoc comparisons were reported as the difference between two group means with 95% confidence intervals. For all analyses, $p < .05$ was considered significant.

Results

Bodyweight

AOM/DSS induced significant bodyweight loss on days 7–16, 28–32, 50–52 and 54–55 compared to saline controls ($p < .05$; Figure 1). However, bodyweight was elevated in AOM/DSS mice on day 84 compared to normal controls ($p < .05$). 5-FU treatment resulted in significant bodyweight loss in AOM/DSS mice on days 65, 73, 78–82 and 85–88 compared to AOM/DSS controls ($p < .05$).

Disease activity index (DAI)

AOM/DSS significantly elevated daily DAI scores on days 6–19, 24–25, 27–28, 30, 44, 47–48, 50, 54, 72 and 81 compared to saline controls ($p < .05$; Figure 2). DAI in AOM/DSS treated mice was lower than saline controls on days 3, 22, 34 and 65 ($p < .05$). In AOM/DSS mice, 5-FU treatment (from day 56) resulted in a further increase in DAI compared to AOM/DSS controls on days 71 and 78 ($p < .05$). Interestingly, 5-FU mice presented with lower DAI scores on days 74, 80, 87 and 89 compared to AOM/DSS controls ($p < .05$).

Colonoscopically-assessed parameters

AOM/DSS significantly increased mean colitis scores on days 20, 41, 62 and 90 compared to saline controls (Figure 3; $p < .05$). 5-FU treatment did not impact colitis score at any time-point compared to AOM/DSS controls ($p > .05$). Colonic tumours were present in all mice treated with AOM/DSS by day 41 and no tumours developed in saline controls over the experimental period. Consequently, the numbers of tumours were increased in AOM/DSS controls compared to saline controls on days 41 (7.1 ± 1.5), 62 (5.6 ± 1.0) and 90 (6.1 ± 1.1; $p < .05$). The numbers of tumours in the 5-FU treatment group were reduced on day 41 (3.5 ± 0.9) compared to AOM/DSS controls; even though 5-FU treatment had not yet commenced at this time ($p < .05$). However, tumour numbers

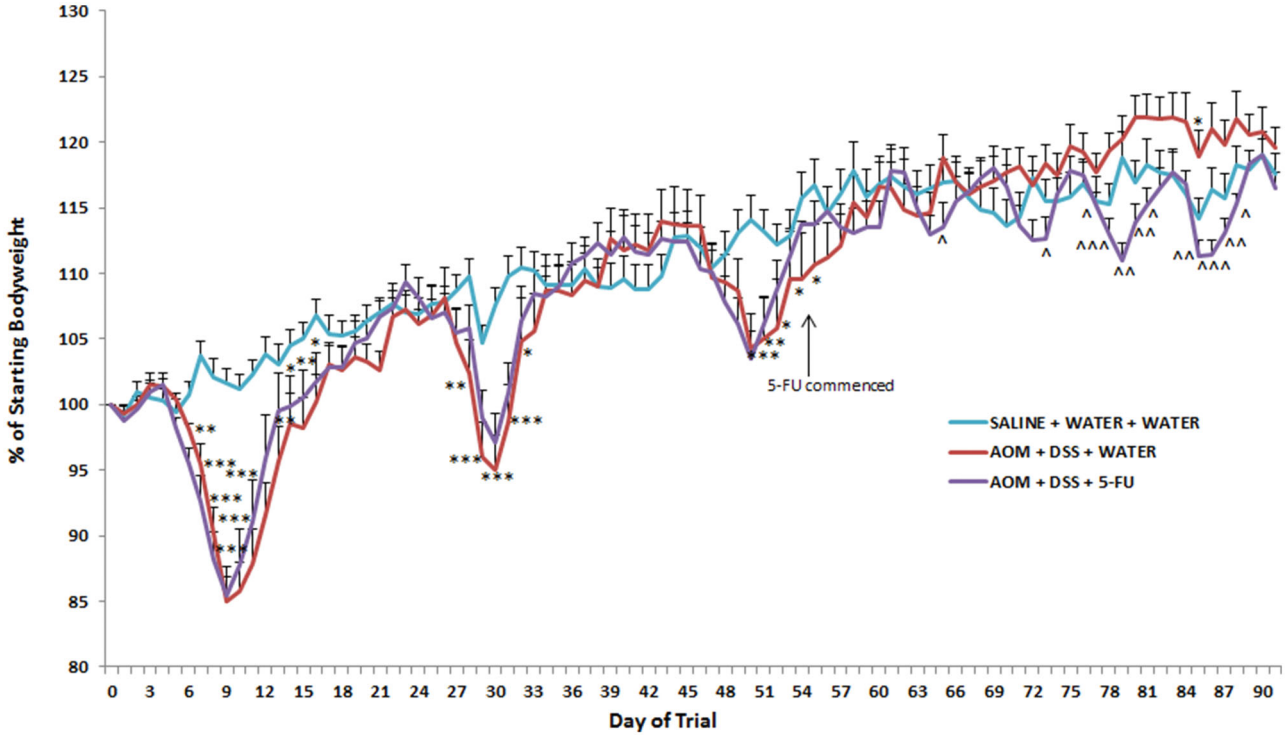


Figure 1. Daily bodyweight change of mice ($n = 10$/group). Data are expressed as mean (% change of starting bodyweight)±SEM (DSS weeks are days: 0-6, 21-27 and 42-48). $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$ compared to Saline + Water + Water, $^{\wedge\wedge\wedge}p < .001$, $^{\wedge\wedge}p < .01$, $^{\wedge}p < .05$ compared to AOM + DSS + Water.

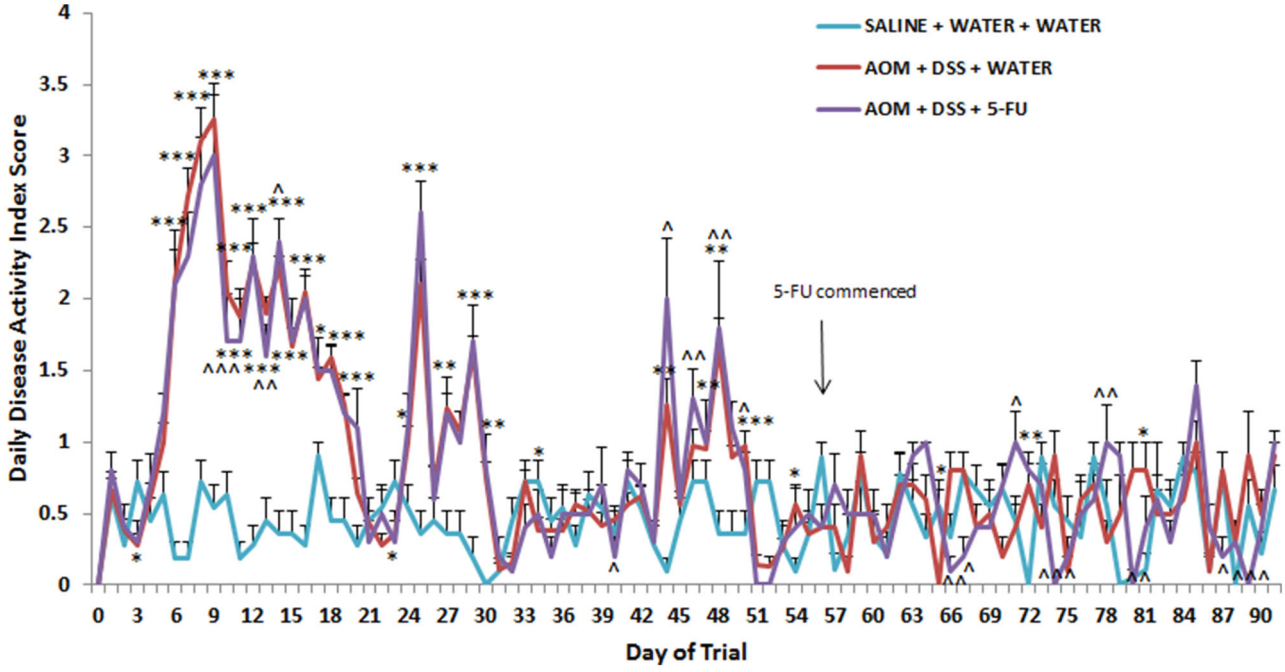


Figure 2. Daily disease activity index (DAI) score ($n = 10$/group). Data are expressed as mean DAI score ± SEM (DSS weeks are days: 0-6, 21-27 and 42-48). $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$ compared to Saline + Water + Water, $^{\wedge\wedge\wedge}p < .001$, $^{\wedge\wedge}p < .01$, $^{\wedge}p < .05$ compared to AOM + DSS + Water.

were identical to AOM/DSS controls (5.6 ± 1.0) at the following time-point (day 62; $p > .05$).

Visceral organ data

AOM/DSS increased spleen weight compared to saline controls (115% increase ± 0.09%; $p < .05$; Supplement Table S1). Thymus weight remained unchanged in AOM/DSS controls compared to saline controls ($p > .05$). However, thymus weight was reduced in 5-FU-treated animals compared to AOM/DSS controls (44% reduction ± 0.02%; $p < .05$). No significant differences were evident in other visceral organ weights, expressed as a proportion of bodyweight (%), amongst treatment groups ($p > .05$).

AOM/DSS resulted in significantly greater colon weight compared to saline controls (64% increase ± 0.1%; $p < .05$;

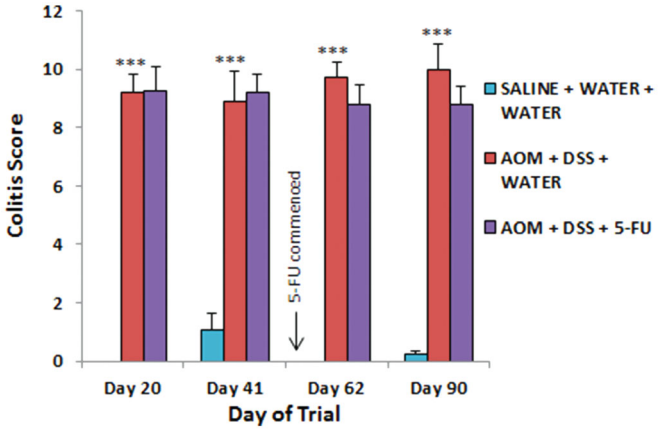


Figure 3. Colitis severity score calculated from colonoscope videos ($n = 10$/group). Data are expressed as mean (colitis score)±SEM. $^{***}p < .001$ compared to Saline + Water + Water.

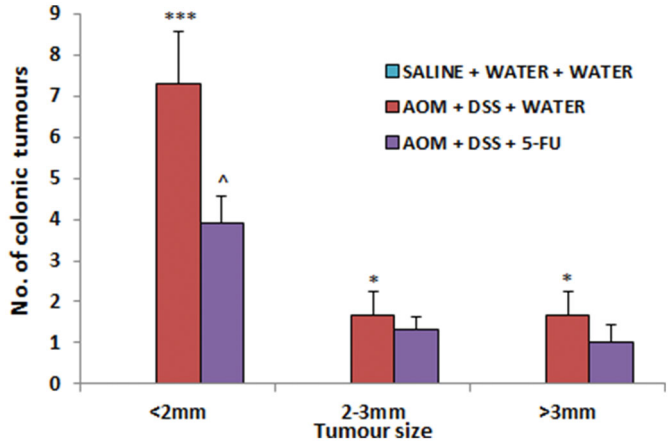


Figure 4. Colonic tumours measured from photographs arranged into sizes ($n = 10$/group). Tumours were categorised depending on their diameters; small (<2mm), medium (2-3mm) and large (>3mm). Data are expressed as mean number of tumours ± SEM. $^{***}p < .001$, $^{*}p < .05$ compared to Saline + Water + Water, $^{\wedge}p < .05$ compared to AOM + DSS + Water.

Supplement Table S1), with no significant impact following 5-FU treatment ($p > .05$). Small intestinal weights remained unchanged between saline and AOM/DSS controls; however, 5-FU-treatment resulted in significantly elevated small intestinal (14% increase ±0.06%) and caecum weight (20% increase ±0.02%) compared to AOM/DSS controls ($p < .05$). Weights of the stomach and duodenum remained unchanged across all treatment groups ($p > .05$). Furthermore, duodenum, small intestine and colon lengths were not significantly impacted by AOM/DSS or 5-FU treatment ($p > .05$).

Tumour photo analyses

Colonic tumours did not develop in saline controls during the experimental period. AOM/DSS resulted in significant colonic tumour development compared to saline controls ($p < .05$). 5-FU treatment did not significantly alter overall tumour numbers assessed at kill compared to AOM/DSS controls ($p > .05$). However, when tumours were classified into size (small: <2 mm; medium: 2-3 mm; large: >3 mm), the number of small tumours significantly decreased following 5-FU-treatment compared to AOM/DSS controls ($p < .05$; Figure 4). Medium and large tumour sizes remained unaffected by 5-FU ($p > .05$).

Complete blood analyses

AOM/DSS significantly reduced red blood cell numbers, haemoglobin and haematocrit count compared to saline controls ($p < .05$; Table 1). Additionally, AOM/DSS increased the variation of red blood cell size and width (red cell distribution) and increased mean platelet volume compared to saline controls ($p < .05$). 5-FU treatment further reduced red blood cell numbers, haemoglobin and haematocrit count compared to AOM/DSS controls ($p < .05$). Platelets and red cell distribution were further increased in 5-FU-treated mice compared to AOM/DSS controls ($p < .05$). No significant differences were evident in any other blood analytes amongst treatment groups ($p > .05$).

Histologically-assessed parameters

Villus height, crypt depth and villus height/crypt depth ratio remained unchanged across treatment groups in the jejunum and ileum ($p > .05$; Table 2(a)). In the proximal colon, crypt depth did not differ significantly amongst treatment groups ($p > .05$). However, crypts were significantly lengthened in the distal colon of AOM/DSS control mice compared to saline controls ($p < .05$). 5-FU treatment did not impact distal colonic crypt depth compared to AOM/DSS controls ($p > .05$). Histologically-assessed damage severity was significantly increased in AOM/DSS mice, compared to saline controls in the ileum, proximal and distal colon, with no further increase by 5-FU ($p < .05$; Table 2(b)). Additionally, 5-FU treatment reduced histologically-assessed damage in both sections of the colon compared to AOM/DSS controls ($p < .05$). There were no significant changes in histologically-assessed disease severity in the jejunum ($p > .05$).

Myeloperoxidase activity

Colonic MPO activity (units/gram of tissue), indicative of acute inflammation, did not differ statistically across all treatment groups (saline control: 35.13 ± 7.5; AOM/DSS control: 38.13 ± 7.5; AOM/DSS + 5-FU: 34.38 ± 7.7; $p > .05$).

Discussion

The characteristics of the AOM/DSS model of colitis-associated CRC displayed in this study were consistent with previous studies [25–27]. These features included loss of bodyweight together with increased colitic disease activity. Similarly, over the period of disease development, colonoscopic examination revealed significant numbers of colonic tumours of various sizes, accompanied by increases in colon and spleen weight. In addition to the contribution of tumour development, histological severity score was increased in the ileum and both the proximal and distal colon, although crypt depth was increased only in the distal colon. However,

Table 1. Complete blood picture analyses.

	Saline + Water + Water	AOM + DSS + Water	AOM + DSS + 5-FU
Red blood cells (10^{-12}/L)	9.33 ± 0.2	8.62 ± 0.2*	7.62 ± 0.2^^
Haemoglobin (g/L)	140.88 ± 3.8	128.11 ± 2.7*	113.33 ± 2.7^^
Haematocrit (L/L)	0.49 ± 0.0	0.45 ± 0.0*	0.39 ± 0.0^^^
Mean cell volume (fL)	52.38 ± 1.0	52.14 ± 1.1	47.74 ± 3.5
Mean cell haemoglobin (pg)	14.83 ± 0.1	14.88 ± 0.2	14.86 ± 0.1
Mean cell haemoglobin concentration (g/L)	283.75 ± 5.0	286.56 ± 4.6	292 ± 5.7
Red cell distribution width (%CV)	18.85 ± 0.6	21.38 ± 0.4**	23.88 ± 0.5^^
Platelet count (10^{-9}/L)	796.75 ± 49.4	656.44 ± 93.9	1814.13 ± 98.5^^^
Mean platelet volume (fL)	5.66 ± 0.2	10.54 ± 1.4***	9.91 ± 0.5
White blood cells (10^{-9}/L)	8.67 ± 1.5	9.62 ± 1.2	11.06 ± 0.6
Neutrophils (%)	4.54 ± 1.5	0.95 ± 0.5	2.73 ± 1.3
Lymphocytes (%)	88.64 ± 1.4	90.46 ± 1.0	87.4 ± 1.5
Monocytes (%)	5.39 ± 1.9	8.05 ± 1.4	8.86 ± 1.5
Eosinophils (%)	0.86 ± 0.3	0.22 ± 0.1	0.45 ± 0.2
Basophils (%)	0.57 ± 0.1	0.32 ± 0.1	0.57 ± 0.3

*$p < .05$, **$p < .01$, ***$p < .001$ compared to Saline + Water + Water; ^^$p < .01$, ^^^$p < .001$ compared to AOM + DSS + Water.

Table 2. Histological assessment of (a) villus height/crypt depth and (b) severity scores of damage. Data are represented as mean value ± SEM.

	Saline + Water + Water	AOM + DSS + Water	AOM + DSS + 5-FU
a)			
Villus height (μm)	Jejunum: 457.0 ± 15.4	Jejunum: 447.0 ± 16.3	Jejunum: 468.6 ± 11.6
	Ileum: 188.9 ± 7.7	Ileum: 206.3 ± 7.3	Ileum: 194.3 ± 4.4
Crypt depth (μm)	Jejunum: 70.8 ± 4.2	Jejunum: 73.3 ± 1.7	Jejunum: 70.5 ± 2.3
	Ileum: 66.9 ± 2.6	Ileum: 73.1 ± 1.9	Ileum: 73.4 ± 1.7
	Proximal colon: 106.1 ± 3.0	Proximal colon: 120.5 ± 3.8	Proximal colon: 130.3 ± 5.3
	Distal colon: 140.7 ± 1.8	Distal colon: 210.3 ± 14.6***	Distal colon: 197.6 ± 7.4
Villus height/crypt depth ratio	Jejunum: 6.6 ± 0.4	Jejunum: 6.1 ± 0.3	Jejunum: 6.7 ± 0.2
	Ileum: 2.8 ± 0.1	Ileum: 2.8 ± 0.1	Ileum: 2.7 ± 0.1
b)			
Jejunum	6.4 ± 0.8	5.2 ± 0.7	7.2 ± 1.5
Ileum	6.8 ± 0.5	9.4 ± 0.8*	7.9 ± 0.5
Proximal colon	0.2 ± 0.1	12.4 ± 0.8***	9.5 ± 1.0^
Distal colon	0 ± 0	19.5 ± 1.1***	16.0 ± 1.0^

*$p < .05$, ***$p < .001$ compared to Saline + Water + Water; ^$p < .05$ compared to AOM + DSS + Water.

perhaps not surprisingly, myeloperoxidase activity, a measure of *acute* inflammation, was not significantly affected in colonic tissues; presumably a consequence of the eight-week lapse between withdrawal of DSS and tissue collection at the end of the experimental period.

In the current study, weekly injections of 5-FU chemotherapy did not affect tumour development or colitis severity on days 62 or 91 of the study; however, 5-FU reduced the overall number of small colonic tumours at necropsy compared to AOM/DSS control groups. Furthermore, 5-FU significantly decreased bodyweight and increased DAI at selected timepoints in mice with colitis-associated CRC; a known response to chemotherapeutic agents. Post-mortem analysis of colonic tissues revealed that 5-FU significantly decreased histologically-assessed damage severity in the colon, although there were no further effects of 5-FU on crypt depth or myeloperoxidase activity. Together, these results suggested that weekly injections of 5-FU may not have been frequent enough, or at a desirable dose, to have a demonstrable effect on tumorigenesis. Moreover, similar to previous studies [16,28,29], 5-FU injection decreased thymus weight whilst increasing small intestinal weight. Organ weights were otherwise unaffected by 5-FU when compared to AOM/DSS controls. Finally, 5-FU in AOM/DSS mice resulted in significantly increased platelet count and red cell distribution, which commonly arise in conditions such as anaemia, chronic liver disease and cancer.

In a recent study by Sougiannis et al., chemotherapy was administered to C57BL/6 mice undergoing AOM/DSS conditions for three cycles consisting of 40 mg/kg 5-FU (cycle 1) and 20 mg/kg 5-FU (cycle 2 and 3) for five consecutive days, followed by nine days recovery [30]. At this dose and frequency, a reduction in total and large adenocarcinomas was observed in 5-FU-treated mice compared to AOM/DSS alone. Histological assessment of the liver also determined that liver toxicity and necrosis with abscess formation was present [30]. While the dose administered in the Sougiannis study was lower than that utilised in the current study, 5-FU did have a significant effect on tumour burden, presumably due to the frequency of administration. However, five consecutive i.p. injections every nine days is a substantially invasive procedure that would likely disrupt behavioural measures of pain and disease and thus we aimed to minimise this by administering a single injection weekly. Despite the reduction in tumour burden, the outcomes of the Sougiannis study indicated that 5-FU significantly reduced bodyweight, increased severity scores and liver weight and decreased epididymal fat, resulting in overall survival [30]. Although these results are characteristic of disease, Sougiannis et al. did not assess for mucositis and therefore cannot conclude that mucositis was established in their model.

Additionally, the tumour-inhibiting effects of 5-FU have been investigated in rats using the AOM/DSS model [31] in

which two doses of 5-FU (50 mg/kg or 75 mg/kg), were administered thrice weekly (intravenous injection) at weeks 9, 10 and 11 of the study. This was followed by one-week recovery and then repeated for weeks 13, 14 and 15, with rats being euthanized at week 16 [31]. Although the number of tumours was not affected by 5-FU, a reduction in the volume of tumours was observed at the 16-week time-point [31]. However, 67% of rats in the group receiving 75 mg/kg thrice weekly did not survive the experiment and died prematurely, primarily due to significant bodyweight loss. Therefore, at this frequency, 50 mg/kg was determined to be the tolerable dose of 5-FU [31].

In an acute murine model of DSS-induced colitis by Xiao et al., 5-FU (15 mg/kg for 3 days) ameliorated disease severity and reportedly modulated the inflammatory response by inhibiting NF-kappaB activation [32]. Moreover, colonic MPO levels were decreased and mRNA levels of pro-inflammatory cytokines (TNF-alpha, IL-1beta and IF-gamma) were downregulated by 5-FU-treatment. Therefore, future studies should assess the impact of 5-FU on pro-inflammatory cytokine levels (TNF-alpha, IL-1beta) in the AOM/DSS model to determine if similar results are obtained in colitis-associated colorectal cancer.

In the current study, 5-FU was unable to significantly impact overall tumour burden in the AOM/DSS mouse model, despite reducing the number of small tumours. Additionally, the dose of 5-FU delivered resulted in undesirable side-effects, such as bodyweight loss, but was insufficient to mimic a model of intestinal mucositis in the AOM/DSS model, as evidenced by insignificant small intestinal severity scoring. Although this study utilised a less-invasive regimen of a single weekly 5-FU injection, it is likely that more pronounced effects of tumour-inhibition and mucositis induction would have been observed if the frequency of 5-FU administration was increased, or the dose increased to 100 mg/kg. Future studies are needed to conclude whether 100 mg/kg is a tolerable dose of 5-FU for weekly injections to induce mucositis in the AOM/DSS model.

Finally, 5-FU-treatment in AOM/DSS mice has the potential to be utilised as a suitable pre-clinical model of colitis-associated CRC with chemotherapy intervention. This model may provide a realistic representation of the disease course in CRC patients as well as being a platform to investigate adjunctive treatments to reduce side-effects of chemotherapy and conventional CRC treatment regimens.

Disclosure statement

The authors report no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author, (SM), upon reasonable request.

References

[1] Hendrickson BA, Gokhale R, Cho JH. Clinical aspects and pathophysiology of inflammatory bowel disease. Clin Microbiol Rev. 2002;15(1):79–94.

[2] Choi PM, Zelig MP. Similarity of colorectal cancer in Crohn's disease and ulcerative colitis: implications for carcinogenesis and prevention. Gut. 1994;35(7):950–954.

[3] Seril DN, Liao J, Yang GY, et al. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. Carcinogenesis. 2003;24(3):353–362.

[4] Biancone L, Armuzzi A, Scribano ML, et al. Cancer Risk in Inflammatory Bowel Disease: A 6-Year Prospective Multicenter Nested Case-Control IG-IBD Study. Inflamm Bowel Dis. 2019.

[5] Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.

[6] De Robertis M, Massi E, Poeta ML, et al. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. J Carcinog. 2011;10:9.

[7] van Hogezand RA, Eichhorn RF, Choudry A, et al. Malignancies in inflammatory bowel disease: fact or fiction? Scand J Gastroenterol Suppl. 2002;37(236):48–53.

[8] McQuade RM, Stojanovska V, Bornstein JC, et al. Colorectal cancer chemotherapy: the evolution of treatment and new approaches. Curr Med Chem. 2017;24(15):1537–1557.

[9] Grem JL. 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. Invest New Drugs. 2000;18(4):299–313.

[10] Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003;3(5):330–338.

[11] Diasio RB, Harris BE. Clinical pharmacology of 5-fluorouracil. Clin Pharmacokinet. 1989;16(4):215–237.

[12] O'Brien ME, Borthwick A, Rigg A, et al. Mortality within 30 days of chemotherapy: a clinical governance benchmarking issue for oncology patients. Br J Cancer. 2006;95(12):1632–1636.

[13] Avallone A, Di Gennaro E, Silvestro L, et al. Targeting thymidylate synthase in colorectal cancer: critical re-evaluation and emerging therapeutic role of raltitrexed. Expert Opin Drug Saf. 2014;13(1):113–129.

[14] Gibson RJ, Keefe DM, Clarke JM, et al. The effect of keratinocyte growth factor on tumour growth and small intestinal mucositis after chemotherapy in the rat with breast cancer. Cancer Chemother Pharmacol. 2002;50(1):53–58.

[15] Lalla RV, Peterson DE. Treatment of mucositis, including new medications. Cancer J. 2006;12(5):348–354.

[16] Mashtoub S, Tran CD, Howarth GS. Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. Exp Biol Med. 2013;238(11):1305–1317.

[17] Sonis ST. Mucositis as a biological process: a new hypothesis for the development of chemotherapy-induced stomatotoxicity. Oral Oncol. 1998;34(1):39–43.

[18] Mitchell EP, Schein PS. Gastrointestinal toxicity of chemotherapeutic agents. Semin Oncol. 1982;9(1):52–64.

[19] Keefe DM. Gastrointestinal mucositis: a new biological model. Support Care Cancer. 2004;12(1):6–9.

[20] Vanhoecke B, Bateman E, Mayo B, et al. Dark Agouti rat model of chemotherapy-induced mucositis: establishment and current state of the art. Exp Biol Med. 2015;240(6):725–741.

[21] Howarth GS, Xian CJ, Read LC. Predisposition to colonic dysplasia is unaffected by continuous administration of insulin-like growth factor-I for twenty weeks in a rat model of chronic inflammatory bowel disease. Growth Factors. 2000;18(2):119–133.

[22] Becker C, Fantini MC, Wirtz S, et al. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. Gut. 2005;54(7):950–954.

[23] Howarth GS, Francis GL, Cool JC, Xu X, et al. Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. J Nutr. 1996;126(10):2519–2530.

[24] Abimosleh SM, Tran CD, Howarth GS. Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a

rat model of indomethacin-induced enteropathy. Evid Based Complement Alternat Med. 2013;2013:1.

[25] Chartier LC, Howarth GS, Lawrance IC, et al. Emu oil improves clinical indicators of disease in a mouse model of colitis-associated colorectal cancer. Dig Dis Sci. 2018;63(1):135–145.

[26] Mashtoub S, Howarth GS, Trinder D, et al. Emu oil attenuates disease severity and results in fewer large colonic tumours in a mouse model of colitis-associated colorectal cancer. Gastroenterology. 2017;152(5):S737–S.

[27] Mashtoub S, Safaeian R, Lawrance IC, et al. Emu oil attenuates disease severity in models of colitis and colitis-associated colorectal cancer. J Gastroen Hepatol. 2016;31:125.

[28] Bajic JE, Eden GL, Lampton LS, et al. Rhubarb extract partially improves mucosal integrity in chemotherapy-induced intestinal mucositis. WJG. 2016;22(37):8322–8333.

[29] Mashtoub S, Lampton LS, Eden GL, et al. Emu oil combined with lyprinol reduces small intestinal damage in a rat model of chemotherapy-induced mucositis. Nutr Cancer. 2016;68(7):1171–1180.

[30] Sougiannis AT, VanderVeen BN, Enos RT, et al. Impact of 5 fluorouracil chemotherapy on gut inflammation, functional parameters, and gut microbiota. Brain Behav Immun. 2019;80:44–55.

[31] Yoshimi K, Hashimoto T, Niwa Y, et al. Use of a chemically induced-colon carcinogenesis-prone Apc-mutant rat in a chemotherapeutic bioassay. BMC Cancer. 2012;12(1):448.

[32] Xiao J, Lu Z, Sheng J, et al. 5-fluorouracil attenuates dextran sodium sulfate-induced acute colitis in mice. Mol Med Rep. 2016;13(3):2821–2828.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS AND FUTURE DIRECTIONS

Inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease, are increasing in prevalence worldwide and represent a significant burden to the health and medical system in Australia. Crohn's and Colitis™ Australia estimate that almost 85,000 Australians are currently living with UC or Crohn's disease, with these conditions affecting one in 250 people between the ages of five and 40. This figure is expected to reach 100,000 cases by 2022, highlighting the need for improved treatment options. Furthermore, UC is a recognised precursor for colorectal cancer (CRC), which is the third most common cancer in Australia, with approximately 15,352 new diagnoses annually.

Aminosalicylates, corticosteroids, immunomodulators and biologics are some of the classes of pharmaceuticals commonly prescribed to UC patients. However, these therapies are associated with significant complications, including suppression of the immune system, overproduction of cortisol and the need for surgical resection of segments of bowel or the entire bowel. Additionally, in patients that develop CRC, chemotherapy is required, which substantially affects patients' quality of life. Thus, there is a need for novel and alternative therapies to treat UC and reduce inflammation, thereby preventing the development of CRC and improving patient outcomes. Currently, the majority of experimental research on IBDs is focused on short-term flare ups. Consequently, there is a need for long-term pre-clinical trials to examine the unremitting nature of disease. The azoxymethane (AOM)/dextran sulphate sodium (DSS) model is an appropriate chronic pre-clinical model for mimicking the pathogenesis of chronic inflammation progressing to CRC development.

In this thesis, Emu Oil was combined with grape seed extract (GSE) and Saireito, a Japanese Kampo medicine, to increase its therapeutic benefits in pre-clinical colitis-associated CRC (CA-CRC). The results revealed that orally administered Emu Oil partially prevented bodyweight loss, reduced diarrhoea and improved other clinical indicators of disease, including colonic inflammation and behaviour. Additionally, mice receiving Emu Oil presented with

fewer small colorectal tumours than untreated mice (**Chapter 2**). Furthermore, the efficacy of Emu Oil was augmented when combined with GSE (**Chapter 3**). Most importantly, mice administered a combination of Emu Oil and GSE displayed a greater a reduction in the total number of colorectal tumours, improvements in clinical indicators and a greater reduction in colonic inflammation than mice in the disease control groups. Interestingly, a similar result was observed when Emu Oil was combined with Saireito, whereby the combination of Emu Oil and Saireito attenuated bodyweight loss, reduced disease activity index, ameliorated colonic inflammation and reduced overall tumour development in mice (**Chapter 4**).

Traditional and indigenous medicines have been used worldwide throughout history; however, these therapies have not been widely accepted in conventional healthcare. More recently, there has been an attraction towards complementary and naturally-sourced medicines, as individuals have begun to seek alternatives to either manage conditions or to treat the side-effects associated with conventional therapies. Thus, rigorous scientific studies into alternative medicines for various health conditions are paramount to validate therapeutic claims and assess their safety.

Accordingly, since 2007, the National Health and Medical Research Council has provided more than $67 million for scientific research into complementary medicines and alternative therapies. This thesis provides additional scientific support for the use of Emu Oil and other nutraceuticals as potential adjuncts to conventional therapies for UC and CA-CRC patients. GSE and Saireito were most beneficial when administered in combination with Emu Oil in mice with CA-CRC, suggesting that Emu Oil may be driving the therapeutic response. To support this claim that Emu Oil was the main contributor to the therapeutic benefit of combined nutraceuticals, the combination of Saireito and GSE could be investigated in future studies of CA-CRC. Furthermore, future studies should also investigate the effects of other Japanese Kampo formulations and mistletoe extract, which has displayed anti-cancer potential *in vitro* (**Appendix 1**).

Indigenous Australian people, encompassing the Aboriginal and Torres Strait Islander people of Australia, are the oldest civilisation with ancestries dating back approximately 75,000 years. In 2016, the Australian Institute of Health and Welfare documented that Indigenous Australian people accounted for 3.3% (798,000) of the total Australian population. The majority of Indigenous Australian people live in cities or populated areas; however, one in five Indigenous Australian people live in remote communities. Those residing in remote communities in Australia have limited access to health and medical facilities and conventional medicines. This is a pertinent issue, as chronic disease is highly prevalent (64%) in Indigenous Australian populations. As compliance with Western medicine is difficult, alternative and traditional medicines are widely appreciated by remote communities. Specifically, in Aboriginal culture, information is scarcely documented; rather, it is passed through generations in the form of song, dance and stories. Investigating the use of Emu Oil in Australian Aboriginal communities through a community-led and locally adapted initiative is important to explore its therapeutic efficacy and accessibility in the treatment of chronic conditions. Furthermore, other native plant-derived bush medicines, for example, eucalyptus (*Eucalyptus sp.*), Billy goat plum (*Terminalia ferdinandiana*) or Snake vine (*Tinospora smilacina*), could be applied in combination with Emu Oil for application in chronic diseases.

A diverse and abundant microbiota has been linked to positive gut health and the prevention of chronic gastrointestinal diseases. Due to a relatively traditional diet and lifestyle, Indigenous Australian people in remote communities have a distinct and diverse microbial profile that results in a relatively low incidence of IBDs. It would be interesting to analyse the microbiota of Indigenous Australian people living in urbanised areas to determine whether a Western diet affects microbial populations. Considering the effects of Emu Oil on the gut microbiota have yet to be analysed in a healthy or CA-CRC setting, shotgun metagenomics and serial faecal lipocalin-2 analyses on samples from pre-clinical investigations is warranted.

To date, Emu Oil research has predominantly involved rodent models of gastrointestinal conditions in which the therapeutic action was primarily analysed through clinical indicators, markers of acute inflammation and histological damage. Consequently, the mechanism of Emu Oil action is not well understood. Recently, the effects of Emu Oil on cell proliferation were investigated immunohistochemically through Ki-67 staining. Crypt lengthening following Emu Oil administration was attributed to hyperplasia and Emu Oil attenuated elevated cell proliferation in the mouse model of CA-CRC (**Appendix 4**). Most importantly, studies of healthy animals determined that the cessation of Emu Oil therapy resulted in the restoration of normal intestinal growth (**Appendix 3**), indicating that Emu Oil therapy does not result in the uncontrolled proliferation of intestinal cells. Following these initial investigations of enterocyte proliferation, the effect of Emu Oil should be investigated on cell apoptosis through the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.

The preliminary effects of Emu Oil on the secretion of pro- and anti-inflammatory cytokines in cell lines were also investigated in this thesis (**Chapter 4**). In mature dendritic cells, Emu Oil resulted in decreased secretion of the pro-inflammatory cytokine, interleukin (IL)-12p40, suggesting an anti-inflammatory response (**Chapter 4**). Emu Oil in combination with Saireito also reduced IL-12p40 secretion; however, it should be noted that this effect was not observed in cells treated with Saireito alone. This preliminary *in vitro* investigation first suggested that Emu Oil could exert therapeutic properties in CA-CRC by reducing levels of pro-inflammatory cytokines and secondly, that Emu Oil was driving the anti-inflammatory action when combined with Saireito. However, the combination of Emu Oil and GSE was not investigated *in vitro*. These preliminary results should be confirmed by performing cytokine analyses on mouse intestinal tissue obtained from CA-CRC trials when the selected nutraceuticals are applied. To further investigate the potential mechanisms of action of Emu Oil and the combined nutraceuticals, histological analyses of proliferating cell nuclear antigen, Ki-67 and Bromodeoxyuridine are needed to determine the phase of the cell cycle that is affected by enterocyte proliferation. Individual components of each nutraceutical could then be compared

in vitro to establish the most effective constituents for clinical application. Additionally, cytokine analyses of intestinal tissue obtained from colonoscopies during the multiple DSS/water cycles should be incorporated in future studies to determine localised effects on inflammatory markers; and compare with those obtained in **Chapter 4**.

Emu Oil is comprised of 98% fatty acids; the remaining 2% non-triglyceride fraction encompasses anti-oxidant and free radical scavenging constituents. In all pre-clinical models investigating therapies for gastrointestinal conditions, Emu Oil has been effective when administered orally to rodents as a whole compound. Moving forward, it is crucial to determine the active constituents of Emu Oil to increase its therapeutic potential and deliver a concentrated synthetic Emu Oil product in the clinical setting.

Pre-clinical research involving animal studies is a requirement for therapies and drugs prior to testing in humans. The AOM/DSS model provides an accurate representation of the development of colorectal tumours on an inflammatory background, as observed in patients with CA-CRC. The AOM/DSS mouse model can differ in its duration, dose and frequency of reagent administration to induce varying degrees of disease severity. Throughout this thesis, a 9-week AOM/DSS model was used, whereby a single injection of the carcinogen, AOM, was administered. Mice underwent DSS/water cycling and were then euthanised at the end of the ninth week (day 63). This timeline mimics the human condition of colitic flare ups followed by a period of recovery; such flare ups ultimately result in significant tumourigenesis. **Appendix 5** utilised a 12-week AOM/DSS study, whereby mice underwent three extra weeks of water recovery following the third DSS cycle. Over this longer timeline (84 days), the numbers of 'large' colonic tumours were decreased by Emu Oil treatment. Suggesting that Emu Oil may have an effect on the development of 'new' tumours, as 'small' or 'younger/newly developed' tumours were not affected at the 12-week time-point. However, the number of 'small' tumours were decreased in **Chapters 2 and 4**.

Colonoscopy procedures allow researchers to monitor and analyse disease progression from inflammation to tumour development throughout long-term pre-clinical studies without the need for multiple end points and increased laboratory animal use. Incorporating biopsy collection throughout this procedure would be substantially beneficial to characterise the formation of tumours to determine whether they are hyperplastic polyps, adenomatous polyps, polyps with dysplasia or other, and to measure the presence of inflammatory cytokines throughout the 9-week period.

Traditional monitoring and scoring methods such as bodyweight and disease activity index are invaluable in animal models. Recently, studies have begun to include species-specific measures of affective states to better understand the benefits of treatments. Burrowing behaviour has routinely been included in mouse studies of gastrointestinal disease due to its simplicity; nonetheless, burrowing results have varied in their outcomes. Nesting was performed for the first time in the AOM/DSS model in **Chapter 4**; however, no significant results were obtained. The study described in **Chapter 5** included retrospective and real-time mouse facial grimace as a pain-monitoring component in mice with CA-CRC. The results from this study suggested that real-time data collection was more accurate than retrospective grimace methods. Importantly, in **Chapter 5**, it was concluded that administration of the analgesic buprenorphine did not affect the experimental study outcomes, hence, it could be used to provide pain relief in future chronic animal studies.

In a clinical setting, patients are prescribed chemotherapy treatment for CA-CRC that can result in the development of mucositis coincident with existing gastrointestinal conditions. The AOM/DSS model is an accurate representation of CA-CRC in mice; however, it is limited, as the model does not represent a realistic setting of disease with chemotherapy treatment. The study described in **Chapter 6** aimed to develop a modified AOM/DSS model that incorporated chemotherapy (5-Fluorouracil) treatment and resulted in mucositis development alongside CA-CRC pathogenesis. However, future studies need to refine the dose and frequency of 5-

Fluorouracil administration in the AOM/DSS model, as the chemotherapy administered in the study did not successfully induce mucositis. Nonetheless, this is an important advancement in developing an accurate clinical representation of current treatment regimens to test potential interactions with alternative medicines. Such investigations will allow progression to clinical trials assessing the therapeutic effect of Emu Oil on chemotherapy-induced mucositis and quality of life in CA-CRC patients.

Finally, following these rigorous pre-clinical studies, Emu Oil has been identified as a safe natural adjunctive therapy for CA-CRC. Thus, these promising findings should be translated into a clinical setting of CA-CRC, whereby Emu Oil is provided to UC patients that are at an increased risk of cancer in order to prevent dysplasia over time. Emu Oil in combination with other plant-sourced nutraceuticals could potentially be used on a global scale for UC patients and could ultimately reduce the substantial health burden and mortality associated with CA-CRC.

APPENDICES

APPENDIX 1

COMPARATIVE EFFECTS OF MISTLETOE EXTRACTS IN COMBINATION WITH 5-FLUOROURACIL ON VIABILITY OF IEC-6 AND CACO-2 INTESTINAL EPITHELIAL CELLS

STATEMENT OF AUTHORSHIP

Title of Paper: Comparative Effects of Mistletoe Extracts in Combination with 5-Flurouracil on Viability of IEC-6 and Caco-2 Intestinal Epithelial Cells

Publication Status: Published

Publication Details: Australian Journal of Herbal and Naturopathic Medicine, 2018, Vol 30, pages 174-179

Principal Author

Name of Principal Author	Zahra Lotfollahi		
Contribution to the Paper	Completed experimental trials and data collection, analyses and interpretation. Prepared manuscript including writing and formatting.		
Signature		Date	03/09/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Data analyses and interpretation, figure preparation, revision and editing of the manuscript.		
Signature		Date	17/9/20

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, data interpretation, revision and editing of the manuscript.		
Signature		Date	17/9/20

Name of Co-Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Data interpretation, figure preparation, editing and submission of the manuscript.		
Overall percentage (%)	10%		
Certification:	This paper reports on original research directly related to my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	9/9/20

Name of Co-Author	Ljubov Simson		
Contribution to the Paper	Conceptualisation and methodology development.		
Signature		Date	7/09/20

Name of Co-Author	Ker Y Cheah		
Contribution to the Paper	Assistance with analyses and data collection.		
Signature		Date	31/08/20

Name of Co-Author	Susan EP Bastian		
Contribution to the Paper	Conceptualisation, methodology development, supervision of analyses and data collection, revision and editing of the manuscript.		
Signature		Date	3/09/2020

Australian Journal of Herbal and Naturopathic Medicine **2018** 30(4)

Comparative effects of mistletoe extracts in combination with 5-Fluorouracil on viability of IEC-6 and Caco-2 intestinal epithelial cells

Zahra Lotfollahi Hons[a], Suzanne Mashtoub PhD[a,b,c], Gordon S Howarth PhD[a,b,d], Lauren C Chartier Hons[a,b], Ljubov Simson PhD[e], Ker Y Cheah PhD[b,f] and Susan EP Bastian PhD[f]

[a] Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, SA, Australia
[b] Gastroenterology Department, Women's and Children's Hospital, North Adelaide, SA, Australia
[c] School of Medicine, The University of Western Australia; Fiona Stanley Hospital, Murdoch, WA, Australia
[d] School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy Campus, SA, Australia
[e] Faculty of Applied Sciences, University of Canberra, Canberra, ACT, Australia
[f] School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, SA, Australia

Contact information

Professor Gordon S Howarth, School of Animal and Veterinary Sciences, The University of Adelaide
Roseworthy Campus, SA 5371, Australia,
Email gordon.howarth@adelaide.edu.au

Abstract

Background: Colorectal cancer is the second most common cancer in Western countries. Mistletoe extract has been used for decades as a complementary cancer therapy in Europe to improve patient general condition, vitality, pain and appetite.

Aim: To examine the effect of mistletoe extracts *(Quercus, Fraxini and Mali)* on the viability of colon cancer and normal intestinal cells.

Materials/methods: Cell viability of IEC-6 (non-transformed) and Caco-2 (colon cancer) cells was determined by MTT assay for mistletoe extracts *(Quercus, Fraxini and Mali;* aqueous; 1–100 µg/mL) alone and in combination with 5-Flurorouracil (5-FU). $p < 0.05$ was considered significant.

Results: IC_{50} values on Caco-2 cells for *Fraxini, Mali* and *Quercus* were 42.7, 65.5 and 84.4 µg/mL, respectively. IC_{50} values for *Fraxini, Mali* and *Quercus* on IEC-6 cells were 71.74, 65.52 and 84.39 µg/mL, respectively. *Fraxini* (50 µg/mL), when combined with 5-FU (5 µM), significantly increased the potency of 5-FU on IEC-6 cells compared to *Fraxini* (50 µg/mL) alone ($p<0.05$). *Quercus* was less effective than *Fraxini* at reducing Caco-2 cell viability. However, effects on IEC-6 cells were also less pronounced. *Mali* was the least effective extract on both cell lines.

Conclusion: *Fraxini* was the most potent mistletoe extract at decreasing colon cancer cell viability.

Keywords: Cell culture, chemotherapy, gastrointestinal diseases, mistletoe extracts.

Introduction

Colorectal cancer is the second most prevalent cause of cancer-related death in Western countries[1,2]. Chemotherapy, particularly 5-Fluorouracil (5-FU), is used for the most advanced stages (III and IV) of colon cancer[1,2]. Unfortunately, the indiscriminate mode of action of 5-FU not only targets cancer cells but also kills the rapidly proliferating cells in human body such as cells lining the gastrointestinal tract (enterocytes)[3-5]. Enterocyte damage results in severe side effects, including mucositis[5,6]. Mucositis is characterised by inflammation and ulceration of mucosal tissue in the gastrointestinal tract[5,6]. The severity of 5-FU-related side-effects can result in the cessation of chemotherapy[7]. Therefore, new therapeutic agents with specific toxicity to colon cancer cells are desirable, without exacerbating the undesirable impact of 5-FU on the normal healthy intestine cells.

Mistletoe is a semi-parasitic plant, which grows on several types of tree such as oak (*Quercus*), pine (*Pinus*), apple (*Mali*) and ash (*Fraxini*)[8,9]. Mistletoe extract (ME; *Viscum album L.*) was introduced in 1920 by Rudolf Steiner as an anticancer substance[9,10]. Aqueous MEs have been used for several decades as an adjunctive complementary cancer therapy in Europe[8,9,11]. A recent review concluded that mistletoe therapy resulted in long-term disease stability, improvements in patient general condition, vitality, strength, pain, sleep, and appetite. Furthermore, chemotherapy was better tolerated and patients displayed improved emotional and mental condition following mistletoe therapy[12]. The therapeutic efficacy is attributed primarily to the mistletoe lectins[13]. Other cytotoxic components of ME include viscotoxin and alkaloids[8,14]. The composition of ME varies depending on factors such as the host tree, the extraction technique and the manufacturing process[8,15].

Mistletoe lectin (ML) is a heterodimeric glycoprotein which belongs to the class of ribosome-inactivating protein type II. ML comprises a toxic chain A (N-glycosidase enzyme) and chain B (galactoside-recognising lectin)[14]. It has been proposed that ML induces tumour death as chain B of ML binds to the cell surface and then chain A inhibits protein synthesis. The glycoprotein expression on the membrane of tumour cells differs from normal cells and has a higher binding affinity for mistletoe lectins. Therefore, ME toxicity to normal cells is less pronounced indicating selective toxicity to tumour cells[16,17]. However, to date the comparative effects of MEs on normal and transformed intestinal epithelial cells have not been investigated.

In the current study, we examined MEs from three different tree species (ash, oak and apple) for their potential to induce cell death in normal (IEC-6) and transformed (Caco-2) colonic epithelial cells and whether the three extracts differentially had an impact on 5-FU induced toxicity.

Materials and methods

Materials

Dimethyl sulfoxide (DMSO) (Sigma Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and tissue culture solutions including Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS), phosphate buffer saline (PBS), antibodies and trypelExpress were from Gibco BRL, Life Technologies Pty Ltd. DBL 5-Fluorouracil for injection was purchased from Mayne Pharma Pty Ltd, Mulgrave, Victoria, Australia. The vented tissue culture flasks (75 cm^2) were from Gibco BRL, Life Technologies Pty Ltd and sterile 96-well tissue culture plates were purchased from Greiner Bio-one. The CO_2 incubator was from SANYO (Japan) and the spectrophotometer was from Dynatech (Germany).

Cell culture

The IEC-6 and Caco-2 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Both cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2, and 90% relative humidity in DMEM supplemented with 10% (v/v) FCS and 1% (v/v) antibiotics (penicillin, gentamicin and streptomycin). The cells were grown in 75 cm^2 vented tissue culture flasks. Culture medium was changed every two days and cells were passaged when they achieved 80–90% confluency.

Mistletoe extracts, *Viscum album*

Ampoules of ME (aqueous; 20 mg/ml; ABNOBA, Viscum) including *Quercus*, *Fraxini* and *Mali* dissolved in physiological saline were kindly provided by A/Prof Ljubov Simson. These samples were initially obtained from ABNOBA Pty Ltd (Germany). The extracts were prepared from mistletoe plant material using a proprietary press developed by ABNOBA. Table 1 presents the lectin and viscotoxin content (µg/mL) of the MEs.

MTT positive control

Grape seed extract (GSE) was used as a positive control as it was shown to be non-toxic to IEC-6 cells in low doses and to protect against 5-FU[18]. Also, GSE (100 µg/mL) decreased Caco-2 cell viability by 20%[18].

Cell viability assay/MTT

MTT assay was used to determine the Caco-2 (passage number: 25–30) and IEC-6 (passage number: 16–19) cell viability[16] according to a previously described method[19], described further[18]. After 24hrs and 48hrs incubation of IEC-6 and Caco-2 cells with the DMEM, respectively, the DMEM was replaced with 100 µl of each ME (1–100 µg/mL)[12] followed by 48 hrs addition of MTT reagent and reading at 570 nm. For the experiments, which examined the effect of ME combined with 5-FU on cell viability, DMEM was replaced with 90 µL of each ME mixed with 10 µL of 5-FU.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using XLSTAT Version 2012.6.03 (Addinsoft) and GraphPad Prism 6. The data for IC_{50} calculation and percentage of cell viability were analysed using one-way ANOVA and two-way ANOVA respectively with a Tukey's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

$IC_{50\ values}$ of MEs on IEC-6 and Caco-2 cells

The IC_{50} of *Quercus* on IEC-6 cells was significantly higher than on Caco-2 cells (84.39 and 64.54 µg/mL respectively; $p < 0.05$; Figure 1). Similarly, the IC_{50} of *Fraxini* was significantly higher on IEC-6 cells compared to Caco-2 cells (71.74 and 42.66 µg/mL respectively; $p < 0.01$; Figure 1). However, the IC_{50} for *Mali* was significantly lower when administered to IEC-6 cells compared to Caco-2 cells (65.52 and 86.75 µg/mL respectively; $p < 0.05$).

Effects of *Quercus* combined with 5-FU on viability of IEC-6 and Caco-2 cells

Efficacy of 5-FU was confirmed as the viability of 5-FU-treated cells decreased significantly by 30% for IEC-6 cells ($p < 0.05$; Figures 2A, 3A and 4A) and 50% for Caco-2 cells ($p < 0.05$; Figures 2B, 3B and 4B). *Quercus* (50µg/mL and 100 µg/mL; 10 µg/mL, 50 µg/mL and 100µg/mL, respectively) significantly decreased cell viability of IEC-6 and Caco-2 cells compared to cell controls ($p < 0.05$; Figures 2A and 2B). In IEC-6 cells, the combination of 5-FU (5 µM) with 100 µg/mL of *Quercus* increased the potency of 5-FU on IEC-6 cells ($p < 0.05$; Figure 2A). Importantly in the context of colon cancer treatment, *Quercus* (50 and 100 µg/mL) combined with 100 µM of 5-FU significantly decreased Caco-2 cell viability compared to 5-FU only treated cells ($p < 0.05$; Figure 2B).

Effects of *Fraxini* combined with 5-FU on viability of IEC-6 and Caco-2 cells

Fraxini (10, 50 and 100 µg/mL) significantly decreased viability of IEC-6 and Caco-2 cells compared to cell controls ($p < 0.05$; Figures 3a and 3b). In IEC-6 cells, *Fraxini* (50 µg/mL) combined with 5-FU (5 µM) significantly increased 5-FU potency compared to IEC-6 cells independently treated with either *Fraxini* or 5-FU (30%, $p < 0.05$, Figure 3A). After 48 hours, treatment of Caco-2 cells with the combination of *Fraxini* (50 and 100 µg/mL) and 5-FU (100 µM), resulted in significantly fewer viable cells (49% and 46% respectively) compared to cells exposed to 5-FU alone (71%, $p < 0.05$). Nonetheless, there was no significant difference between the viability of Caco-2 cells treated with *Fraxini* (50 and 100 µg/mL) compared to *Fraxini* in combination with 5-FU (100 µM; Figure 3B).

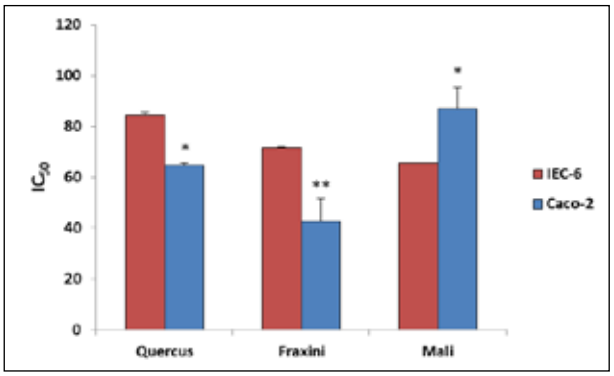
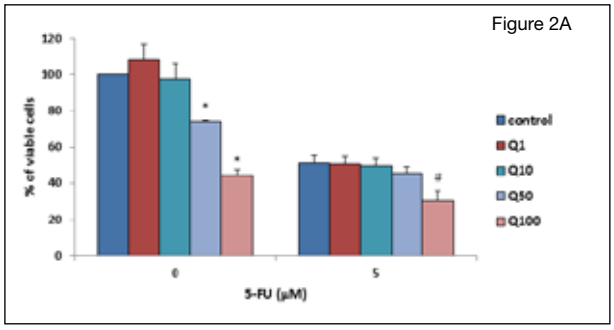


Figure 1: IC_{50} values of Quercus, Fraxini and Mali for IEC-6 and Caco-2 cells. IC50s were calculated by MTT assay after 48hrs incubation. Data are expressed as mean (IC50) ± SEM of triplicate wells from two independent experiments. * indicates $p < 0.05$, ** indicates $p < 0.01$ compared to IEC-6 treated with same ME.



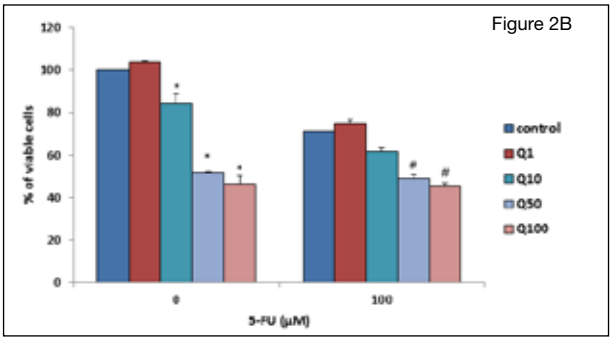
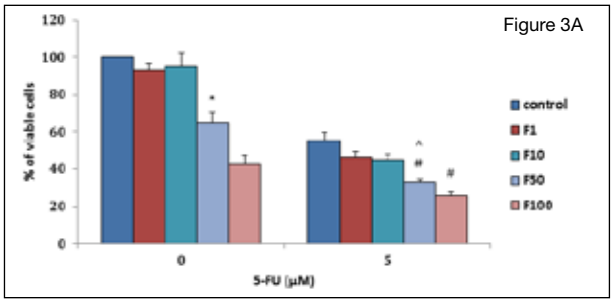


Figure 2: Effects of *Quercus* (1–100 µg/ml) and 5-FU (µM) in combination on viability of IEC-6 (A) and Caco-2 cells (B) after 48hrs. Data are expressed as mean (percentage of cell viability relative to serum free only treated cells) ± SEM of triplicate wells from 2–3 independent experiments. * indicates $p < 0.05$ compared to cell control (0 5-FU); # indicates $p < 0.05$ compared to cell control (5 and 100 µM 5-FU).

Effects of *Mali* combined with 5-FU on viability of IEC-6 and Caco-2 cells

Mali (50 µg/mL and 100 µg/mL) significantly decreased viability of IEC-6 and Caco-2 cells compared to cell controls ($p < 0.05$; Figures 4a and 4b). The combination of *Mali* (50 µg/mL and 100 µg/mL) with 5-FU (5 µM) significantly decreased IEC-6 cell viability compared to cells treated with 5-FU alone ($p < 0.05$, Figure 4A). Nevertheless, in IEC-6 cells, there was no significant difference in viability between *Mali* (50 µg/mL) and the combination of *Mali* and 5-FU (5 µM; Figure 4A). After 48hrs treatment of Caco-2 cells with *Mali* (50 and 100 µg/mL) and 5-FU (100 µM), cell viability was decreased (47% and 44% respectively) compared to cells exposed to 5-FU alone (77%). However, there was no significant difference between the viability of Caco-2 cells treated with *Mali* (50 and 100 µg/mL) compared to *Mali* in combination with 5-FU (100 µM; Figure 4B).



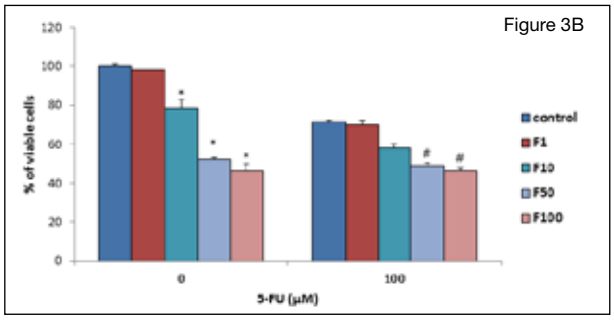
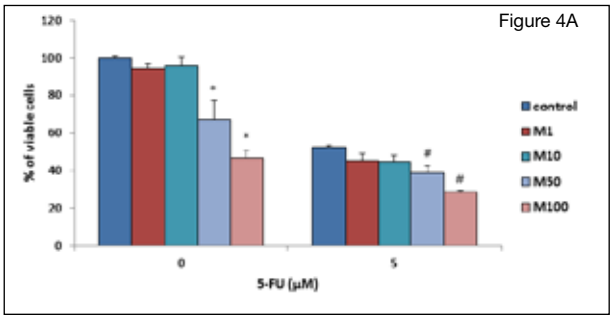


Figure 3: Effects of *Fraxini* (1–100 µg/ml) combined with 5-FU (µM) on viability of IEC-6 (A) and Caco-2 cells (B) after 48hrs. Data are expressed as mean (percentage of cell viability relative to serum free only treated cells) ± SEM of triplicate wells from 2–3 independent experiments. * indicates $p < 0.05$ compared to cell control (0 5-FU); # indicates $p < 0.05$ compared to cell control (5 and 100µM 5-FU); ^ indicates $p < 0.05$ between cells treated with 50 µg/ml of *Fraxini* and cells treated with *Fraxini* + 5µM of 5-FU.

Discussion

Despite advances in chemical-based medications, cancer patients are inclined to use naturally-sourced toxins due to adverse effects associated with chemotherapy and radiation[20]. We investigated the effects of three different MEs (*Quercus*, *Fraxini* and *Mali*), alone and in combination with 5-FU, on the viability of normal and



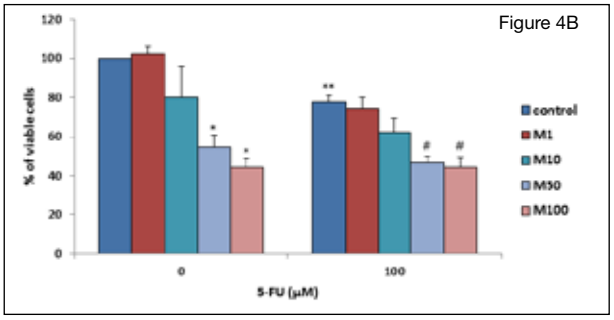


Figure 4: Effects of *Mali* (1–100 µg/ml) and 5-FU (µM) in combination on viability of IEC-6 (A) and Caco-2 cells (B) after 48 hours. Data are expressed as mean (percentage of cell viability relative to serum free only treated cells) ± SEM of triplicate wells from 2–3 independent experiments. * and ** indicate $p < 0.05$ and $p < 0.01$ compared to cell control (0 5-FU); # indicates $p < 0.05$ compared to cell control (5 and 100 5-FU µM).

transformed intestinal epithelial cells. *Fraxini* was the most potent ME on Caco-2 cells; however, it significantly increased the potency of 5-FU chemotherapy on IEC-6 cells compared to *Fraxini* alone (50 µg/mL). With the exception of *Fraxini*, none of the MEs had a significant impact on IEC-6 cell viability when combined with 5-FU, compared to IEC-6 cells treated with 5-FU alone. Furthermore, *Fraxini* was the only ME to increase 5-FU efficacy on Caco-2 cells above that of the same concentration of ME alone.

In the current study, *Fraxini* had the greatest effect on Caco-2 cells compared to *Mali* and *Quercus*. This presumably reflected the highest concentration of lectin and viscotoxin in *Fraxini* compared to *Quercus* and *Mali* (Table 1). In a study conducted by Ding and colleagues[21], the anti-proliferative activity of Fraxini was shown to be almost 10 and 6 times stronger than that of Iscador M and Iscador Q, respectively, in both Hep3B and HepG2 cells. Furthermore, *Quercus* (with slightly higher lectin and viscotoxin levels; as shown in Table 1) was more toxic than *Mali* when applied to Caco-2 cells.

Table 1: Main active constituents (lectin and viscotoxin) in *Quercus, Fraxini* and *Mali*.

Mistletoe extract	Lectin (µg/mL)	Viscotoxin (µg/mL)
Quercus	6.37	50.33
Fraxini	10.38	61.0
Mali	6.02	45.95

Lectin and viscotoxin are believed to be the main biological active components of MEs contributing to their potency to cancer cells[13,22]. These active components are reported to show anti-neoplastic effects by different mechanism of action including cell cycle arrest, induction of apoptosis, altering tumour angiogenesis and anti-inflammatory effects[21-24]. However, the exact underlying mechanism of action of MEs is not clearly understood yet[24].

It is proposed that 5-FU and MEs induce cell death through distinct molecular mechanisms of action, which include inhibition of protein synthesis, activation of apoptotic cascades, such as p-53 independent apoptosis and inhibition of telomerase[15,16,25]. Engdal (2009) demonstrated that MEs (Iscador M series II) inhibited the p-glycoprotein function of Caco-2 cells[26]. On the other hand, 5-FU induces cell death by interfering with thymidylate synthase (TS) enzyme function and consequently inhibition of DNA synthesis[27]. The MTT assay, employed in the current study, was limited in terms of distinguishing apoptosis from necrosis of cells treated with MEs. Future assays which measure direct DNA binding and determine cell apoptosis, such as ^{3}H-thymidine and Edu-IT, respectively, are recommended.

Another interesting observation was the greater efficacy of *Mali* on IEC-6 cells compared to Caco-2 cells. Previous *in vitro* studies have mainly focused on the impact of MEs on tumour cell lines and not the normal human cell lines. Further experiments are necessary to investigate the factors influencing the higher potency of *Mali* on normal intestine cells compared to colon cancer cells.

Apart from possessing the lowest IC_{50}, *Fraxini* (50 µg/mL combined with 5-FU) significantly potentiated 5-FU on IEC-6 cells compared to the same concentration of *Fraxini* alone. The study by Cazacu and his colleagues (2003) showed that mistletoe therapy (Isorel) as an adjunct to 5-FU chemotherapy after surgery improved survival rate and also alleviated chemotherapy side-effects (digestive and/or hematological toxicity) of colon cancer patients[16].

In the current study, none of the MEs protected the normal intestinal cells from 5-FU toxicity. Similar results were obtained in a recent study done by Weissenstein and colleagues, as *Viscum album* extraction did not inhibit chemotherapy-induced toxicity on 5 different cell lines[22]. In clinical settings, internal factors, such as the

immune system; a purported target of ME could have contributed to alleviation of chemotherapy side-effects after mistletoe therapy[13,22,28]. Clearly these factors were absent in the present *in-vitro* study.

The most likely explanation for IC_{50} variations in *Quercus, Fraxini* and *Mali* is due to different magnitudes of toxic components in each. The aforementioned extracts originated from mistletoe bushes, which grow on different host trees and possess distinct compositions resulting in varying magnitudes of toxicity[8,15]. Also, as mentioned previously lectins can induce apoptosis through different pathways, some being more effective than others in specific cell lines, therefore resulting in different toxicity values for different cell lines[29].

Recently, Huber and colleagues conducted a maximum tolerable dose and safety investigation of intravenous mistletoe application[30]. ME (200, 400, 700, 1200 or 2000mg) infusions were administered once weekly for 3 weeks in advanced cancer patients. A dose-limiting toxicity was not reached and no serious adverse events or suspected unexpected serious adverse events occurred as a result of mistletoe treatment. Furthermore, it was concluded that weekly infusions of ME at a starting dose of up to 2000mg is well-tolerated; however, at 2000mg dose, there is a minor risk of fever or allergic reaction[30]. These results support the safety of mistletoe therapy in advanced cancer patients.

In summary, the current study indicated that all MEs significantly reduced viability of colon cancer cells (Caco-2), while not inhibiting 5-FU efficacy. However, all MEs differentially reduced IEC-6 cell viability. Importantly *Quercus* and *Fraxini* at lower doses exhibited greater toxicity to Caco-2 cells when compared to IEC-6 cells. These observations support previous studies describing ME as an 'anticancer' substance and warrant future studies in animal models of colon cancer. Interestingly, both *Quercus* and *Fraxini* potentiated the effects of 5-FU at higher doses. On the basis of the impact on IEC-6 and Caco-2 cells displayed in this study, *Quercus* would likely be the preferred candidate for further clinical development. MEs could represent a promising new adjunct to conventional chemotherapy regimens. Further studies are required to determine the optimal host tree species, optimal dosing regimen and the specific bioactive factors responsible.

Acknowledgements

This study was not funded or sponsored by any organisation, grants or any other resources. The full raw data are available and we are able to provide them in Excel format if requested by the journal. The authors have no other acknowledgements to disclose.

Declaration of interest

The authors have no conflicts of interest to report.

References

1. André T, Boni C, Mounedji-Boudiaf L *et al.* Oxaliplatin, Fluorouracil, and Leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004;350:2343–2351.
2. Markle B, May EJ, Majumdar APN. Do nutraceutics play a role in the prevention and treatment of colorectal cancer? Cancer Metastasis Rev 2010;29:395–404.
3. Fata F, Ron IG, Kemeny N *et al.* 5-fluorouracil-induced small bowel toxicity in patients with colorectal carcinoma. Cancer 1991;86:1129–1134.
4. Mauger CA, Butler RN, Geier MS *et al.* Probiotic effects on 5-fluorouracil-induced mucositis assessed by the sucrose breath test in rats. Dig Dis Sci 2007;52:612–619.
5. Wright TH, Yazbeck R, Lymn KA *et al.* The herbal extract, Iberogast, improves jejunal integrity in rats with 5-Fluorouracil (5-FU)-induced mucositis. Cancer Biol Ther 2009;8:923–929.
6. Bowen JM, Gibson RJ, Cummins AG *et al.* Intestinal mucositis: the role of the Bcl-2 family, p53 and caspases in chemotherapy-induced damage. Support Care Cancer 2006;14:713–731.
7. Lindsay RJ, Geier MS, Yazbeck R *et al.* Orally administered emu oil decreases acute inflammation and alters selected small intestinal parameters in a rat model of mucositis. Br J Nutr 2010;104:513–519.
8. Kelter G, Schierholz JM, Fischer IU *et al.* Cytotoxic activity and absence of tumor growth stimulation of standardized mistletoe extracts in human tumor models in vitro. Anticancer Res 2007;27:223–233.
9. Mabed M, El-Helw L, Shamaa S. Phase II study of viscum fraxini-2 in patients with advanced hepatocellular carcinoma. Br J Cancer 2004;90:65–69.
10. Beuth J, Schneider B, Schierholz JM. Impact of complementary treatment of breast cancer patients with standardized mistletoe extract during aftercare: a controlled multicenter comparative epidemiological cohort study. Anticancer Res 2008;28:523–527.
11. Elsasser-Beile U, Leiber C, Wetterauer U *et al.* Adjuvant intravesical treatment with a standardized mistletoe extract to prevent recurrence of superficial urinary bladder cancer. Anticancer Res 2005;25:4733–4736.
12. Kienle GS, Mussler M, Fuchs D *et al.* Intravenous mistletoe treatment in integrative cancer care: A qualitative study exploring the procedures, concepts, and observations of expert doctors. Evid Based Complement Alternat Med 2016;4628287.
13. Heinzerling L, Von Baehr V, Liebenthal C *et al.* Immunologic effector mechanisms of a standardized mistletoe extract on the function of human monocytes and lymphocytes *in vitro*, *ex vivo*, and *in vivo*. J Clin Immunol 2006;26:347–359.
14. Duong Van Huyen J-P, Delignat S, Bayry J *et al.* Interleukin-12 is associated with the *in vivo* anti-tumor effect of mistletoe extracts in B16 mouse melanoma. Cancer Letters 2006;243:32–37.
15. Eggenschwiler J, Von Balthazar L, Stritt B *et al.* Mistletoe lectin is not the only cytotoxic component in fermented preparations of *Viscum album* from white fir (*Abies pectinata*). BMC Complement Alter Med 2007;7:14.
16. Cazacu M, Oniu T, Lungoci C *et al.* The influence of isorel on the advanced colorectal cancer. Cancer Biother Radiopharm 2003;18:27–34.
17. Guo Q, Xia B, Zhang F *et al.* Tetraspanin CO-029 inhibits colorectal cancer cell movement by deregulating cell-matrix and cell–cell adhesions. PLoS ONE 2012;7:e38464.
18. Cheah KY, Howarth GS, Yazbeck R *et al.* Grape seed extract protects IEC-6 cells from chemotherapy-induced cytotoxicity and improves parameters of small intestinal mucositis in rats with experimentally-induced mucositis. Cancer Biol Ther 2009;8:382–390.
19. Huynh-Delerme C, Huet H, Noël L *et al.* Increased functional expression of P-glycoprotein in Caco-2 TC7 cells exposed long-term to cadmium. Toxicol in Vitro 2005;19:439–47.
20. Molassiotis A, Scott JA, Kearney N *et al.* Complementary and alternative medicine use in breast cancer patients in Europe. Support Care Cancer 2005;14:260–267.
21. Ding X, Cartwright C, Tan L *et al.* Abstract 3206: Mistletoe extract inhibits the proliferation of human hepatocellular carcinoma cells by induction of apoptosis and downregulation of c-MYC. Cancer Research 2014;74:3206–3206.

22. Weissenstein U, Kunz M, Urech K *et al.* Interaction of standardized mistletoe (*Viscum album*) extracts with chemotherapeutic drugs regarding cytostatic and cytotoxic effects in vitro. BMC Complement and Altern Med 2014;14:1–9.
23. Von Schoen-Angerer T, Wilkens J, Kienle GS *et al.* High-dose *Viscum album* extract treatment in the prevention of recurrent bladder cancer: A retrospective case series. Perm J 2015;19:76–83.
24. Saha C, Hegde P, Friboulet A *et al. Viscum album*-mediated COX-2 inhibition implicates destabilization of COX-2 mRNA. PLoS One 2015;10:e0114965.
25. Hostanska K, Vuong V, Rocha S *et al.* Recombinant mistletoe lectin induces p53-independent apoptosis in tumour cells and cooperates with ionising radiation. Br J Cancer 2003;88:1785–1792.
26. Engdal S, Nilsen OG. *In vitro* inhibition of CYP3A4 by herbal remedies frequently used by cancer patients. Phytother Res 2009;23:906–912.
27. Miyazaki K, Shibahara T, Sato D *et al.* Influence of chemotherapeutic agents and cytokines on the expression of 5-fluorouracil-associated enzymes in human colon cancer cell lines. J Gastroenterol 2006;41:140–50.
28. Lee JY, Kim JY, Lee YG *et al. In vitro* immunoregulatory effects of Korean mistletoe lectin on functional activation of monocytic and macrophage-like cells. Biol Pharm Bull 2007;30:2043–2051.
29. Yau T, Dan X, Ng CC *et al.* Lectins with potential for anti-cancer therapy. Molecules 2015;20:3791–3810.
30. Huber R, Schlodder D, Effertz C *et al.* Safety of intravenously applied mistletoe extract — results from a phase I dose escalation study in patients with advanced cancer. BMC Complement Altern Med 2017;17:465.

APPENDIX 2

ORALLY ADMINISTERED EMU OIL ATTENUATES DISEASE IN A MOUSE MODEL OF CROHN'S-LIKE COLITIS

STATEMENT OF AUTHORSHIP

Title of Paper: Orally-Administered Emu Oil Attenuates Disease in a Mouse Model of Crohn's-like Colitis

Publication Status: Published

Publication Details: Experimental Biology and Medicine, 2020, pages 1–11, *DOI: 10.1177/1535370220951105*

Principal Author

Name of Principal Author	Chloe J Mitchell		
Contribution to the Paper	Completed animal trials, sample and data collection, data analyses and interpretation. Manuscript preparation including writing, formatting and submission.		
Signature		Date	17/9/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, supervision of analyses and data collection, revision and editing of the manuscript		
Signature		Date	9/10/20

Name of Co-Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Supervision and assistance with animal trials, data interpretation, editing and submission of the manuscript		
Overall percentage (%)	20%		
Certification:	This paper reports on original research directly related to my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	28/9/20

Name of Co-Author	Debbie Trinder		
Contribution to the Paper	Conceptualisation, methodology development and approval of the manuscript		
Signature		Date	16 Sept 2020

Name of Co-Author	Ian C Lawrance		
Contribution to the Paper	Conceptualisation, methodology development		
Signature		Date	23 Sept 2020

Name of Co-Author	Li San Huang		
Contribution to the Paper	Histological data collection and interpretation		
Signature		Date	21/9/20

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, methodology development, supervision of analyses and data collection, revision and editing of the manuscript		
Signature		Date	8/10/20

Original Research

Orally administered emu oil attenuates disease in a mouse model of Crohn's-like colitis

Chloe J Mitchell[1,2], Gordon S Howarth[1,2,3], Lauren C Chartier[1,3], Debbie Trinder[4,5], Ian C Lawrance[4,6], Li San Huang[2] and Suzanne Mashtoub[1,3,4]

[1]Gastroenterology Department, Women's and Children's Hospital, North Adelaide, South Australia 5006, Australia; [2]School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, South Australia 5371, Australia; [3]Adelaide Medical School, University of Adelaide, Adelaide, South Australia 5005, Australia; [4]School of Medicine, University of Western Australia, Murdoch, Western Australia 6150, Australia; [5]Harry Perkins Institute of Medical Research, Murdoch, Western Australia 6150, Australia; [6]Saint John of God Hospital, Centre for Inflammatory Bowel Disease, Subiaco, Western Australia 6008, Australia

Corresponding author: Suzanne Mashtoub. Email: suzanne.mashtoub@adelaide.edu.au

Impact statement

The submitted work details novel research to contribute to the field of inflammatory bowel diseases, specifically Crohn's disease and alternative therapies. This work is important as current therapies for Crohn's disease are variably effective and often significantly compromise patient quality of life. Emu oil, used in the current study, has the potential to alleviate disease severity and promote intestinal repair in a mouse model of Crohn's-like colitis. The new findings from this manuscript, whereby emu oil attenuated disease severity from clinical scores and colonoscopy results, add to the literature of inflammatory bowel disease mouse models and support the therapeutic potential of emu oil. This research may advance the progression to clinical trials and ultimately the commercialization of emu oil as an adjunctive or alternative therapy for the detrimental inflammatory bowel diseases.

Abstract

Crohn's disease is a severe, incurable inflammatory bowel disease. Orally administered emu oil has demonstrated anti-inflammatory properties in previous models of gastrointestinal disease. We aimed to determine whether orally administered emu oil could attenuate disease in a mouse model of Crohn's-like colitis. Female ARC(s) mice (CD-1 equivalent, $n = 10$/group) were intra-rectally administered water (120 μL) or trinitrobenzene sulfonic acid (TNBS; 3 mg in 50% ethanol; 120 μL bolus) on day 0. Mice were orally administered water (80 μL) or emu oil (80 μL or 160 μL) daily for five days and euthanized on day six. Bodyweight and disease activity were recorded daily. Colonoscopy, burrowing activity, facial grimace, histological parameters (damage severity, small intestinal villus height/crypt depth and colonic crypt depth), myeloperoxidase activity and intestinal permeability were assessed. $P < 0.05$ was considered statistically significant. TNBS decreased bodyweight (days 1, 2, 4; $P < 0.05$) and increased disease activity (days 1–6; $P < 0.01$), compared to normal controls. Emu oil (80 μL) attenuated disease activity on days 5–6 ($P < 0.05$), although bodyweight loss was not significantly impacted ($P > 0.05$). Facial grimace and colonoscopy scores were significantly increased in TNBS-control mice; effects attenuated by both volumes of emu oil ($P < 0.001$). TNBS increased histological damage severity compared to normal controls ($P < 0.05$); an effect attenuated by 80 μL emu oil (proximal and distal colon; $P < 0.05$) and 160 μL emu oil (distal colon; $P < 0.01$). In the ileum, villus height and crypt depth were unaffected by TNBS or emu oil treatment compared to normal ($P > 0.05$). TNBS-induced distal colonic crypt lengthening was unaffected following emu oil administration ($P > 0.05$). Remaining parameters, including burrowing, myeloperoxidase activity and intestinal permeability, were unchanged across all treatment groups ($P > 0.05$). In normal mice, emu oil treatment did not significantly impact any parameter compared to normal controls. In conclusion, emu oil reduced overall disease severity and facial grimace scores in TNBS mice. These results suggest therapeutic potential for orally administered emu oil in the management of Crohn's disease.

Keywords: Emu oil, inflammatory bowel disease, Crohn's disease, mouse model, nutraceutical, gastroenterology

***Experimental Biology and Medicine* 2020; 0: 1–11. DOI: 10.1177/1535370220951105**

Introduction

Inflammatory bowel disease (IBD) is the collective term for a group of incurable inflammatory conditions of the small intestine and colon, encompassing Crohn's disease, ulcerative colitis, and indeterminate colitis. Crohn's disease is a chronic inflammatory condition characterized by transmural inflammation of the gastrointestinal tract occurring in alternating active and quiescent periods, distinguishing it from colitis which affects only the colonic mucosa.[1] Crohn's disease may affect all layers of the gastrointestinal tract from mucosa to serosa in a discontinuous pattern of inflamed and healthy segments.[2,3]

Common treatments for Crohn's disease include corticosteroids (prednisolone, budesonide) and targeted anti-TNF-α antibody therapies (infliximab, adalimumab) with the aim to alleviate inflammation and induce remission.[1] There are detrimental aspects to the use of commonly prescribed medications which limit their efficacy, particularly with prolonged use. Reported side-effects of adverse extra-intestinal manifestations include ocular and dermatological reactions, metabolic and nervous system interference, as well as increased risk of opportunistic infections resulting from immunosuppression.[4,5] Surgical intervention is often required to resect severely inflamed sections of bowel, yet this is not curative and post-operative recurrence is common. The variable efficacy and side-effects of conventional IBD treatments emphasize the need for new and novel adjunctive treatment options, and naturally derived orally administered therapies (nutraceuticals) have been of recent interest.[6]

Emu oil is one such animal-derived product investigated for its potential therapeutic applications. Traditionally used by Indigenous Australian people as a topical anti-inflammatory treatment for wound healing and arthritis relief,[7,8] emu oil is rendered from the subcutaneous and retroperitoneal adipose tissue of the emu (*Dromaius novaehollandiae*). Modern emu oil refinement involves heating and filtration of adipose tissue to remove solids, and centrifugation to further separate contents.[9] The refined oil comprises approximately 98% fatty acids: 49% *n*-9 oleic acid, 24% palmitic acid, 4% *n*-7 palmitoleic acid, 1% *n*-3 α-linoleic acid, with lower levels of other fatty acids.[6] The content and ratio of *n*-3, *n*-6 and *n*-9 fatty acids have been of particular interest. *n*-3 fatty acids have demonstrated anti-inflammatory potential by downregulating pro-inflammatory eicosanoid pathways and reducing levels of pro-inflammatory cytokines including TNF-α and interleukin-12 in a rodent model of colitis.[10] Topically applied emu oil has exhibited greater anti-inflammatory and reparative properties than other animal-derived oils with lower *n*-6 and higher *n*-3 fatty acid content[11] as well as exhibiting a greater antioxidant effect than other ratite oils with similar fatty acid profiles. Dietary fish oil has previously been investigated in IBD attributed to its high *n*-3 content; however, it has been ineffective at long-term maintenance of disease remission.[12]

Oral administration of emu oil has effectively attenuated disease parameters in preclinical models of gastrointestinal disease, including non-steroidal anti-inflammatory drug (NSAID)-induced enteropathy,[13] ulcerative colitis,[6,14] colitis-associated colorectal cancer,[15–17] and chemotherapy-induced mucositis.[18–21] Although emu oil has demonstrated efficacy in rodent models of colitis, it has yet to be investigated in a Crohn's disease-specific model. The trinitrobenzene sulfonic acid (TNBS) rodent model is a well-established IBD model that mimics the histopathology and clinical presentation of Crohn's disease.[22]

In the current study, it was hypothesized that orally administered emu oil would attenuate disease severity in a mouse model of TNBS-induced Crohn's-like colitis. The aim of the study was to determine whether emu oil could reduce the severity of TNBS-induced Crohn's-like colitis as assessed by clinical indicators, biochemical and histological analyses.

Materials and methods

Animals

All studies were conducted in accordance with the 'Australian code of practice for the care and use of animals for scientific purposes 8th edition (2013)' (National Health and Medical Research Council; Canberra, 2013) under approval from the Animal Ethics Committees of the University of Adelaide and the Women's and Children's Health Network (approval number 1024/2/2018). Female ARC(s) mice (12 weeks of age, mean day 0 bodyweight 33.7 g) were sourced from the Animal Resource Centre (Perth, Western Australia, Australia) and housed at the Women's and Children's Hospital Animal Care Facility (North Adelaide, South Australia, Australia). Animals were group-housed in standard open-top cages with litter and shredded newspaper bedding at room temperature with a light:dark cycle of 14:10 h. All animals underwent a one-week acclimatization period and were provided *ad libitum* access to standard mouse chow and drinking water throughout the trial period.

Experimental trial

The experimental trial duration was determined from a seven-day pilot study. Female ARC(s) mice ($n = 50$) were assigned to five groups ($n = 10$/group) by random stratification based on initial bodyweight and burrowing ability: Group 1: normal control; mice administered water enema and water gavage (Water+Water), Group 2: mice administered water enema and emu oil gavage (Water+emu oil (160 μL)), Group 3: Crohn's-like colitis control; mice administered TNBS enema and water gavage (TNBS+Water), Group 4: mice administered TNBS enema and low volume emu oil gavage (TNBS+emu oil (80 μL)), Group 5: mice administered TNBS enema and high volume emu oil gavage (TNBS+emu oil ((160 μL)). On day 0, mice were anaesthetized (1.5–2% isoflurane; AbbVie; Mascot, New South Wales, Australia) and administered an intra-rectal enema of Water (120 μL) or TNBS (3 mg in 50% ethanol; 120 μL bolus; Sigma, Castle Hill, New South Wales, Australia) while in the Trendelenburg position. Water or

Table 1. Fatty acid composition of emu oil (Emu Tracks, Marleston, South Australia, Australia; batch #09171018) analyzed by gas chromatography.

Analyte	Common name	Total lipids (%)
Total saturates		**44.0**
14:00	Myristic acid	5.0
16:00	Palmitic acid	22.7
18:00	Stearic acid	12.7
20:00	Arachidonic acid	0.2
Total monounsaturated		**44.7**
16: 1n-7 Omega 7	Palmitoleic acid	6.3
18: 1n-9 Omega 9	Oleic acid	36.4
18: 1n-7 Omega 7	Vaccenic acid	3.0
20: 1n-9 Omega 9	Gondoic acid	0.4
Total Omega 9		**36.6**
Total Omega 7		**8.1**
Total Omega 3		**2.9**
18: 3n-3	Alpha-linolenic acid	0.9
Total Omega 6		**7.6**
18: 2n-6	Linoleic acid	8.0
20: 2n-6	Eicosadienoic acid	0.8
20: 4n-6	Arachidonic acid	0.8

Bold characters signify the total lipids for that category of Analytes. Non-bold characters listed below the bold characters represent the breakdown of the lipids within that Analyte category.

emu oil (100% emu oil) was administered by oral gavage once daily from day 1 to 5 of the trial (total five gavages).

Emu Oil

Commercially available pure emu oil was purchased from Emu Tracks (Marleston, South Australia, Australia; batch #09171018). Aliquots (5 mL) of emu oil were stored in darkness at 4°C. Fatty acid composition (Table 1) was analyzed by gas chromatography by the Waite Lipid Analysis Service (Urrbrae, South Australia, Australia).

Colonoscopy

Disease progression was monitored using a high-resolution Karl Storz colonoscope (1.9 mm outer diameter; Tuttlingen, Germany). On day 0 and 5, mice were anaesthetized using isoflurane inhalant (AbbVie; Mascot, New South Wales, Australia) and colonoscopy was conducted to obtain baseline data and visualize disease progression. Colitis severity was scored by a blinded assessor using five parameters scored from 0 to 3 (maximal severity): colon wall thickening, changes in vasculature pattern, fibrin, mucosal granularity, and stool consistency.[23]

Disease activity index and daily monitoring

Mice were monitored daily for changes in body weight and condition throughout the experimental period. Disease activity index (DAI) was used to assess colitis severity based on four parameters scored from 0 to 3 (maximal severity): daily body weight change, rectal bleeding, stool consistency and general condition.[24] Rectal bleeding was scored as 0 for not present, 1; if identified in the feces, 2; if present around the anus, and 3; when bleeding was obviously wet/smeared along the mouse's tail. Additionally, general condition was scored by observing grooming, activity, alertness and movement of each mouse. Mice with severely impaired general condition appeared with a ruffled coat, hunched and immobile.

Behavioral analyses

Burrowing behavior was recorded pre- and post-enema as an indicator of welfare and pain due to disease progression. Burrowing studies were conducted on days −2, −1 and 4 of the experimental period. On day −2, animals were burrowed in pairs to encourage burrowing behavior and familiarize the animals with the burrowing apparatus, with subsequent burrowing conducted individually (day −1 being the baseline recording of individual burrowing ability). Burrows were pre-filled with 400 g of clay substrate gravel (cat litter, Black and Gold; Adelaide, South Australia, Australia). Mice were allowed to acclimatize in darkness for 1 h before being moved to test cages, where they remained in darkness to burrow for an additional hour. At the end of the test period, the burrow was removed and weighed to determine the volume of displaced substrate.

TNBS-induced pain was measured using the validated mouse facial grimace criteria.[25] Animals were individually placed in a transparent plastic enclosure and video recorded (JVC Everio Hard Disk Camcorder GZ-MG330; Yokohama, Japan) for a 10-min period. Ten still images were retrieved from the video footage when animals were facing the camera front-on, or when a clear side-on image could be acquired. Images were cropped to show only the face, and five images were randomly selected for scoring by two treatment-blinded observers.

Tissue collection and histological analysis

On day 6, animals were euthanized by CO_2 asphyxiation and whole blood was obtained by cardiac puncture. The small intestine and colon were removed, measured, emptied of contents and weighed before being separated into segments for further processing and histological analysis. Organs (thymus, heart, lungs, liver, stomach, spleen, kidneys) were removed, weighed and discarded.

Sections of the ileum and colon (4 μm) were stained with hematoxylin and eosin. Proximal and distal colonic crypt depth measurements were obtained from 40 crypts per tissue section per mouse. Histological damage severity scoring was assessed using a quantitative scoring system.[26] Additionally, measurements of 10 well-orientated villi and crypts for each ileum section per mouse were recorded. Sections were analyzed in a blinded fashion using a light microscope (Olympus Corporation; Tokyo, Japan) and Olympus Soft Imaging Solutions GmbH software analysis version 5.2 (Tokyo, Japan).

Myeloperoxidase assay

Colonic myeloperoxidase (MPO) activity was determined as an indicator of acute inflammation by neutrophil

infiltration using techniques previously described.[13] Absorbance (450 nm) was measured at 1-min intervals for 15 min with a BioTek Synergy Mx Microplate Reader (BioTek; Winooski, Vermont, USA) and Gen5 version 2.00.18 software.

Intestinal permeability assay

Three hours prior to euthanasia, mice from Water+Water, TNBS+Water and TNBS+emu oil (160 µL) treatment groups received a 500 mg/kg dose of fluorescein isothiocyanate (FITC)-dextran (mol wt 4000, 75 mg/mL; Sigma; Castle Hill, New South Wales, Australia) by oral gavage. Blood samples were centrifuged (11,000*g* at 23°C) for 12 min and serum collected. Serum samples were diluted 1:3 with 0.2 M PBS and FITC-dextran was quantified using a BioTek Synergy Mx Microplate Reader (BioTek; Winooski, Vermont, USA) and Gen5 version 2.00.18 software relative to a standard curve (0.001–100 µg/mL).

Statistics and sample size justification

Sample size calculations (SigmaPlot 12.3) based upon the primary outcome of MPO activity (indicative of acute inflammation) indicated that a total of 10 mice per group would enable the detection of a 3.6-fold difference between groups with 95% power (alpha = 0.05). Statistical analyses were conducted using SPSS version 17 for Windows (SPSS Inc.; Chicago, Illinois, USA). Shapiro–Wilk test and residual plots were used to test data for normality. Bodyweight, DAI, burrowing activity and colonoscopically-assessed disease severity were analyzed using a repeated measures ANOVA with least significant difference (LSD) *post hoc*. Facial grimace score, intestinal villus height/crypt depth, MPO activity, FITC-dextran and organ data were analyzed by one-way ANOVA with Tukey's *post hoc* test. Data were presented as mean±standard error of the mean (SEM). Histological severity score data were analyzed by non-parametric Kruskal–Wallis test with Mann–Whitney U *post hoc* and were presented as median and interquartile range (IQR). $P < 0.05$ was considered statistically significant.

Results

Bodyweight

Bodyweight was determined as a percentage change from starting weight (day 0, 100% bodyweight). TNBS administration significantly decreased bodyweight compared to normal controls (days 1, 2, and 4; maximum 4.5% bodyweight reduction [(95.5% starting bodyweight); $P < 0.05$; Figure 1). There was no statistically significant effect of emu oil administration (80 µL or 160 µL) on bodyweight when compared to both normal controls and TNBS-treated controls ($P > 0.05$; Figure 1).

Disease activity index

Compared to normal controls, emu oil treatment did not increase any DAI parameters in normal animals ($P > 0.05$; Figure 2). TNBS increased DAI compared to normal controls throughout the trial period (days 1–6; maximum 11-fold increase; $P < 0.01$; Figure 2). In TNBS-treated mice, 80 µL emu oil treatment significantly reduced DAI scores compared to controls on days 5 and 6 (maximum 55% reduction, $P < 0.05$; Figure 2). There was no statistically significant effect of 160 µL emu oil treatment on DAI score ($P > 0.05$; Figure 2).

Colonoscopy

Water and emu oil (160 µL) treated normal controls did not develop Crohn's-like colitis throughout the trial (Figure 3). TNBS administration induced colitis, increasing colonoscopically assessed disease severity scores (2.9 ± 0.3) compared to normal controls (0.1 ± 0.1; $P < 0.001$; Figure 4). In TNBS-treated mice, both 80 µL and 160 µL volumes of emu oil significantly reduced colonoscopically assessed disease severity (80 µL: 1.7 ± 0.3; 160 µL: 2.1 ± 0.3; $P < 0.01$; Figure 4) compared to TNBS-treated controls.

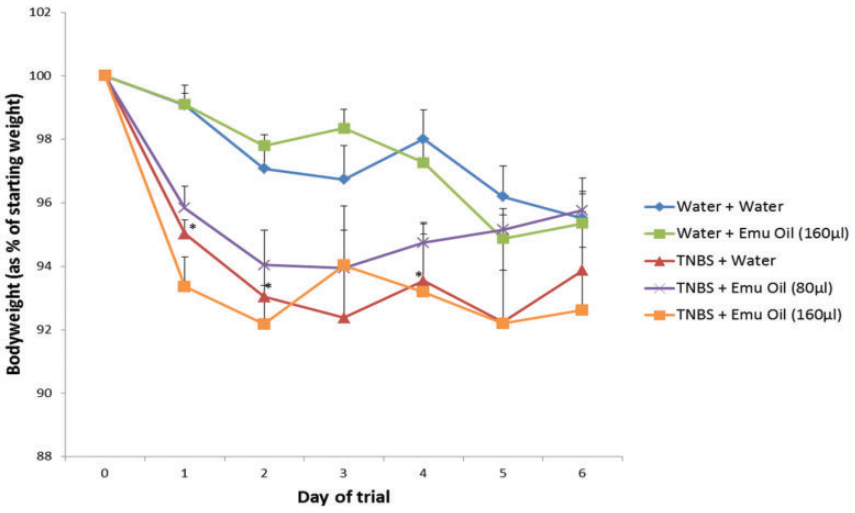


Figure 1. Daily bodyweight change of mice ($n = 10$/group). Data analyzed by repeated measures ANOVA with LSD *post hoc*, expressed as mean (% change from starting weight)+SEM. *$P < 0.05$ compared to Water+Water. (A color version of this figure is available in the online journal.)

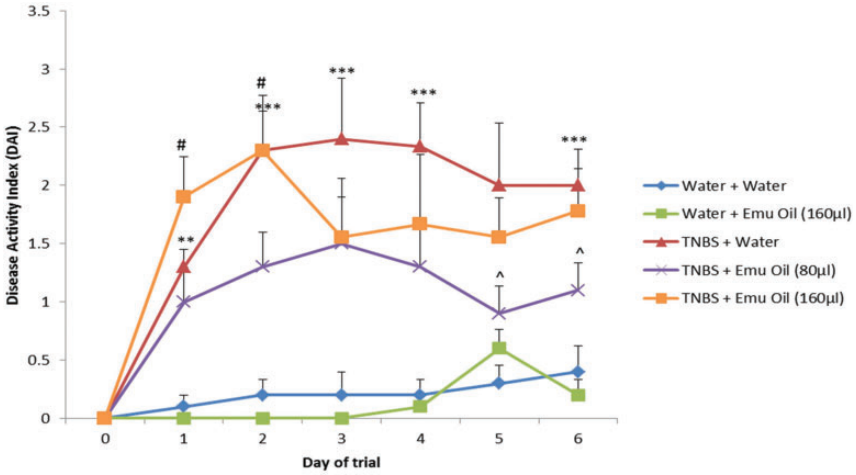


Figure 2. Daily disease activity index (DAI) score ($n = 10$/group). DAI score calculated from bodyweight loss, general condition, stool consistency, and rectal bleeding. Data analyzed by repeated measures ANOVA with LSD *post hoc*, expressed as mean (disease activity index total)+SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to Water+Water. $^{\wedge}P < 0.05$ compared to TNBS+Water. $^{\#}P < 0.05$ compared to TNBS+emu oil (80 µL). (A color version of this figure is available in the online journal.)

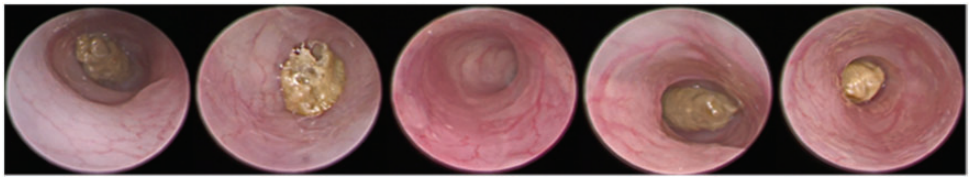


Figure 3. Images of mouse colon taken from day 5 colonoscopy. From left to right: Water+Water, Water+emu oil (160 µL), TNBS+Water, TNBS+emu oil (80 µL), TNBS+emu oil (160 µL). (A color version of this figure is available in the online journal.)

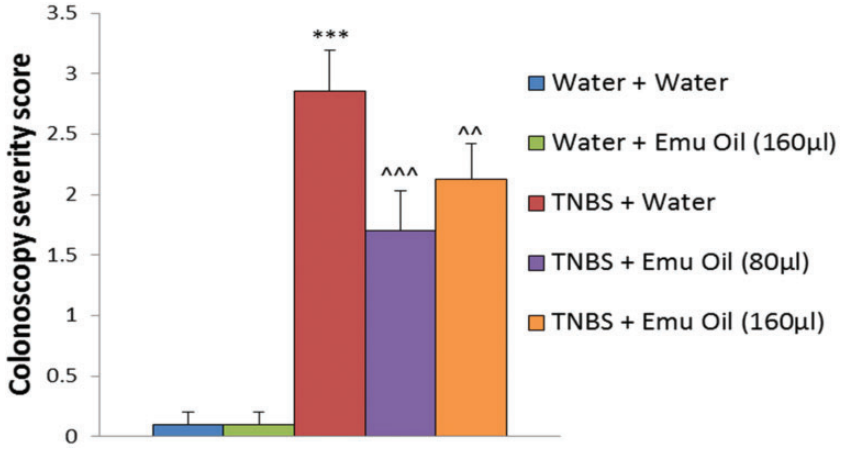


Figure 4. Colonoscopically assessed colitis severity scores ($n = 10$/group). Colitis severity score was calculated from parameters including; mucosal thickening, vasculature pattern, stool consistency, granularity of mucosal surface, and presence of fibrin. Data analyzed by repeated measures ANOVA with LSD *post hoc*, expressed as mean (colitis score)+SEM. $^{***}P < 0.001$ compared to Water+Water. $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ compared to TNBS+Water. (A color version of this figure is available in the online journal.)

Facial grimace score

TNBS administration significantly increased facial grimace scores compared to normal controls (11.5-fold increase; $P < 0.001$; Figure 5). Both 80 µL and 160 µL volumes of emu oil significantly reduced facial grimace score compared to TNBS-treated controls (11.5-fold and 5.4-fold decrease respectively, $P < 0.001$; Figure 5). There were no statistically significant differences between the two volumes of emu oil ($P > 0.05$).

Visceral and gastrointestinal organ weights and lengths

Organ weights were expressed as a percentage of day 6 bodyweight. TNBS administration did not affect visceral

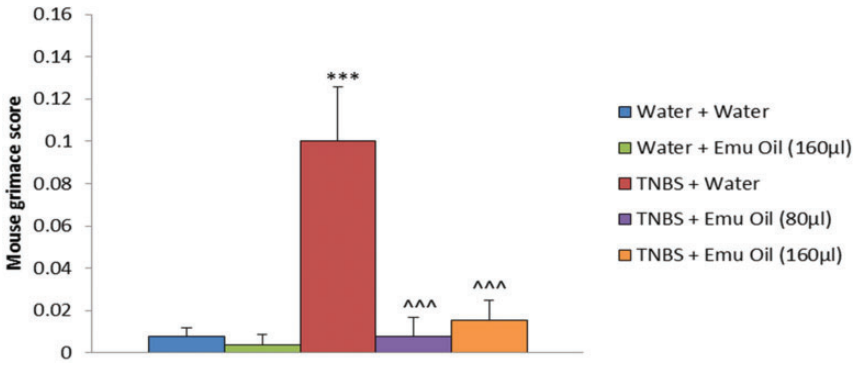


Figure 5. Facial grimace score ($n = 10$/group). Data analyzed by one-way ANOVA with Tukey's *post hoc*, expressed as mean (grimace score)+SEM. ***$P < 0.001$ compared to Water+Water. ^^^$P < 0.001$ compared to TNBS+Water. (A color version of this figure is available in the online journal.)

organ weights across treatment groups, with the exception of liver and left kidney weight. Liver weight was increased in TNBS-treated mice receiving 80 µL emu oil treatment compared to both TNBS-treated controls and 160 µL emu oil treatment ($P < 0.05$; data not shown). Left kidney weight was increased in normal mice receiving 160 µL emu oil compared to normal controls ($P < 0.05$; data not shown).

TNBS administration did not significantly impact gastrointestinal lengths or weights. Emu oil administration also had no impact on gastrointestinal lengths or weights in either water- or TNBS-treated animals ($P > 0.05$; data not shown).

Histological analyses

Histological damage severity score was increased in TNBS-treated animals compared to normal controls ($P < 0.05$; Figure 6); an effect significantly attenuated by 80 µL emu oil in both the proximal and distal colon ($P < 0.05$), and by 160 µL emu oil in the distal colon ($P < 0.01$). Importantly, there was no effect of emu oil treatment on histological severity score in normal animals compared to normal controls ($P > 0.05$; Figure 6).

TNBS induced compensatory intestinal crypt lengthening in the distal colon (190.0 ± 8.6 µm) compared to normal controls (161.1 ± 3.9 µm; $P < 0.05$; Figure 7). Emu oil administration in TNBS-treated animals had no impact on proximal or distal colonic crypt depth compared to TNBS-treated controls (80 µL: 206.6 ± 10.7 µm; 160 µL: 202.4 ± 4.9 µm; $P > 0.05$).

In the ileum, emu oil did not affect villus height (181 ± 14 µm), crypt depth (76 ± 2 µm), or villus height:crypt depth ratio (2 ± 0.2 µm) in normal mice compared to normal controls (165 ± 8 µm; 74 ± 2 µm; 2 ± 0.1 µm respectively; $P > 0.05$; Figures 8 and 9). Furthermore, TNBS did not affect villus height (154 ± 10 µm), crypt depth (78 ± 4 µm), and villus height: crypt depth ratio (2 ± 0.1 µm) compared to normal controls ($P > 0.05$). Moreover, emu oil (high dose: 153 ± 12 µm; low dose: 194 ± 19 µm) did not alter villus height or crypt depth of TNBS-treated animals compared to TNBS controls ($P > 0.05$; Figures 8 and 9). Finally, villus height: crypt depth ratio remained unchanged in TNBS-mice treated with emu oil (high dose: 2 ± 0.1 µm; low dose: 2 ± 0.2 µm) compared to TNBS controls ($P > 0.05$; Figure 9).

Burrowing, myeloperoxidase activity, and intestinal permeability

There was no demonstrable impact of TNBS administration or emu oil treatment on burrowing ability, tissue MPO, or serum FITC-dextran levels.

Discussion

In the present study, TNBS administration induced clinical, macroscopic and histological features of Crohn's-like colitis. Importantly, orally administered emu oil attenuated selected disease parameters as evidenced by reduced disease activity, decreased colonoscopically- and histologically-assessed disease severity and normalized behavioral analyses. Moreover, histological measurements of villus height/crypt depth in the ileum were unaffected by TNBS or emu oil, highlighting that the current model induced damage that was confined to the colon. Therefore, the current study mimicked a Crohn's-like colitis disease phenotype compared to a traditional Crohn's model, in which damage can be observed in the small intestine.

The safety of emu oil for intestinal application has been confirmed in both acute and chronic models of gastrointestinal disease.[6,13–15,17–21,27–29] Abimosleh *et al.*[13] reported that emu oil administration did not adversely affect small intestinal function in normal animals, as assessed by the non-invasive ^{13}C-sucrose breath test.[6] Chartier *et al.*[15] were the first to demonstrate the safety of emu oil for long-term use in normal mice, demonstrating consistent biological parameters in normal animals. Additionally, Mashtoub *et al.*[28] confirmed that indicators of intestinal proliferation in rodent models of mucositis and colitis returned to normal levels following cessation of emu oil treatment. In the current study, emu oil administration in normal animals did not elicit significant biological changes across any parameters investigated, supporting these previous studies detailing the safety of emu oil for application in healthy individuals.

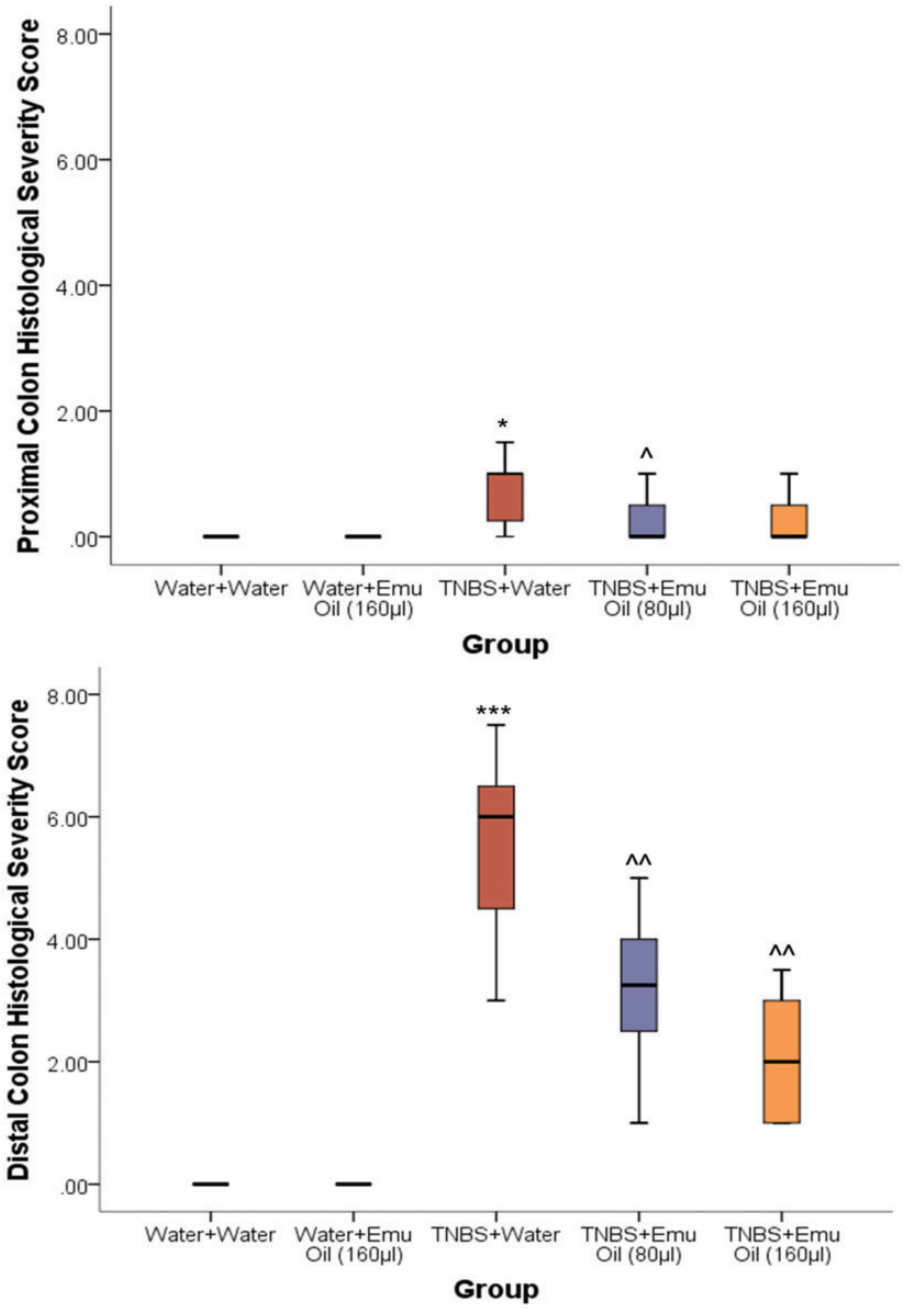


Figure 6. Histologically assessed colitis severity score for (a) proximal and (b) distal colon ($n = 10$/group). Data analyzed by Kruskal–Wallis test with Mann–Whitney U *post hoc*, expressed as median and IQR. *$P < 005$, ***$P < 0.001$ compared to Water+Water. ^$P < 0.05$, ^^$P < 0.01$ compared to TNBS+Water. (A color version of this figure is available in the online journal.)

Previously, oral administration of emu oil was effective at attenuating clinically assessed disease severity in rodent models of gastrointestinal disease, including both bodyweight and disease activity index parameters.[15,17,29] Nonetheless, Abimosleh *et al.*[13] reported that bodyweight was not significantly affected by emu oil administration in a rat model of NSAID enteropathy.[13] Similar results are reflected in the current study, whereby emu oil administration did not impact bodyweight, though significantly reduced clinically-assessed disease severity compared to TNBS controls. Moreover, in normal animals, emu oil did not significantly impact bodyweight, indicating that the observed restoration of bodyweight may not have been attributed to dietary lipid intake by emu oil ingestion, rather, a protective or reparative effect of emu oil on the intestinal barrier.[20]

It is well documented that communication occurs between the central and enteric nervous system; hence, inflammation and altered microbiota in the gut could impact not only intestinal physiology but also affective state and behavior.[30] Burrowing is a behavior used as a measure of wellbeing in mice. In a previous study of colitis-associated colorectal cancer in mice, disease control animals displayed impaired burrowing activity during

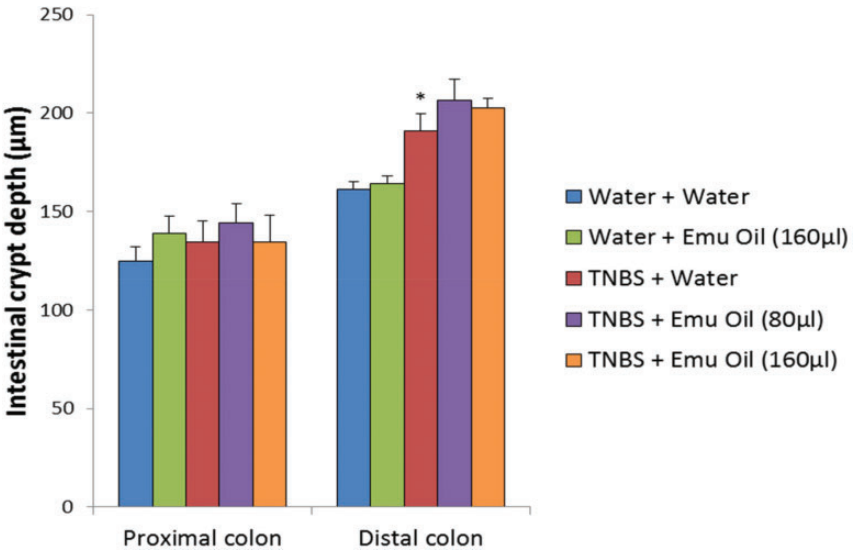


Figure 7. Intestinal crypt depth of the proximal and distal colon ($n = 10$/group). Data analyzed by one-way ANOVA with Tukey's *post hoc*, expressed as mean crypt depth (µm)+SEM. *$P < 0.05$ compared to Water+Water. (A color version of this figure is available in the online journal.)

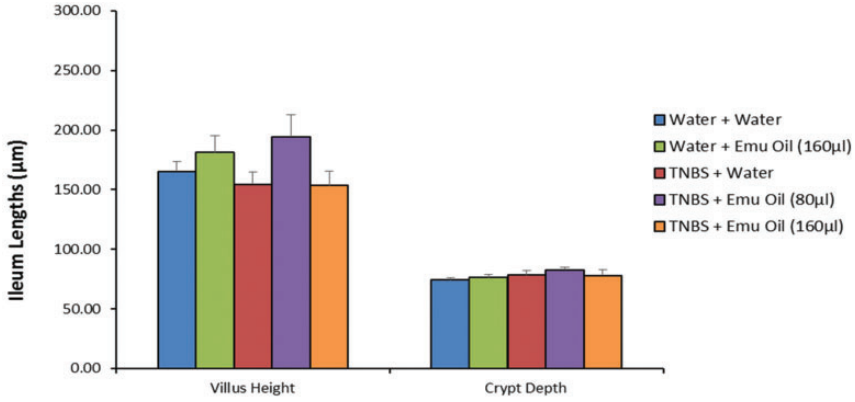


Figure 8. Small intestinal villus height and crypt depth measurements ($n = 10$/group). Data analyzed by one-way ANOVA with Tukey's *post hoc*, expressed as mean crypt depth (µm)+SEM. (A color version of this figure is available in the online journal.)

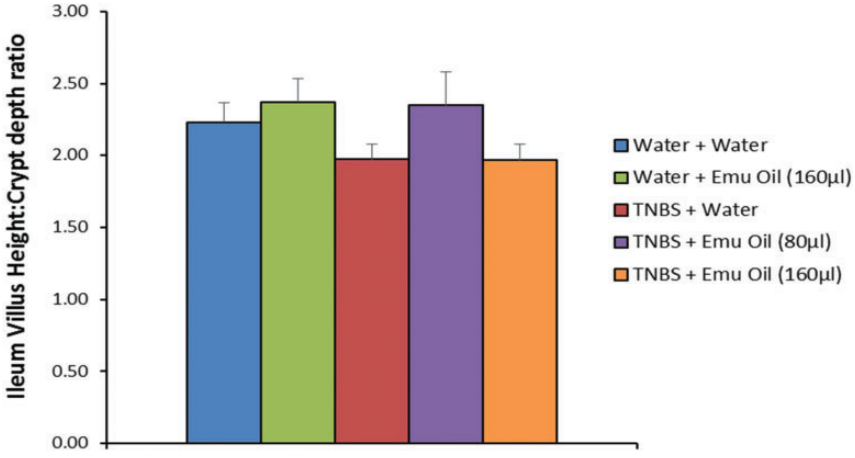


Figure 9. Small intestinal villus height:crypt depth ratio ($n = 10$/group). Data analyzed by one-way ANOVA with Tukey's *post hoc*, expressed as mean crypt depth (µm)+SEM. (A color version of this figure is available in the online journal.)

periods of peak disease; an effect which was attenuated by emu oil.[15] Additionally, Jirkof *et al*.[31] reported that a decrease in burrowing behavior coincided with an increase in colitis severity in mice. Interestingly, neither TNBS administration nor emu oil impacted burrowing activity in the current study. Animals were allocated to treatment groups by stratified randomization based upon their initial burrowing ability and bodyweight; however, increasing the number of baseline tests conducted prior to TNBS or water treatment may have benefited the current preselection criteria by allowing the animals to familiarize themselves with the burrowing apparatus. An insult to the gut microbiota can negatively impact behavior, as demonstrated by anxiety-like behaviors in a mouse model of DSS colitis during a light/dark box test[30]; hence, further analyses on cecal content would be useful to determine the effect of emu oil treatment on the gut microbiota in future studies.

Pain has been assessed by "rodent grimace scales" in various studies as a method of evaluating acute pain.[25,32,33] In the present study, TNBS administration induced an increase in the mouse grimace scale compared to normal controls; an effect which was attenuated by both doses of emu oil. The grimace test was conducted at baseline and repeated at four days post TNBS administration, allowing time for inflammation to progress as confirmed by colonoscopy the following day. Importantly, the current study is the first to incorporate the mouse grimace scale as a measure of pain in a model of TNBS-induced inflammation, and the results indicate potential for the scale to be utilized in other models of gastrointestinal disease.

In a model of acute colitis, emu oil treatment reduced histologically assessed colonic damage severity up to threefold and induced colonic crypt lengthening compared to DSS controls.[6] Chartier *et al*.[15] reported that colonic crypt depth was increased in a chronic mouse model of colitis-associated colorectal cancer, with no further effect following long-term emu oil administration. In the current acute study, there was compensatory crypt lengthening following TNBS administration, with no further effect of emu oil treatment. In future studies, it would be useful to conduct a time-course study to determine if emu oil treatment in mice administered TNBS could result in compensatory crypt lengthening at an earlier time point than the endpoint of the current study.

In the present study, intestinal permeability, as quantified by serum FITC-dextran uptake, was unaffected by both TNBS administration and emu oil treatment. This may have been attributed to the timing of blood collection, upon which the site of FITC-dextran uptake within the gastrointestinal tract is highly dependent.[34] It would be useful to employ a multi-sugar permeability assay in future studies using the TNBS model to allow separate determinations of small intestinal and colonic permeability.[35]

Prior studies have reported an increase in MPO activity in animal models of small intestinal disease including chemotherapy-induced mucositis[18,20] and NSAID-induced enteropathy.[13] Despite a reduction in ileal MPO following emu oil treatment in these models of small intestinal disease, MPO levels remained unchanged across all treatment groups in the current model of colonic disease.

TNBS colitis increases levels of circulating IL-12 and IL-17 as inflammation becomes chronic.[36] Additionally, serum levels of the pro-inflammatory cytokines IL-6, IL-11 and IL-22 are known to increase in human IBD patients.[37]IL-6, IL-11 and IL-22 are of particular interest as they are responsible for activation of the Signal Transducer and Activator of Transcription 3 (STAT3) gene, known to be associated with IBD susceptibility.[38] These interleukins have both protective and antagonistic action, as they can confer a protective effect on intestinal epithelial cells, yet may simultaneously elicit an inflammatory immune response.[37] It would be beneficial in further studies to analyze tissue activity of pro-inflammatory cytokines known to be involved in both the pathogenesis of human Crohn's disease and the inflammatory pathways associated with TNBS-induced Crohn's-like colitis.

While the exact mechanism of emu oil action is not well understood, it has been suggested that its protective and reparative effects may be attributed to its fatty acid profile. A higher ratio of *n*-6 to *n*-3 FAs has been associated with colitis development[11] Dietary *n*-3 FAs eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) regulate multiple inflammatory pathways, inhibiting the transcription of genes responsible for production of pro-inflammatory cytokines.[39] Interestingly, emu oil has exhibited greater anti-inflammatory action than other animal-derived oils with both similar FA profiles and profiles with higher *n*-3 content.[40] This suggests that the properties of emu oil cannot be attributed to the FA profile alone. It is therefore important to consider the composition of the 2% non-triglyceride fraction as well as the ratio of unsaturated to saturated FAs. Previous studies suggest that a high unsaturated to saturated FA ratio is indicative of greater radical scavenging activity (RSA), thereby protecting against oxidative damage.[40] However, Mashtoub *et al*.[41] demonstrated that this ratio was greater for olive oil than for emu oil, despite emu oil exhibiting far greater RSA.[40,41] This further supports the theory of the 2% non-triglyceride fraction of emu oil comprising compounds responsible for its antioxidant properties.

This study is the first to investigate emu oil administration in a Crohn's-specific animal model, providing encouraging results for the potential application of emu oil in the management of Crohn's disease. Exploring microbiome analyses, relevant cytokine profiles and intestinal permeability measures will further elucidate the mechanism of emu oil action. Moreover, although there were no overt indications of extra-intestinal effects of emu oil, future studies could include serological and urine tests to determine renal and hepatic function. Finally, it would be worthwhile investigating emu oil in a model of chronic Crohn's disease which is more reflective of the long-term relapsing disease course.

Authors' contributions: CJM performed all animal work and analyses detailed, as well as writing the final manuscript. GSH and SM supervised and conceptualized the study. GSH and SM also critically reviewed and edited the manuscript. DT and ICL assisted in the planning and conceptualization of the study. LCC assisted with animal work and analyses involved

in the study and contributed to editing of the final manuscript. LSH contributed to data analyses. All authors have read and approved the final version of this manuscript.

ACKNOWLEDGMENTS

The authors would like to acknowledge Dr Alexandra Whittaker for assistance with the mouse facial grimace analysis and burrowing input.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Lauren C Chartier https://orcid.org/0000-0001-7068-627X

REFERENCES

1. Akobeng AK. Crohn's disease: current treatment options. *Arch Dis Child* 2008;**93**:787–92
2. Laass MW, Roggenbuck D, Conrad K. Diagnosis and classification of Crohn's disease. *Autoimmun Rev* 2014;**13**:467–71
3. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006;**3**:390–407
4. Mocci G, Marzo M, Papa A, Armuzzi A, Guidi L. Dermatological adverse reactions during anti-TNF treatments: focus on inflammatory bowel disease. *J Crohns Colitis* 2013;**7**:769–79
5. Vavricka SR, Schoepfer AM, Scharl M, Rogler G. Steroid use in Crohn's disease. *Drugs* 2014;**74**:313–24
6. Abimosleh SM, Lindsay RJ, Butler RN, Cummins AG, Howarth GS. Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. *Dig Dis Sci* 2012;**57**:887–96
7. Snowden JM, Whitehouse MW. Anti-inflammatory activity of emu oils in rats. *Inammopharmacology* 1997;**5**:127–32
8. Whitehouse MW, Turner AG, Davis CK, Roberts MS. Emu oil(s): a source of non-toxic transdermal anti-inflammatory agents in aboriginal medicine. *Inammopharmacology* 1998;**6**:18
9. Beckerbauer LM, Thiel-Cooper R, Ahn DU, Sell JL, Parrish FC, Jr., Beitz DC. Influence of two dietary fats on the composition of emu oil and meat. *Poult Sci* 2001;**80**:187–94
10. Whiting CV, Bland PW, Tarlton JF. Dietary n-3 polyunsaturated fatty acids reduce disease and colonic proinflammatory cytokines in a mouse model of colitis. *Inamm Bowel Dis* 2005;**11**:340–9
11. Yoganathan S, Nicolosi R, Wilson T, Handelman G, Scollin P, Tao R, Binford P, Orthoefer F. Antagonism of croton oil inflammation by topical emu oil in CD-1 mice. *Lipids* 2003;**38**:603–7
12. Aberra FN. Omega-3 fatty acids for maintenance of remission of Crohn's disease. *Gastroenterology* 2008;**135**:1005–6; discussion 06–7
13. Abimosleh SM, Tran CD, Howarth GS. Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. *Evid Based Complement Alternat Med* 2013;**2013**:429706–16
14. Safaeian R, Howarth GS, Lawrance IC, Trinder D, Mashtoub S. Emu oil reduces disease severity in a mouse model of chronic ulcerative colitis. *Scand J Gastroenterol* 2019;**54**:273–80
15. Chartier LC, Howarth GS, Lawrance IC, Trinder D, Barker SJ, Mashtoub S. Emu oil improves clinical indicators of disease in a mouse model of colitis-associated colorectal cancer. *Dig Dis Sci* 2018;**63**:135–45
16. Chartier LC, Maiolo KE, Howarth GS, Lawrance I, Trinder D, Barker SJ, Scherer B, Mitchell CJ, Mashtoub S. Emu oil improves clinical indicators of disease and reduces proximal colonic crypt hyperplasia in a murine model of colitis-associated colorectal cancer. *Gastroenterology* 2018;**154**:S875–S75
17. Mashtoub S, Howarth GS, Trinder D, Lawrance I. Emu oil attenuates disease severity and results in fewer large colonic tumours in a mouse model of colitis-associated colorectal cancer. *Gastroenterology* 2017;**152**:S737–S37
18. Lindsay RJ, Geier MS, Yazbeck R, Butler RN, Howarth GS. Orally administered emu oil decreases acute inflammation and alters selected small intestinal parameters in a rat model of mucositis. *Br J Nutr* 2010;**104**:513–9
19. Mashtoub S, Lampton LS, Eden GL, Cheah KY, Lymn KA, Bajic JE, Howarth GS. Emu oil combined with lyprinol reduces small intestinal damage in a rat model of chemotherapy-induced mucositis. *Nutr Cancer* 2016;**68**:1171–80
20. Mashtoub S, Tran CD, Howarth GS. Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. *Exp Biol Med* 2013;**238**:1305–17
21. Raghu Nadhanan R, Abimosleh SM, Su YW, Scherer MA, Howarth GS, Xian CJ. Dietary emu oil supplementation suppresses 5-fluorouracil chemotherapy-induced inflammation, osteoclast formation, and bone loss. *Am J Physiol Endocrinol Metab* 2012;**302**:E1440–9
22. Neurath M, Fuss I, Strober W. TNBS-colitis. *Int Rev Immunol* 2000;**19**:51–62
23. Becker C, Fantini MC, Wirtz S, Nikolaev A, Kiesslich R, Lehr HA, Galle PR, Neurath MF. In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut* 2005;**54**:950–4
24. Howarth GS, Xian CJ, Read LC. Predisposition to colonic dysplasia is unaffected by continuous administration of insulin-like growth factor-I for twenty weeks in a rat model of chronic inflammatory bowel disease. *Growth Factors* 2000;**18**:119–33
25. Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. *Nat Methods* 2010;**7**:447–9
26. Yazbeck R, Howarth GS, Geier MS, Demuth HU, Abbott CA. Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice. *Front Biosci* 2008;**13**:6850–8
27. Barker SJ, Howarth GS, Scherer BL, Chartier LC, Cheah KY, Lymn KA, Mashtoub S. Mucosal thickening following oral administration of emu oil represents a process of normal intestinal growth in rats. *J Gastroen Hepatol* 2017;**32**:15
28. Mashtoub S, Cheah KY, Lymn KA, Howarth GS. Intestinal homeostasis is restored in mice following a period of intestinal growth induced by orally administered emu oil. *Exp Biol Med* 2018;**243**:945–52
29. Mashtoub S, Ghaemi R, Lawrance I, Trinder D, Howarth GS. Emu oil attenuates disease severity in mouse models of colitis and inflammation-associated colorectal cancer. *Gastroenterology* 2016;**150**:S1154–S54
30. Emge JR, Huynh K, Miller EN, Kaur M, Reardon C, Barrett KE, Gareau MG. Modulation of the microbiota-gut-brain axis by probiotics in a murine model of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* 2016;**310**:G989–98
31. Jirkof P, Leucht K, Cesarovic N, Caj M, Nicholls F, Rogler G, Arras M, Hausmann M. Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice. *Lab Anim* 2013;**47**:274–83
32. Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, Mapplebeck JC, Wei P, Zhan S, Zhang S, McDougall JJ, King OD, Mogil JS. The rat grimace scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. *Mol Pain* 2011;**7**:55–65

33. Whittaker AL, Leach MC, Preston FL, Lymn KA, Howarth GS. Effects of acute chemotherapy-induced mucositis on spontaneous behaviour and the grimace scale in laboratory rats. *Lab Anim* 2016;**50**:108–18
34. Wang L, Llorente C, Hartmann P, Yang AM, Chen P, Schnabl B. Methods to determine intestinal permeability and bacterial translocation during liver disease. *J Immunol Methods* 2015;**421**:44–53
35. Anderson AD, Poon P, Greenway GM, MacFie J. A simple method for the analysis of urinary sucralose for use in tests of intestinal permeability. *Ann Clin Biochem* 2005;**42**:224–6
36. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inamm Bowel Dis* 2009;**15**:341–52
37. Nguyen PM, Putoczki TL, Ernst M. STAT3-Activating cytokines: a therapeutic opportunity for inflammatory bowel disease? *J Interferon Cytokine Res* 2015;**35**:340–50
38. Atreya R, Atreya I, Neurath MF. Novel signal transduction pathways: analysis of STAT-3 and rac-1 signaling in inflammatory bowel disease. *Ann N Y Acad Sci* 2006;**1072**:98–113
39. Novak TE, Babcock TA, Jho DH, Helton WS, Espat NJ. NF-kappa B inhibition by omega -3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. *Am J Physiol Lung Cell Mol Physiol* 2003;**284**:L84–9
40. Bennett DCC, WE, Godin David V, Cheng, Kimberly M. Comparison of the antioxidant properties of emu oil with other avian oils. *Aust J Exp Agric* 2008;**48**:1345–50
41. Abimosleh SM, Tran CD, Howarth GS. Emu oil: a novel therapeutic for disorders of the gastrointestinal tract? *J Gastroenterol Hepatol* 2012;**27**:857–61

(Received May 18, 2020, Accepted July 28, 2020)

APPENDIX 3

MUCOSAL STIMULATION FOLLOWING THE ORAL ADMINISTRATION OF EMU OIL REPRESENTS A PROCESS OF NORMAL INTESTINAL GROWTH IN RATS

STATEMENT OF AUTHORSHIP

Title of Paper: Mucosal Stimulation Following Oral Administration of Emu Oil Represents a Process of Normal Intestinal Growth in Rats

Publication Status: Published

Publication Details: Australian Journal of Herbal and Naturopathic Medicine, 2020, Vol 32, pages 15-23

Principal Author

Name of Principal Author	Scott J Barker		
Contribution to the Paper	Completed all assays, data collection, analyses and interpretation. Prepared manuscript including writing, formatting and submission.		
Signature		Date	21 Aug 2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, revision and editing of the manuscript.		
Signature		Date	17/9/20

Name of Co-Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Data interpretation, editing and submission of the manuscript.		
Overall percentage (%)	15%		
Certification:	This paper reports on original research directly related to my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	31/8/20

Name of Co-Author	Benjamin L Scherer		
Contribution to the Paper	Methodology development.		
Signature		Date	28/8/20

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, supervision of analyses, data interpretation, revision and editing of the manuscript.		
Signature		Date	17/9/20

Australian Journal of Herbal and Naturopathic Medicine **2020** 32(1)

Mucosal stimulation following oral administration of emu oil represents a process of normal intestinal growth in rats

Scott J Barker Hons[1,2], Gordon S Howarth PhD[2,3], Lauren C Chartier Hons[1,2], Benjamin L Scherer BSc[4], and Suzanne Mashtoub PhD[1,2,5]

[1]Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, SA 5000
[2]Department of Gastroenterology, Women's and Children's Hospital, North Adelaide, SA 5006
[3]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy campus, Roseworthy, SA 5371
[4]CSIRO Health and Biosecurity, Adelaide, SA 5000
[5]School of Medicine, The University of Western Australia, Fiona Stanley Hospital, Murdoch, WA 6150

Contact information

Dr Suzanne Mashtoub
Gastroenterology Department, Women's and Children's Hospital, 72 King William Road, North Adelaide, SA 5006
Email suzanne.mashtoub@adelaide.edu.au

Keywords intestinal growth, cell proliferation, emu oil, Ki67

For referencing Barker SJ et al. Mucosal stimulation following oral administration of emu oil represents a process of normal intestinal growth in rats The Australian Journal of Herbal and Naturopathic Medicine 2020;32(1):15-23

DOI https://doi.org/10.33235/ajhnm.32.1.15-23

Abstract

Background The therapeutic properties of emu oil have been attributed to its omega fatty acid composition. Orally-administered emu oil has previously been shown to increase intestinal crypt depth following chemotherapy-induced mucositis. However, the impact of emu oil on cell kinetics remains unclear. It was hypothesised that 10 days' oral-administration of emu oil to normal rats would increase crypt cell proliferation, returning to normal levels on day 17 following emu oil withdrawal.

Aim To determine the impact of emu oil on intestinal cell proliferation in normal rats.

Methods Female Dark Agouti (DA) rats (n=8/group) were treated with either water (1ml), olive oil (1ml) or emu oil (0.5ml or 1 ml) via gavage for 10 days and were euthanised on days 10 and 17. Crypt depth measurements and crypt cell counts were conducted on jejunal and ileal specimens. To assess proliferation, sections were immunohistochemically stained for Ki67.

Results On day 10, emu oil increased crypt depth (22%) compared to water control ($p<0.05$). Following oil withdrawal, crypt depths returned to normal values. Cell numbers per crypt increased in olive oil (10%) and emu oil (11%) groups compared to water control ($p<0.05$). Cell diameter was unaffected in all oil-treated rats ($p>0.05$). Emu oil increased Ki67 positive cells per crypt (40%) compared to water control (30%; $p<0.05$).

Conclusion Emu oil administration increased crypt depth by stimulating cell proliferation as opposed to the process of hypertrophy. Restoration of normal intestinal growth following cessation of emu oil administration supports its safety for application in intestinal disorders.

Introduction

Anti-inflammatory, antioxidant and reparative properties have been described following oral administration of emu oil for a range of experimentally-induced gastrointestinal disorders including rat models of: ulcerative colitis[1], chemotherapy-induced mucositis[2,3] and NSAID induced enteropathy[4]. Emu oil is derived from the subcutaneous and retroperitoneal adipose tissue of the emu (*Dromaius novaehollandiae*), a large flightless bird native to Australia which belongs to the Ratite family of birds. The lipid content of emu oil is approximately 98%, comprising a unique blend of essential and non-essential fatty acids including: oleic acid (omega-9; 48%), palmitic acid (25%), stearic acid (10%), linoleic acid (omega-6; 7%) and low levels of α-linolenic acid (omega-3; 1%)[5]. The constituents of the remaining 2% non-triglyceride fraction is proposed to contain carotenoids and anti-oxidants, although this is yet to be fully determined[6]. Emu oil has long been used by Indigenous Australians as a traditional medicine for treating topical wounds,

burns and arthritis. Whitehouse et al. (1998) observed the skin permeability and anti-inflammatory properties of topically applied emu oil in rat models of arthritis[7].

Emu oil has been reported to reduce histologically-assessed intestinal damage severity and inflammation, and to restore intestinal mucosal architecture, as evidenced by lengthened crypts and/or villi[2]. Mashtoub et al. (2013) demonstrated the capacity for emu oil to preserve villus height and maintain the surface area of the intestine in a 5-Fluorouracil (5-FU)-induced model of intestinal mucositis, although the mechanism is unknown. The intestino-trophic effects of emu oil could potentially be mediated by a hyperplastic effect on cell kinetics (increased proliferation or reduced apoptosis) and/or an effect on enterocyte hypertrophy (increase in cell volume),

It was hypothesised that daily oral administration of emu oil in normal Dark Agouti (DA) rats would increase small intestinal crypt cell proliferation on day 10, returning to normal levels following withdrawal of emu oil treatment for 7 days. It was further hypothesised that the increase in crypt growth induced by emu oil would be the result of a hyperplastic process as opposed to the development of hypertrophy. Accordingly, we aimed to determine the impact of orally-administered emu oil on small intestinal crypt cell proliferation and cell size in normal female DA rats using Ki67 immunohistochemistry and related histologically-assessed parameters.

Materials and methods

Experimental design

All animal studies were conducted in compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committees of the Children, Youth and Women's Health Service and The University of Adelaide. Healthy female DA rats (n=64) were group housed with *ad libitum* access to water and rat chow (18% casein-based diet) with a 12 hour light–dark cycle. Rats were orally-gavaged daily with either water (1ml), olive oil (1ml) or emu oil (0.5ml or 1ml) for 10 days, and were culled on day 10 or 17 (n=8/group). At the end of the experimental period, rats were euthanised and jejunal and ileal tissues were collected for histological analyses.

Oil treatments

Emu oil was sourced from Emu Tracks Pty Ltd, (Marleston, South Australia, Australia). Emu oil was processed from the subcutaneous and retroperitoneal adipose tissue, by first heating then centrifuging and filtering the oil to remove bacteria and contaminants.

Tissue staining

Crypt depth

Jejunal and ileal tissue sections were routinely processed, embedded in paraffin wax and cut in series to a thickness of 4µm, then mounted on normal glass slides and stained with haematoxylin and eosin (H&E).

Immunohistochemical detection of Ki67

The Ki67 protein is consistently produced by proliferating cells within the nucleus in all phases of interphase (G1, S and G2) and mitosis. Ki67 is not produced by cells in the resting state (G0)[8]. This renders Ki67 as a robust marker for cellular proliferation. Jejunal and ileal sections were mounted on poly L-lysine coated microscope slides. The immunohistochemical staining method utilised was a modified version of the Ki67 protocol used by Clarke et al. (2012)[9]. Ki67 monoclonal primary antibody (Abcam Anti-Ki67 [SP6] – ab16667), derived from rabbit. Sections were placed in xylene and rehydrated in graded series of ethanol. For antigen retrieval, the slides were immersed in Tris-EDTA buffer and placed in an antigen retrieval system (2100 retriever, Prestige Medical) for 75 minutes. Endogenous peroxidase activity was blocked by immersing slides in 3% hydrogen peroxide for 10 minutes. Slides were then incubated in Acuity advanced pre-block solution (Australian Biosearch, Karrinyup, Western Australia) to block non-specific binding before Ki67 primary antibody was applied (1:1500 dilution, Abcam Australia, Melbourne, Victoria, Australia) and allowed to incubate overnight in a refrigerator at 4ºC. The following day, slides were washed and Acuity poly-HRP anti-mouse/rabbit IgG secondary antibody (Australian Biosearch, Karrinyup, Western Australia) was applied. The antigen–antibody complex is made visible by incubation with 3, 3'-Diaminobenzidine tetrahydrochloride (DAB; Rowe Scientific, Lonsdale, South Australia) for 1.5 minutes followed by counterstaining with haematoxylin for 1 minute.

Crypt depth and enterocyte counts

Both H&E and Ki67 stained sections were assessed using an Olympus CX41 light microscope (Olympus Industries, Tokyo, Japan) and analySIS Five imaging software (Olympus, version 5.2, Munster, Germany). Crypt depths of 40 well-orientated crypts per rat per section were measured and cells within counted. Crypt depths and cell counts from the four treatment groups were measured against each other as a comparison for crypt and cell growth changes. The average cell count was divided by average crypt depth per rat to give an average number of cells per micro-meter. Furthermore, average crypt depth was divided by average cell count per rat to determine average cell diameter in micro-meters.

Ki67

Well-orientated crypts (n=40) per rat were selected for analysis. Each section was counted for presence of Ki67 positive cells per crypt. The proliferation index was calculated as the mean number of Ki67 positive cells per crypt column divided by the total number of cells in the column multiplied by 100.

Statistical analysis

The statistical program SPSS (SPSS Inc. Chicago, Illinois, USA; version 19, 2010) was used for all statistical analyses. A normality test was first conducted

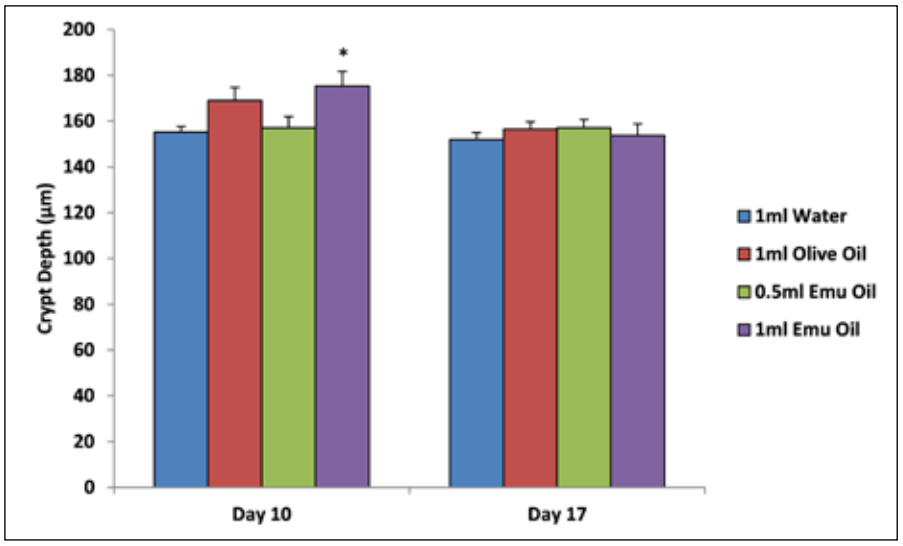


Figure 1. Crypt depth in the (a) jejunum and (b) ileum of rats on days 10 and 17. Data are expressed as mean (µm) ± SEM. *p<0.05 compared to 1ml water control group.

Figure 1a.

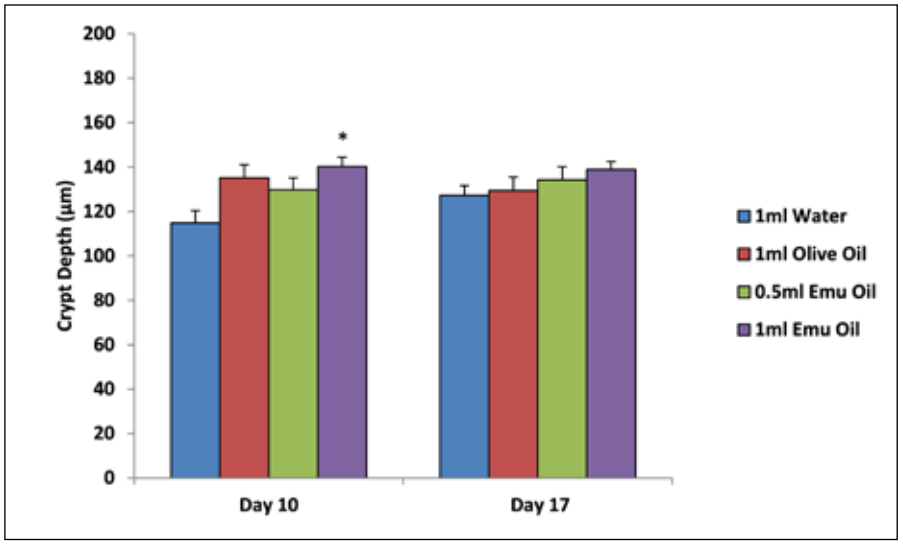


Figure 1b.

using the Shapiro-Wilk test. Data were analysed using a one-way ANOVA. Significance was determined with a Tukey's post hoc test. A two-way ANOVA was used for cell position group analysis and a least significant difference (LSD) was used to determine significance. For all statistical analysis, $p<0.05$ was considered significant.

Results

Crypt depth

Jejunal H&E data indicated no change in crypt depth in the olive oil (1ml) treated rats at the day 10 time point when compared to water treated controls ($p>0.05$; Figure 1a). High dose emu oil (1ml) demonstrated an increase in crypt depth in both the jejunum ($p<0.05$) and ileum ($p<0.05$) compared to water control (Figures 1a and 1b). No change in jejunal crypt depth was evident following low dose emu oil treatment (0.5ml; $p>0.05$). High dose emu oil increased jejunal and ileal crypt depth by 13% and 22% respectively ($p<0.05$) when compared to olive oil ($p<0.05$). Crypt depth in all oil treatment groups returned to water control levels by the day 17 time point.

Enterocyte count

Jejunum crypt cell numbers increased 11% following olive oil (1ml) administration at day 10 compared to water controls ($p<0.05$). High dose emu oil increased cell counts in both the jejunum (12%) and ileum (19%) in comparison to water controls ($p<0.05$; Figures 2a and 2b). No difference in jejunal or ileal crypt cell numbers was observed following low dose emu oil treatment compared to water treated controls. Crypt cell numbers in all oil treatments returned to water control levels by day 17.

Cell diameter

No change in cell diameter (µm) in the jejunum or ileum was observed in any of the oil treated groups compared

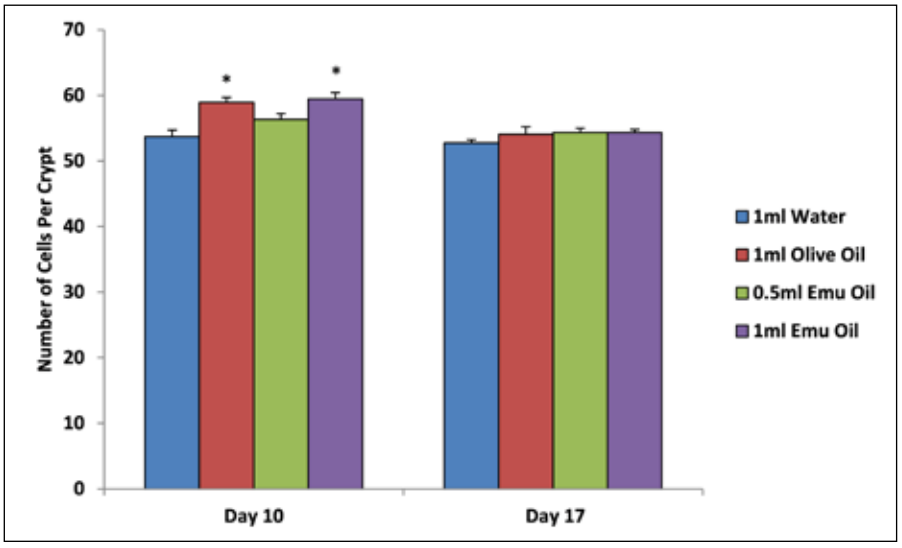


Figure 2a.

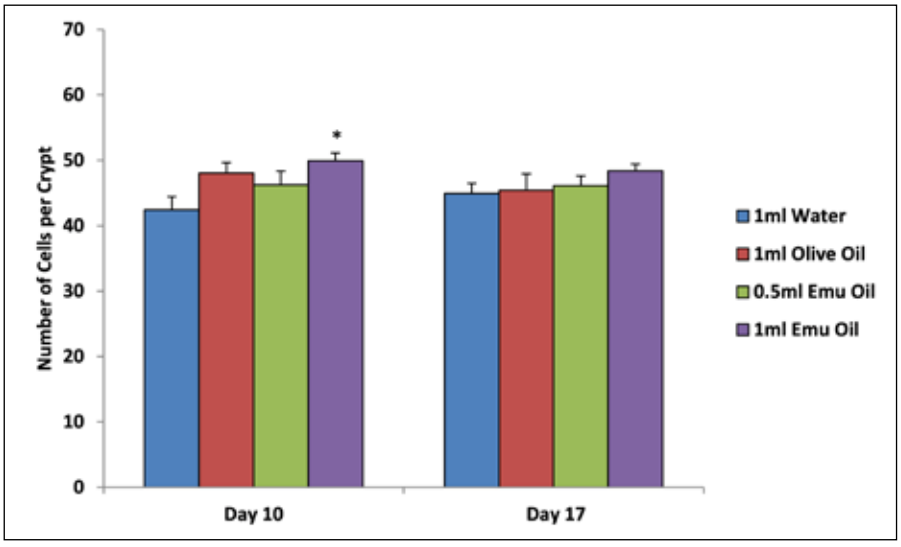


Figure 2b.

Figure 2. Average number of cells within (a) jejunum and (b) ileum crypts on days 10 and 17. Data are expressed as mean (μm) ± SEM. *p<0.05 compared to 1ml water control group.

to water controls at either time point (p>0.05; Figures 3a and 3b).

Ki67 proliferation

Olive oil and high dose emu oil increased the number of Ki67 positive cells in the jejunal crypts at day 10 compared to water treated controls (p<0.05; Figure 4a). Additionally, low dose emu oil demonstrated an increase in Ki67 positive cells (lower average than high dose emu oil or olive oil) in the jejunum when compared to water control (p<0.05). Ki67 proliferating cells in the jejunum in all oil treatment groups returned to water control levels by day 17 after cessation of oil treatments (p>0.05). Interestingly, in the ileum, only high dose emu oil significantly increased the number of Ki67 positive cells compared to water control on days 10 and 17 (p<0.05; Figure 4b). Olive oil and low dose emu oil did not affect Ki67 positive cell counts at either time-point (p>0.05).

Position of Ki67 positive cells

Olive oil demonstrated an increase in Ki67 positive cells in the jejunum at cell positions 11 to 20 compared to water control on day 10 (p<0.05; Figure 5a). At the day 10 time-point, high dose emu oil treatment had increased numbers of jejunal Ki67 positive cells at positions 1–10 and 11–20 compared to water control (p<0.05) In the jejunum, low dose emu oil did not demonstrate a change in the number of Ki67 positive cells at any position compared to water treated controls (p>0.05). In the ileum, high dose emu oil increased the percentage of Ki67 positive cells at all three regions (1–10, 11–20 and 21–30) compared to water controls on day 10 (p<0.05; Figure 5b). Additionally, in the ileum, olive oil treatment resulted in greater Ki67 positive cell numbers on day 10 at position 11–20 compared to water controls (p<0.05; Figure 5b). Low dose emu oil did not affect ileal Ki67 numbers at any cell position on day 10 (p>0.05; Figure 5b). Moreover,

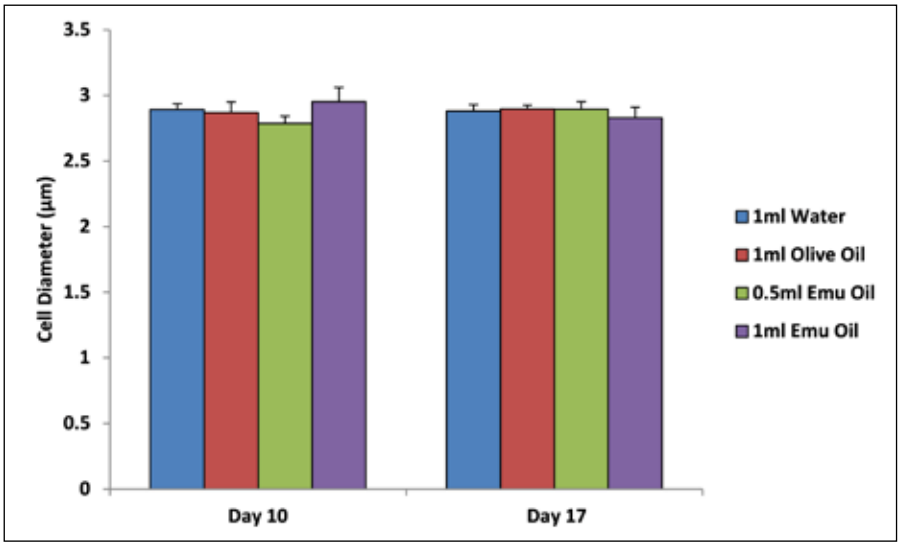


Figure 3. Average crypt cell diameter in (a) jejunum and (b) ileum on days 10 and 17. Data are expressed as mean (µm) ± SEM.

Figure 3a.

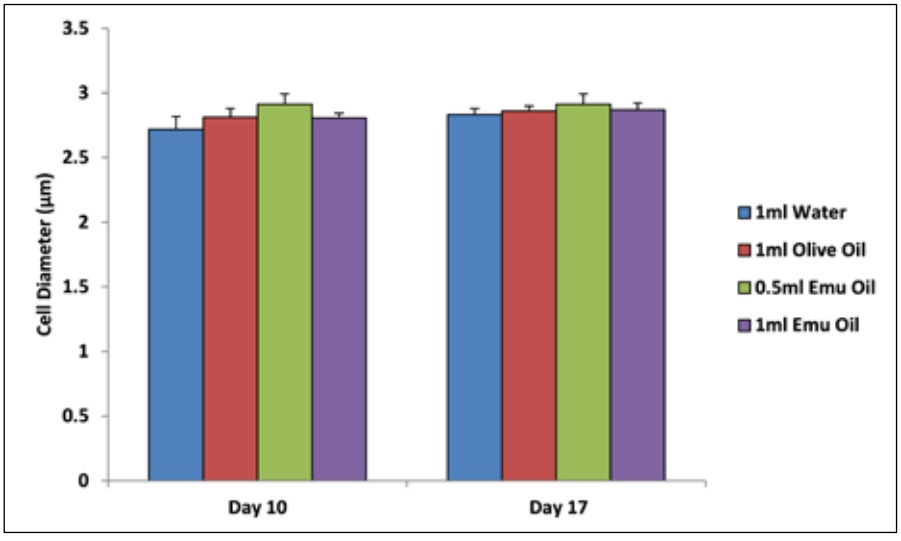


Figure 3b.

on day 17, no difference was observed in Ki67 positive cells in the jejunum or ileum in all oil treatment groups compared to water controls ($p>0.05$; Figures 6a and 6b).

Discussion

In previous animal models of intestinal disorders, emu oil reduced inflammation and exhibited therapeutic properties within the intestinal tract[1,2]. Whilst these studies demonstrated the effects of emu oil in a disease state, the mode of emu oil action in a normal intestine has remained unclear.

Inducing intestinal disease impacts homeostasis which can influence hormone levels regulating cellular proliferation or the response to fatty acids. The current study demonstrated that emu oil reproduced similar findings to the chemotherapy (5-FU)-induced mucositis study by Mashtoub et al. (2013)[2]. In the current study, jejunal and ileal crypts were lengthened by olive oil and high dose emu oil treatment in healthy rats compared with normal controls. Crypt depths were greater in the jejunum and ileum following treatment with high dose emu oil compared to water administration at day 10. These crypt depths decreased and returned to water control levels by day 17. Crypt cell diameter remained unchanged amongst all treatment groups, suggesting that stimulation of mucosal growth was more likely attributed to the process of hyperplasia rather than hypertrophy, as confirmed by immunohistochemical staining of Ki67. This data supports the increase in crypt depth and villus height observed in studies of chemotherapy-induced mucositis, reflecting increased cell proliferation by emu oil treatment[2,3,10]. Courtney et al. (2007) investigated the effects of daily fish oil (high in omega-3) supplementation on crypt cell proliferation and apoptosis in patients with colorectal adenomas[11], revealing that crypt cell proliferation was significantly reduced whilst apoptosis was significantly increased after supplementation. Neither crypt cell proliferation nor apoptosis were altered in the control group[11]. Whilst the Courtney study was focused on the colon, it does demonstrate the impact of fatty acids on crypt cell proliferation.

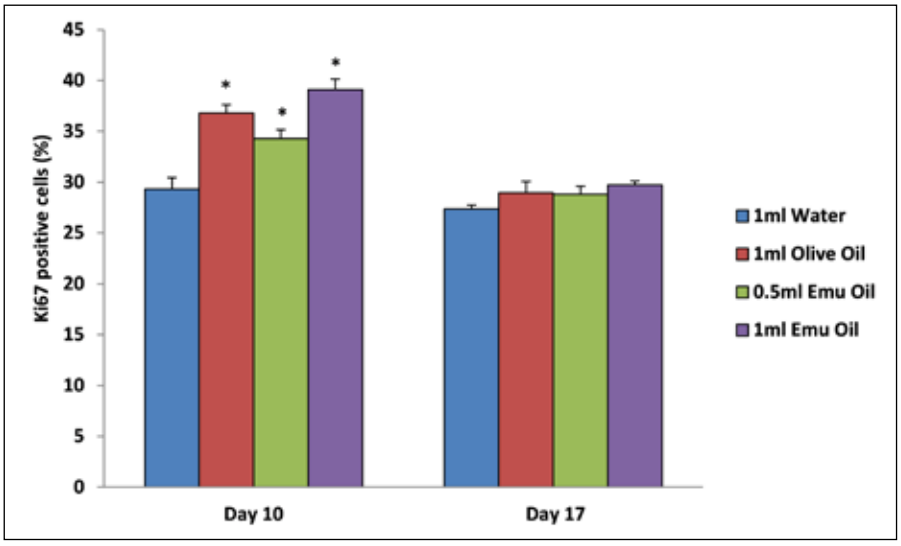


Figure 4. Overall percentage of Ki67 positive cells per treatment group in a) jejunum and b) ileum on days 10 and 17. Data are expressed as mean (%) ± SEM.
*p<0.05 compared to water group.

Figure 4a.

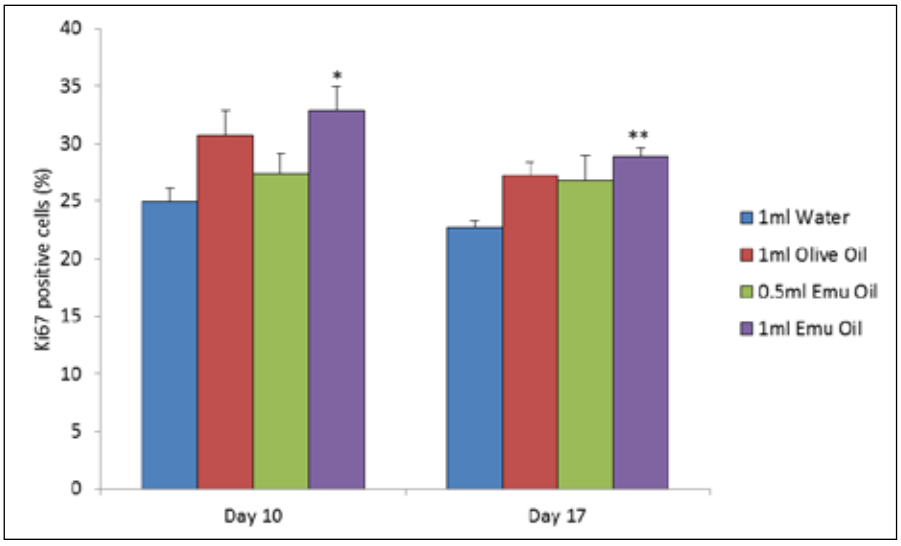


Figure 4b.

In the current study, an increase in cell numbers within the crypts was observed in both olive oil and high dose emu oil groups at the day 10 time point in the jejunum, compared to the water control. Cell numbers returned to water control levels after cessation of all oil gavages by day 17, a result similar to that observed by Mashtoub et al. (2018)[3]. Both oils contain high levels of oleic acid (omega-9) (emu oil 48% and olive oil 74%)[5]. Oleic acid has been reported to elicit anti-inflammatory properties and stimulate cell proliferation by down-regulating pro-inflammatory pathways, including tumour necrosis factor alpha (TNFα), and up regulating enzymes involved in tissue remodelling and repair[12].

In the current study, the overall percentage of Ki67 positive cells increased across all oil treatment groups compared to water controls. Water treated control rats averaged approximately 30% Ki67 positive cells per crypt, congruent with similar studies examining crypt cell numbers in control DA rats[13]. This increase in proliferation may have been due to the presence of lipids within the small intestine. Lipid absorption in the proximal small intestine stimulates intestinal mucosal growth. When lipids reach the duodenum, cholecystokinin (CCK) is released from I-cells lining the intestinal mucosa which stimulate bile release[14]. The hydrophobic bile binds to lipids allowing lipase to break down the lipid into smaller monoglycerides for absorption[15,16]. Furthermore, gastrin is secreted by G-cells upon digestion, competing with CCK in binding to CCK_B receptors lining the intestine. Activation of the CCK_B receptor activates pathways involved in early response genes[17]. This process aids in cellular migration, adhesion and proliferation. Tripathi et al. (2015) reported that apoptosis was inhibited by gastrin via B-cell lymphoma-mediated repression of pro-apoptotic caspases[18]. Furthermore, lipid ingestion induces secretion of glucagon-like peptide 1 and 2 (GLP-1 and GLP-2). Both are secreted by intestinal endocrine L-cells upon nutrient ingestion. The GLP-2 receptor is a G protein-coupled receptor which stimulates and upregulates villus height, increases cell proliferation within crypts, and decreases enterocyte apoptosis[19]. In the

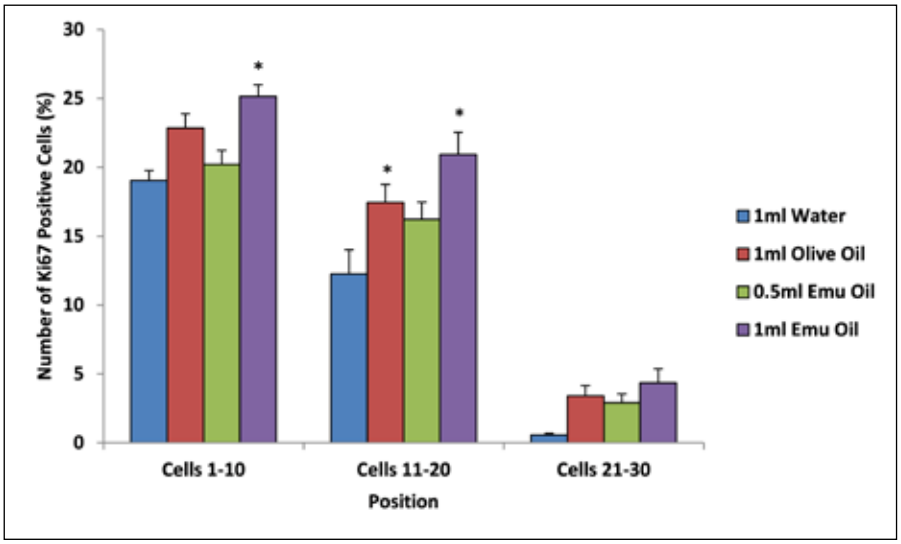


Figure 5. Average cell numbers at positions at day 10 in a) jejunum and b) ileum. Data are expressed as mean (%) ± SEM.
*p<0.05 compared to water group.

Figure 5a.

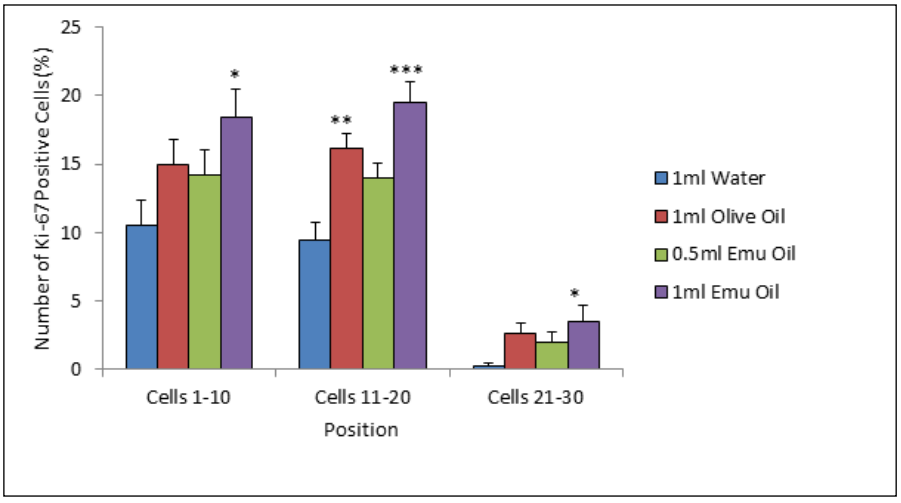


Figure 5b.

current study, high dose emu oil resulted in an increase in Ki67 positive cell percentage compared to olive oil treatment, which further translated to significantly deeper crypts compared to water controls. The emu oil specific effects may have been attributed to the unique ratio of fatty acids and components within the 2% non-triglyceride fraction, including antioxidants and skin-permeation enhancing factors; or indeed a combination of these factors.

Following high dose emu oil treatment, jejunal and ileal cell position data demonstrated an increase of Ki67 positive cells at all positions along the crypt lining at day 10 compared to water treated controls. Olive oil and low dose emu oil also demonstrated an increase in Ki67 positive cells at all crypt cell positions compared to water controls. Ki67 positive cell counts for all oil treatment groups returned to water control levels after oil treatment cessation. Increased cell proliferation at the lower levels of the crypt causes cells still undergoing cell differentiation to move further up the crypt wall. This contributes to the deepening of the crypt which most likely contributed to the villus elongation observed by Mashtoub et al. (2013)[2]. Emu oil contains the omega-6 fatty acids linoleic (7.7%) acid and arachidonic acid (0.1%)[5]. Linoleic acid is converted into arachidonic acid by enzymes in the 6Δ/5Δ desaturase pathway[12]. Arachidonic acid is converted into prostaglandin H2 (PGH2) by membrane bound enzymes and is further converted into prostaglandin E2 (PGE2) by COX1 and COX2 enzymes[20]. PGE2 is an eicosanoid linked to inflammation and found in elevated levels within cancer cells[21]. All cell types within the body can produce PGE2; however, it is primarily produced by immune cells during inflammatory responses to infection or tissue damage[22]. PGE2 has been observed to increase mucosal growth[23], aiding in cell migration and increasing cell adhesion[24]. This could be an important aspect in the ability of emu oil to maintain villus height under disease conditions as described by Mashtoub et al. (2013), as cells are not exfoliated from the mucosa so readily due to increased levels of PGE2 increasing cell adhesion.

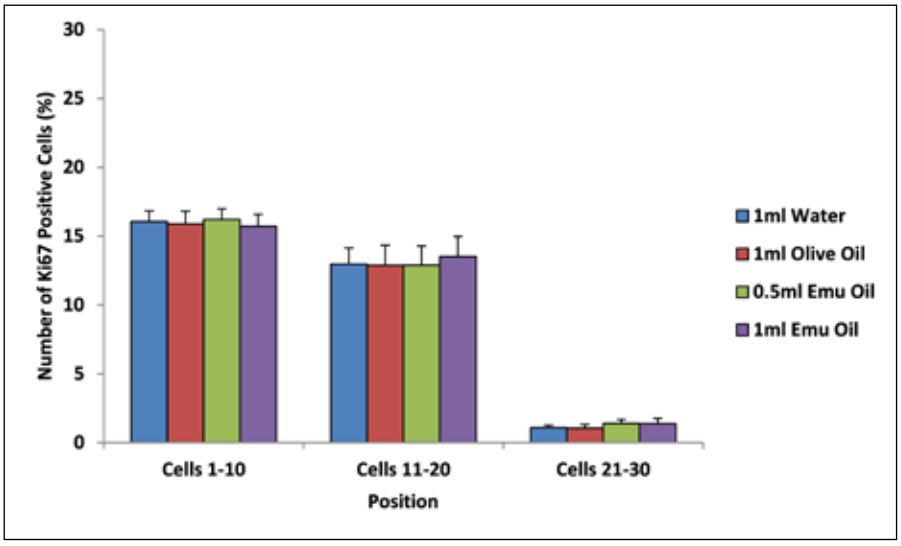


Figure 6a.

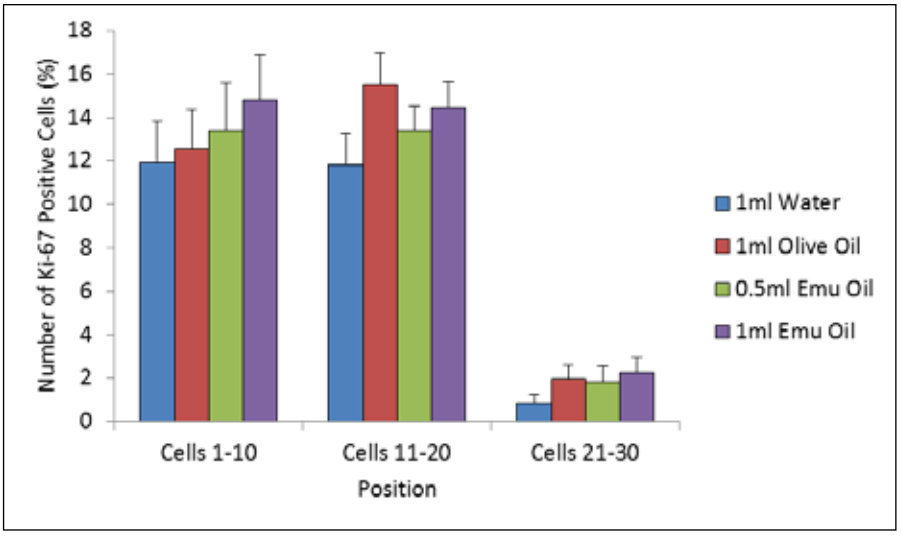


Figure 6b.

Figure 6. Average cell numbers at positions at day 17 in a) jejunum and b) ileum. Data are expressed as mean (%) ± SEM.

Conclusions

The current study demonstrated that emu oil increased crypt depth by increasing cell proliferation as opposed to the process of hypertrophy. Further research is required to investigate the impact of emu oil on expression of genes regulating mucosal growth in addition to or pro- and anti-inflammatory cytokine genes. Future studies should also investigate the impact of emu oil on apoptosis. Such a decrease in cell death rates could potentially lead to cells accumulating along the villus, contributing to the increase in villus height. Further studies could also define the optimal fatty acid ratio to induce maximal enterocyte proliferation. Perhaps most importantly, the restoration of normal intestinal growth following cessation of emu oil therapy, demonstrated in the current study, supports its safety for application in a broad range of clinically relevant gastrointestinal disorders.

Conflicts of interest / funding disclosures

None

References

1. Abimosleh SM, Lindsay RJ, Butler RN, Cummins AG, Howarth GS. Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. Dig Dis Sci 2012 Apr;57(4):887–896.
2. Mashtoub S, Tran CD, Howarth GS. Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. Exp Biol Med (Maywood) 2013 Nov;238(11):1305–1317.
3. Mashtoub S, Cheah KY, Lymn KA, Howarth GS. Intestinal homeostasis is restored in mice following a period of intestinal growth induced by orally administered emu oil. Exp Biol Med (Maywood) 2018 Jan; 1535370218787457.
4. Abimosleh SM, Tran CD, Howarth GS. Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. Evid Based Complement Alternat Med 2013:429706.
5. Abimosleh SM, Tran CD, Howarth GS. Emu oil: a novel therapeutic for disorders of the gastrointestinal tract? J Gastroenterol Hepatol 2012 May;27(5):857–861.
6. Yoganathan S, Nicolosi R, Wilson T, Handelman G, Scollin P, Tao R, Binford P, Orthoefer F. Antagonism of croton oil inflammation by topical emu oil in CD-1 mice. Lipids 2003 Jun;38(6):603–607.

7. Whitehouse MW, Turner AG, Davis CK, Roberts MS. Emu oil(s): a source of non-toxic transdermal anti-inflammatory agents in aboriginal medicine. Inflammopharmacol 1998;6(1):1–8.
8. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000 Mar;182(3):311–322.
9. Clarke JM, Young GP, Topping DL, Bird AR, Cobiac L, Scherer BL, Winkler JG, Lockett TJ. Butyrate delivered by butyrylated starch increases distal colonic epithelial apoptosis in carcinogen-treated rats. Carcinogenesis 2012 Jan;33(1):197–202.
10. Mashtoub S, Lampton LS, Eden GL, Cheah KY, Lymn KA, Bajic JE, Howarth GS. Emu oil combined with lyprinol reduces small intestinal damage in a rat model of chemotherapy-induced mucositis. Nutr Cancer 2016 Oct;68(7):1171–1180.
11. Courtney ED, Matthews S, Finlayson C, Di Pierro D, Belluzzi A, Roda E, Kang JY, Leicester RJ. Eicosapentaenoic acid (EPA) reduces crypt cell proliferation and increases apoptosis in normal colonic mucosa in subjects with a history of colorectal adenomas. Int J Colorectal Dis 2007 Jul;22(7):765–776.
12. Lamy S, Ben Saad A, Zgheib A, Annabi B. Olive oil compounds inhibit the paracrine regulation of TNF-alpha-induced endothelial cell migration through reduced glioblastoma cell cyclooxygenase-2 expression. J Nutr Biochem 2016 Jan;27:136–145.
13. Al-Dasooqi N, Bowen JM, Gibson RJ, Logan RM, Stringer AM, Keefe DM. Irinotecan-induced alterations in intestinal cell kinetics and extracellular matrix component expression in the Dark Agouti rat. Int J Exp Pathol 2011 Oct;92(5):357–365.
14. Wang HH, Liu M, Portincasa P, Tso P, Wang DQ. Lack of endogenous cholecystokinin promotes cholelithogenesis in mice. Neurogastroenterol Motil 2016 Mar;28(3):364–375.
15. Ninomiya R, Matsuoka K, Moroi Y. Micelle formation of sodium chenodeoxycholate and solubilization into the micelles: comparison with other unconjugated bile salts. Biochim Biophys Acta 2003 Nov;1634(3):116–125.
16. Patton JS, Carey MC. Inhibition of human pancreatic lipase-colipase activity by mixed bile salt-phospholipid micelles. Am J Physiol 1981 Oct;241(4):G328–336.
17. Yassin RR. Signaling pathways mediating gastrin's growth-promoting effects. Peptides 1999;20(7):885–898.
18. Tripathi S, Flobak A, Chawla K, Baudot A, Bruland T, Thommesen L, Kuiper M, Laegreid A. The gastrin and cholecystokinin receptors mediated signaling network: a scaffold for data analysis and new hypotheses on regulatory mechanisms. BMC Syst Biol 2015 Jul;9:40.
19. Burrin DG, Petersen Y, Stoll B, Sangild P. Glucagon-like peptide 2: a nutrient-responsive gut growth factor. J Nutr 2001 Mar;131(3):709–712.
20. Agard M, Asakrah S, Morici LA. PGE(2) suppression of innate immunity during mucosal bacterial infection. Front Cell Infect Microbiol 2013;3:45.
21. Nardone G, Rocco A, Vaira D, Staibano S, Budillon A, Tatangelo F, Sciulli MG, Perna F, Salvatore G, Di Benedetto M, De Rosa G, Patrignani P. Expression of COX-2, mPGE-synthase1, MDR-1 (P-gp), and Bcl-xL: a molecular pathway of H pylori-related gastric carcinogenesis. J Pathol 2004 Mar;202(3):305–312.
22. Kalinski P. Regulation of immune responses by prostaglandin E2. J Immunol 2012 Jan;188(1):21–28.
23. DuBois RN, Awad J, Morrow J, Roberts LJ, 2nd, Bishop PR. Regulation of eicosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-alpha and phorbol ester. J Clin Invest 1994 Feb;93(2):493–498.
24. Dormond O, Bezzi M, Mariotti A, Ruegg C. Prostaglandin E2 promotes integrin alpha Vbeta 3-dependent endothelial cell adhesion, rac-activation, and spreading through cAMP/PKA-dependent signaling. J Biol Chem 2002 Nov;277(48):45838–45846.

APPENDIX 4

EMU OIL REDUCES PROXIMAL COLONIC CRYPT HYPERPLASIA IN A MURINE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

STATEMENT OF AUTHORSHIP

Title of Paper: Emu Oil reduces proximal colonic crypt hyperplasia in a murine model of colitis-associated colorectal cancer

Publication Status: Under Review

Publication Details: Prostaglandins, Leukotrienes & Essential Fatty Acids

Principal Author

Name of Principal Author	Kayla E Maiolo		
Contribution to the Paper	Completed all experimental assays, data analyses and interpretation. Prepared manuscript including writing and formatting.		
Signature		Date	7 October 2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodological development, data interpretation, revision and editing of the manuscript.		
Signature		Date	9/10/20-

Name of Co-Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Completed animal trials related to manuscript and sample collection. Involved in editing, formatting and submission of the manuscript.		
Overall percentage (%)	20%		
Certification:	This paper reports on original research directly related to my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	8/10/20

Name of Co-Author	Debbie Trinder		
Contribution to the Paper	Conceptualisation and methodology development.		
Signature		Date	16 Sept 2020

Name of Co-Author	Ian C Lawrance		
Contribution to the Paper	Conceptualisation and methodology development.		
Signature		Date	23 Sept 2020

Name of Co-Author	Scott J Barker		
Contribution to the Paper	Assistance in methodology development.		
Signature		Date	01 Oct 2020

Name of Co-Author	Benjamin L Scherer		
Contribution to the Paper	Assistance in methodology development.		
Signature		Date	29/9/20

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodological development, data interpretation, revision and editing of the manuscript.		
Signature		Date	8/10/20

EMU OIL REDUCES PROXIMAL COLONIC CRYPT HYPERPLASIA IN A MURINE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

Kayla E Maiolo[1,2], Gordon S Howarth[1,2,3], Lauren C Chartier[1,2], Debbie Trinder[4,5], Ian C Lawrance[4,6], Scott J Barker[1], Benjamin L Scherer[7] and Suzanne Mashtoub[1,2,4*]

[1]Gastroenterology Department, Women's and Children's Hospital, North Adelaide, South Australia, Australia, 5006; [2]Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, Australia, 5000; [3]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, South Australia, Australia, 5371; [4]Medical School, The University of Western Australia, Fiona Stanley Hospital, Murdoch, Western Australia, Australia, 6150; [5]Harry Perkins Institute of Medical Research, Murdoch, Western Australia, Australia, 6150; [6]Centre for Inflammatory Bowel Diseases, Saint John of God Hospital, Subiaco, Western Australia, Australia, 6008; [7]CSIRO Health and Biosecurity, Adelaide, South Australia, Australia, 5000.

***Corresponding Author:**

Dr Suzanne Mashtoub

Department of Gastroenterology

Women's and Children's Hospital

North Adelaide 5006

South Australia, Australia

E: suzanne.mashtoub@adelaide.edu.au

Ph: +61 434 539 797

Sources of funding: None

Declaration of Interest: None

Summary: The work detailed in this manuscript is important to the field of inflammatory bowel disease and colitis-associated cancer as it discusses the possible mechanism involved in crypt lengthening as identified in a pre-clinical study of colitis-associated colorectal cancer.

Furthermore, cell proliferation is concluded as a contributing factor of cancer development in models of gastrointestinal inflammation. This advances the field as it strengthens our understanding of the association between inflammation, cell response and cancer development. Additionally, this manuscript discusses the influence of Emu Oil on cell proliferation and histologically-assessed measures in a mouse model of colitis-associated colorectal cancer. The new information presented in this manuscript significantly impacts the field of gastrointestinal disease and the use of naturally-sourced therapies, such as Emu Oil, in colitis-associated cancer management.

Authors' contributions: Conceptualization and supervision (Gordon S Howarth and Suzanne Mashtoub), Methodology (Debbie Trinder, Ian C Lawrance, Gordon S Howarth, Suzanne Mashtoub, Benjamin L Scherer, Scott J Barker, Lauren C Chartier), Formal analysis and investigation (Kayla E Maiolo, Lauren C Chartier), Writing – full preparation (Kayla E Maiolo), Writing – review and editing (Lauren C Chartier, Suzanne Mashtoub, Gordon S Howarth). All authors approve the final manuscript.

Abstract

Background: Ulcerative colitis, an inflammatory bowel disease, is characterised by chronic colonic inflammation which can result in uncontrolled crypt lengthening and the development of colitis-associated colorectal cancer. Orally-administered Emu Oil has demonstrated protective and reparative properties in models of bowel disease; however, its long-term impact on colonocyte proliferation in mice with colitis-associated colorectal cancer (CA-CRC) has not been determined. **Aim:** To investigate the impact of Emu Oil on colonocyte kinetics in a mouse model of CA-CRC. **Methods:** Female C57BL/6 mice were injected with saline/azoxymethane (7.4mg/kg, i.p.) and underwent three dextran sulphate sodium (DSS)/water cycles (each cycle consisting of seven days DSS [2%w/v] and 14 days water). Mice were gavaged with water or Emu Oil. Colonic sections were stained with haematoxylin and eosin for crypt depth, cell count and size measurements and immunohistochemically-stained for the proliferation marker Ki-67 to determine colonocyte proliferation. $p<0.05$ was considered statistically significant. **Results:** Crypt depth and cell count increased in CA-CRC controls compared to normal controls (37.4%; $p<0.05$). Proximal colonocyte proliferation increased in colitis-associated colorectal cancer controls, compared to normal mice; an effect that was attenuated by Emu Oil (47.3% reduction; $p<0.05$). Distal colonocyte proliferation increased in the crypt upper region in CA-CRC controls compared to normal mice (157.4%). **Conclusions:** Crypt lengthening in colitis-associated colorectal cancer was attributed to crypt cell hyperplasia. Emu Oil attenuated proximal colonic cell proliferation in mice with colitis-associated colorectal cancer but did not affect intestinal growth in normal mice, suggesting safety for long-term usage.

Keywords: Emu Oil, ulcerative colitis, colorectal cancer, mouse model, cell proliferation, Ki-67

1.1 Introduction

The inflammatory bowel diseases, Crohn's disease and ulcerative colitis (UC), are lifelong intermittently-relapsing diseases of the gastrointestinal tract. Ulcerative colitis is characterised by continuous areas of inflammation and ulceration of the mucosa in the colon and rectum [1]. Its aetiology is not well understood; however, UC is proposed to develop due to a combination of a genetic predisposition, an abnormal host immune response to the intestinal microbiota and less well-defined environmental factors [1]. Symptoms of UC are unpredictable and can include pain, diarrhoea, bloody stools, weight loss, decreased appetite and fever [1]. UC is primarily managed using aminosalicylates, corticosteroids and thiopurines. However, these treatments are unable to maintain long-term remission in a significant proportion of patients and often present with undesirable side-effects [1]. Uncontrolled colonic inflammation can result in additional complications including tumorigenesis. Indeed, UC patients have a 60% greater risk of developing colorectal cancer than that of the general population [2]. Therefore, an effective treatment for UC could significantly inhibit, or possibly prevent the onset of colitis-associated colorectal cancer (CA-CRC).

Inflammatory cells migrate to the site of damage in UC and release pro-inflammatory mediators such as interleukin-6 and tumour necrosis factor-α (TNF-α) [3] and subsequent free radical production may induce oxidative damage to DNA [4]. In the early stages of CA-CRC pathogenesis, uncontrolled proliferation occurs in colonocytes during migration up the crypt [5]. Inactivation of adenomatous polyposis coli (*APC*) is usually the first mutation causing hyper-proliferation and aberrant crypt foci [4]. This increased proliferation may be a compensatory mechanism or evidence of early mutation and neoplastic development. The accumulation of other mutations such as K-ras, abnormal DNA methylation and inactivation of p53 follows, causing the progression of an adenoma to carcinoma with high-grade dysplasia [4].

Emu Oil is derived from the subcutaneous and retroperitoneal adipose tissue of the Australian Emu and is commercially available in capsule or liquid form. It primarily comprises a unique

blend of essential and non-essential fatty acids including oleic acid (omega-9, 48%), linoleic acid (omega-6, 7%), palmitic acid (25%), stearic acid (10%) and α-linolenic acid (omega-3, 1%) [6]. The 2% non-triglyceride fraction comprises antioxidant components (carotenoids and flavones) [7]. The reparative properties of topically-applied Emu Oil have been documented in many studies [8-11]. More recently, Emu Oil has been investigated as a potential therapy for intestinal disorders. Emu Oil exhibited anti-inflammatory and reparative properties in intestinal disease models including mucositis [7, 12-14], non-steroidal anti-inflammatory drug enteropathy [15], UC [16, 17] and CA-CRC [18-22].

Chartier et al. [18] investigated the effect of Emu Oil in normal mice and in an azoxymethane/dextran sulphate sodium (AOM/DSS) mouse model of CA-CRC. Emu Oil significantly reduced the number of small colonic tumours present and reduced clinically-assessed disease severity by 20% compared to colorectal cancer controls [18]. Colonic crypts were lengthened in CA-CRC controls compared to normal mice. However, there was no further impact of Emu Oil treatment.

Throughout the intestine, stem cells are confined to the lower region of crypts and differentiated daughter cells migrate up to the surface to replace senescent epithelial cells [23] in the process of hyperplasia. It remains unclear whether the villous and crypt lengthening observed in the AOM/DSS model of CA-CRC was due to hypertrophy or hyperplasia [18]. Furthermore, the impact of long-term Emu Oil treatment (nine weeks) on colonocyte kinetics has not yet been identified in either normal mice or mice with CA-CRC. Therefore, the current study firstly aimed to determine the impact of Emu Oil on cell proliferation in normal and CA-CRC mice, and secondly, whether crypt lengthening in CA-CRC was to the result of hypertrophy or hyperplasia.

1.2 Materials and Methods

1.2.1 Animal trial

Archived tissue was used from a previous study by Chartier et al. (2018). All 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 (approval #AE966/1/2017), The University of Adelaide (approval #M-2013-228) and The University of Western Australia (approval #RA/3/900/88). The composition of Emu Oil was analyzed by gas chromatography at the Waite Lipid Analysis Service – FOODPlus Research Centre (Urrbrae, South Australia) [18].

Female C57BL/6 mice (n=45) were randomly assigned to 5 groups (n=9/group):

(Injection + *ad libitum* + gavage)

1) Saline + water + water
2) Saline + water + Emu Oil (160µl)
3) AOM + DSS + Water
4) AOM + DSS + Emu Oil (80µl)
5) AOM + DSS + Emu Oil (160µl)

Mice were injected intraperitoneally with either saline or AOM (7.4mg/kg) on day 0. Groups 3-5 underwent three DSS/water cycles (7 days DSS [2% w/v in drinking water] followed by 14 days of water). Mice were gavaged thrice weekly with water or Emu Oil (low [80µl] or high [160µl] dose) and euthanized by CO_2 asphyxiation followed by cervical dislocation on day 63 [18]. The colon was removed, emptied of contents, cut into proximal and distal sections, fixed in formalin for 24 hours and then stored in 70% ethanol for processing.

1.2.2 Crypt depth, cell count and cell size

Proximal and distal colon sections were embedded in paraffin wax, sectioned at 4µm in series and mounted on glass slides. Tissues were stained with haematoxylin and eosin (H&E) for visualization of mucosal architecture. Crypt depth was measured using an Olympus CX41 light microscope (Tokyo, Japan) with attached camera and analySIS imaging software (Olympus,

version 5.2, Munster, Germany). Forty well-oriented crypts were measured per colonic section for each mouse. For each crypt measured, the number of cells was counted on one half of the crypt. The mean cell count was divided by the mean crypt depth per mouse to give the average number of cells per 100μm. Conversely, the mean crypt depth was divided by the mean cell count per mouse to give the average cell diameter in μm.

1.2.3 Analysis of cell proliferation by immunohistochemical stain

The proximal and distal colon sections were embedded in paraffin wax, sectioned at 4μm in series and mounted on poly-L-lysine coated slides. They were stained for the presence of Ki-67, a nuclear protein associated with cell proliferation. Ki-67 is present through G1, S, G2 phases and mitosis of the cell cycle and absent from resting cells and is an excellent protein marker for proliferation and determining the growth fraction of a cell population [24]. The immunohistochemical staining procedure was adapted from the protocol used by Clarke et al. (2012) [25]. Slides were washed in xylene to remove paraffin wax and were then rehydrated. They were immersed in Tris-EDTA buffer and placed in an antigen retrieval system (2100 retriever, Prestige Medical) for 75 minutes. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 10 minutes. Slides were incubated in Acuity advanced pre-block solution to prevent non-specific binding of the Ki-67 antibody. Ki-67 monoclonal primary antibody (Abcam anti-Ki67 [SP6], ab16667, derived from rabbit) was applied at a 1:1000 dilution and left to incubate overnight at 4°C. The following day, the slides were incubated with Acuity boost solution followed by poly-HRP anti-mouse/rabbit IgG secondary antibody. 3,3'-Diaminobenzidine chromogen was applied for 2 minutes to visualise the antigen-antibody complex followed by counterstaining with haematoxylin for 2 minutes. Slides were dehydrated and coverslips were applied.

Forty well-oriented crypts were measured per colonic section for each mouse. Half of each crypt was counted and the position of positive and negative cells was recorded. The proliferation index was calculated by dividing the mean number of Ki-67 positive cells per crypt by the total number of cells and multiplying by 100.

1.2.3 Statistical methods

Chartier et al. (2018) determined the appropriate sample size (n=9/group) in the original study to assume a power of 95% as determined by myeloperoxidase outcomes (intestinal inflammation) for statistical power [18]. Statistical analyses were performed using SPSS software (version 19.0 for Windows). Data were tested for normality by the Shapiro-Wilk normality test and analysed by one-way ANOVA with Tukey's *post hoc* test. Cell position data were analysed by two-way repeated measures ANOVA with least significant difference (LSD) to find significance. Data are expressed as mean ± standard error of the mean (SEM). $p<0.05$ was considered statistically significant.

1.3 Results

1.3.1 Crypt depth

Crypt depth in the proximal colon (20.2%; $p<0.01$; Figure 1a) and distal colon (37.4%; $p<0.01$; Figure 1b) was increased in mice with CA-CRC compared to normal controls. There was no further increase in crypt depth following Emu Oil treatment in mice with CA-CRC. Moreover, Emu Oil did not impact crypt depth in normal mice.

1.3.2 Cell count

No change in cell count was observed in the proximal colon ($p>0.05$; Figure 2a) in mice with CA-CRC compared to normal controls. Cell count in the distal colon (42.7%; $p<0.001$; Figure 2b) was increased in mice with CA-CRC compared to normal controls. There was no further increase in cell count following Emu Oil treatment in mice with CA-CRC. Neither did Emu Oil impact cell count in normal mice.

1.3.3 Cell Diameter

No change in cell diameter (μm) was observed in the proximal or distal colon ($p>0.05$; Figures 3a and 3b) of mice with CA-CRC compared to normal controls. Similarly, there was no impact of Emu Oil on cell diameter in normal mice or mice with CA-CRC.

1.3.4 Number of cells per 100μm

No change in the number of cells per 100μm was observed in the proximal or distal colon ($p>0.05$; Figures 4a and 4b) of mice with CA-CRC compared to normal controls and there was no impact of Emu Oil on the number of cells per 100μm in normal mice or mice with CA-CRC.

1.3.5 Percentage of Ki-67 positive cells

Proximal and distal colon sections were immunohistochemically stained for the presence of Ki-67 (Figure 5). No change in the percentage of Ki-67 positive cells present was observed in the proximal or distal colon ($p>0.05$; Figures 6a and 6b) of mice with CA-CRC compared to normal controls. There was no impact of Emu Oil on the overall percentage of Ki-67 positive cells in normal mice or mice with CA-CRC.

1.3.6 Ki-67 positive cell position

The number of Ki-67 positive cells was increased in the proximal colon ($p<0.05$; Figure 7a) at cell positions 1-10 (16%) and 11-20 (100%) in mice with CA-CRC compared to normal controls, which was ameliorated by low dose Emu Oil ($p<0.05$) at cell positions 1-10 (-15.5%) and 11-20 (-47.3%). The number of Ki-67 positive cells was increased at cell positions 11-20 (157.4%) in CA-CRC controls compared to normal controls in the distal colon ($p<0.001$; Figure 7b), with no further impact following Emu Oil treatment. Moreover, Emu Oil did not impact the position of Ki-67 positive cells in normal mice.

1.4 Discussion

In previous experimental studies of UC and CA-CRC, Emu Oil reduced disease severity and displayed reparative properties [14, 16-21]. In the current study, in CA-CRC controls colonic crypts lengthened and cell numbers increased in the distal colon, with no change in the cell diameter and number of cells per μm compared to normal control mice. Furthermore, in CA-CRC mice numbers of Ki-67+ proliferating cells were increased at the base of crypts in the proximal colon and at the upper regions of crypts in the proximal and distal colon. Emu Oil treatment had no demonstrable impact on crypt depth, cell number, cell diameter and number of cells per 100μm in the proximal and distal colon of mice with CA-CRC. In the proximal colon, low dose Emu Oil treatment reduced proliferation at the base and upper region of crypts in mice with CA-CRC compared to CA-CRC controls. In normal mice, Emu Oil did not impact colonic crypt depth, cell number, cell diameter, number of cells per 100μm nor Ki-67 expression.

In an acute ten-day study of DSS-induced colitis in rats, distal colonic crypts were lengthened in the DSS treated controls in the distal colon compared to normal controls during the recovery phase [16]. This is thought to be a compensatory repair mechanism of the colonic mucosa after injury [16]. Chartier *et al*. (2018) studied a mouse model of CA-CRC and demonstrated that colonic crypts lengthened in control mice with CA-CRC compared to normal controls [18]. A similar effect was observed in the current study in which the number of cells per crypt was increased in CA-CRC controls, with no change in cell diameter and the number of cells present per 100μm. Considered together, this suggests that the crypt lengthening reported in the study by Chartier *et al.* (2018) was primarily to the result of hyperplasia as opposed to hypertrophy [18].

In the current study, CA-CRC controls displayed significant crypt lengthening and increased Ki-67 positive cells in the upper region of crypts, indicating proliferating cells were not confined to the crypt base. In the early stages of human CA-CRC pathogenesis, proliferation is no longer suppressed in colonocytes and S-phase cells are distributed throughout crypts.

Increased cell proliferation and differentiation is followed by the development of aberrant crypt foci and carcinoma [5]. Arvai *et al.* (2015) demonstrated a pattern of discontinuous proliferating cells in primary colorectal cancer tumours throughout the crypt [26]. In the current study, cell proliferation not confined to the base of crypts was likely indicative of disease. However, Ki-67 expression was not increased overall in CA-CRC controls compared to normal controls. This may have been due to increased proliferation during early tumour formation (prior to the nine-week end of trial time point), resulting in the observed increased cell number but no change in the number of Ki-67 positive cells. The results of the current study suggest that colorectal cancer development was more related to the dissemination of proliferating cells than their number in the crypts.

Abimosleh *et al.* (2012) investigated orally-administrated Emu Oil in a rat model of acute UC [16]. In colitic rats, Emu Oil further lengthened crypts in the proximal (24%) and distal colon (30%) compared to untreated disease controls. This study suggested that Emu Oil lengthened crypts to further stimulate intestinal recovery and repair. Safaeian *et al.* (2019) also investigated the effect of orally-administered Emu Oil in a seven week mouse study of DSS-induced UC [17]. DSS-treated mice exhibited reduced bodyweight compared to normal controls, which was attenuated by Emu Oil treatment. Additionally, Mashtoub *et al.* (2016) investigated the effect of Emu Oil in a twelve-week study of CA-CRC in mice [27]. In the CA-CRC mice, the number of large tumours was reduced by Emu Oil treatment. Moreover, clinically-assessed disease activity and histologically-assessed damage severity were increased in UC and CA-CRC controls [23, 27]. However, following Emu Oil administration, both measures of disease severity improved significantly. In the current study, Emu Oil therapy did not affect crypt length and the number of proliferating cells, implying that Emu Oil may affect CA-CRC tumorigenesis through other processes.

Emu Oil has been shown to stimulate intestinal growth but may have a different action in reducing CA-CRC disease activity. Apoptosis, or programmed cell death, is important to maintain the steady-state in cell proliferation levels, especially in highly proliferative tissues

such as the mucosa of the intestine, to prevent under- or over- growth [28]. Thus, apoptosis is commonly down-regulated in cancers, allowing these cells to survive longer and with DNA damage that would usually activate the cell death pathway. TNF-α can provide pro- or anti-apoptotic signals and potentially promote cell survival through binding with nuclear factor kappa beta (NFκβ). Emu Oil treatment may restore apoptosis to normal levels by reducing cell death via anti-apoptotic pathway activation. Future studies would benefit from assessment of apoptosis by techniques such as terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) to detect DNA fragmentation.

Mashtoub *et al.* (2014) demonstrated increased ileal crypt depth and villus height following ten days of Emu Oil administration in healthy rats, which returned to normal after cessation of treatment [29]. Furthermore, the villus height:crypt depth ratio, which is altered in neoplasia, was unchanged. Barker et al. (2020) also demonstrated that Emu Oil increased crypt depth in the jejunum and ileum of normal rats via increased cell proliferation [30]. Importantly, crypt depth and proliferation returned to normal levels after Emu Oil administration was withdrawn, indicating a return to the normal homeostatic state in the intestine. The current study represents the first investigation of the effect of Emu Oil on cell proliferation in normal animals for a longer duration. The results of the current study confirm that Emu Oil has no lasting effect on mucosal architecture and thus support its safety for use long-term. In the current study, long-term Emu Oil administration did not increase colonic crypt depth, [consistent with findings from Chartier et al. (2018), cell count, cell size or the number of Ki-67 positive cells in crypts in normal mice. Considered together, the results from these studies suggest that increased crypt depth observed following ten days of Emu Oil treatment may have been due to an initial increase in cell proliferation with Emu Oil treatment that later normalised. Alternatively, crypts may have lengthened and returned to normal in a cyclical pattern throughout the duration of administration. The effects of Emu Oil in studies of short-term administration have been attributed to the unique ratio of fatty acids and other components within the non-triglyceride fraction.

1.5 Conclusion

The current study demonstrated that crypt lengthening in CA-CRC was primarily due to hyperplasia, determined by increased cell numbers with no change in cell size. Proliferating cells not confined to the base of crypts is a feature of aberrant intestinal growth. Despite the observed increase in colonic growth, the number of Ki-67 positive cells was not increased overall in AOM/DSS mice. This suggests that increased proliferation may have occurred earlier than the nine-week time point. Emu Oil administration in CA-CRC controls did not affect crypt depth or cell size; however, low dose Emu Oil treatment reduced proliferation at the base and upper region of crypts in the proximal colon. Importantly, in normal mice, Emu Oil did not affect any parameters of intestinal growth, supporting its safety for long-term use as an orally-administered treatment for intestinal disorders.

Future studies would benefit from an assessment of the effect of Emu Oil on expression levels of the oncogene *Kras*, tumour suppressor genes *p53* and *Apc*, inflammatory cytokine TNF-α and NFκβ, which are implicated in colorectal cancer progression. Further understanding of the mechanism by which Emu Oil affects the gastrointestinal tract could lead to identification of the bioactive oil constituents. A deeper understanding of Emu Oil and its effects on colonocyte kinetics in CA-CRC could facilitate the development of new nutraceutical treatments for UC and CA-CRC.

1.6 References

[1] K.A. Head, J.S. Jurenka. Inflammatory bowel disease Part 1: ulcerative colitis--pathophysiology and conventional and alternative treatment options. Altern Med Rev. 8 (2003) 247-283.

[2] L.J. Herrinton, L. Liu, T.R. Levin, J.E. Allison, J.D. Lewis, F. Velayos. Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1998 to 2010. Gastroenterology. 143 (2012) 382-389.

[3] N. Kamada, T. Hisamatsu, S. Okamoto, et al. Abnormally differentiated subsets of intestinal macrophage play a key role in Th1-dominant chronic colitis through excess production of IL-12 and IL-23 in response to bacteria. J Immunol. 175 (2005) 6900-6908.

[4] M. Ponz de Leon, A. Percesepe. Pathogenesis of colorectal cancer. Dig Liver Dis. 32 (2000) 807-821.

[5] E.E. Deschner, J. Godbold, H.T. Lynch. Rectal epithelial cell proliferation in a group of young adults. Influence of age and genetic risk for colon cancer. Cancer. 61 (1988) 2286-2290.

[6] J.M. Snowden, M.W. Whitehouse. Anti-inflammatory activity of emu oils in rats. Inflammopharmacology. 5 (1997) 127-132.

[7] S. Mashtoub, C.D. Tran, G.S. Howarth. Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. Exp Biol Med (Maywood). 238 (2013) 1305-1317.

[8] M.W. Whitehouse, A.G. Turner, C.K. Davis, M.S. Roberts. Emu oil(s): a source of non-toxic transdermal anti-inflammatory agents in aboriginal medicine. Inflammopharmacology. 6 (1998) 1-8.

[9] V. Zanardo, D. Giarrizzo, L. Maiolo, G. Straface. Efficacy of Topical Application of Emu Oil on Areola Skin Barrier in Breastfeeding Women. J Evid Based Complementary Altern Med. 21 (2016) 10-13.

[10] M.K. Jeengar, S. Shrivastava, S.C. Mouli Veeravalli, V.G. Naidu, R. Sistla. Amelioration of FCA induced arthritis on topical application of curcumin in combination with emu oil. Nutrition. 32 (2016) 955-964.

[11] D.C. Rollmann, P.J. Novotny, I.A. Petersen, et al. Double-Blind, Placebo-Controlled Pilot Study of Processed Ultra Emu Oil Versus Placebo in the Prevention of Radiation Dermatitis. International journal of radiation oncology, biology, physics. 92 (2015) 650-658.

[12] S. Mashtoub, K.Y. Cheah, N.L.C. Lansdown, G.S. Howarth. Emu oil source does not significantly alter therapeutic efficacy, while ostrich oil has no beneficial effect, in a rat model of chemotherapy-induced mucositis. J Gastroen Hepatol. 29 (2014) 17-17.

[13] S. Mashtoub, L.S. Lampton, G.L. Eden, et al. Emu Oil Combined with Lyprinol Reduces Small Intestinal Damage in a Rat Model of Chemotherapy-Induced Mucositis. Nutr Cancer. 68 (2016) 1171-1180.

[14] R.J. Lindsay, M.S. Geier, R. Yazbeck, R.N. Butler, G.S. Howarth. Orally administered emu oil decreases acute inflammation and alters selected small intestinal parameters in a rat model of mucositis. British Journal of Nutrition. 104 (2010) 513-519.

[15] S.M. Abimosleh, C.D. Tran, G.S. Howarth. Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. Evid Based Complement Alternat Med. 2013 (2013) 10.

[16] S.M. Abimosleh, R.J. Lindsay, R.N. Butler, A.G. Cummins, G.S. Howarth. Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. Dig Dis Sci. 57 (2012) 887-896.

[17] R. Safaeian, G.S. Howarth, I.C. Lawrance, D. Trinder, S. Mashtoub. Emu Oil reduces disease severity in a mouse model of chronic ulcerative colitis. Scand J Gastroenterol. (2019) 1-8.

[18] L.C. Chartier, G.S. Howarth, I.C. Lawrance, D. Trinder, S.J. Barker, S. Mashtoub. Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. Dig Dis Sci. 63 (2018) 135-145.

[19] L.C. Chartier, K.E. Maiolo, G.S. Howarth, et al. Emu Oil Improves Clinical Indicators of Disease and Reduces Proximal Colonic Crypt Hyperplasia in a Murine Model of Colitis-Associated Colorectal Cancer. Gastroenterology. 154 (2018) S875-S875.

[20] S. Mashtoub, G.S. Howarth, D. Trinder, I. Lawrance. Emu Oil Attenuates Disease Severity and Results in Fewer Large Colonic Tumours in a Mouse Model of Colitis-Associated Colorectal Cancer. Gastroenterology. 152 (2017) S737-S737.

[21] S. Mashtoub, R. Safaeian, I.C. Lawrance, D. Trinder, G.S. Howarth. Emu oil attenuates disease severity in models of colitis and colitis-associated colorectal cancer. J Gastroen Hepatol. 31 (2016) 125-125.

[22] L.C. Chartier, G.S. Howarth, D. Trinder, S. Mashtoub. Emu Oil And Grape Seed Extract Reduce Tumour Burden And Disease Parameters In Murine Colitis-Associated Colorectal Cancer. Carcinogenesis. (2020).

[23] M. Lipkin, W.E. Blattner, J.F. Fraumeni, Jr., H.T. Lynch, E. Deschner, S. Winawer. Tritiated thymidine (phi p, phi h) labeling distribution as a marker for hereditary predisposition to colon cancer. Cancer Res. 43 (1983) 1899-1904.

[24] J. Gerdes, H. Lemke, H. Baisch, H.H. Wacker, U. Schwab, H. Stein. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol. 133 (1984) 1710-1715.

[25] J.M. Clarke, G.P. Young, D.L. Topping, et al. Butyrate delivered by butyrylated starch increases distal colonic epithelial apoptosis in carcinogen-treated rats. Carcinogenesis. 33 (2012) 197-202.

[26] K.J. Arvai, Y.H. Hsu, L.A. Lee, D. Jones. A Transition Zone Showing Highly Discontinuous or Alternating Levels of Stem Cell and Proliferation Markers Characterizes the Development of PTEN-Haploinsufficient Colorectal Cancer. PLoS One. 10 (2015) e0131108.

[27] S. Mashtoub, R. Safaeian, I.C. Lawrance, D. Trinder, G.S. Howarth. Emu oil attenuates disease severity in models of colitis and colitis-associated colorectal cancer: Abstract of the Australian Gastroenterology Week,10-12 October 2016, Adelaide, Australia- Inflammatory Bowel Disease Basic. J Gastroenterol Hepatol2016. p. 125.

[28] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter. Programmed Cell Death (Apoptosis). New York: Garland Science2002.

[29] S. Mashtoub, K.Y. Cheah, G.S. Howarth. Safety of emu oil for intestinal applications. J Gastroen Hepatol. 29 (2014) 17-17.

[30] S.J. Barker, G.S. Howarth, L.C. Chartier, B.L. Scherer, S. Mashtoub. Mucosal stimulation following oral administration of emu oil represents a process of normal intestinal growth in rats. The Australian Journal of Herbal and Naturopathic Medicine. 32 (2020) 15-23.

Figure Legends

Fig. 1 Crypt depth in the (a) proximal colon and (b) distal colon, measured from H&E stained tissue. Data are expressed as mean (μm) ± SEM. **p<0.01 compared to saline + water + water

Fig. 2 Cell count per half crypt in the (a) proximal colon and (b) distal colon, measured from H&E stained tissue. Data are expressed as mean ± SEM

Fig. 3 Cell diameter in the (a) proximal colon and (b) distal colon, calculated by crypt depth divided by cell count. Data are expressed as mean ± SEM

Fig. 4 Number of cells per 100μm in the (a) proximal colon and (b) distal colon, calculated by cell count divided by crypt depth. Data are expressed as mean ± SEM

Fig. 5 Ki-67 immunohistochemical staining of distal colonic sections with cell nuclei (blue) and Ki-67 positive cells (brown) in A) normal controls, B) normal mice with Emu Oil treatment, C) colitis-associated colorectal cancer controls, D) colitis-associated colorectal cancer with low dose Emu Oil treatment (80μl), E) colitis-associated colorectal cancer with high dose Emu Oil treatment (160μl)

Fig. 6 Percentage of Ki-67 positive cells in the (a) proximal colon and (b) distal colon, measured from immunohistochemically stained tissue. Data are expressed as mean ± SEM

Fig. 7 Percentage of Ki-67 positive cells in the (a) proximal colon and (b) distal colon at cell positions within crypts, measured from immunohistochemically stained tissue. Data are expressed as mean ± SEM. *p<0.05 compared to saline + water + water, ^p<0.05 compared to AOM + DSS + water

Figure 1a.

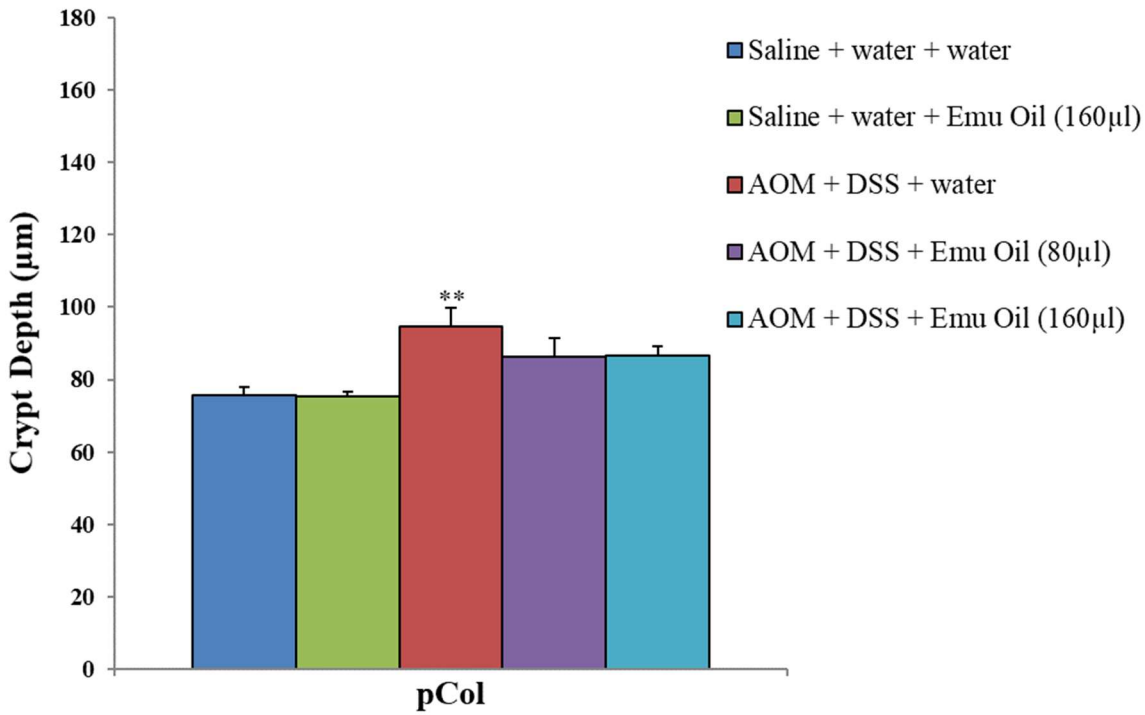


Figure 1b.

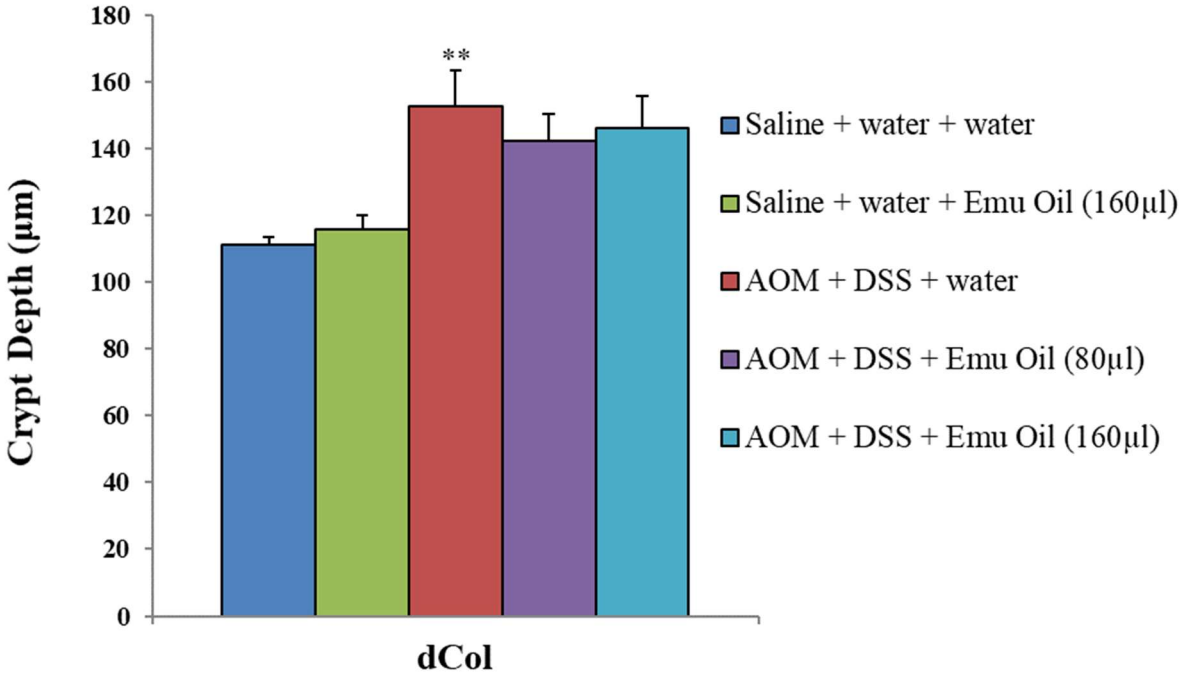


Figure 2a.

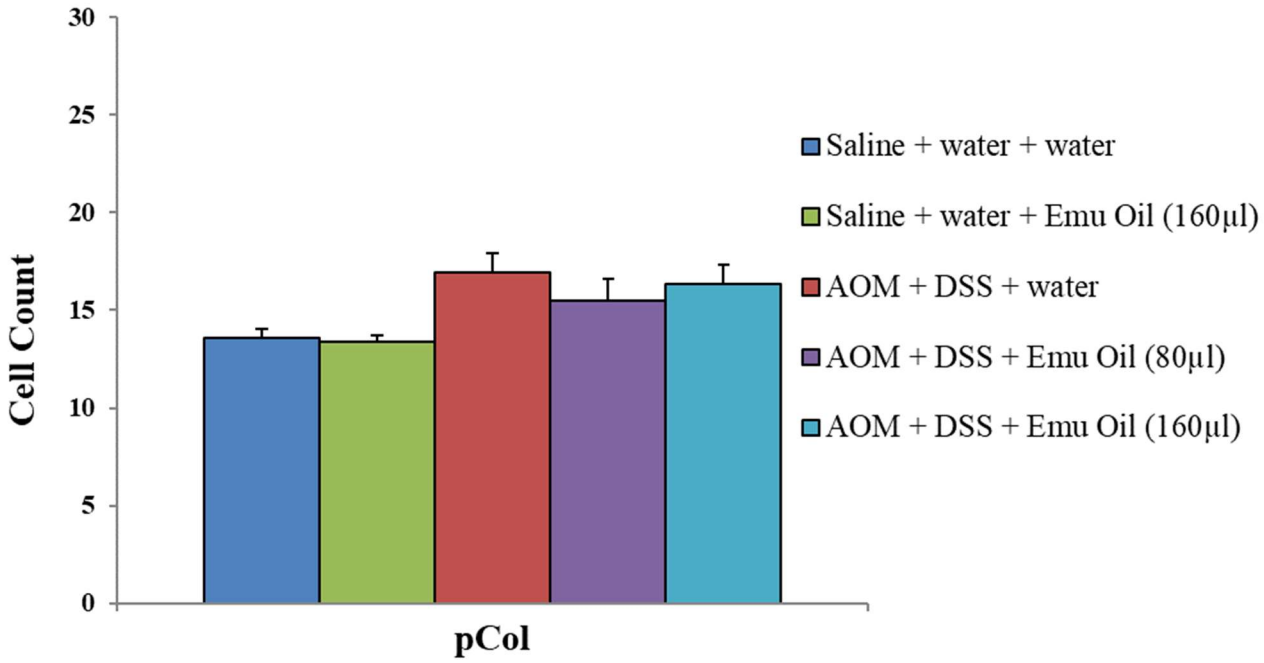


Figure 2b.

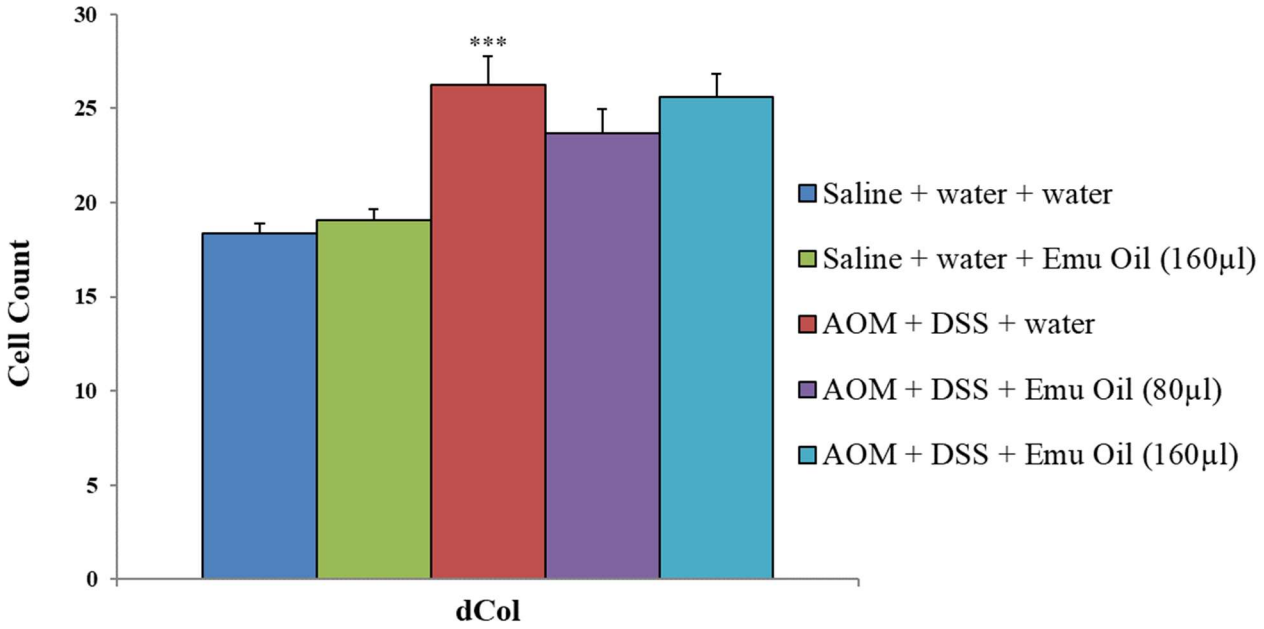


Figure 3a.

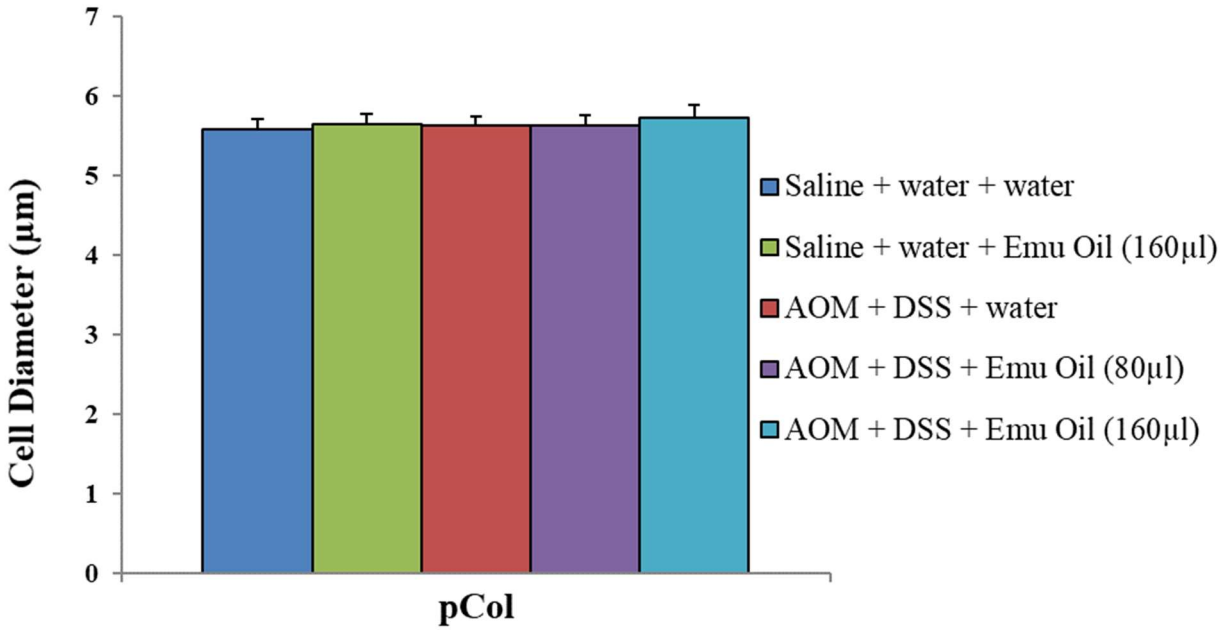


Figure 3b.

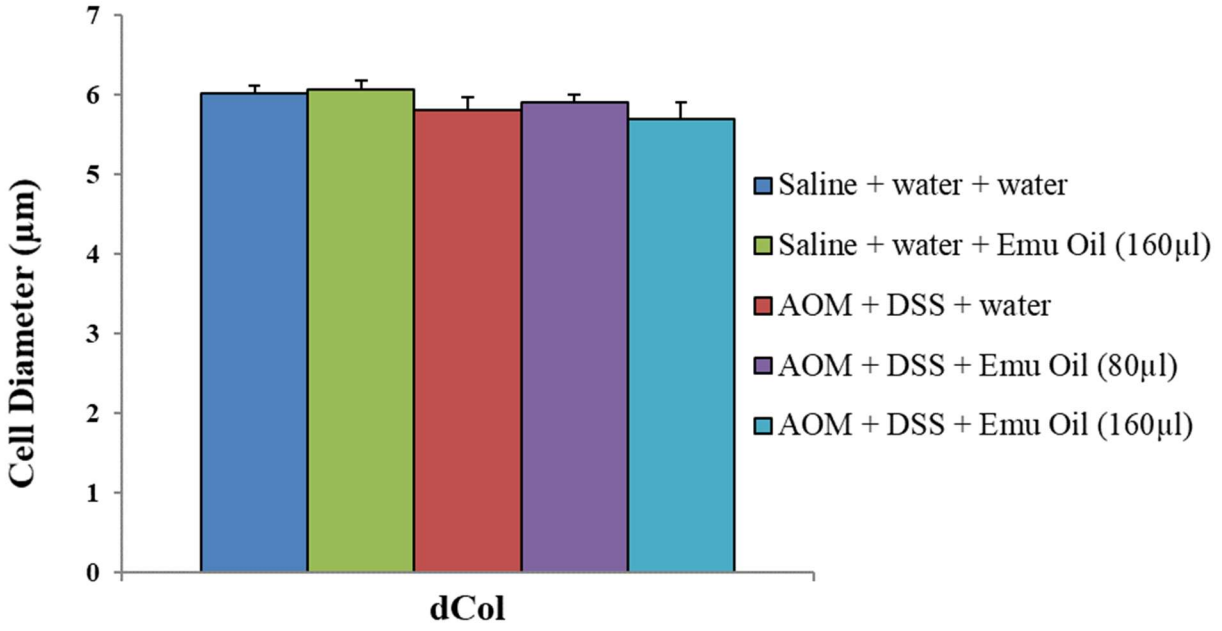


Figure 4a.

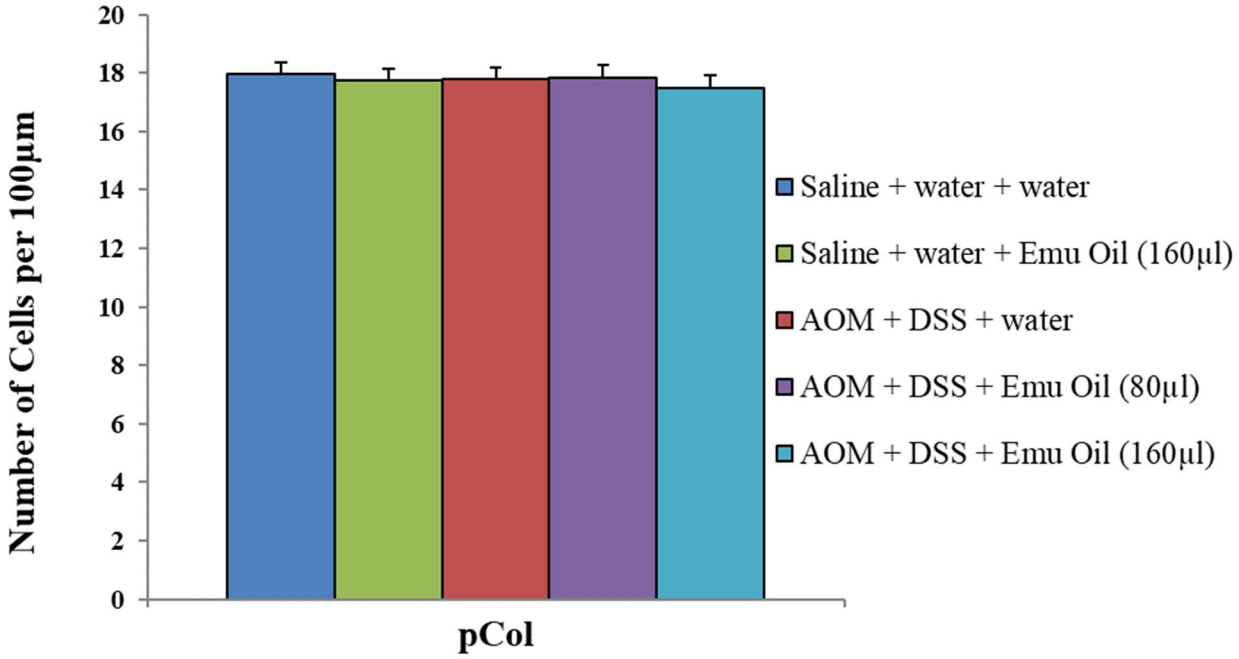


Figure 4b.

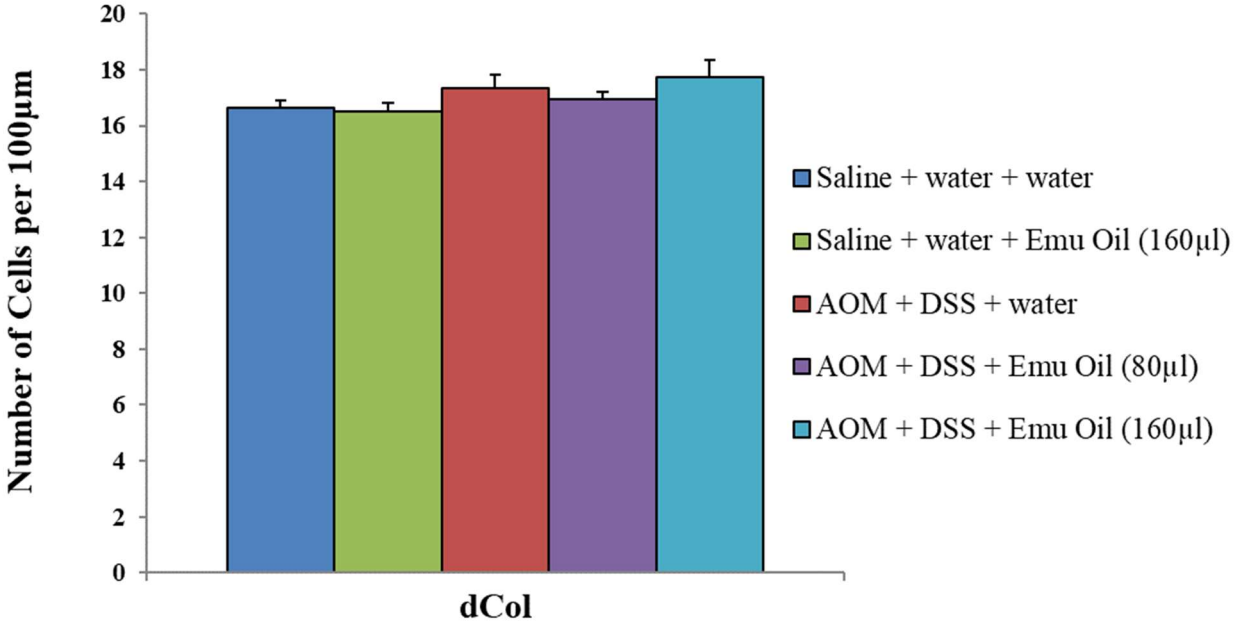


Figure 5.

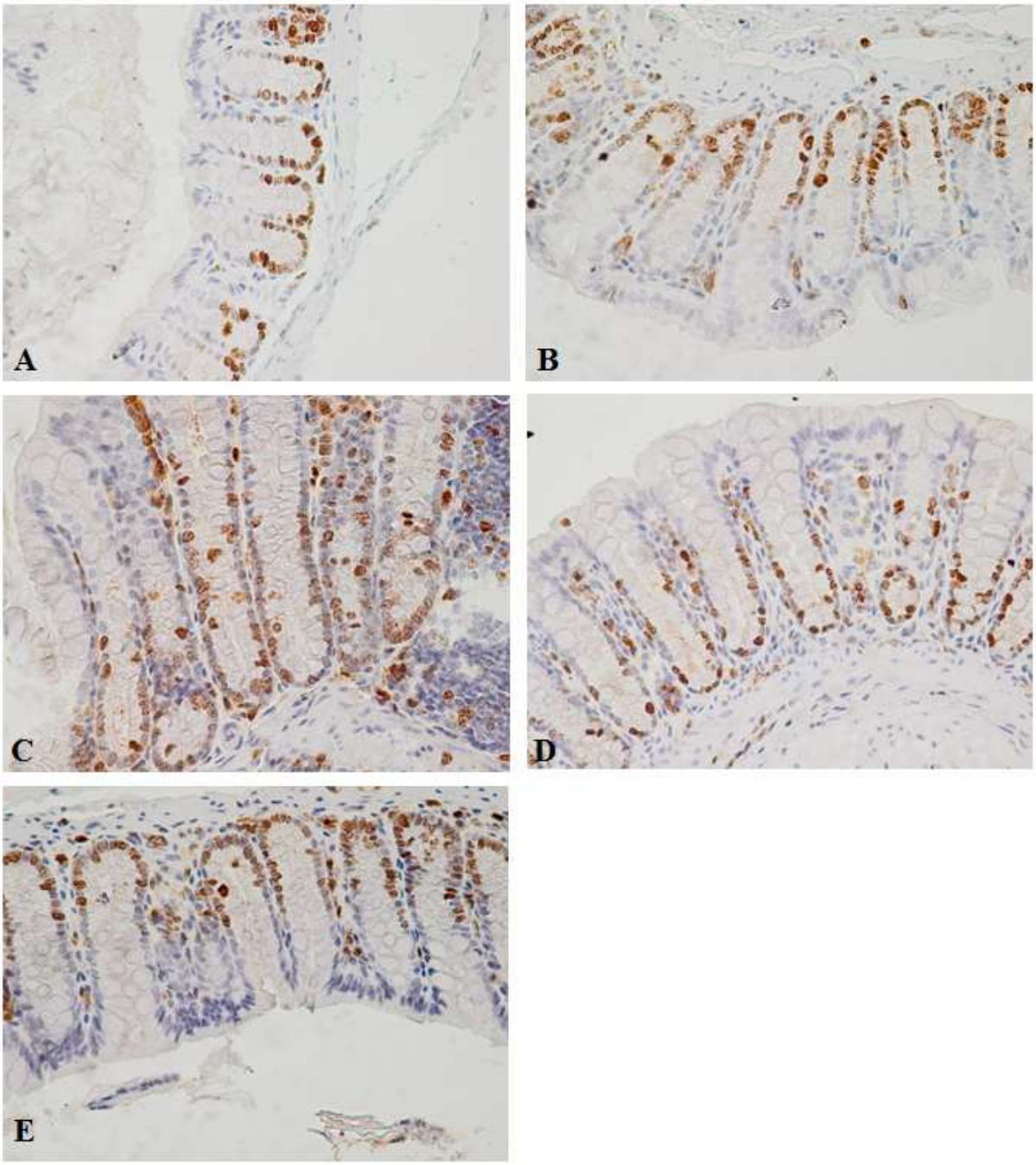


Figure 6a.

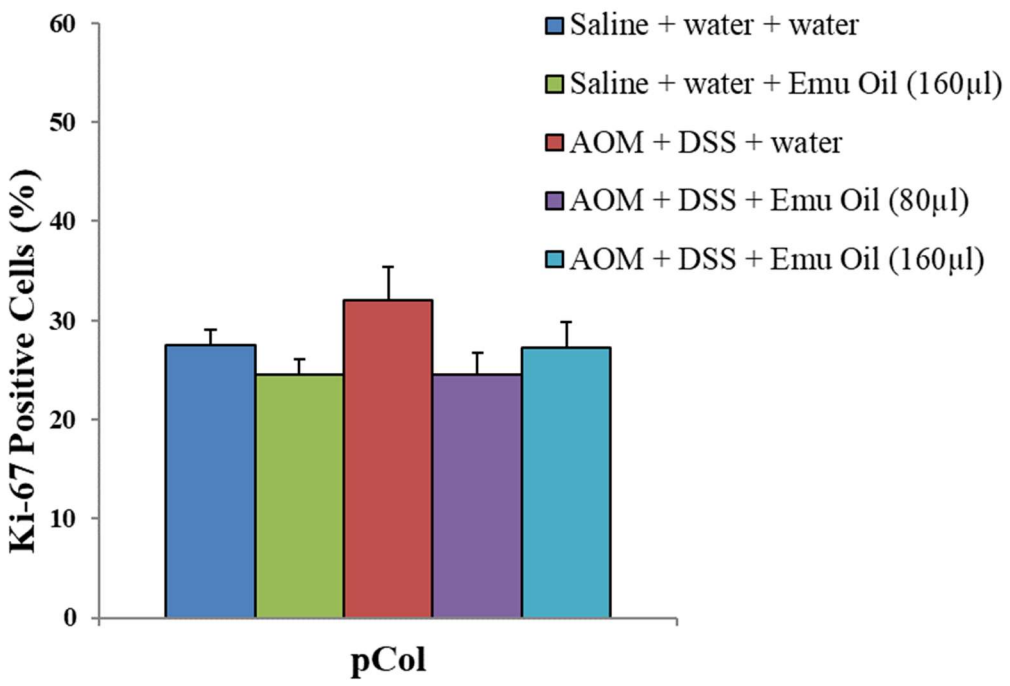


Figure 6b.

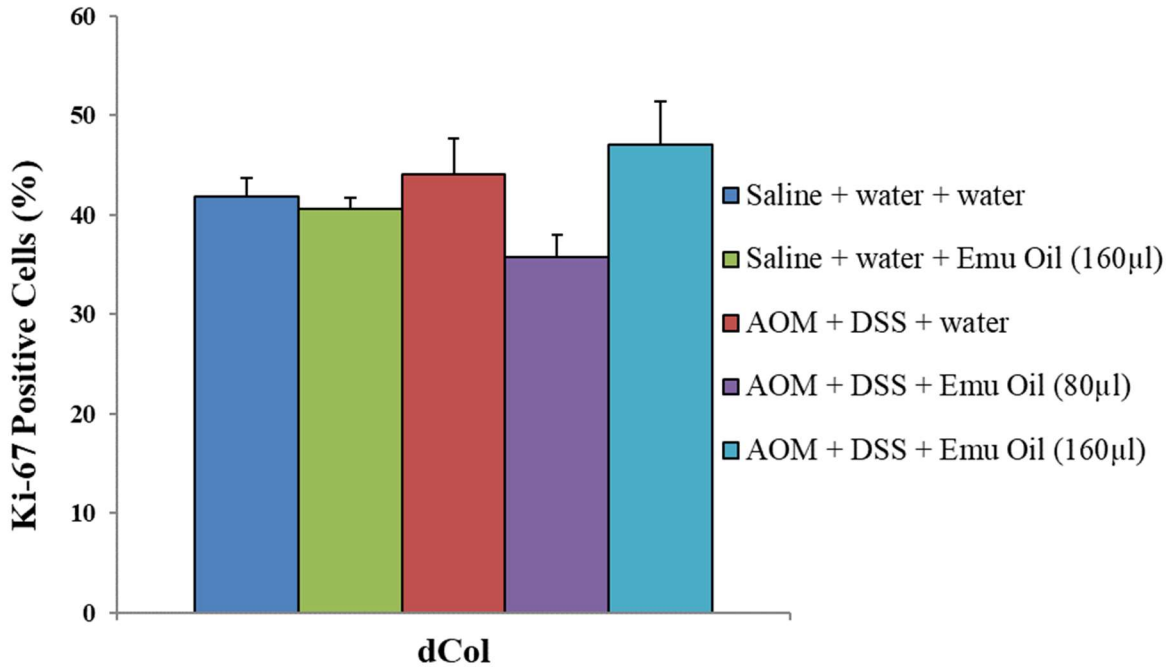


Figure 7a.

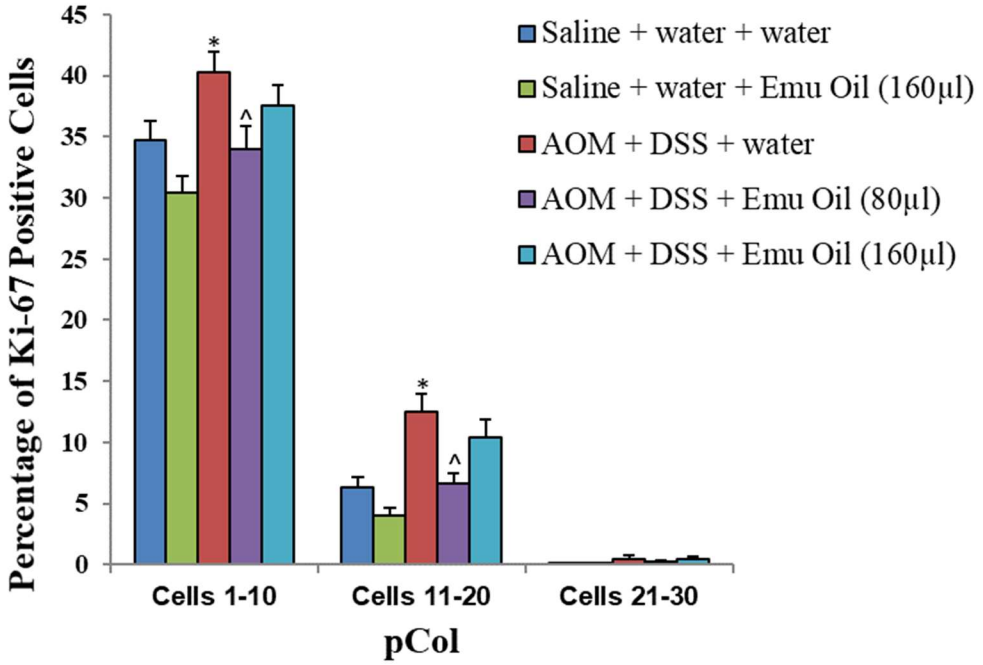
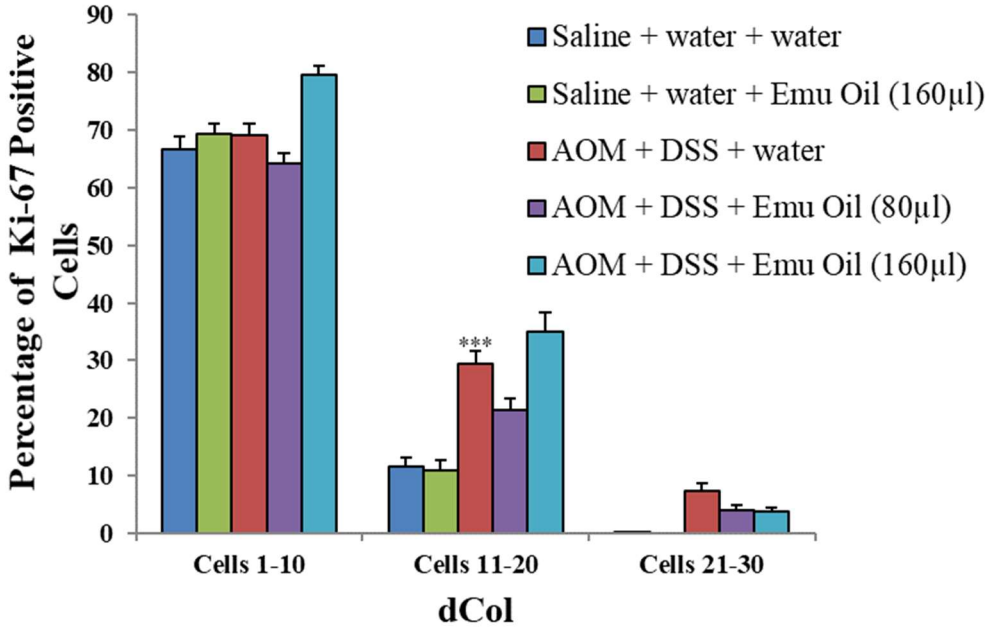

Percentage of Ki-67 Positive Cells
45
40
35
30
25
20
15
10
5
0
*
^
*
^
Cells 1-10
Cells 11-20
Cells 21-30
pCol
Saline + water + water
Saline + water + Emu Oil (160µl)
AOM + DSS + water
AOM + DSS + Emu Oil (80µl)
AOM + DSS + Emu Oil (160µl)

Figure 7b.


Percentage of Ki-67 Positive Cells
90
80
70
60
50
40
30
20
10
0
***
Cells 1-10
Cells 11-20
Cells 21-30
dCol
Saline + water + water
Saline + water + Emu Oil (160µl)
AOM + DSS + water
AOM + DSS + Emu Oil (80µl)
AOM + DSS + Emu Oil (160µl)

APPENDIX 5

EMU OIL ATTENUATES DISEASE SEVERITY AND RESULTS IN FEWER LARGE COLONIC TUMOURS IN A MOUSE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

STATEMENT OF AUTHORSHIP

Title of Paper: Emu Oil Attenuates Disease Severity and Results in Fewer Large Colonic Tumours in a Mouse Model of Colitis-Associated Colorectal Cancer

Publication Status: Under Review

Publication Details: Nutrition and Cancer

Principal Author

Name of Principal Author	Suzanne Mashtoub		
Contribution to the Paper	Completed animal trials, sample and data collection, data analyses and interpretation. Prepared manuscript including writing and formatting.		
Signature		Date	8/10/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author (Candidate)	Lauren Claire Chartier
Contribution to the Paper	Data interpretation and histological analyses, editing and submission of the manuscript.
Overall percentage (%)	20%
Certification:	This paper reports on original research directly related to my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.
Signature	Date 28/9/20

Name of Co-Author	Debbie Trinder
Contribution to the Paper	Conceptualisation and methodology development.
Signature	Date 16 Sept 2020

Name of Co-Author	Ian C Lawrance
Contribution to the Paper	Conceptualisation and methodology development.
Signature	Date 23 Sept 2020

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodological development, data interpretation, revision and editing of the manuscript.		
Signature		Date	9/10/20

EMU OIL ATTENUATES DISEASE SEVERITY AND RESULTS IN FEWER LARGE COLONIC TUMOURS IN A MOUSE MODEL OF COLITIS-ASSOCIATED COLORECTAL CANCER

S Mashtoub,[1, 2, 3*] LC Chartier,[2, 3] D Trinder,[1, 5] IC Lawrance,[1, 6] GS Howarth[2, 3, 4]

1. Medical School, The University of Western Australia, Fiona Stanley Hospital, Murdoch, Western Australia, 6150
2. Department of Gastroenterology, Women's and Children's Hospital, North Adelaide, South Australia, 5006
3. Discipline of Physiology, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, 5005
4. School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy Campus, Roseworthy, South Australia, 5371
5. Harry Perkins Institute of Medical Research, Murdoch, Western Australia, 6150
6. Centre for Inflammatory Bowel Diseases, Saint John of God Hospital, Subiaco, Western Australia, 6008

*Corresponding author: Dr Suzanne Mashtoub; Email: suzanne.mashtoub@adelaide.edu.au; Tel: +61 08 8161 6991

Word count: 3,483 words

ABSTRACT

Background: Ulcerative colitis (UC) patients have an increased risk of developing colorectal cancer (CRC). **Aim:** To determine whether Emu Oil (EO) could reduce the severity of colitis, thereby inhibiting colitis-associated CRC (CA-CRC) development. **Methods:** Female C57BL/6 mice (n=8/group) were injected (i.p.) with saline or AOM (7.4mg/kg). Mice then underwent three DSS/water cycles. Mice were orally-administered either water (160µL) or EO (80µL or 160µL), thrice weekly. Mice were euthanised after 12 weeks. **Results:** AOM/DSS decreased bodyweight compared with normal controls (max. 20%; $p<0.05$). In AOM/DSS mice, EO (160µL) increased bodyweight compared with untreated and 80µL EO-treated mice (max. 10%; $p<0.05$). Both volumes of EO reduced DAI scores on days 49, 56–63 (max. 40%; $p<0.05$), compared with AOM/DSS controls. Histological disease severity was increased in the distal colon of AOM/DSS mice, and reduced by EO (160µL; $p<0.05$). Mucin-secreting goblet cells were increased by AOM/DSS compared to normal, with no effect observed following EO treatment ($p>0.05$). Importantly, large tumour numbers were decreased in EO-treated mice (160µL; 2±0.6) compared with AOM/DSS controls (5±0.7; $p<0.05$), although EO did not impact overall tumour number ($p>0.05$). Other analyses remained unchanged across groups ($p>0.05$). **Conclusions:** EO demonstrates promise as an adjunct to conventional treatment options for colitis management.

Keywords: Emu Oil, IBD, ulcerative colitis, colitis-associated colorectal cancer, AOM/DSS.

INTRODUCTION

The inflammatory bowel diseases (IBDs), ulcerative colitis (UC) and Crohn's disease (CD), are life-long, incurable diseases that are increasing in frequency with high morbidity (1). The IBDs are characterised by chronic inflammatory damage to the gastrointestinal mucosa with features of ulceration, haemorrhage, fibrosis and sometimes, perforation. The management of UC involves both induction (to induce remission) and maintenance (to prevent further flares) therapies using aminosalicylates, steroids, thiopurines, immunotherapy, biologic agents and vedolizumab (2, 3). However, these treatments are inadequate to maintain long-term remission in a substantial proportion of patients.

A significant consequence of chronic intestinal inflammation is the development of colorectal cancer (CRC) (4, 5). The prevalence of CRC is increasing worldwide, and in 2018 alone, there were approximately 1.8 million new cases and 881,000 CRC-related deaths globally (6). The link between colonic inflammation and carcinogenesis is well established, exemplified by an increased risk of CRC development in IBD patients (7, 8). Whilst the pathogenesis of colitis-associated CRC remains unclear, significant contributing factors include modifications to the intestinal microbiota and mucosal inflammatory mediators (interleukin-6 [IL-6], IL-11, tumour necrosis factor-α [TNF-α], nuclear factor-κB [NF-κB], cyclooxygenase 2 and chemokines) (9–11). Activation of the NF-κB pathway contributes to CRC by preventing epithelial cell apoptosis in the colon and promoting growth factor production by inflammatory cells. Simultaneously, there is a significant infiltration of neutrophils into the lamina propria and sub-mucosa in the progression of colitis-associated CRC. Furthermore, oxidative stress plays a role in carcinogenesis, as prolonged generation of cellular oxidants and reactive oxygen species (ROS) can damage cells and promote mutagenic processes (4, 12).

Agents capable of decreasing inflammation and neutralising ROS would be ideal candidates to prevent or reduce the development of colitis-associated CRC. Recently, attention has been

directed toward animal-derived oils with anti-inflammatory and antioxidant properties, such as that derived from the Australian bird, the Emu (*Dromaius novaehollandiae*) (13, 14).

Emu Oil is extracted from the subcutaneous and retroperitoneal fat of the emu by first rendering the macerated tissue, and then passing the liquefied fat through a series of filters to extract a purified oil (15). Indigenous Australian people first used Emu Oil for healing of wounds and burns, pain alleviation and treatment of inflamed joints. Fatty acids represent the predominating component of Emu Oil, with a lipid content of 98.0%, comprising approximately 42% oleic acid (18:1 n-9), 21% linoleic acid (18:2 n-6), and 21% palmitic acid (16:0), with lower levels of other fatty acids, including 1% α-linolenic acid (18:3 n-3) (15, 16). Emu Oil also contains variable levels of compounds including carotenoids, flavones, polyphenols, tocopherol and phospholipids in the non-triglyceride fraction, which may confer therapeutic benefits including antioxidant properties (16, 17). Bennett *et al.* (2008) and Mashtoub *et al.* (2014) demonstrated that Emu Oil has both antioxidant properties *in vitro* (radical scavenging activities) (17) and a protective role against oxidative damage (assessed by measuring the ability to inhibit lipid peroxidation of erythrocytes) in a biological membrane model system (18).

Emu Oil has been investigated for its therapeutic potential in various preclinical models of intestinal inflammation. In a model of dextran sulphate sodium (DSS)-induced acute colitis, orally-administered Emu Oil improved mucosal architecture and reduced histologically-assessed colonic damage severity (19). More recently, orally-administered Emu Oil increased bodyweight and decreased clinically- and histologically-assessed disease severity in a mouse model of chronic (51 days) colitis (20). Additionally, Emu Oil decreased disease severity in models of chemotherapy (5-Fluorouracil)-induced mucositis (21, 22) and non-steroidal anti-inflammatory drug-enteropathy (23). Finally, in an azoxymethane (AOM)/DSS mouse model of colitis-associated CRC, Emu Oil reduced clinical indicators and the number of *small* colonic tumours over a 9-week timeline (24), without affecting overall tumour burden. Therefore, the current study aimed to investigate Emu Oil in a more protracted 12-week mouse model of

colitis-associated CRC. It was hypothesised that orally administered Emu Oil would reduce the severity of colitis and inhibit the development of colonic tumours over this extended timeline of colitis-associated CRC.

MATERIALS AND METHODS

General Experimental Procedures

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, The University of Adelaide and The University of Western Australia. Female C57BL/6 mice were sourced from Animal Resource Centre (Perth, Western Australia) and group housed at room temperature with a light:dark cycle of 14:10 hours. Mice were acclimatised for one week prior to trial commencement and provided with *ad libitum* access to standard mouse chow (meat-free mouse diet; Specialty Feeds, Glen Forrest, Western Australia) and drinking water.

Experimental Groups

Female C57BL/6 mice (n=32) were randomly assigned to four groups (n=8/group):

(1) Saline + Water + Water [normal control]

(2) AOM + DSS + Water [CRC control]

(3) AOM + DSS + Emu Oil (80µL)

(4) AOM + DSS + Emu Oil (160µL).

Test Compound

Commercially available 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. Emu Oil (batch no. 0214660) was aliquoted into 5ml opaque tubes and stored in darkness at 4°C. The composition of Emu Oil was analyzed by gas chromatography at the Waite Lipid Analysis Service – FOODPlus Research Centre (Urrbrae, South Australia; Table 1).

Experimental trial

On Day 0, mice were intraperitoneally injected with saline (Group 1; 7.4mg/kg; InterPharma, Manly, NSW, Australia) or the carcinogen azoxymethane (AOM) (Groups 2–4; 7.4mg/kg; Sigma-Aldrich, Castle Hill, NSW, Australia). Mice then underwent three cycles, whereby each cycle consisted of *ad libitum* access to water (Group 1) or a DSS solution (Groups 2–4; 2%w/v; MP Biomedicals LLC, Santa Ana, California, USA) for seven days, followed by 14 days of plain water in drinking bottles. All mice were then subjected to a further 3 weeks of plain drinking water after the third cycle. Water (160µL/gavage) or Emu Oil (80µL or 160µL/gavage) was administered via oral-gastric gavage thrice weekly throughout the duration of the trial. All mice were euthanised after the 12-week experimental period (day 84).

Trial analyses

Daily Measurements

Body weight was recorded daily. A disease activity index (DAI) which scored body weight loss, rectal bleeding, stool consistency and overall general condition of the animal was utilized to monitor the progression and resolution of disease. Each parameter was scored from 0–3 (increasing in severity), and summed to achieve an overall daily DAI, as described previously (25).

Post-mortem analyses

Tissue collection

On day 84, following an overnight fast, mice were gavaged with a lactulose/rhamnose solution (0.3 ml; 100 mg lactulose, 50 mg rhamnose; Sigma Aldrich, Castle Hill, NSW, Australia) for intestinal permeability assessment and were sacrificed via CO_2 asphyxiation 90 min post-gavage. Whole blood was collected via cardiac puncture for complete blood and intestinal permeability analyses. Colons were excised, opened longitudinally, and photographed alongside a ruler (1mm increments), using a Canon EOS 600 D camera with 17–40 mm lens attached. Weights and lengths of gastrointestinal (duodenum, small intestine and colon) and weights of visceral (heart, liver, spleen, thymus, lung and kidneys) organs were recorded. Segments of colon (2cm; proximal and distal) were removed and placed in 10% buffered formalin for histological analyses. Additionally, mid-colon segments (2cm) were collected, snap-frozen in liquid nitrogen and stored individually at -80^{o}C for analysis of myeloperoxidase activity.

Tumour analyses

Photographs of colons were analysed in a blinded manner using Olympus Soft Imaging Solutions GmbH computer software analySIS version 5.2 (Tokyo, Japan). Colonic tumours were counted, measured (following appropriate calibration) and categorised into size based upon their diameters (< 2mm 'small', 2–3 mm 'medium', >3 mm 'large').

Histological analyses

Histologically-assessed damage severity score. Proximal and distal colon samples were routinely processed and embedded in paraffin wax. Sections (4µm) were then stained with haematoxylin and eosin (H&E). Colonic damage severity was assessed using a semi-quantitative analysis as described by Howarth *et al.* (1996) and Yazbeck *et al.* (2008) (26, 27).

A score from 0 (unaffected) to 3 (severe) was recorded to provide a maximum damage severity score of 24.

Mucin-Secreting Goblet Cell Count.

Following processing and embedding in paraffin wax, additional 4µm sections were mounted on poly-L-lysine-coated slides. Sections were deparaffinised 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, Missouri, 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 mouse.

All histological analyses were performed in a blinded fashion, using a light microscope (Olympus Corporation, Tokyo, Japan) and Image J Software Version 1.44p (National Institutes of Health, USA) for mucin-secreting goblet cell counts.

MPO activity

Myeloperoxidase (MPO) activity in the colon was determined as an indicator of neutrophil infiltration and hence, acute inflammation, using techniques described by Mashtoub *et al.* (2013) (21, 27). Data were expressed as MPO units per gram of tissue.

Small intestinal permeability

Whole blood was centrifuged at room temperature for 10 minutes at 13,000 g for serum collection. The concentrations of lactulose and rhamnose (representing paracellular and transcellular pathways, respectively), and hence lactulose/rhamnose ratio, was quantified in serum using high performance liquid chromatography, indicative of small intestinal permeability.

Statistical Analyses

Statistical comparisons were performed using SPSS version 22.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Data were tested for normality using a Shapiro-Wilk test. All data were expressed as mean ± standard error of the mean (SEM). Organ data, tumour analyses, histologically assessed parameters, MPO activity and small intestinal permeability were analysed using a one-way analysis of a variance and a Tukey's *post-hoc* test. Bodyweight and disease activity index were analysed using a linear mixed effects model with mouse treated as a random factor and *post-hoc* comparisons were reported as the difference between two group means with 95% confidence intervals. For all analyses, $p<0.05$ was considered significant.

RESULTS

Bodyweight

AOM/DSS resulted in significant bodyweight loss (maximum 20%) on days 7, 9, 12, 14, 26, 28, 30, 33, 35, 37, 40, 42, 44 and 51 ($p<0.05$) compared with normal controls (Figure 1). However, in AOM/DSS mice, Emu Oil (160µL) increased bodyweight compared with untreated disease control mice on days 14, 28, 33, 40, 42, 44, 68, 70, 72, 75 and 82 ($p<0.05$; Figure 1); and compared with low volume Emu Oil (80µL)-treated mice on days 14, 47, 48, 51,

54, 56, 58, 61, 63, 65, 68, 70, 72, 75 and 77 ($p<0.05$; Figure 1). Furthermore, 80µL of Emu Oil did not significantly attenuate bodyweight loss compared with disease controls ($p>0.05$).

Disease activity index (DAI) scores

AOM/DSS resulted in significantly elevated DAI on days 6, 7, 9, 26, 28, 30, 44, 47, 49, 56, 61, 65, 68, 70, 75, 77, 79, 82 ($p<0.05$) compared with normal controls (Figure 2). In AOM/DSS mice, Emu Oil (80µL) treatment significantly attenuated DAI on days 26, 49, 56, 68, 70, 79 and 82 ($p<0.05$) compared with disease controls. Moreover, treatment with 160µL Emu Oil significantly reduced DAI on days 6, 7, 26, 30, 33, 44, 49, 56, 70 and 82 ($p<0.05$; Figure 2), compared with AOM/DSS controls. In AOM/DSS mice, Emu Oil (160µL) resulted in significantly reduced DAI on days 28, 30, and 44 ($p<0.05$) and significantly elevated DAI on days 6, 16 and 70 ($p<0.05$) compared to low volume Emu Oil (80µL) administration.

Organ data

AOM/DSS increased spleen weight and decreased thymus weight, compared with normal controls ($p<0.05$; Figure 3a), which was not impacted by either volume of Emu Oil. No significant differences were evident in other visceral organ weights, expressed as a proportion of bodyweight (%), amongst treatment groups ($p>0.05$; Figure 3a). Additionally, AOM/DSS resulted in significantly greater colon weight compared with normal controls ($p<0.05$; Figure 3b), with no significant effects following Emu Oil treatment ($p>0.05$). Stomach, duodenum, caecum, and small intestinal weights remained unchanged across all treatment groups ($p>0.05$; Figure 3b). Furthermore, duodenum, small intestine and colon lengths were not significantly impacted by AOM/DSS or Emu Oil ($p>0.05$; Figure 3c).

Tumour analyses

AOM/DSS resulted in significant tumour development in the colon compared with normal controls ($p<0.05$; Figure 4a). The total number of tumours remained unchanged in Emu Oil-

treated mice compared with AOM/DSS controls ($p>0.05$; Figure 4a). However, when classified into tumour size (small: <2 mm; medium: 2–3 mm; large: >3 mm), there were significantly fewer large colonic tumours in high volume (160µL) Emu Oil-treated mice compared with AOM/DSS controls ($p<0.05$; Figure 4b). The number of small and medium tumours remained unaffected following Emu Oil treatment, compared with AOM/DSS controls ($p>0.05$; Figure 4b).

Histologically-assessed parameters

Histologically-assessed damage severity in the distal colon was significantly elevated in AOM/DSS mice, compared with normal controls ($p<0.01$; Figure 5). In AOM/DSS mice, 160µL Emu Oil treatment resulted in significantly decreased histologically-assessed damage severity in the distal colon compared with disease controls ($p<0.05$; Figure 5). Neither AOM/DSS nor either volume of Emu Oil impacted proximal colonic damage severity ($p>0.05$; Figure 5).

Average number of mucin-secreting goblet cells in the distal colon was significantly elevated in AOM/DSS mice, compared with normal controls ($p<0.05$; Figure 6); however, neither volume of Emu Oil significantly impacted mucin counts in AOM/DSS mice compared to disease controls ($p>0.05$; Figure 6). Average number of proximal colonic mucin-secreting goblet cells remained unchanged across all treatment groups ($p>0.05$; Figure 6).

MPO activity

Colonic MPO activity, indicative of acute inflammation, was not significantly affected by AOM/DSS compared to saline controls (MPO units per gram of tissue [mean±SEM]; saline+water+water: 75±16; AOM+DSS+water: 89±19; $p> 0.05$). Furthermore, Emu Oil-treatment in AOM/DSS mice resulted in approximately 30% lower colonic MPO activity, however, failed to achieve statistical significance (AOM+DSS+Emu Oil [80µL]: 65±8; AOM+DSS+Emu Oil [160µL]: 58±13; $p>0.05$).

Small intestinal permeability

The lactulose/rhamnose (L/R) ratio, indicative of small intestinal permeability, remained unchanged across treatment groups (L/R ratio [mean±SEM]; saline+water+water: 44±7; AOM+DSS+water: 45±2; AOM+DSS+Emu Oil [80µL]: 44±3; AOM+DSS+Emu Oil [160µL]: 40±3; $p>0.05$).

DISCUSSION

The AOM/DSS model is the preferred pre-clinical method to study colitis-associated CRC, as this model induces tumours rapidly and shares histopathological characteristics with human CRC, including mutations in K-ras and ß-catenin (28). Although the combination of induction reagents remains the same, the doses of AOM/DSS and the timeline of *in vivo* studies can vary (28, 29). In the current study, a single dose (7.4mg/kg) of the carcinogen AOM was administered followed by three cycles of 2%w/v DSS, including a final three weeks of water recovery prior to sacrifice on day 84 (12-week trial). This timeline induced severe colitis-associated CRC with significant bodyweight loss, clinical scores of disease and the development of colorectal tumours in all AOM/DSS-treated mice. In the current study, despite a reduction in clinical and histological indicators of disease and decreased 'large' tumour numbers, there was no therapeutic effect on *total* tumour number following Emu Oil administration. Interestingly, in a 9-week AOM/DSS study, Emu Oil decreased tumour burden by reducing the number of 'small' colorectal tumours (24), which corresponds to the reduction of 'large' tumours observed in the current 12-week study.

In the current study, Emu Oil-administration at the largest volume resulted in a reduction of 'large' colonic tumours, further highlighting the potential for Emu Oil to reduce tumour burden in colitis-associated CRC. Recently, black lentil water, containing beneficial polyphenols, was administered to AOM/DSS mice, resulting in a reduction of DAI. The black lentil water further

maintained bodyweight, reduced overall number of tumours and, similar to that observed in the current study, reduced large (>3mm) colonic tumours (30). Furthermore, Damazo-Lima *et al.* (2020) histologically assessed lesions in the distal colon to determine the nature of polyps developed in an AOM/DSS trial, revealing that AOM/DSS control mice developed protuberant-type lesions that were characterised as adenocarcinomas (31). Moreover, in AOM/DSS mice administered a phenolic-extract derived from germinated oats, lesions were primarily pedunculated polyps; which are considered less malignant compared with protruding polyps of greater colonic mucosal surface area (31, 32). In the current study, tumour burden and size were measured via longitudinally-opened colonic photographs obtained at euthanasia. Future studies would benefit from a histological assessment of tumour characteristics to determine whether Emu Oil affects degree of malignancy. Furthermore, this could also be investigated via colonoscopic assessment; a technique incorporated in other AOM/DSS studies (24, 33–35).

It is well documented that diet and lifestyle factors significantly influence IBD and CRC burden globally (36). In 1981, Doll and Peto suggested that 90% of stomach and bowel cancer-related deaths could be attributed to dietary factors (37), and numerous studies have investigated food (grain, plant and animal-derived) compounds and their effects on inflammation and CRC (36). Recently, mixed grain cereals (MGC), mainly derived from brown rice, were tested in the AOM/DSS mouse model (31, 38). In AOM/DSS mice, oral-administration of MGC reduced the increase in colon weight-to-length ratio. Additionally, histologically-assessed neoplasia and inflammatory cell infiltration in the colonic mucosa were decreased (38). These results were supported by ELISA and mRNA analysis of colonic tissue, which revealed that markedly-increased pro-inflammatory cytokines (TNF, IL-1ß, IL-6 and IFN-gamma) in AOM/DSS mice were significantly reduced following MCG treatment (38).

In the current study, inflammation was assessed primarily by MPO activity in colonic tissue, as an indicator of acute inflammation. However, there were no differences observed in MPO levels between normal, AOM/DSS controls or Emu Oil-treated AOM/DSS mice. Furthermore, this

was an unsurprising result as the timeline in the current study induced a chronic inflammatory environment over the 12-week period. Previous studies in the AOM/DSS model have observed similar results in MPO activity (24, 35), compared with acute models of inflammation such as chemotherapy-induced mucositis (21). Consequently, future studies of the AOM/DSS model should include cytokine and mRNA analyses of pro-inflammatory markers (for example, TNF-alpha, IL-1ß, IL-6 and IFN-gamma) to determine if Emu Oil can affect levels in mice with colitis-associated CRC.

Histological assessments of disease severity utilised in the current study identified a protective action of high volume (160µL) Emu Oil in the distal colon compared to AOM/DSS controls. Interestingly, a parameter of this severity score was a reduction in goblet cells. Goblet cells secrete mucins involved in protecting the mucosal barrier of the gastrointestinal tract and regulating innate immune responses (39). However, when analysed independently, AOM/DSS increased the abundance of mucin-secreting goblet cells, although, there was no significant effect in either group of Emu Oil-treated mice. The particular mucins expressed along the intestinal tract vary between the small and large intestine; however, MUC4 is expressed in relatively similar levels in both regions, and increased expression of MUC4 has been linked to poorer survival in CRC patients (40, 41). Das *et al.* (2016) developed a MUC4 knockout mouse to investigate the role of this mucin in DSS-induced colitis and colitis-associated CRC (42). The authors concluded that $MUC4^{-/-}$ mice demonstrated resistance to DSS-colitis and significant reduction in the number and size of adenocarcinomata developed in the AOM/DSS model compared to wild-type mice (42). Furthermore, $MUC4^{-/-}$ mice indicated restoration of histologically-assessed intestinal damage (42). Therefore, as mucin-secreting goblet cells were increased by AOM/DSS in the current study, it would be beneficial to undertake specific investigation into the levels of the particular mucins secreted by these goblet cells to better understand the potential role that Emu Oil could have on protecting the mucosal barrier in this model.

Additionally, small intestinal permeability, measured by lactulose/rhamnose ratio, was also unaffected by Emu Oil in the current study. Interestingly, Chen *et al.* (2016) concluded that dietary fish oil improved intestinal permeability, measured by urinary lactulose/mannitol ratio, in a rat model of ethanol-fed hepatic injury (43). Moreover, in a mouse study of methotrexate-induced mucositis, administration of an omega-3 docosahexaenoic acid ethyl ester (DHA)-supplement significantly reduced fluorescein isothiocyanate (FITC)-dextran uptake, indicating reduced small intestinal permeability (44). It would be of interest in future studies to investigate the effect of Emu Oil on large intestinal permeability, potentially via FITC-dextran uptake or sucralose absorption, to determine if there is a potential protective effect.

In the current study, Emu Oil was therapeutic in colitis-associated CRC by attenuating bodyweight loss, reducing clinical scores of disease and histologically-assessed damage to intestinal architecture. High volume (160µL) Emu Oil exhibited a greater effect than 80µL Emu Oil, evidenced by reduced DAI scores and a decrease in numbers of large colorectal tumours. By modifying the volume, frequency of administration and utilising the truncated 9-week study timeline, Emu Oil may exhibit a more pronounced effect on total tumour burden. Finally, cytokine analysis and proliferation studies are necessary to understand the potential mechanisms that underpin the efficacy of Emu Oil in colitis-associated CRC.

Funding details: N/A

Declaration of interest statement: The authors report no conflict of interest

Author contribution statement: SM. DT, ICL and GSH contributed to the intellectual conceptualisation and methodological development of the current study. All experimental analyses including animal trial, biochemical and histological analyses were conducted by SM. SM and GSH interpreted data and revised the manuscript. LCC assisted in histological analyses, interpretation of data and manuscript preparation. All authors reviewed and approved the final version of this manuscript.

REFERENCES

1 Hendrickson BA, Gokhale R, Cho JH: Clinical aspects and pathophysiology of inflammatory bowel disease. *Clin Microbiol Rev* **15,** 79–94, 2002. doi: 10.1128/cmr.15.1.79-94.2002

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

3 Ungaro R, Mehandru S, Allen PB, Peyrin-Biroulet L, Colombel JF: Ulcerative colitis. *Lancet* **389,** 1756–1770, 2017. doi: 10.1016/S0140-6736(16)32126-2

4 Seril DN, Liao J, Yang GY, Yang CS: Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. *Carcinogenesis* **24,** 353–62, 2003. doi: 10.1093/carcin/24.3.353

5 Choi PM, Zelig MP: Similarity of colorectal cancer in Crohn's disease and ulcerative colitis: implications for carcinogenesis and prevention. *Gut* **35,** 950-4, 1994. doi: 10.1136/gut.35.7.950

6 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, et al.: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **68,** 394–424, 2018. doi: 10.3322/caac.21492

7 Dulai PS, Sandborn WJ, Gupta S: Colorectal Cancer and Dysplasia in Inflammatory Bowel Disease: A Review of Disease Epidemiology, Pathophysiology, and Management. *Cancer Prev Res (Phila)* **9,** 887–894, 2016. doi: 10.1158/1940-6207.CAPR-16-0124

8 Kinugasa T, Akagi Y: Status of colitis-associated cancer in ulcerative colitis. *World J Gastrointest Oncol* **8,** 351–7, 2016. doi: 10.4251/wjgo.v8.i4.351

9 Brenner H, Kloor M, Pox CP: Colorectal cancer. *Lancet* **383,** 1490–1502, 2014. doi: 10.1016/S0140-6736(13)61649-9

10 Herszenyi L, Barabas L, Miheller P, Tulassay Z: Colorectal cancer in patients with inflammatory bowel disease: the true impact of the risk. *Dig Dis* **33,** 52–7, 2015. doi: 10.1159/000368447

11 Lucas C, Barnich N, Nguyen HTT: Microbiota, Inflammation and Colorectal Cancer. *Int J Mol Sci* **18**, 2017. doi: 10.3390/ijms18061310

12 Zheng H, Lu Z, Wang R, Chen N, Zheng P: Establishing the colitis-associated cancer progression mouse models. *Int J Immunopathol Pharmacol* **29,** 759–763, 2016. doi: 10.1177/0394632016670919

13 Whitehouse MW, Turner AG, Davis CK, Roberts MS: Emu oil(s): a source of non-toxic transdermal anti-inflammatory agents in aboriginal medicine. *Inflammopharmacology* **6,** 1–8, 1998. doi: 10.1007/s10787-998-0001-9

14 Snowden JM, Whitehouse MW: Anti-inflammatory activity of emu oils in rats. *Inflammopharmacology* **5,** 127–32, 1997. doi: 10.1007/s10787-997-0021-x

15 Beckerbauer LM, Thiel-Cooper R, Ahn DU, Sell JL, Parrish FC, Jr., et al.: Influence of two dietary fats on the composition of emu oil and meat. *Poult Sci* **80,** 187–94, 2001

16 Abimosleh SM, Tran CD, Howarth GS: Emu Oil: a novel therapeutic for disorders of the gastrointestinal tract? *J Gastroenterol Hepatol* **27,** 857–61, 2012. doi: 10.1111/j.1440-1746.2012.07098.x

17 Mashtoub S, Bennett DC, Tran CD, Howarth GS: Processing and storage of ratite oils affects primary oxidation status and radical scavenging ability. *Animal Production Science* **55,** 1332–1337 2014. doi: http://dx.doi.org/10.1071/AN13556

18 Bennett DC, Code, WE, Godin, DV, Cheng, KM: Comparison of the antioxidant properties of emu oil with other avian oils. *Australian Journal of Experimental Agriculture* **48,** 1345–1350, 2008

19 Abimosleh SM, Lindsay RJ, Butler RN, Cummins AG, Howarth GS: Emu oil increases colonic crypt depth in a rat model of ulcerative colitis. *Dig Dis Sci* **57,** 887–96, 2012. doi: 10.1007/s10620-011-1979-1

20 Safaeian R, Howarth GS, Lawrance IC, Trinder D, Mashtoub S: Emu Oil reduces disease severity in a mouse model of chronic ulcerative colitis. *Scand J Gastroenterol,* 1–8, 2019. doi: 10.1080/00365521.2019.1581253

21 Mashtoub S, Tran CD, Howarth GS: Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. *Exp Biol Med (Maywood)* **238,** 1305–17, 2013. doi: 10.1177/1535370213493718

22 Mashtoub S, Lampton LS, Eden GL, Cheah KY, Lymn KA, et al.: Emu Oil Combined with Lyprinol Reduces Small Intestinal Damage in a Rat Model of Chemotherapy-Induced Mucositis. *Nutr Cancer* **68,** 1171–80, 2016. doi: 10.1080/01635581.2016.1208829

23 Abimosleh SM, Tran CD, Howarth GS: Emu oil reduces small intestinal inflammation in the absence of clinical improvement in a rat model of indomethacin-induced enteropathy. *Evid Based Complement Alternat Med* **2013,** 429706, 2013. doi: 10.1155/2013/429706

24 Chartier LC, Howarth GS, Lawrance IC, Trinder D, Barker SJ, et al.: Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. *Dig Dis Sci* **63,** 135–145, 2018. doi: 10.1007/s10620-017-4876-4

25 Howarth GS XC, Read LC: Predisposition to colonic dysplasia is unaffected by continuous administration of insulin-like growth factor-I for twenty weeks in a rat model of chronic inflammatory bowel disease. *Growth Factors* **18,** 119–133, 2000

26 Yazbeck R, Howarth GS, Geier MS, Demuth HU, Abbott CA: Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice. *Front Biosci* **13,** 6850–8, 2008. doi: 10.2741/3193

27 Howarth GS, Francis GL, Cool JC, Xu X, Byard RW, et al.: Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. *J Nutr* **126,** 2519–30, 1996. doi: 10.1093/jn/126.10.2519

28 De Robertis M, Massi E, Poeta ML, Carotti S, Morini S, et al.: The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. *J Carcinog* **10,** 9, 2011. doi: 10.4103/1477-3163.78279

29 Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, et al.: A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* **94,** 965–73, 2003. doi: 10.1111/j.1349-7006.2003.tb01386.x

30 Mazewski C, Luna D, Berhow M, Gonzalez de Mejia E: Reduction of colitis-associated colon carcinogenesis by a black lentil water extract through inhibition of inflammatory and immunomodulatory cytokines. *Carcinogenesis*, 2020. doi: 10.1093/carcin/bgaa008

31 Damazo-Lima M, Rosas-Perez G, Reynoso-Camacho R, Perez-Ramirez IF, Rocha-Guzman NE, et al.: Chemopreventive Effect of the Germinated Oat and its Phenolic-AVA Extract in Azoxymethane/Dextran Sulfate Sodium (AOM/DSS) Model of Colon Carcinogenesis in Mice. *Foods* **9**, 2020. doi: 10.3390/foods9020169

32 Neergheen VS, Bahorun T, Taylor EW, Jen LS, Aruoma OI: Targeting specific cell signaling transduction pathways by dietary and medicinal phytochemicals in cancer chemoprevention. *Toxicology* **278,** 229–41, 2010. doi: 10.1016/j.tox.2009.10.010

33 Becker C, Fantini MC, Wirtz S, Nikolaev A, Kiesslich R, et al.: In vivo imaging of colitis and colon cancer development in mice using high resolution chromoendoscopy. *Gut* **54,** 950–4, 2005. doi: 10.1136/gut.2004.061283

34 Chartier LC, Hebart ML, Howarth GS, Whittaker AL, Mashtoub S: Affective state determination in a mouse model of colitis-associated colorectal cancer. *PLoS One* **15,** e0228413, 2020. doi: 10.1371/journal.pone.0228413

35 Chartier LC, Howarth GS, Mashtoub S: Chemotherapy-induced mucositis development in a murine model of colitis-associated colorectal cancer. *Scand J Gastroenterol* **55,** 47–54, 2020. doi: 10.1080/00365521.2019.1699601

36 Song M, Garrett WS, Chan AT: Nutrients, foods, and colorectal cancer prevention. *Gastroenterology* **148,** 1244–60 e16, 2015. doi: 10.1053/j.gastro.2014.12.035

37 Doll R, Peto R: The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* **66,** 1191–308, 1981

38 Song JL, Lee JS, Kim HY, Jeong BJ, Jeong JS, et al.: Dietary Mixed Cereal Grains Ameliorate the Azoxymethane and Dextran Sodium Sulfate-Induced Colonic Carcinogenesis in C57BL/6J Mice. *J Med Food*, 2020. doi: 10.1089/jmf.2019.4540

39 Ma J, Rubin BK, Voynow JA: Mucins, Mucus, and Goblet Cells. *Chest* **154,** 169–176, 2018. doi: 10.1016/j.chest.2017.11.008

40 Zhang J, Yasin M, Carraway CA, Carraway KL: MUC4 expression and localization in gastrointestinal tract and skin of human embryos. *Tissue Cell* **38,** 271–5, 2006. doi: 10.1016/j.tice.2006.06.004

41 Shanmugam C, Jhala NC, Katkoori VR, Wan W, Meleth S, et al.: Prognostic value of mucin 4 expression in colorectal adenocarcinomas. *Cancer* **116,** 3577–86, 2010. doi: 10.1002/cncr.25095

42 Das S, Rachagani S, Sheinin Y, Smith LM, Gurumurthy CB, et al.: Mice deficient in Muc4 are resistant to experimental colitis and colitis-associated colorectal cancer. *Oncogene* **35,** 2645–54, 2016. doi: 10.1038/onc.2015.327

43 Chen JR, Chen YL, Peng HC, Lu YA, Chuang HL, et al.: Fish Oil Reduces Hepatic Injury by Maintaining Normal Intestinal Permeability and Microbiota in Chronic Ethanol-Fed Rats. *Gastroenterol Res Pract* **2016,** 4694726, 2016. doi: 10.1155/2016/4694726

44 Horie T, Nakamaru M, Masubuchi Y: Docosahexaenoic acid exhibits a potent protection of small intestine from methotrexate-induced damage in mice. *Life Sci* **62,** 1333–8, 1998. doi: 10.1016/s0024-3205(98)00067-8

Table 1. Fatty acid composition of Emu Oil used in the current study

ANALYTE	COMMON NAME	TOTAL LIPIDS (%)
Total Saturates		**34.4**
14:00	Myristic acid	0.3
16:00	Palmitic acid	24.1
18:00	Stearic acid	9.8
20:00	Arachidonic acid	0.1
Total Monos		**55.7**
16:1n-7 *Omega 7*	Palmitoleic acid	4
18:1n-9 *Omega 9*	Oleic acid	48.8
18:1n-7 *Omega 7*	Vaccenic acid	2.6
20:1n-9 *Omega 9*	Gondoic acid	0.3
Total *Omega 9*		49.1
Total *Omega 7*		6.6
Total *Omega 3*		**0.4**
18:3n-3	alpha-Linolenic acid	0.4
Total *Omega 6*		**9.5**
18:2n-6	Linoleic acid	9.3
20:2n-6	Eicosadienoic acid	0.1
20:4n-6	Arachidonic acid	0.1

Monos-monounsaturated fatty acids

FIGURE CAPTIONS

Figure 1. Bodyweight. Data expressed as a percentage of starting bodyweight ± SEM. ***p<0.001, **p<0.01, *p<0.05 compared to Saline + water + water; ^^p<0.01, ^p<0.05 compared to AOM + DSS + water; ##p<0.01, #p<0.05 compared to low dose Emu Oil.

Figure 2. Disease Activity Index (DAI). Data expressed as mean DAI score ± SEM. ***p<0.001, **p<0.01, *p<0.05 compared to Saline + water + water; ^^^p<0.001, ^^p<0.01 compared to AOM + DSS + water; ##p<0.01 compared to low dose Emu Oil.

Figure 3. (a) Visceral organ weights, (b) Intestinal organ weights, and (c) lengths of the intestinal organs. Weight data are expressed as mean weight (relative to bodyweight) ± SEM and lengths are expressed as mean length (cm) ± SEM. *p<0.05 compared to Saline +water + water.

Figure 4. Colonic tumour analysis (a) total number of colonic tumours, and (b) tumours arranged in size. Data are expressed as mean tumour no. ± SEM. ***p<0.001 compared to Saline + water + water; ^p<0.05 compared to AOM + DSS + water.

Figure 5. Histological severity scoring of the proximal and distal colon. Data are expressed as mean severity score ± SEM. **p<0.01 compared to Saline + water + water; ^p<0.05 compared to AOM + DSS + water.

Figure 6. Mucin-secreting goblet cell count in the proximal and distal colon. Data are expressed as mean no. mucin-secreting goblet cell ± SEM. ***p<0.001 compared to Saline + water + water.

Figure 1.

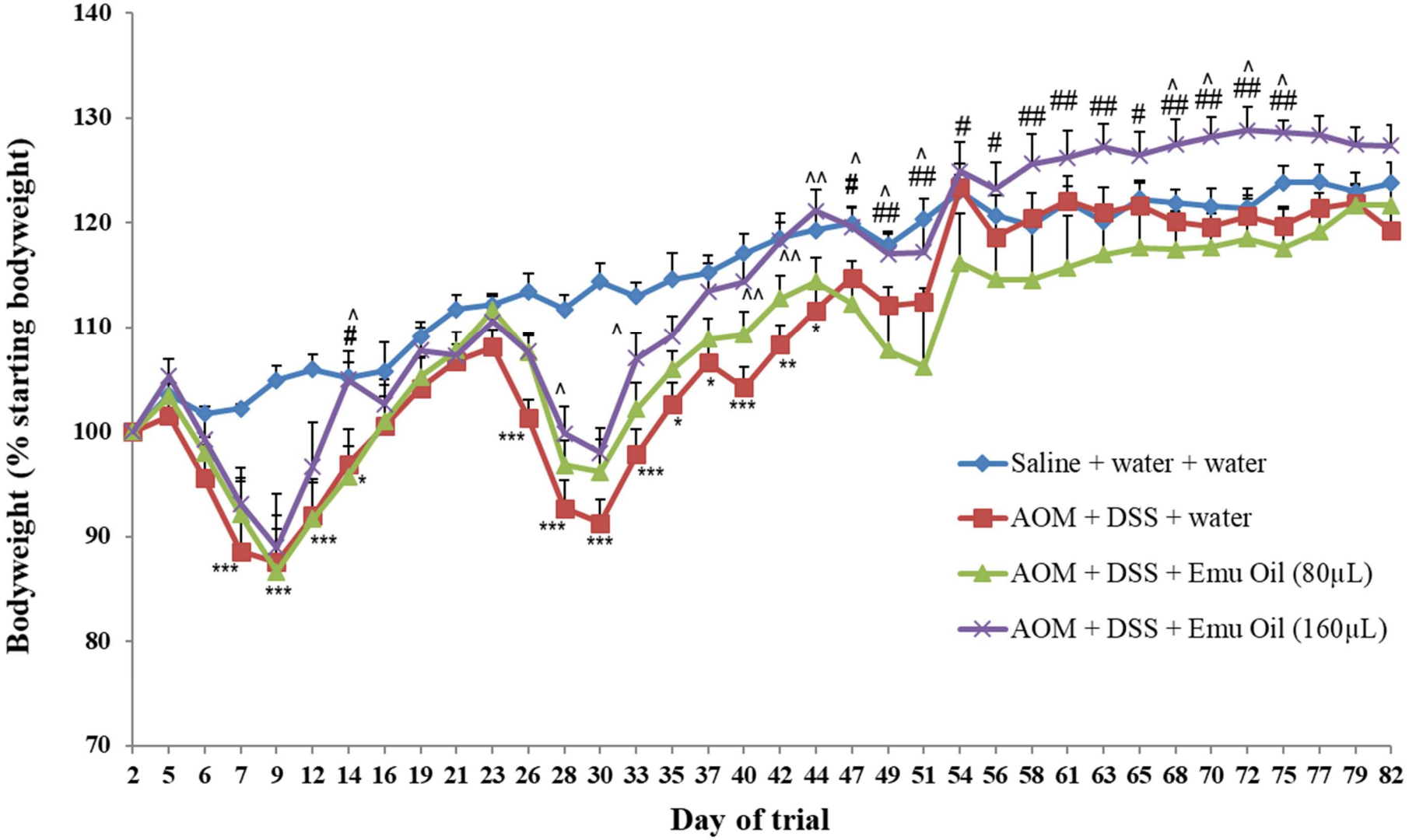

Bodyweight (% starting bodyweight)
140
130
120
110
100
90
80
70
2 5 6 7 9 12 14 16 19 21 23 26 28 30 33 35 37 40 42 44 47 49 51 54 56 58 61 63 65 68 70 72 75 77 79 82
Day of trial
Saline + water + water
AOM + DSS + water
AOM + DSS + Emu Oil (80µL)
AOM + DSS + Emu Oil (160µL)

Figure 2.

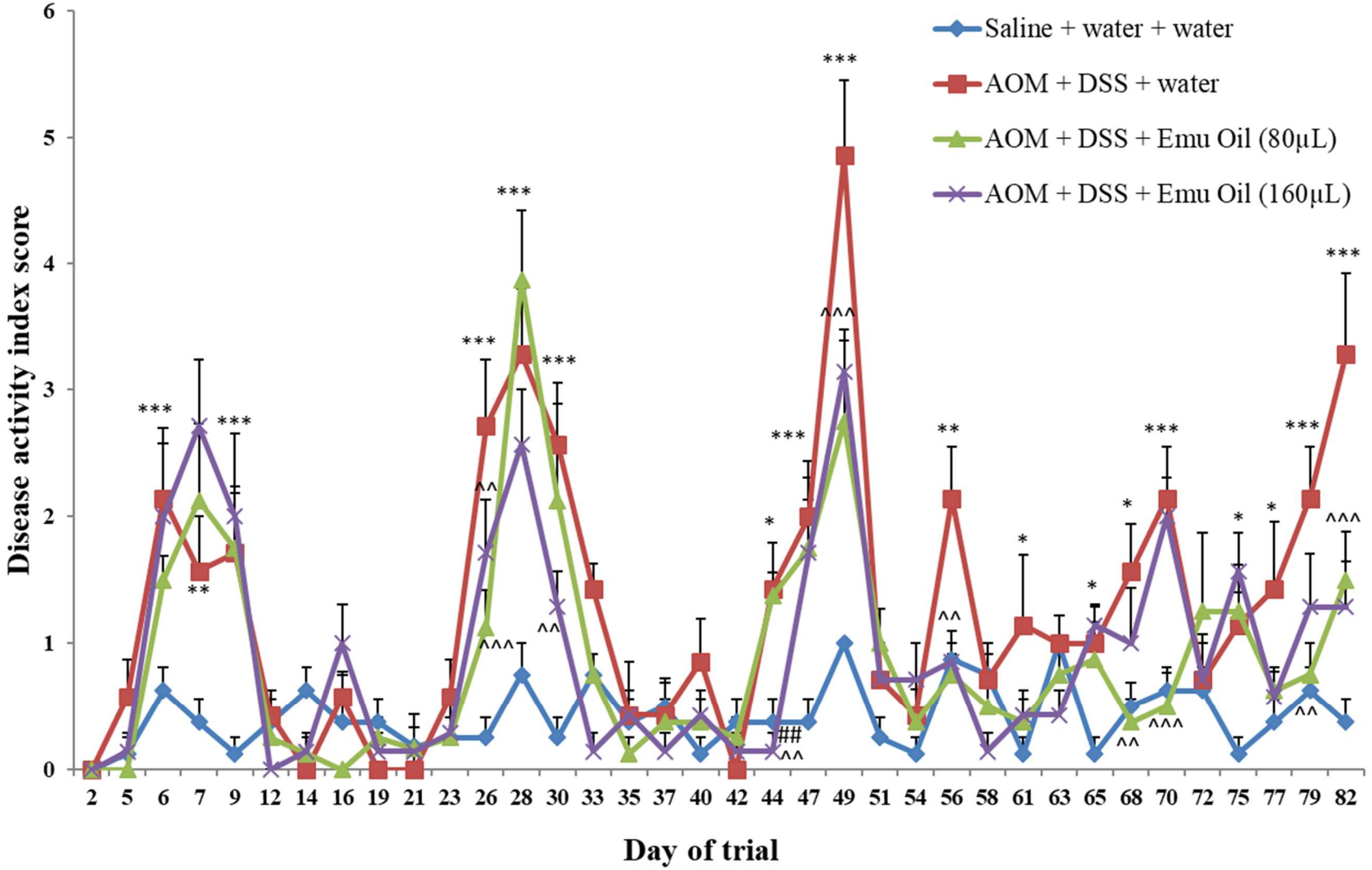

Saline + water + water
AOM + DSS + water
AOM + DSS + Emu Oil (80µL)
AOM + DSS + Emu Oil (160µL)
Disease activity index score
Day of trial
0
1
2
3
4
5
6
2 5 6 7 9 12 14 16 19 21 23 26 28 30 33 35 37 40 42 44 47 49 51 54 56 58 61 63 65 68 70 72 75 77 79 82

Figure 3a.

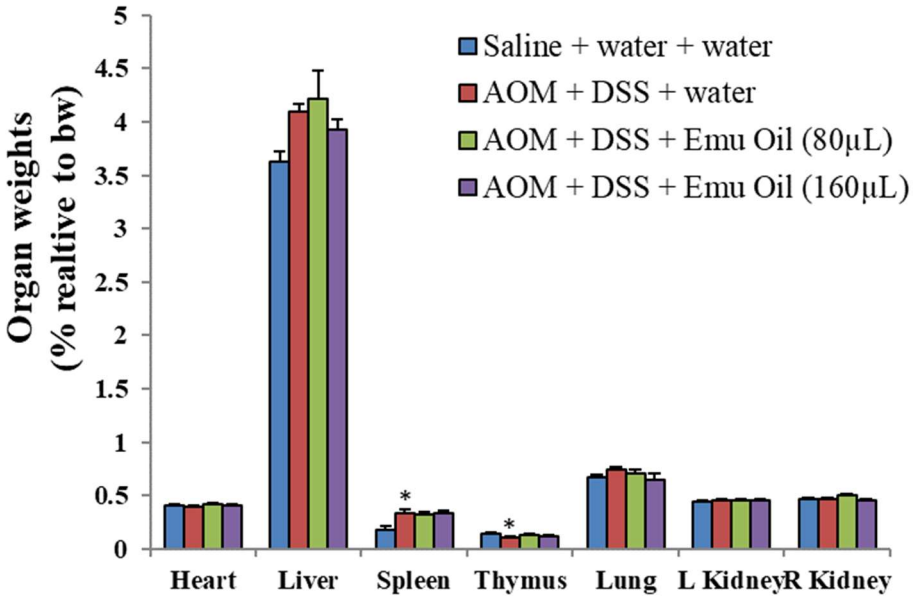


Figure 3b.

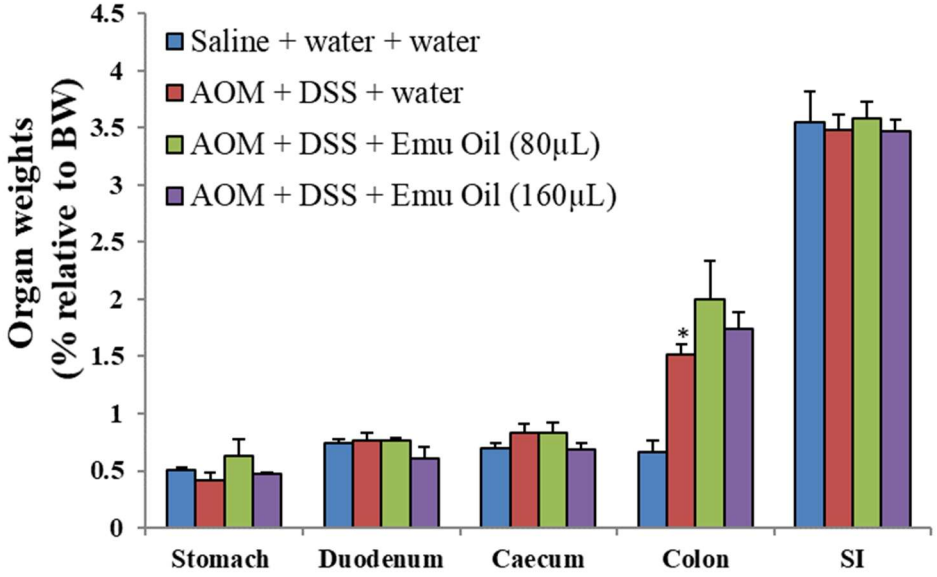


Figure 3c.

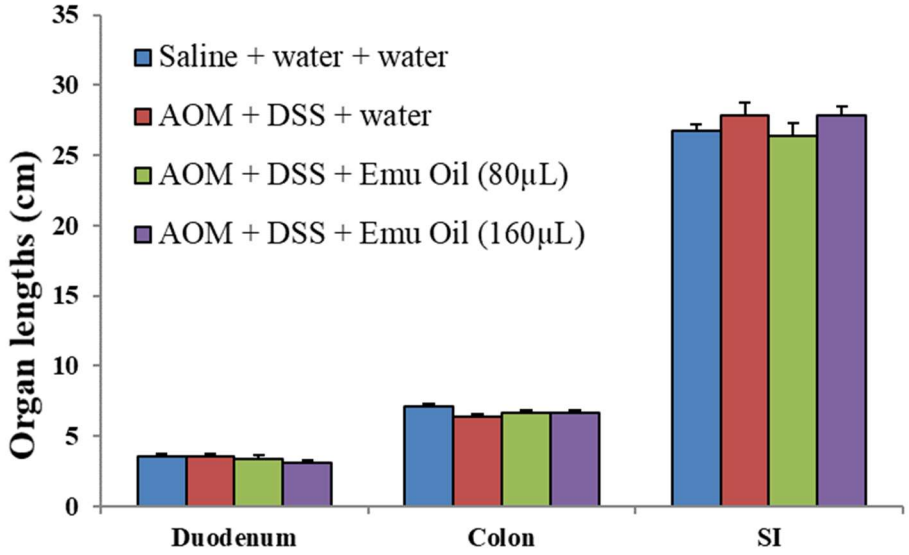


Figure 4a.

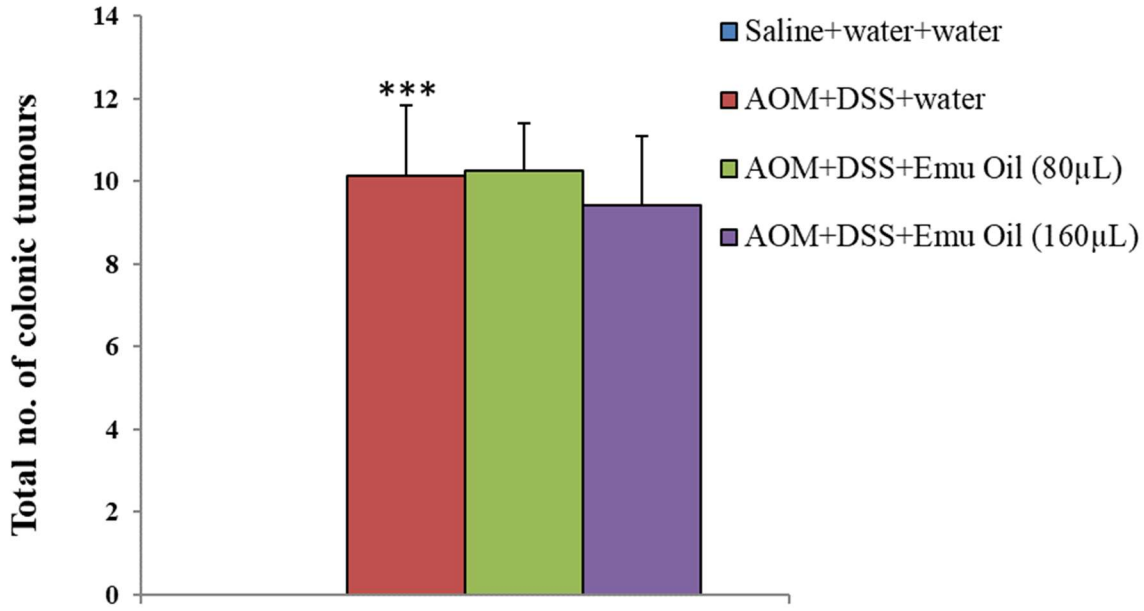


Figure 4b.

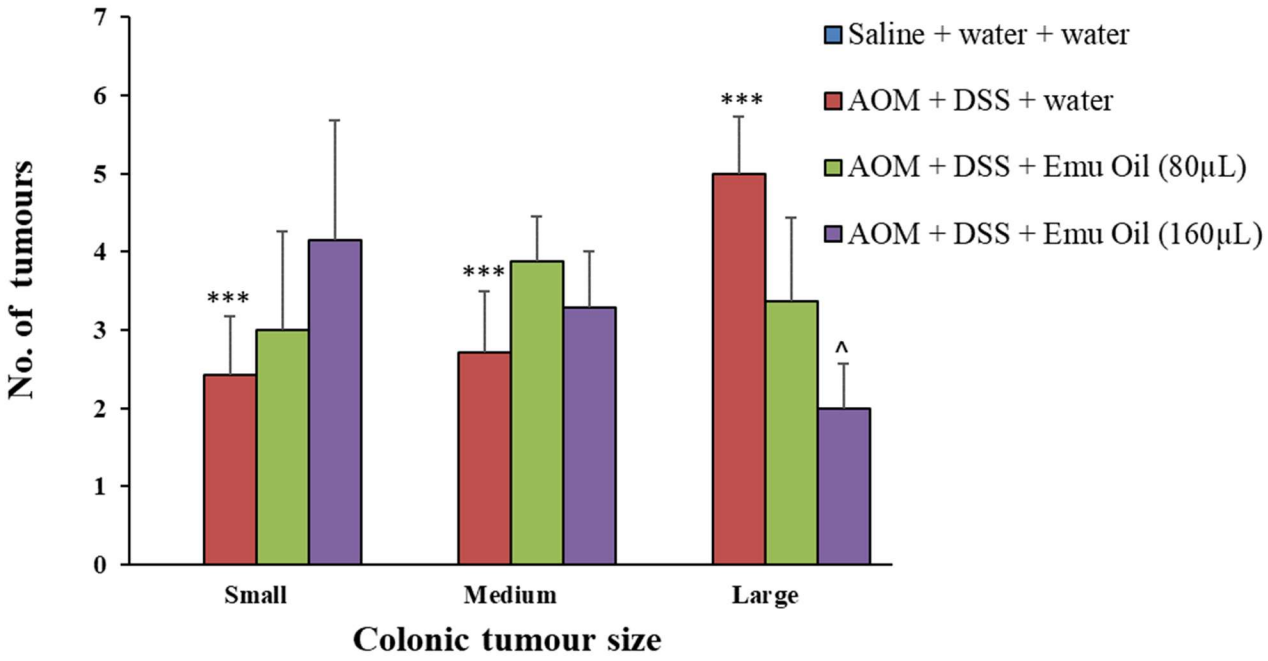


Figure 5.

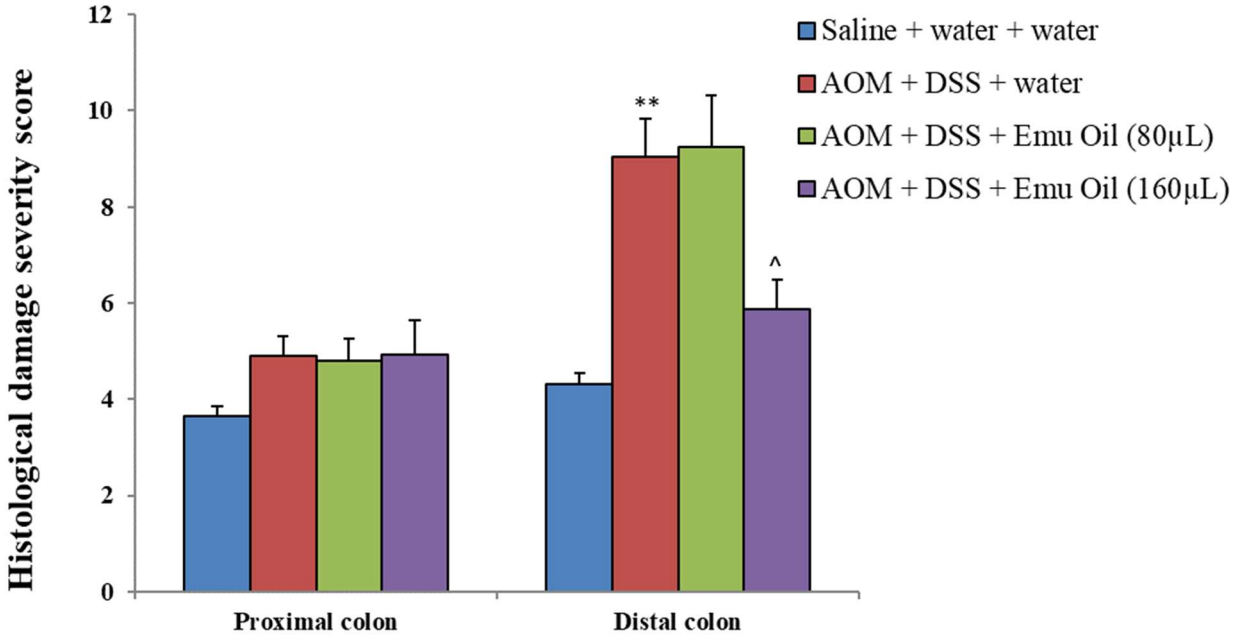
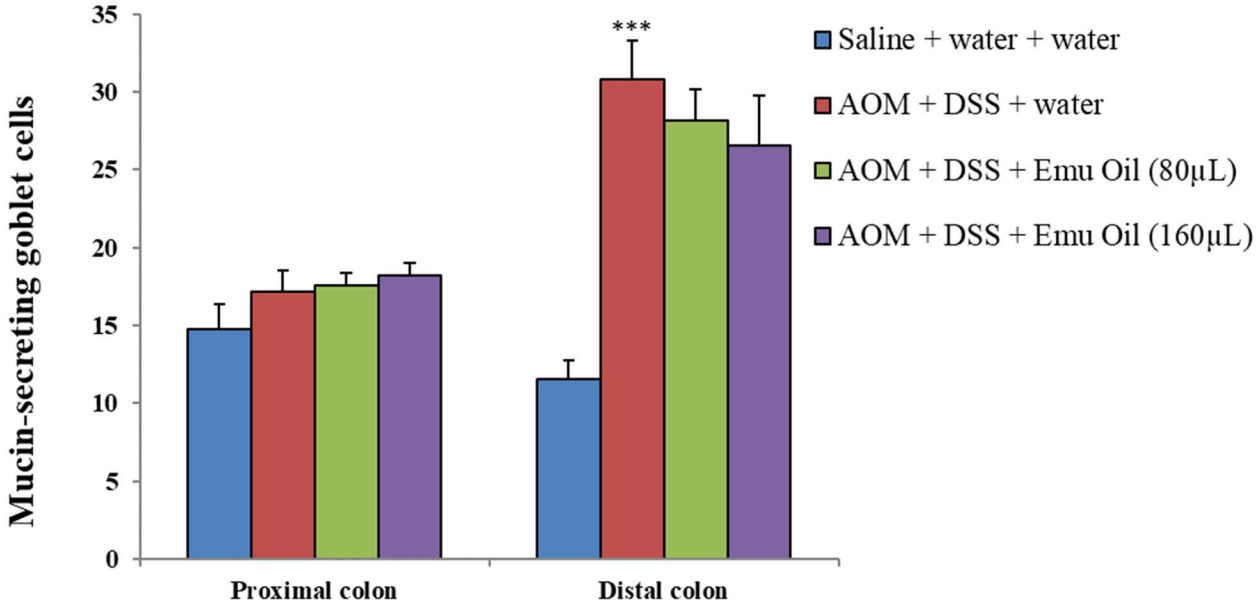


Figure 6.


Mucin-secreting goblet cells
35
30
25
20
15
10
5
0
***
Proximal colon
Distal colon
Saline + water + water
AOM + DSS + water
AOM + DSS + Emu Oil (80µL)
AOM + DSS + Emu Oil (160µL)

APPENDIX 6

KAMPO MEDICINE (ORENGEDOKUTO) IMPROVES STOOL CONSISTENCY IN A MOUSE MODEL OF 5-FLUOROURACIL-INDUCED MUCOSITIS

STATEMENT OF AUTHORSHIP

Title of Paper: Kampo medicine (Orengedokuto) improves stool consistency in a mouse model of 5-Fluorouracil-induced mucositis

Publication Status: Under Review

Publication Details: Ethnopharmacology

Principal Author

Name of Principal Author	Tahlia L Kennewell		
Contribution to the Paper	Completed all experimental trials, assays, data collection and interpretation. Prepared manuscript including writing, formatting and submission.		
Signature		Date	28-09-20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon S Howarth		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, revision and editing of the manuscript.		
Signature		Date	9/10/20

Name of Co-Author (Candidate)	Lauren Claire Chartier		
Contribution to the Paper	Supervision and assistance with animal trials, data interpretation and editing of the manuscript.		
Overall percentage (%)	10%		
Certification:	This paper reports on original research directly related to my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	2/10/20

Name of Co-Author	Junko Fujino		
Contribution to the Paper	Intellectual and methodology development.		
Signature		Date	23/9/2020

Name of Co-Author	Chloe J Mitchell		
Contribution to the Paper	Assistance with animal trials.		
Signature		Date	2/10/20

Name of Co-Author	Alexandra L Whittaker		
Contribution to the Paper	Assistance with selected methodology development.		
Signature		Date	28/09/20

Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Conceptualisation, intellectual and methodology development, supervision of analyses, data interpretation, revision and editing of the manuscript.		
Signature		Date	8/10/20

KAMPO MEDICINE (ORENGEDOKUTO) IMPROVES STOOL CONSISTENCY IN A MOUSE MODEL OF 5-FLUOROURACIL-INDUCED MUCOSITIS

Tahlia L Kennewell.[1, 2] Gordon S Howarth,[1, 2] Lauren C Chartier,[2, 3] Junko Fujino,[4] Chloe J Mitchell,[1, 2] Alexandra L Whittaker,[1] Suzanne Mashtoub*[2, 3, 5]

[1]School of Animal and Veterinary Sciences, University of Adelaide, South Australia, Australia; [2]Gastroenterology Department, Women's and Children's Hospital, South Australia, Australia; [3]Adelaide Medical School, University of Adelaide, South Australia, Australia; [4]Department of Paediatric Surgery, Saitama Medical Centre, Dokkyo Medical University, Saitama, Japan; [5]School of Medicine, The University of Western Australia, Murdoch, Western Australia, Australia

* Corresponding author

Keywords: Mucositis, 5-Fluorouracil, Orengedokuto, Balb/c, mouse model

Abbreviations: 5-Fluorouracil, 5-FU; reactive oxygen species, ROS; tumour necrosis factor-alpha, TNF-α; interferon-gamma, IFN-γ; interleukin-1beta, IL-1β; interleukin-6, IL-6; nuclear factor-κβ, NF-κβ; intraperitoneal i.p.; disease activity index, DAI; myeloperoxidase, MPO.

Corresponding author

Suzanne Mashtoub: Email: suzanne.mashtoub@adelaide.edu.au; Phone: 8161 6991; Postal address: 72 King William Road, North Adelaide SA 5006

Author e-mails

Tahlia L. Kennewell: tahlia.kennewell@mymail.unisa.edu.au

Gordon S Howarth: gordon.howarth@adelaide.edu.au

Lauren C Chartier: lauren.chartier@adelaide.edu.au

Junko Fujino: fujunko@dokkyomed.ac.jp

Chloe J Mitchell: chloe.mitchell@adelaide.edu.au

Alexandra L Whittaker: alexandra.whittaker@adelaide.edu.au

Author contributions

Tahlia L. Kennewell—Investigation, formal analysis, writing draft manuscript, reviewing and editing manuscript

Gordon S. Howarth—Methodology, supervision, reviewing and editing manuscript

Lauren C Chartier—Methodology

Junko Fujino—Conceptualisation

Chloe J Mitchell—Investigation

Alexandra L Whittaker—Methodology

Suzanne Mashtoub—Conceptualisation, funding acquisition, methodology, supervision, formal analysis and reviewing and editing manuscript

Abstract

Ethnopharmacological relevance: Orengedokuto is a traditional Japanese herbal medicine composed of Phellodendri cortex, Coptidis rhizoma, Scutellariae radix and Gardenia fructus. It is utilised in Japan to treat conditions such as dermatitis, gastric ulcers, and gastritis. Previously, Orengedokuto has been given in combination with chemotherapy treatment where it reduced the incidence of chemotherapy-induced diarrhoea, highlighting a potential preventative effect on mucositis. Mucositis is a common, debilitating side-effect of chemotherapy treatment for cancer. Patients often terminate chemotherapy to facilitate repair of the damaged mucosa, as currently no truly effective mucositis treatment is available. This study investigated the effect of Orengedokuto in a mouse model of mucositis.

Methods: Female Balb/c mice (8 weeks) were injected (intraperitoneal; day 0) with 5-Fluorouracil (5-FU). Mice (n=10/group) were gavaged daily with water (160µl) or Orengedokuto (0.5mg/kg; 1g/kg) for four days. Bodyweight and disease activity index (DAI) were measured daily. Mice were euthanized on day 4 and intestinal samples collected for histological and biochemical analyses. $p<0.05$ was considered statistically significant.

Results: 5-FU significantly increased DAI (days 3-4) and stool-consistency (days 3-4) compared to normal control ($p<0.05$). Additionally, thymus weight and jejunal-ileal length decreased, while colon weight and crypt depth increased, compared to normal control ($p<0.05$). Importantly, Orengedokuto, at the 1g/kg dose improved stool consistency compared to 5-FU treated control (days 3-4; $p<0.05$) . Orengedokuto did not impact other indicators of mucositis.

Conclusion: Orengedokuto at a dose of 1g/kg improved stool consistency in mice with 5-FU-induced mucositis. Future studies should explore higher doses and different treatment periods to further investigate the potential for Orengedokuto to alleviate mucositis symptomatology.

Introduction

Mucositis is one of the most common side-effects of chemotherapy and radiotherapy, occurring in approximately 40% of cancer patients (Gibson et al., 2002; Mashtoub et al., 2013; Tooley et al., 2009). The condition manifests by the indiscriminate destruction of rapidly dividing cells within the gastrointestinal tract resulting in a loss of mucosal integrity (McCreath and Delgoda, 2017; Sonis, 2004). The oral and small intestinal mucosa are most commonly impacted (Sonis, 2004) although all regions of the gastrointestinal tract can be affected by mucositis. The small intestine is particularly susceptible to mucositis due to its rapid cell replacement cycle of 3-4 days (Song et al., 2013).

The incidence and severity of mucositis can vary, depending on the chemotherapeutic agent used (Lalla and Peterson, 2006). For example, 5-Fluorouracil (5-FU), used widely in the treatment of colorectal cancer (Longley et al., 2003; Song et al., 2013), results in the development of intestinal mucositis in approximately 80% of patients (Cool et al., 2005; Song et al., 2013). Intestinal mucositis is extremely painful with symptoms including severe abdominal pain, nausea, bloating and diarrhoea (Gibson et al., 2002; Lalla and Peterson, 2006; Tooley et al., 2009), which can impair food intake, communication, sleep and mental status and can result in dehydration and malnutrition (Tooley et al., 2009) as well as increasing hospital stay, use of resources and cost of treatment (Sonis, 2004).

The primary factors associated with mucositis development are the generation of reactive oxygen species (ROS), and the up-regulation of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1beta (IL-1β) and interleukin-6 (IL-6) (Mashtoub et al., 2013; Sonis, 2004). ROS production occurs rapidly following the administration of radiotherapy or chemotherapy, causing DNA strands to break, resulting in cell damage (Sonis, 2004). Breakage of DNA strands leads to the activation of transduction pathways that activate transcription factors such as nuclear factor-κβ (NF-κβ). NF-κβ activation leads to the production of pro-inflammatory cytokines, such as TNF-α and IL-1β,

resulting in both tissue damage and a positive feedback loop that amplifies cell damage by activating NF-κβ in other cells (Sonis, 2004). Intestinal damage is characterised by apoptosis of regenerative stem cells, blunted villi and crypts and perturbed brush-border hydrolase activity (Cool et al., 2005). Damage to the mucosa can range from lesions on the mucosal surface to ulcerations that penetrate the submucosa with a loss of integrity, thereby allowing pathogenic bacteria to enter the bloodstream (Sonis, 2004).

At present, there are no effective therapies to prevent mucositis (Kato et al., 2015; Mashtoub et al., 2015; Tooley et al., 2009), or to facilitate healing of the mucosa. Often, chemotherapy is ceased or reduced to allow healing to occur, as mucositis is generally acute and self-resolving, however, this can impact on patient prognosis (Mashtoub et al., 2015). Most available therapies are only prescribed for oral mucositis (Wright et al., 2009), and those that are used for intestinal mucositis typically focus on the control of pain and symptoms (Keefe et al., 2008). For example, following treatment with standard dose cyclophosphamide, methotrexate and 5-FU chemotherapy it is recommended that Ranitidine or Omeprazole be used to prevent epigastric pain (Peterson et al., 2011). Ranitidine is used to treat and prevent ulcers in the stomach and intestine, and Omeprazole is used to treat gastric and duodenal ulcers.

Naturally sourced therapies such as Emu Oil (Mashtoub et al., 2013), grape seed extract (Cheah et al., 2009) and the herbal extract Iberogast (Wright et al., 2009) have shown potential in mucositis treatment through acceleration of intestinal repair and reduced intestinal damage. These effects have been attributed to their anti-inflammatory and anti-oxidative properties (Cheah et al., 2009; Mashtoub et al., 2013; Wright et al., 2009). The traditional Japanese herbal medicine (Kampo), Orengedokuto, comprised of *Phellodendri cortex, Coptidis rhizoma*, *Scutellariae radix* and *Gardeniae fructus*, has been credited with these features in addition to anti-ulcerative, anti-microbial and anti-diarrhoeal properties (Chen et al., 2016; Fujii et al., 2017; Zhou and Mineshita, 1999). Orengedokuto is utilised in Japan to treat conditions such as dermatitis, gastric ulcers, and gastritis (Fujii et al., 2017) and has been used alongside leukaemia treatment where it has been reported to reduce the incidence of chemotherapy-induced

diarrhoea and stomatitis, suggesting a preventative effect on both intestinal and oral mucositis (Yuki et al., 2003).

The aim of the current study was to determine the effect of Orengedokuto on 5-FU-induced intestinal mucositis in mice. It was hypothesised that Orengedokuto would ameliorate mucositis by reducing intestinal inflammation, restoring intestinal architecture and reducing clinically assessed disease severity.

Materials and methods

Animal ethics

All animal studies were conducted in compliance with the 'Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013)' (National Health and Medical Research Council: Canberra, 2013) and were approved by both the Children, Youth and Women's Health Service and University of Adelaide animal ethics committees.

Animals

Female Balb/c mice (n=60) at 7 weeks of age were obtained from the Animal Resources Centre (ARC), Western Australia, and group-housed in cages (n=5/cage) with a light:dark cycle of 14:10 hour. Mice were acclimatised for one week before being randomly allocated to 6 groups (n=10/group). Group 1: (injection + gavage) saline + water (normal control), Group 2: saline + 80µl Orengedokuto, Group 3: saline + 160µl Orengedokuto, Group 4: 5-FU + water (5-FU control), Group 5: 5-FU + 80µl Orengedokuto, Group 6: 5-FU + 160µl Orengedokuto. Mice were fed standard mouse chow and provided drinking water throughout the trial.

On day 0, all mice received an intraperitoneal (i.p.) injection of either saline or 200mg/kg 5-FU (Mayne Pharma Pty Ltd, Victoria, Australia). Day 0 to day 3, all mice were gavaged with either 80µl (0.5g/kg; low dose) Orengedokuto, 160µl (1g/kg; high dose) Orengedokuto or 160µl water. Orengedokuto (Tsumura & Co, Tokyo, Japan) is a powder which comprises four herbs

Phellodendri cortex (0.75g), *Coptidis rhizome* (1.0g), *Scutellariae radix* (1.5g) and *Gardeniae fructus* (1.0g) dissolved in water. On day 3, mice were fasted overnight with *ad libitum* access to water. On day 4, all mice were euthanized by CO_2 asphyxiation followed by cervical dislocation. Mice from groups 1, 4 and 6 were gavaged with Fluorescein Isothiocyanate-Dextran (FITC-D; 500mg/kg body weight; mol wt. 4000; 75mg/ml; Sigma; Castle Hill, New South Wales) 3 hours prior to euthanasia.

Disease activity index

Disease activity index (DAI) was recorded daily for each mouse during the trial, comprising four parameters (stool consistency, rectal bleeding, weight loss and general condition) scored from 0 – 3 (normal – maximal severity). The scores were totalled for overall DAI with maximum score of 12. Stool consistency was scored as normal stool (0), soft stool (1), soft and wet stool (Broughton et al.) and watery stool (3). Weight loss was scored based on the percentage weight lost from day 0; <5% (0), <10% (1), <15% (2) and >15% (3) weight loss, respectively. General condition was scored based on fur quality: healthy mice presented with well-groomed fur flat on their bodies, while unhealthy mice were more ruffled in appearance; activity level: healthy mice would move around the cage while unhealthy would be less willing to move; and eyes: healthy mice would have wide open eyes while unhealthy mice would have squinted eyes.

Behavioural analyses

On days -1 and 3, mice underwent behavioural analyses (facial grimace and burrowing activity) to indicate changes in pain and affective state during the experimental period. Prior to behavioural analyses, mice were acclimatised to the burrowing setup by burrowing in pairs on day -2. Burrowing activity is used as an indicator of well-being as it is often reduced in challenging situations due to it being a non-essential behaviour typically expressed in mice (Jirkof, 2014; Jirkof et al., 2013). During the dark period of the circadian cycle, mice were individually placed in cages with a burrow containing 400g pebbles (Kitty litter, Black and

Gold, Adelaide, Australia) then allowed to burrow for one hour. The burrow was then re-weighed, and the gravel removed through burrowing was calculated.

Facial grimace, using the validated mouse grimace scale (Langford et al., 2010) is used as an indicator of pain in mice. This was scored both in real time and retrospectively. Five facial features: orbital tightening, nose bulge, cheek bulge, ear position and whisker change; were each scored 0 – 2 (not present – severe). For real time facial grimace assessment, mice were individually placed in clear cages for five minutes and facial features scored every 30 seconds by a treatment-blinded observer. The average scores of each facial feature across all timepoints were then added together to a total real time score (maximum score of 10), this was repeated for both days -1 and 3. The facial feature scores were summed to produce a total score for each time point, then the overall mean score were calculated and used for analysis.

Simultaneously, video footage of the mice was obtained by two cameras placed in front and to the side of the cage. Six still images were obtained from the video footage when mice were facing the camera front-on, or clear side-on images could be acquired. Images were cropped to only show the face and scored by two treatment-blinded observers. As with real time the facial feature scores were summed to produce a total score, this time for each image. The mean score for the six images were calculated. The mean observer score for each mouse was used for analysis.

Tissue collection

Mice were euthanized by CO_2 asphyxiation on day 4 post 5-FU injection, followed by cervical dislocation. Cardiac puncture was performed on mice from groups 1, 4 and 6, to collect blood for FITC-D analysis. The gastrointestinal tract was removed measured, emptied and weighed. Jejunal and ileal segments of 2 cm were placed in 10% buffered formalin for histological analyses and jejunal and ileal segments of 2 cm were collected and frozen in liquid nitrogen for biochemical analyses. Visceral organ lengths and weights were recorded, and organs discarded.

Histological analyses

Jejunal and ileal specimens were transferred from 10% buffered formalin to 70% ethanol 24hr post-collection. Samples were then processed, embedded and stained with haematoxylin and eosin (H&E). Measurements of 30 villi and crypts were obtained from each section and the mean villus height and crypt depth (μm) calculated. Jejunal sections were then histologically-assessed and allocated a total damage severity score based on eleven parameters (villus fusion and stunting/villus:crypt ratio, enterocyte distribution, reduction in goblet cells, reduction in mitotic figures, crypt abscesses, crypt disruption, crypt cell disruption, neutrophil infiltration, capillary lymphatic dilation, submucosal thickening, muscularis externa thickening) each scored from 0-3 (maximal severity) for a maximum damage severity score of 33 (Howarth et al., 1996). Jejunal sections were assessed using a light microscope (Olympus corporation; Tokyo, Japan) and AnalySIS version 5.2 (Olympus; Tokyo, Japan).

Biochemical analyses

Myeloperoxidase (MPO) is an indicator of acute inflammation. Jejunal and ileal sections were homogenised with 10mM phosphate buffer, separated into 200μl aliquots and stored at -80°C. Thawed homogenised aliquots were centrifuged at 13000g for 12 minutes at 4°C and supernatant discarded. The pellet was resuspended in 200μl hexadecyltrimethylammonium bromide (HTAB). Samples were then vortexed for two minutes to release MPO from the tissue then centrifuged at 15000g for two minutes at 4°C. The supernatant was added to two wells (50μl/well) per sample followed by 200μl of reaction solution (4.2mg O-dianisidine dichloride, 12.5ml 30% (w/v) H_2O_2, 2.5ml potassium phosphate buffer pH 6.0 and 22.5ml distilled water). Absorbance was measured at 450nm using a BioTek synergy MX microplate reader (BioTek; Winooski, Vermont, USA) and Gen5 version 2.00.18 software every minute for 15 minutes.

The FITC-D test is a non-invasive method for monitoring changes in permeability of the paracellular pathway in the small and large intestine. Mice were gavaged with FITC-D (500mg/kg body weight; mol wt. 4000; 75mg/ml; Sigma; Castle Hill, New South Wales) 3

hours prior to euthanasia. Mice were then euthanized by CO_2 asphyxiation and cervical dislocation and blood was collected via cardiac puncture. Blood was centrifuged at 11000g at room temperature for 12 minutes to separate the serum. Serum was diluted with 0.2M PBS (1:3 dilution) and FITC-D was quantified using a BioTek synergy MX microplate reader and Gen5 version 2.00.18 software relative to a PBS standard curve (0.001 - 100µl).

Statistical analyses

All data were analysed using SPSS version 17 for windows (SPSS Inc.; Chicago, Illinois, USA) and tested for normality with a Shapiro-Wilk test. Body weight, DAI, facial grimace and burrowing were analysed using a linear mixed model with a least significant difference (LSD) *post hoc* test and presented as mean ± standard error of the mean (SEM). Organ lengths and weights, villus height and crypt depth, MPO and FITC-D were analysed using a one-way ANOVA with Tukey's *post hoc* test and presented as mean ± SEM. Intestinal severity scores were analysed using a Kruskal Wallis with Mann Whitney U test and presented as median and interquartile range. $p<0.05$ was considered significant.

Results

Daily data

Body weight was calculated as a percentage of initial weight (day 0 body weight). Body weight of all mice decreased following i.p. injection on day 0. In normal mice treated with low dose Orengedokuto, weight loss was reduced compared to normal mice treated with high dose Orengedokuto (day 1 and 4; $p<0.05$) and normal controls (day 3; $p<0.01$). Following administration of 5-FU weight loss was increased compared to normal control (day 3 and 4; $p<0.001$). There was no impact of Orengedokuto on weight loss in mice administered 5-FU ($p>0.05$; figure 1).

Overall DAI scores were reduced in normal mice treated with low dose Orengedokuto, primarily attributed to reduced weight loss, compared to normal controls (day 2; $p<0.05$) and

high dose Orengedokuto (day 4; $p<0.05$). Administration of 5-FU increased DAI compared to normal controls (days 3-4; $p<0.001$), with an increase in scores for general condition, weight loss and stool consistency. In mice administered 5-FU, there was no effect of Orengedokuto at both high and low dose on DAI ($p>0.05$; figure 2).

Stool consistency, a parameter of the overall DAI, was scored from 0 – 3 (normal – watery stool). In normal mice, there was no effect of Orengedokuto on stool consistency compared to normal controls. Surprisingly, stool consistency scores in mice administered 5-FU were significantly lower than normal controls (day 2; $p<0.01$), however, on the following days, 5-FU administration resulted in increased stool consistency scores compared to normal controls (day 3 and 4; $p<0.01$). Importantly, in mice administered 5-FU, high dose Orengedokuto reduced stool consistency scores compared to 5-FU controls (days 3-4; $p<0.01$) and low dose Orengedokuto (day 3; $p<0.01$; figure 3).

Behavioural analyses

In all behavioural analyses (burrowing and grimace scoring) there was no difference between groups for baseline measurements (day -1; $p>0.05$). Burrowing activity of normal mice was unaffected by Orengedokuto compared to normal controls (day 3: $p>0.05$). Administration of 5-FU significantly decreased burrowing activity compared to normal controls (day 3; $p<0.05$). Additionally, there was no effect of Orengedokuto on the burrowing activity of mice administered 5-FU compared to 5-FU controls (day 3; $p>0.05$; figure 4). Real time grimace scores of normal mice treated with Orengedokuto were not significantly different to normal controls (day 3; $p>0.05$). These scores were increased in mice administered 5-FU compared to normal controls (day 3: $p<0.01$) whilst there was no effect of Orengedokuto on the grimace scores of mice administered 5-FU compared to 5-FU controls ($p>0.05$). Interestingly real time and retrospective grimace scores did not show the same result, with no difference in the retrospective grimace scores of all treatment groups (day 3; $p>0.05$; table 1).

Visceral organ weights

Visceral organ weights were calculated as a percentage of body weight on the day of euthanasia (day 4). Thymus weight was reduced in mice administered 5-FU compared to normal controls ($p<0.01$); however, there was no difference following Orengedokuto administration compared to 5-FU controls ($p>0.05$). There was no difference in the remaining visceral organ weights between treatment groups ($p>0.05$; table 2).

Gastrointestinal organ weights and lengths

Gastrointestinal weights were calculated as a percentage of body weight on the final experimental day (day 4). There was no effect of Orengedokuto on the gastrointestinal organ weights of normal mice compared to normal controls ($p>0.05$). Colon weight was increased in mice administered 5-FU compared to normal controls ($p<0.001$) and remaining gastrointestinal organ weights were unaffected. Additionally, there was no effect of Orengedokuto on the gastrointestinal organ weights of mice administered 5-FU compared to 5-FU controls ($p>0.05$; table 3). Length of the duodenum and colon did not differ between treatment groups ($p>0.05$). jejunoileal (JI) length was decreased in mice administered 5-FU compared to normal controls ($p<0.05$). In both normal mice and mice administered 5-FU, there was no effect of Orengedokuto on JI length compared to normal controls and 5-FU controls ($p>0.05$; table 4).

Histological analyses

In normal mice, Orengedokuto did not impact either jejunal or ileal crypt depth compared to normal controls ($p>0.05$). Jejunal crypt depth was significantly increased by 5-FU compared to normal control ($p<0.001$); additionally, ileal crypt depth was not affected by 5-FU ($p>0.05$). Additionally, there was no impact of Orengedokuto on jejunal and ileal crypt depth of mice administered 5-FU compared to 5-FU controls ($p>0.05$; figure 5). Villus height did not differ among treatment groups ($p>0.05$; figure 6).

Small intestinal severity scores were not significantly impacted in normal mice by Orengedokuto, compared to normal control ($p<0.05$). Severity scores were significantly

increased by 5-FU compared to normal controls ($p<0.01$), and scores were unaffected by Orengedokuto ($p>0.05$; figure 7).

Biochemical analyses

There was no significant difference in jejunal and ileal MPO activity between any of the treatment groups ($p>0.05$; figure 8). Additionally, intestinal permeability, calculated as concentration of FITC-D in serum, was not different between treatment groups ($p>0.05$; figure 9).

Discussion

The present study investigated the effect of the Kampo medicine, Orengedokuto, on 5-FU-induced intestinal mucositis in mice. Orengedokuto improved stool consistency in 5-FU-injected mice; however, no effect was observed on MPO activity, intestinal architecture or clinically assessed disease severity. In Japan Orengedokuto has been used to treat various conditions (Fujii et al., 2017) and is considered safe to use. The current study supported this postulate, demonstrating no negative impact of Orengedokuto in normal mice.

In response to pain and disease, physiological and behavioural responses can alter metabolic and behavioural states (Ulrich-Lai et al., 2015), which could present in rodent studies as a loss of weight, decrease in burrowing activity (Jirkof, 2014; Jirkof et al., 2013) or increase in grimace score (Langford et al., 2010). Consequently, a decrease in bodyweight was detected in all mice following saline and 5-FU injections resulting in all mice having a measurable DAI. Bodyweight is commonly reduced following 5-FU administration compared to normal controls (Chartier et al., 2019; Mashtoub et al., 2013; Whittaker et al., 2017; Wu et al., 2011). This reduction was evident in the current study as 5-FU mice continued to lose weight beyond day 1 such that 5-FU controls weighed significantly less than normal controls on days 3 and 4. Orengedokuto has previously been shown to improve weight recovery in mice with trinitrobenzene-sulphonic acid (TNBS)-induced colitis (Zhou and Mineshita, 1999); however,

increased recovery of bodyweight was not observed in 5-FU control mice in the current study. Weight loss combined with increased scores for stool consistency led to an increased DAI score in 5-FU mice. Previously, increased DAI score in mucositis has been attributed to an increase in all scoring parameters (Mashtoub et al., 2013). In the present study, DAI was impacted by three of the four parameters; general condition, stool consistency and weight loss scores, with weight loss and stool consistency having the most impact on the overall DAI score.

One of the most common side-effects of 5-FU administration is diarrhoea, resulting from damage to the small intestine (Song et al., 2013; Tooley et al., 2009; Zhang et al., 2018). This was reflected in the current study, with 5-FU inducing a higher incidence of diarrhoea compared to normal controls. Orengedokuto has been reported to have an anti-diarrhoeal effect on chemotherapy-induced diarrhoea in leukemia patients being treated with chemotherapeutic agents (Yuki et al., 2003). In the present study this anti-diarrhoeal effect was observed in mice treated with high dose Orengedokuto. Reduced diarrhoea was not observed at the low Orengedokuto dose suggesting that the effect was dose dependent. A previous study by Miura et al. (2007), demonstrated Orengedokuto to have a dose-dependent effect in mice with indomethacin-induced enteropathy. In the study by Miura et al. (2007), mice survival increased when administered a dose of Orengedokuto equivalent to 2.8g/kg compared to doses equivalent to 0.7g/kg and 1.5g/kg. By increasing the dose of Orengedokuto used in the current study there exists potential for Orengedokuto to alleviate mucositis symptoms.

Damage to the intestinal mucosa by 5-FU, characterised by decreased villus height and crypt depth has been observed in rodents following 5-FU administration (Mashtoub et al., 2015; Whittaker et al., 2015). Consistent with previous studies, jejunal damage severity in 5-FU controls was increased compared to normal controls; however, villus height was unchanged and crypt depth was significantly increased. Decreased villus height and increased crypt depth have been observed in mouse models of mucositis in the duodenum, jejunum and jejunal-ileal sections (Chang et al., 2012; Justino et al., 2015; Soares et al., 2013). Intestinal inflammation can lead to an increase in crypt length (Chang et al., 2012). With increased intestinal damage,

jejunal-ileal and colon lengths can become reduced to increase surface area within the intestine (Whittaker et al., 2015; Whittaker et al., 2017; Wright et al., 2009). However, increases in colon weight (Whittaker et al., 2017) and small intestinal weight (Chartier et al., 2019) have also been reported. Orengedokuto has been demonstrated to have an effect in the colon when administered orally. In a model of TNBS-induced colitis, Orengedokuto was reported to reduce colon weight, reduce the thickness of the colonic wall and reduce the size of ulcerations within the colon compared to disease control at a dose of 2g/kg in rats (Zhou and Mineshita, 1999). However, in the current mucositis study there was no impact of Orengedokuto on the histological damage severity, length or weight of the small intestine or colon.

Intestinal mucositis is characterised by lesions in the mucosa of the small intestine that can fully penetrate the submucosa and allow bacteria access to the bloodstream (Sonis, 2004). The increased permeability of the damaged intestinal mucosa allows FITC-D to pass through (Nagahama et al., 2002). While the current study demostrated no significant increase in the intestinal permeability and no effect of Orengedokuto in mice administered 5-FU. Increased intestinal permeability has previously been demostrated by Horie et al. (2001) in rats administered 5-FU and by Nagahama et al. (2002) in rats administered uracil.

In mucositis studies MPO, found in azurophilic neutrophil granules (Justino et al., 2015), is used as an indicator of acute inflammation, as it is often elevated in the small intestine of rodents with 5-FU-induced mucositis (Chang et al., 2012; Justino et al., 2015). Studies in Balb/c mice have demonstrated elevated jejunal MPO activity in mice administered 5-FU compared to normal mice two days post 100mg/kg 5-FU injection (Chang et al., 2012) and in the duodenum three days after 450mg/kg 5-FU injection (Soares et al., 2011). In the present study there was no significant difference in MPO activity in either the jejunum or ileum between normal controls and 5-FU controls four days after 200mg/kg 5-FU injection. Orengedokuto in a model of TNBS-induced colitis has been reported to reduce MPO activity (Zhou and Mineshita, 1999). However, the current study saw no effect Orengedokuto on the MPO activity of mice administered 5-FU.

The grimace scale, developed by Langford et al. (2010), can be used to assess pain in mice. Studies have confirmed the reliability of grimace scoring when scored retrospectively, in various disease models (Akintola et al., 2017; Leach et al., 2012). In a rat model of mucositis, 5-FU did not significantly impact grimace scores (Whittaker et al., 2016). The current study, when scored retrospectively, presented similar results to Whittaker et al. (2016), with no significant difference in scores between 5-FU controls and normal controls on day 3. Despite no significance in retrospective analysis, real time analysis demonstrated a significant increase in the grimace score of 5-FU controls compared to normal controls on day 3. Real time and retrospective grimace scores have previously been compared in mice (Miller and Leach, 2015) and rats (Leung et al., 2016). Real time grimace scoring has not previously been conducted in a mucositis model; however, it has been conducted in mice to compare baseline scores of several strains (Miller and Leach, 2015), a mouse model of colitis associated colorectal cancer (Chartier et al., 2020) and rats with carrageenan-induced pain (Leung et al., 2016) where it was demonstrated to be scored lower than retrospective scoring. Lower scores in real time grimace scoring were suggested to be due to the constant changing of facial features such that an image may be taken mid-blink and therefore would retrospectively be scored as orbital tightening (Miller and Leach, 2015). In the current study, as with those by Miller and Leach (2015) and Chartier et al. (2020) real time grimace scores tended to be lower. Additionally in the study by Chartier et al. (2020) real time scoring demonstrated an increase in the grimace scores of disease control mice compared to normal control at one time point, while retrospective scoring demonstrated no difference between treatment groups at all time-points.

Another test used as an indicator of well-being in mice is burrowing activity. Burrowing is a luxury behaviour, typically expressed in healthy mice, and is among the first behaviours to be reduced in distressed mice (Jirkof, 2014; Jirkof et al., 2013). Burrowing activity has been applied in gastrointestinal disease models including colitis (Jirkof et al., 2013), colitis-associated colorectal cancer (Chartier et al., 2018) and mucositis (Whittaker et al., 2017) demonstrating a reduction in burrowing activity in disease controls compared to normal

animals. Consistent with Whittaker et al. (2017), in the current study, 5-FU reduced burrowing activity compared to normal control mice, indicating a reduction in the well-being of 5-FU control mice. However, there was no restoration of burrowing activity in mice treated with Orengedokuto implying a psychological effect of the treatment was not apparent.

In summary, high dose Orengedokuto improved stool consistency in mice with 5-FU-induced mucositis, although there was no impact on other indicators of disease severity. Importantly, there were no deleterious effects of Orengedokuto in normal mice nor mice with 5-FU induced mucositis. Future studies should explore the effects of different Orengedokuto treatment periods together with increased doses to determine its potential to further alleviate mucositis symptomatology.

References

Akintola, T., Raver, C., Studlack, P., Uddin, O., Masri, R., Keller, A., 2017. The grimace scale reliably assesses chronic pain in a rodent model of trigeminal neuropathic pain. Neurobiology of pain (Elsevier) 2, 13-–17.

Broughton, G., 2nd, Janis, J.E., Attinger, C.E., 2006. Wound healing: an overview. Plast Reconstr Surg 117(7 Suppl), 1e-S–32e-S.

Chang, C.T., Ho, T.Y., Lin, H., Liang, J.A., Huang, H.C., Li, C.C., Lo, H.Y., Wu, S.L., Huang, Y.F., Hsiang, C.Y., 2012. 5-Fluorouracil induced intestinal mucositis via nuclear factor-kappaB activation by transcriptomic analysis and in vivo bioluminescence imaging. PLoS One 7(3), e31808.

Chartier, L.C., Hebart, M.L., Howarth, G.S., Whittaker, A.L., Mashtoub, S., 2020. Affective state determination in a mouse model of colitis-associated colorectal cancer. PLoS One 15(1), e0228413.

Chartier, L.C., Howarth, G.S., Lawrance, I.C., Trinder, D., Barker, S.J., Mashtoub, S., 2018. Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. Digestive Diseases and Sciences 63(1), 135–145.

Chartier, L.C., Howarth, G.S., Mashtoub, S., 2019. Chemotherapy-induced mucositis development in a murine model of colitis–associated colorectal cancer. Scand J Gastroenterol, 1–8.

Cheah, K.Y., Howarth, G.S., Yazbeck, R., Wright, T.H., Whitford, E.J., Payne, C., Butler, R.N., Bastian, S.E., 2009. Grape seed extract protects IEC-6 cells from chemotherapy-induced cytotoxicity and improves parameters of small intestinal mucositis in rats with experimentally-induced mucositis. Cancer Biol Ther 8(4), 382–390.

Chen, Y., Xian, Y., Lai, Z., Loo, S., Chan, W.Y., Lin, Z.X., 2016. Anti-inflammatory and anti-allergic effects and underlying mechanisms of Huang-Lian-Jie-Du extract: Implication for atopic dermatitis treatment. Journal of ethnopharmacology 185, 41–52.

Cool, J.C., Dyer, J.L., Xian, C.J., Butler, R.N., Geier, M.S., Howarth, G.S., 2005. Pre-treatment with insulin-like growth factor-I partially ameliorates 5-fluorouracil-induced intestinal mucositis in rats. Growth Hormone & Igf Research 15(1), 72–82.

Fujii, A., Okuyama, T., Wakame, K., Okumura, T., Ikeya, Y., Nishizawa, M., 2017. Identification of anti-inflammatory constituents in Phellodendri Cortex and Coptidis Rhizoma by monitoring the suppression of nitric oxide production. Journal of Natural Medicines 71(4), 745–756.

Gibson, R.J., Keefe, D.M., Clarke, J.M., Regester, G.O., Thompson, F.M., Goland, G.J., Edwards, B.G., Cummins, A.G., 2002. The effect of keratinocyte growth factor on tumour growth and small intestinal mucositis after chemotherapy in the rat with breast cancer. Cancer Chemotherapy and Pharmacology 50(1), 53–58.

Horie, T., Awazu, S., Itakura, Y., Fuwa, T., 2001. Alleviation by garlic of antitumor drug-induced damage to the intestine. J Nutr 131(3s), 1071S–1074S.

Howarth, G.S., Francis, G.L., Cool, J.C., Xu, X., Byard, R.W., Read, L.C., 1996. Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. J Nutr 126(10), 2519–2530.

Jirkof, P., 2014. Burrowing and nest building behavior as indicators of well-being in mice. Journal of Neuroscience Methods 234, 139–146.

Jirkof, P., Leucht, K., Cesarovic, N., Caj, M., Nicholls, F., Rogler, G., Arras, M., Hausmann, M., 2013. Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice. Laboratory animals 47(4), 274–283.

Justino, P.F., Melo, L.F., Nogueira, A.F., Morais, C.M., Mendes, W.O., Franco, A.X., Souza, E.P., Ribeiro, R.A., Souza, M.H., Soares, P.M., 2015. Regulatory role of Lactobacillus acidophilus on inflammation and gastric dysmotility in intestinal mucositis induced by 5-fluorouracil in mice. Cancer Chemotherapy and Pharmacology 75(3), 559–567.

Kato, S., Hayashi, S., Kitahara, Y., Nagasawa, K., Aono, H., Shibata, J., Utsumi, D., Amagase, K., Kadowaki, M., 2015. Saireito (TJ–114), a Japanese traditional herbal medicine, reduces 5-fluorouracil-induced intestinal mucositis in mice by inhibiting cytokine-mediated apoptosis in intestinal crypt cells. PLoS One 10(1), e0116213.

Keefe, D.M., Sonis, S.T., Bowen, J.M., 2008. Emerging drugs for chemotherapy-induced mucositis. Expert Opinion on Emerging Drugs 13(3), 511–522.

Lalla, R.V., Peterson, D.E., 2006. Treatment of mucositis, including new medications. Cancer journal (Sudbury, Mass.) 12(5), 348–354.

Langford, D.J., Bailey, A.L., Chanda, M.L., Clarke, S.E., Drummond, T.E., Echols, S., Glick, S., Ingrao, J., Klassen-Ross, T., Lacroix-Fralish, M.L., Matsumiya, L., Sorge, R.E., Sotocinal, S.G., Tabaka, J.M., Wong, D., van den Maagdenberg, A.M., Ferrari, M.D., Craig, K.D., Mogil, J.S., 2010. Coding of facial expressions of pain in the laboratory mouse. Nature Methods 7(6), 447–449.

Leach, M.C., Klaus, K., Miller, A.L., di Perrotolo, M.S., Sotocinal, S.G., Flecknell, P.A., 2012. The Assessment of Post-Vasectomy Pain in Mice Using Behaviour and the Mouse Grimace Scale. Plos One 7(4).

Leung, V., Zhang, E., Pang, D.S., 2016. Real-time application of the Rat Grimace Scale as a welfare refinement in laboratory rats. Sci Rep 6, 31667.

Longley, D.B., Harkin, D.P., Johnston, P.G., 2003. 5-fluorouracil: mechanisms of action and clinical strategies. Nature Reviews Cancer 3(5), 330–338.

Mashtoub, S., Feo, B., Whittaker, A.L., Lymn, K.A., Martinez-Puig, D., Howarth, G.S., 2015. Oral Nucleotides Only Minimally Improve 5-Fluorouracil-Induced Mucositis in Rats. Nutrition and Cancer 67(6), 994–1000.

Mashtoub, S., Tran, C.D., Howarth, G.S., 2013. Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. Experimental Biology and Medicine 238(11), 1305–1317.

McCreath, S.B., Delgoda, R., 2017. Chemotherapeutics, in: McCreath, S.B., Delgoda, R. (Eds.), Pharmacognosy. Elsevier Inc., pp. 295–313.

Miller, A.L., Leach, M.C., 2015. The Mouse Grimace Scale: A Clinically Useful Tool? PLoS One 10(9), e0136000.

Miura, N., Fukutake, M., Yamamoto, M., Ohtake, N., Iizuka, S., Imamura, S., Tsuchiya, N., Ishimatsu, M., Nakamura, Y., Ishige, A., Watanabe, K., Kase, Y., Takeda, S., 2007. An herbal medicine orengedokuto prevents indomethacin-induced enteropathy. Biological & pharmaceutical bulletin 30(3), 495–501.

Nagahama, S., Korenaga, D., Honda, M., Inutsuka, S., Sugimachi, K., 2002. Assessment of the intestinal permeability after a gastrectomy and the oral administration of anticancer drugs in rats: nitric oxide release in response to gut injury. Surgery 131(1 Suppl), S92–97.

Peterson, D.E., Bensadoun, R.J., Roila, F., Esmo Guidelines Working Grp, 2011. Management of oral and gastrointestinal mucositis: ESMO Clinical Practice Guidelines. Ann. Oncol. 22, vi78–vi84.

Soares, P.M., Lima-Junior, R.C., Mota, J.M., Justino, P.F., Brito, G.A., Ribeiro, R.A., Cunha, F.Q., Souza, M.H., 2011. Role of platelet-activating factor in the pathogenesis of 5–fluorouracil-induced intestinal mucositis in mice. Cancer Chemother Pharmacol 68(3), 713–720.

Soares, P.M., Mota, J.M., Souza, E.P., Justino, P.F., Franco, A.X., Cunha, F.Q., Ribeiro, R.A., Souza, M.H., 2013. Inflammatory intestinal damage induced by 5-fluorouracil requires IL-4. Cytokine 61(1), 46–49.

Song, M.K., Park, M.Y., Sung, M.K., 2013. 5-Fluorouracil-induced changes of intestinal integrity biomarkers in BALB/c mice. Journal of cancer prevention 18(4), 322–329.

Sonis, S.T., 2004. The pathobiology of mucositis. Nature Reviews Cancer 4(4), 277–284.

Tooley, K.L., Howarth, G.S., Butler, R.N., 2009. Mucositis and non-invasive markers of small intestinal function. Cancer Biology & Therapy 8(9), 753–758.

Ulrich-Lai, Y.M., Fulton, S., Wilson, M., Petrovich, G., Rinaman, L., 2015. Stress exposure, food intake and emotional state. Stress (Amsterdam, Netherlands) 18(4), 381–399.

Whittaker, A.L., Leach, M.C., Preston, F.L., Lymn, K.A., Howarth, G.S., 2016. Effects of acute chemotherapy-induced mucositis on spontaneous behaviour and the grimace scale in laboratory rats. Laboratory animals 50(2), 108–118.

Whittaker, A.L., Lymn, K.A., Nicholson, A., Howarth, G.S., 2015. The assessment of general well-being using spontaneous burrowing behaviour in a short-term model of chemotherapy-induced mucositis in the rat. Laboratory animals 49(1), 30–39.

Whittaker, A.L., Zhu, Y., Howarth, G.S., Loung, C.S., Bastian, S.E.P., Wirthensohn, M.G., 2017. Effects of commercially produced almond by-products on chemotherapy-induced mucositis in rats. World journal of gastrointestinal pathophysiology 8(4), 176–187.

Wright, T.H., Yazbeck, R., Lymn, K.A., Whitford, E.J., Cheah, K.Y., Butler, R.N., Feinle-Bisset, C., Pilichiewicz, A.N., Mashtoub, S., Howarth, G.S., 2009. The herbal extract, Iberogast, improves jejunal integrity in rats with 5-Fluorouracil (5-FU)-induced mucositis. Cancer Biology & Therapy 8(10), 923–929.

Wu, Z., Han, X., Qin, S., Zheng, Q., Wang, Z., Xiang, D., Zhang, J., Lu, H., Wu, M., Zhu, S., Yu, Y., Wang, Y., Han, W., 2011. Interleukin 1 receptor antagonist reduces lethality and intestinal toxicity of 5-Fluorouracil in a mouse mucositis model. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 65(5), 339–344.

Yuki, F., Kawaguchi, T., Hazemoto, K., Asou, N., 2003. Preventive effects of oren-gedoku-to on mucositis caused by anticancer agents in patients with acute leukemia. Gan To Kagaku Ryoho 30(9), 1303–1307.

Zhang, S., Liu, Y., Xiang, D., Yang, J., Liu, D., Ren, X., Zhang, C., 2018. Assessment of dose-response relationship of 5-fluorouracil to murine intestinal injury. Biomed Pharmacother 106, 910–916.

Zhou, H., Mineshita, S., 1999. The effect of Oren-gedoku-to on experimental colitis in rats. Journal of Pharmacy and Pharmacology 51(9), 1065–1074.

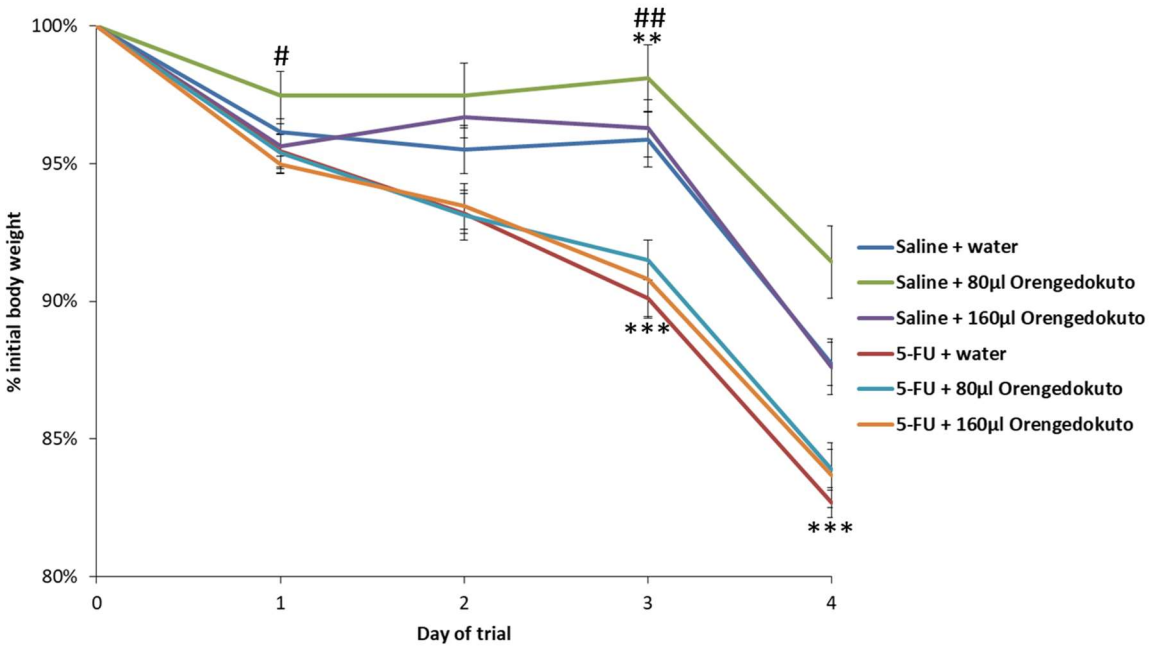


Figure 1. Bodyweight of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto, on all days of trial (0-4). Expressed as mean (% initial bodyweight) ± SEM. **p<0.01, ***p<0.001 compared to saline + water; #p<0.05, ##p<0.01 compared to saline + 160µl Orengedokuto.

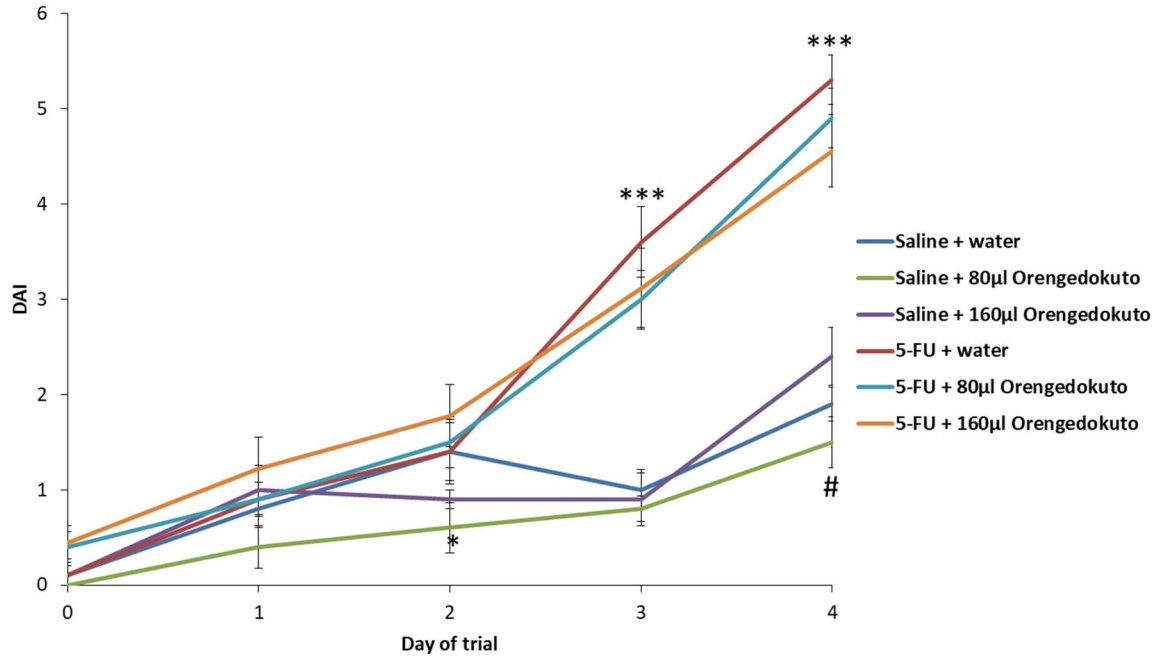


Figure 2. Disease activity index (DAI) of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto, on all days of trial (0-4). Expressed as

mean (DAI) ± SEM. *p<0.05, ***p<0.001 compared to saline + water; #p<0.05 compared to saline + 160µl Orengedokuto.

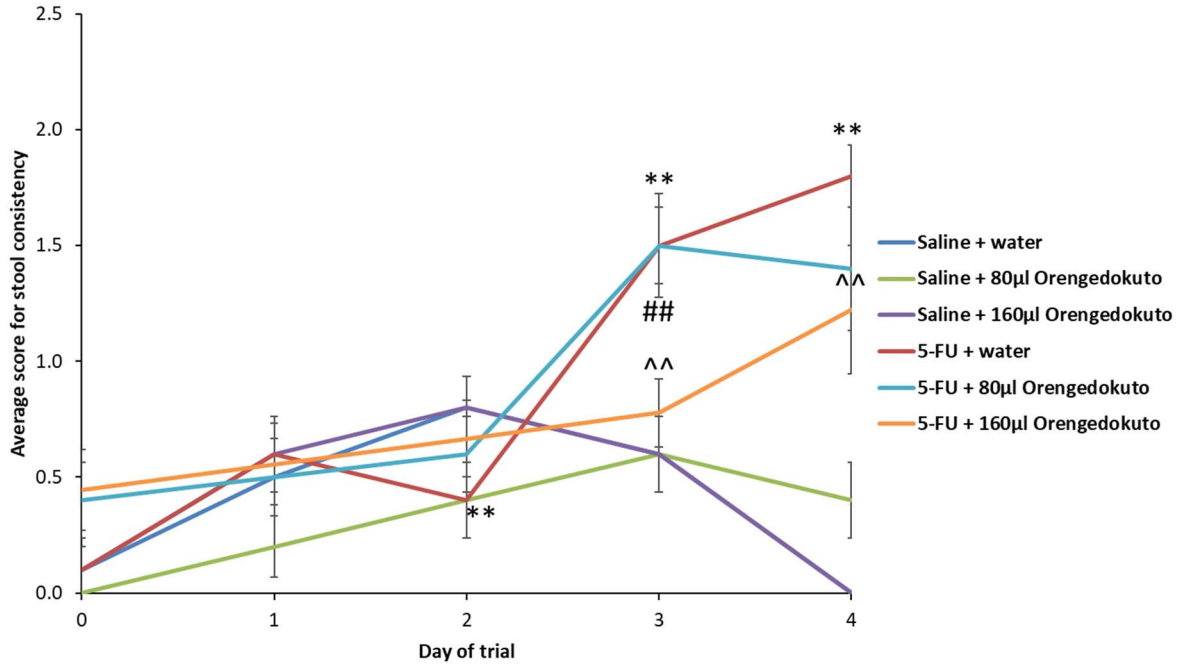


Figure 3. Stool consistency of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto, on all days of trial (0-4). Expressed as mean (score) ± SEM. **p<0.01 compared to saline + water; ^^p<0.01 compared to 5-FU + water; ##p<0.05 compared to saline + 160µl Orengedokuto.

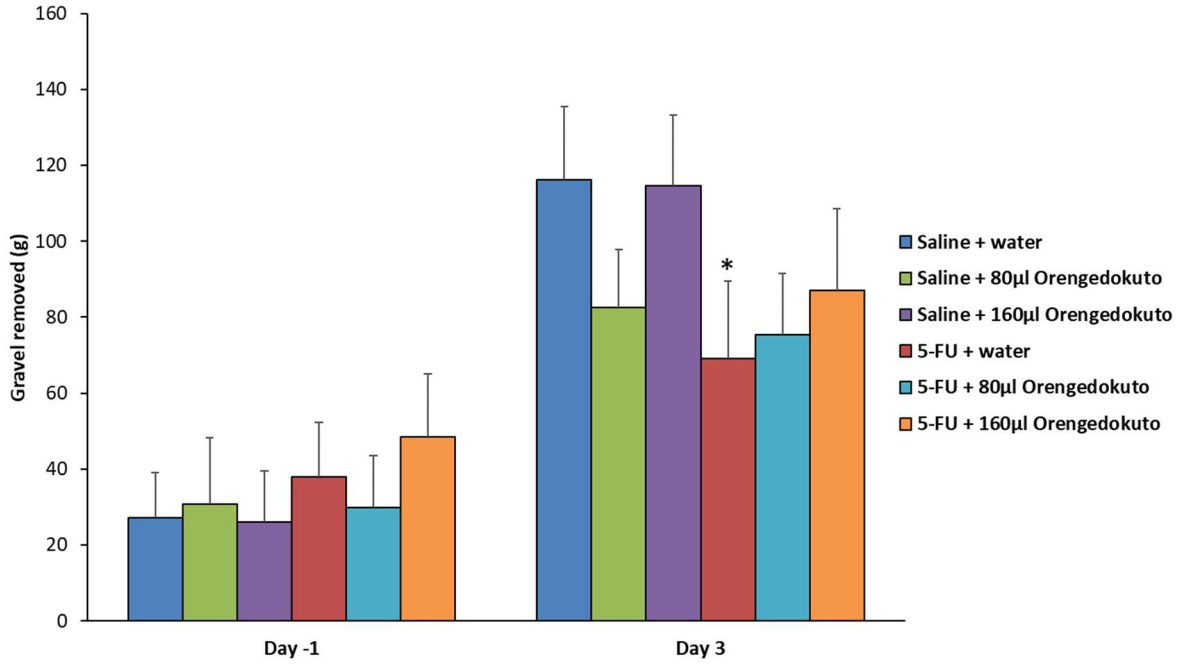


Figure 4. Gravel removed by burrowing activity of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto, on days -1 and 3 of trial. Expressed as mean (g) + SEM. *p<0.05 compared to saline + water.

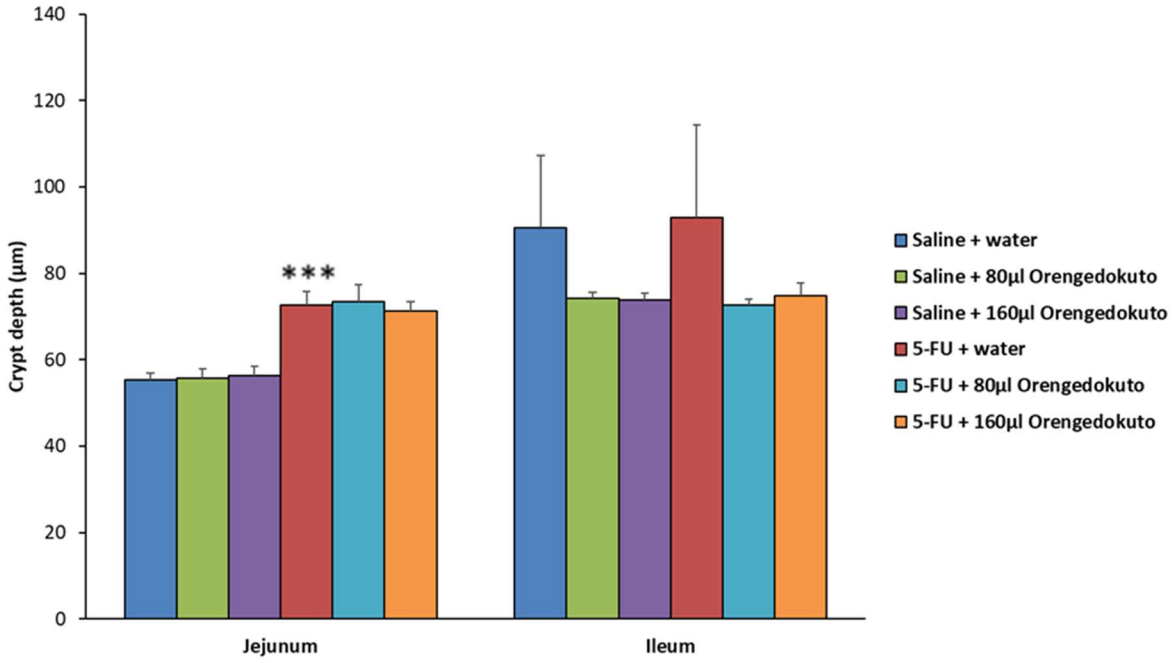


Figure 5. Jejunal and ileal crypt depth of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto. Expressed as mean (µm) + SEM. ***p<0.001 compared to saline + water.

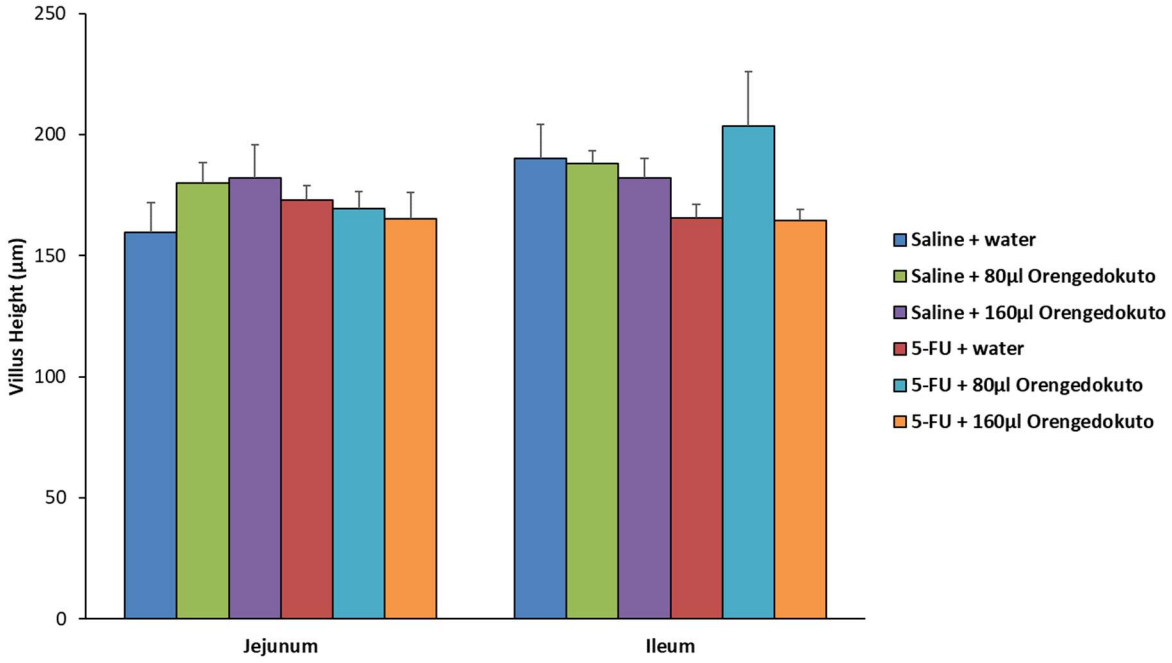


Figure 6. Jejunal and ileal villus height of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto. Expressed as mean (µm) + SEM.

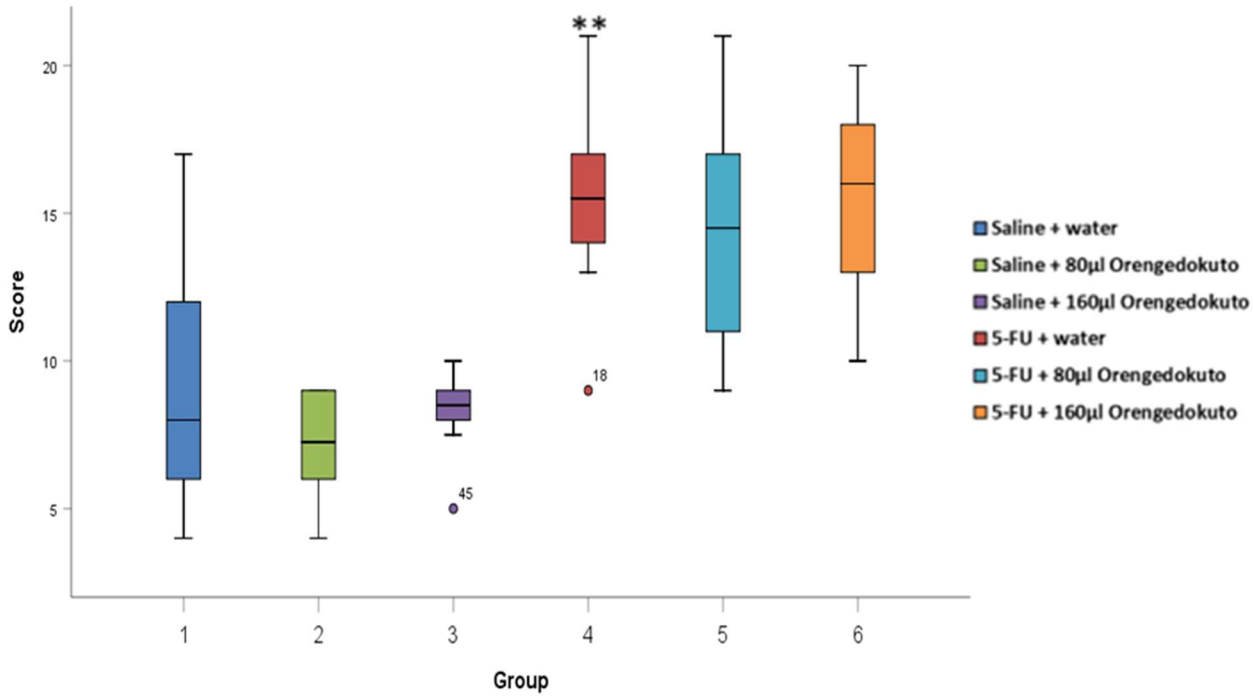


Figure 7. Small intestinal severity scores of saline and 5-FU-injected mice on day 0, gavaged daily (0–3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto. Expressed as median and IQR. **p<0.01 compared to saline + water.

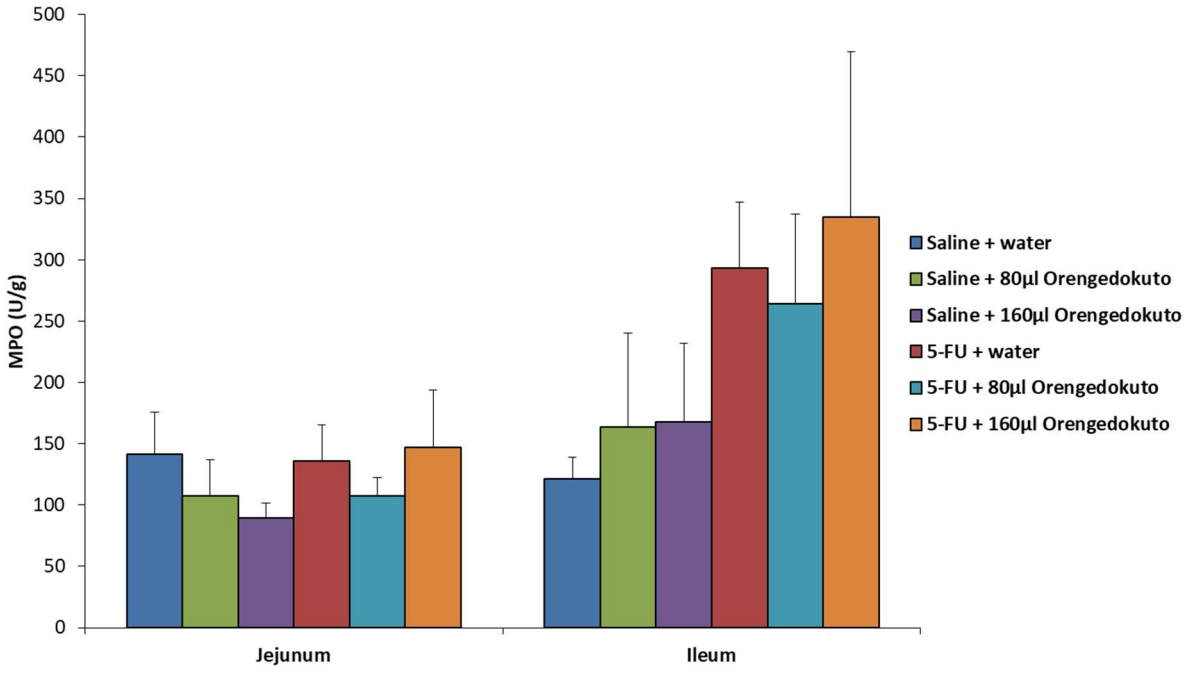


Figure 8. MPO (U/g) measured at 450nm in the jejunum and ileum of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto. Expressed as mean(u/g) + SEM.

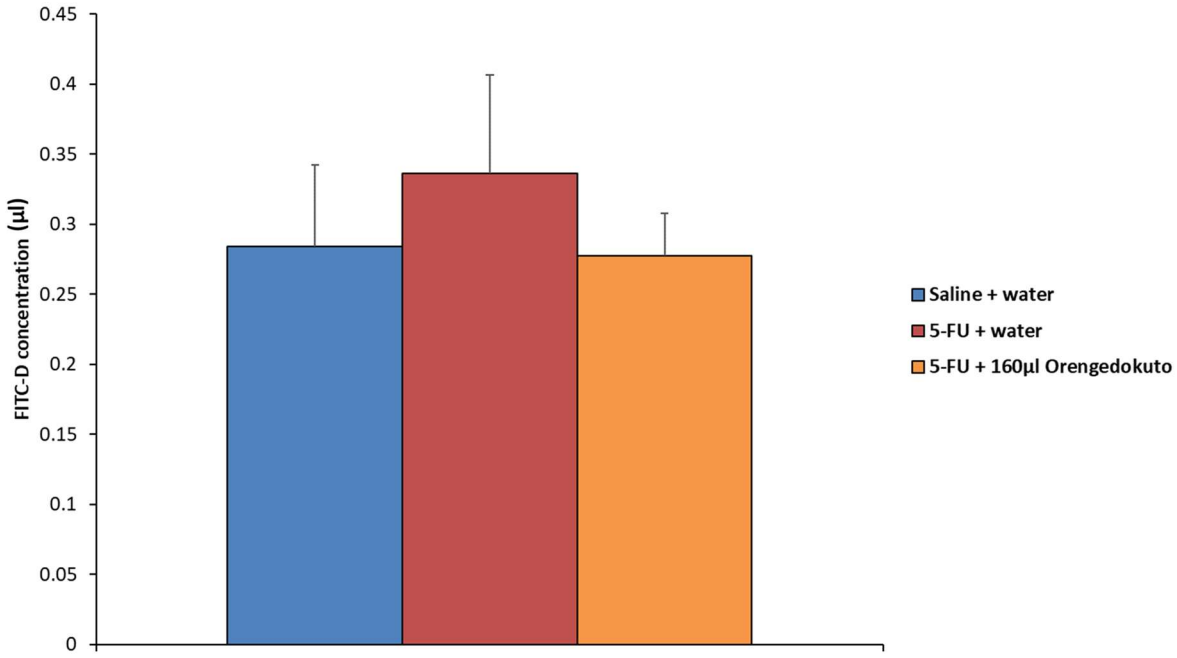


Figure 9. FITC-D concentration *(*µl) in blood serum of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water or 160µl Orengedokuto and 500mg/kg FITC-D day 4. Expressed as mean(concentration) + SEM.

Table 1. Day -1 and day 3 grimace scores. Mice were injected with either saline or 5-FU on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto. Scores are expressed as mean (score) ± SEM. **p<0.01 compared to saline + water.

	Real time		**Retrospective**	
	Day -1	**Day 3**	**Day -1**	**Day 3**
Saline + water	0.19±0.05	0.80±0.20	1.30±0.24	1.31±0.15
Saline + 80µl Orengedokuto	0.30±0.05	0.62±0.20	1.07±0.11	0.96±0.09
Saline + 160µl Orengedokuto	0.24±0.05	0.12±0.05	1.33±0.21	1.14±0.13
5-FU + water	0.20±0.03	1.97±0.42**	1.13±0.09	1.34±0.17
5-FU + 80µl Orengedokuto	0.22±0.05	1.46±0.29	1.44±0.16	1.02±0.13
5-FU + 160µl Orengedokuto	0.19±0.05	2.02±0.43	1.11±0.15	1.01±0.21

Table 2. Visceral organ weights of saline and 5-FU-injected mice on day 0, gavaged daily (0-3) with 160µl water, 80µl Orengedokuto or 160µl Orengedokuto. Expressed as mean (% day 4 body weight) ± SEM x 10^2. **p<0.01 compared to saline + water.

	Heart	Liver	Spleen	Thymus	Lung	Left kidney	Right kidney
Saline + water	50±1	404±8	32±1	19±3	89±6	56±1	59±02
Saline + 80µl Orengedokuto	50±1	412±9	30±1	18±2	96±7	55±1	59±2
Saline + 160µl Orengedokuto	51±1	422±9	33±2	16±2	89±5	56±2	59±1
5-FU + water	52±2	448±5	26±2	10±1**	105±5	60±02	61±1
5-FU + 80µl Orengedokuto	53±2	460±17	25±1	12±1	98±5	59±2	62±2
5-FU + 160µl Orengedokuto	51±2	436±12	27±3	12±1	105±6	57±2	63±3

Table 3. Gastrointestinal weights of saline and 5-FU-injected mice on day 0, gavaged daily (0–3) with 160µl µl water, 80µl µl Orengedokuto or 160µl µl Orengedokuto. Expressed as mean (% day 4 body weight) ± ± SEM x 10^2. ***p< < 0.001 compared to saline + water

	Duodenum	**JI**	**Colon**	**Stomach**	**Caecum**
Saline + water	45±3	219±4	92±4	65±2	67±5
Saline + 80µl Orengedokuto	50±7	340±24	82±4	67±2	71±5
Saline + 160µl Orengedokuto	48±6	304±15	88±6	69±2	78±6
5-FU + water	41±3	264±8	126±4***	69±2	57±2
5-FU + 80µl Orengedokuto	47±3	272±13	121±7	69±1	69±11
5-FU + 160µl Orengedokuto	46±3	290±7	124±4	72±2	62±3

Table 4. Gastrointestinal lengths of saline and 5-FU-injected mice on day 0, gavaged daily (0-–3) with 160µl µl water, 80µl µl Orengedokuto or 160µl µl Orengedokuto. Expressed as mean (cm) ± ± SEM. *p< < 0.05 compared to saline + water.

	Duodenum	**JI**	**Colon**
Saline + water	4.3±0.2	29.8±0.4	8.2±0.5
Saline + 80µl Orengedokuto	4.1±0.1	28.6±0.9	8.0±0.3
Saline + 160µl Orengedokuto	4.1±0.2	29.6±0.5	7.7±0.3
5-FU + water	4.0±0.1	26.5±0.9*	7.6±0.2
5-FU + 80µl Orengedokuto	3.6±0.1	27.7±0.9	8.0±0.3
5-FU + 160µl Orengedokuto	3.8±0.1	28.1±0.6	7.8±0.2