DETECTION OF SMALL INTESTINAL MUCOSITIS UTILISING THE NON-INVASIVE ^{13}C-SUCROSE BREATH TEST

BY

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TABLE OF CONTENTS

ABSTRACT

Mucositis is a common side-effect of chemotherapy, which is characterised by ulceration to the epithelium lining the gastrointestinal tract. This epithelium is susceptible to damage due to its rapid cellular turn-over rate and the inability of the treatment to distinguish between tumour and normal tissue. Assessment of small intestinal chemotherapy-induced mucositis has largely been hindered due to the unavailability of a suitable non-invasive diagnostic tool. Recently, a non-invasive breath test, the ^{13}C-sucrose breath test (SBT), has been developed and applied as a biomarker to detect small intestinal damage associated with methotrexate (MTX)-induced mucositis in rats.

This thesis extended this work, firstly optimising the sucrose dose (0.25 g/mL) for the application of the SBT in the rat versus the previously reported 1.0 g/mL dose. The SBT was shown to provide non-invasive monitoring of the time-course of MTX-induced small intestine damage in rodents, where maximal damage occurred 72 h post-MTX, with repair commencing 96 h post-MTX. In conjunction with biochemical sucrase activity analyses, the SBT quantified the capacity of the small intestine to adapt in response to damage, where ileal sucrase activity was elevated 144 h post-MTX to compensate for damage to the proximal small intestine. This phenomenon may have been due to elevated luminal carbohydrate levels in the ileum due to duodenal damage. The SBT was also successfully applied as a non-invasive biomarker of small intestinal health and integrity in rats receiving chemotherapy with other classes of agents. Here, the SBT demonstrated strong concordance with biochemical sucrase activity. The SBT was also capable of determining the efficacy of *Streptococcus thermophilus* (TH-4), a

potential probiotic, in attenuating MTX-induced mucositis. Specifically, TH-4 attenuated damage to the proximal jejunum as shown by the SBT, and corroborated by sucrase and myeloperoxidase activities, and histological analyses. In contrast, TH-4 treatment in a tumour-bearing model of MTX-induced mucositis did not protect the small intestine. This may be due to elevated pro-inflammatory circulating cytokines or the dose of TH-4 may need to be increased.

The SBT and the small intestinal permeability (SIP) test were applied in a cohort of paediatric cancer patients undergoing chemotherapy to determine small intestinal function. It was demonstrated that the SBT was a superior biomarker of small intestinal function capable of detecting small intestinal changes in patients diagnosed with mucositis, characterised by a significantly lower SBT, whereas SIP was not significantly altered throughout a cycle of chemotherapy. A small pilot study was performed to determine the capability of the SBT to monitor small intestinal function throughout multiple regimes of chemotherapy, where it was demonstrated that there was a gradual decline in small intestinal integrity due to multiple cycles of chemotherapy.

In conclusion, the non-invasive SBT is a biomarker of small intestinal function that can be applied easily and cost-effectively, in both animals and humans, to monitor gut function in relation to chemotherapy agents and/or potential anti-mucositis treatments. This thesis has illustrated the important potential application of the SBT in the arena of supportive cancer care, where new chemotherapy and anti-mucositis agents can be assessed in relation to small intestinal toxicity.

DECLARATION

I declare that this thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan in the University Library, if applicable, should it be accepted for award of the degree of a PhD.

Katie Louise Tooley

Signature: ..... Date: 10/5/07.....

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LIST OF ABBREVIATIONS

% L:	percentage lactulose
% R:	percentage rhamnose
% S:	percentage sucrose
$\%CD_{90}$:	percentage ^{13}C cumulative dose over 90 min
5-FU	5-Fluorouracil
ALL:	acute lymphoblastic leukaemia
bwt:	bodyweight
C+E:	cyclophosphamide + etoposide
cfu:	colony forming units
cm:	centimetre
DAMA:	dark agouti mammary adenocarcinoma
DAR:	dark agouti rat
Dox:	doxorubicin
EGF:	epidermal growth factor
Etop:	etoposide
g:	grams
GIT:	gastrointestinal tract
GPT:	gastric (sucrose) permeability test
h:	hour
HD:	high-dose

HPLC:	high performance liquid chromatography
i.m.:	intra-muscular
i.p.:	intra-peritoneal
i.v.:	intravenous
IFNγ:	interferon gamma
IGF-1:	insulin-like growth factor-1
Irino:	irinotecan
IRMS:	isotope ratio mass spectrometer
kg:	kilograms
KGF-1:	keratinocyte growth factor-1 (Palifermin)
L/R:	lactulose/rhmanose ratio
mg:	milligrams
min:	minute
mL:	millilitres
MPO:	myeloperoxidase
mRNA:	messenger ribonucleic acid
MTX:	methotrexate
nmol:	nano moles
o/n:	overnight
OCTT:	oro-caecal transit time
PBS:	phosphate buffer solution

ROS:	reactive oxygen species
s.c.:	subcutaneous
SBT:	^{13}C-sucrose breath test
SD:	standard deviation
SEM:	standard error of the mean
SIP:	small intestinal permeability
t:	time
TGFβ:	transforming growth factor-β
TH-4:	*Streptococcus thermophilus*
TNFα:	tumour necrosis factor-α
U:	units
w/v:	weight per volume
WGFE:	whey-derived growth factor extract
WHO:	World Health Organisation
wt:	weight
μL:	micro litre
μm:	micro metre
ω-3 PUFAs:	omega-3 polyunsaturated fatty acids

PUBLICATIONS AND PRESENTATIONS

PEER REVIEWED PUBLICATIONS:

1. **Tooley KL** , Howarth GS, Lymn K, Lawrence A, Butler RN. Oral ingestion of *Streptococcus thermophilus* diminishes severity of small intestinal mucositis in methotrexate treated rats. *Cancer Biology & Therapy*. 2006; 5(6): 593-600.

2. Howarth GS, **Tooley KL**, Davidson GP, Butler RN. A non-invasive method for detection of intestinal mucositis induced by different classes of chemotherapy drugs in the rat. *Cancer Biology & Therapy*. 2006; 5(6): 1198-1195.

3. **Tooley KL**, Saxon BR, Webster J, Zacharakis B, McNeil Y, Davidson GP, Butler RN. A novel non-invasive biomarker for assessment of small intestinal mucositis in children with cancer undergoing chemotherapy. *Cancer Biology & Therapy*. 2006; 5(10): 1275-1281.

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3. **Tooley KL**, Howarth GS, Lymn K, Butler RN. The effect of *Streptococcus thermophilus* on the small intestine and tumour growth after methotrexate in the rat with mammary adenocarcinoma.

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   Inglis KL, Saxon B, Webster J, Zacharakis B, Davidson, GP, Butler RN. Non-invasive assessment of chemotherapy-induced intestinal mucositis in children. Journal of Gastroenterology and Hepatology, 2003, 18 (Suppl): B36. (Abstract)

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   Inglis K, Penning K, Howarth G, Butler R. Oral ingestion of Streptococcus thermophilus partially attenuates small intestinal mucositis in methotrexate-treated rats. Supportive Care in Cancer; 13 (6): 445, 2005. (Abstract; poster)

5. *Accepted poster presentations at the MASCC symposium, Toronto (CAN), June 2006.*

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Howarth GS, **Tooley KL**, Davidson GP, Lymn KA, Butler RN. Non-invasive monitoring of intestinal mucositis using the 13C-sucrose breath test (SBT): A new biomarker for drug discovery. Supportive Care in Cancer; 14 (6): 638, 2006. (Abstract)

Butler RN, Kritas S, **Tooley KL**, Lymn K, Howarth GS. Combinations of non-invasive tests to assess gut dysfunction induced by chemotherapy and infection. Supportive Care in Cancer; 14 (6): 611, 2006. (Abstract)

Shillabeer T, Dyer S, Downes M, Collins C, Pincombe J, **Tooley K**, Davidson G, Butler R. Chemotherapy induced gastrointestinal mucositis: Development of a clinical assessment tool in paediatric oncology. Supportive Care in Cancer; 14 (6): 639, 2006. (Abstract)

PART I: INTRODUCTION

CHAPTER 1: INTRODUCTION, LITERATURE REVIEW AND AIMS

1.1 INTRODUCTION

Mucositis is a common and debilitating side effect of chemotherapy, affecting up to 60% of patients receiving high-dose chemotherapy, and almost 100% of patients undergoing pre-conditioning chemotherapy regimens for stem cell transplant.[1] No truly effective therapy is currently available to treat mucositis, which is characterised by ulcerating lesions lining the gastrointestinal tract (GIT). This phenomenon occurs due to the inability of chemotherapy agents, such as methotrexate, irinotecan, etoposide, cyclophosphamide, melphalan and 5-fluorouracil, to distinguish between normal and target tissue.[2,3] Cells that divide rapidly such as tumour cells and the cells that line all mucosal membranes are equally as sensitive to damage. Much research to date has been confined to assessing mucositis associated with the oral cavity, primarily due to its accessibility. However, lower GIT toxicities to the patient are becoming increasingly more apparent with the utilisation of higher chemotherapy doses and the more toxic new chemotherapies for maximal cancer kill. This review will focus on the small intestine as a target for chemotherapy side-effects.

In the small intestine, chemotherapy-induced mucositis causes villus blunting, hypoproliferation of crypt cells, and shallow crypts, due primarily to an increase in apoptosis and a decrease in proliferation.[1,4,5] Patients who develop intestinal mucositis

may experience symptoms ranging from mild nausea, vomiting and abdominal bloating through to painful cramping and diarrhoea, with intense abdominal pain (requiring narcotic administration). In its more severe form, mucositis can lead to intestinal bacterial translocation, resulting in sepsis, which can be fatal.[6] In these cases patient chemotherapy regimens are often postponed or drug doses are reduced, leading to sub-maximal tumour kill. Patients spend extended or unplanned stays in hospital, often requiring parenteral feeding, leading to a significantly decreased quality of life. Overall, other side-effects of chemotherapy, such as neutropaenia and thrombocytopaenia, are usually well managed and systems are in place to treat them. The development of efficacious treatments for the development of small intestinal mucositis has been hindered as the pathobiology of mucositis is still not fully understood. This has led to increased costs for the public hospital system. There is therefore a clear need to develop agents to protect the intestine during cancer treatment. Reducing the severity of mucositis would also improve quality of life for the patient and cancer treatment, potentially enabling tolerance of higher doses of chemotherapy.

1.2 The Small Intestine

The small intestine represents a large proportion of the gastrointestinal system, measuring six to seven metres in length from the gastro-duodenal sphincter to the ileo-caecal sphincter.[7] It consists of three regions: the duodenum, jejunum and ileum. Its primary functions are to aid in the propulsion of chyme (biochemically and mechanically digested food) and to facilitate efficient digestion and absorption of food.

Throughout the intestinal lumen there are circular folds of tissue (rugae). Additionally, on these folds lay finger-like projections (villi; Figure 1.1), which have microvilli on the luminal side of the cell. The combination of these three parameters increases the surface area of the small intestine, and thus its functional capacity to digest and absorb nutrients.[8] The small intestine, and specifically the villus, is lined with a highly specialised simple columnar epithelium, on which brush-border enzymes are located on the microvilli membrane of small intestinal enterocytes. Crypts, located at the base of the villi, contain the stem cells for maintaining villus integrity.[9] The passage of luminal contents is maintained by the presence of tight junctions, located between adjacent cells on the apical side of the enterocyte. Additionally, a layer of mucus coats the small intestinal epithelium and acts as a barrier to the luminal contents, which consists of enzymes, acidic pH, cytokines and bacteria.[10]

1.2.1 Small Intestinal Cell Kinetics

Small intestinal enterocytes are in direct contact with the hostile luminal contents. To overcome this, these cells are replaced by new cells that are generated by progenitor cells located in the crypts (Figure 1.1). Stem cells have the capacity to produce a large progeny of highly differentiated cells.[11] In the crypt, stem cells divide and the resulting cells migrate up the crypt to the villus where they have a life span of 3 days in the rodent, and 5 days in humans.[12] The cells then slough off into the lumen of the intestine.[13] This is suggested to be a mechanism of protecting the tissue from ongoing hazardous exposure to the luminal contents.[14] There are contradicting views as to whether the cells undergo programmed cell death (apoptosis) whilst still attached [15]to the villus, or once they have sloughed off into the lumen once the cell is at the apex of

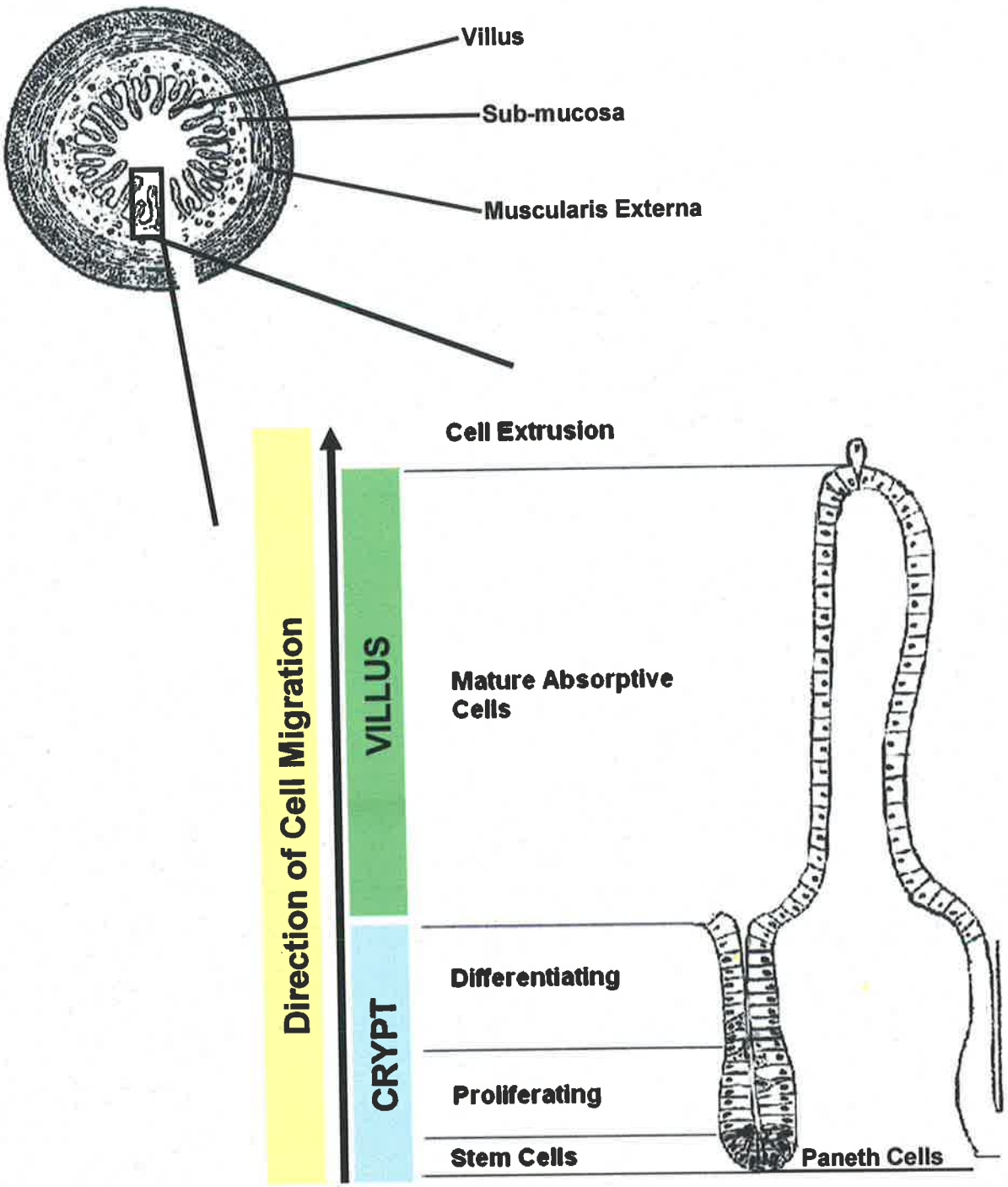

Figure 1.1: Illustration depicting the crypt-villus functional unit of the small intestine. As cells migrate in the direction of crypt to villus, proliferative cells arise from basal stem cells. These then lose their proliferative abilities and begin to differentiate (specialise). The specialised villus cells migrate toward the tip of the villus and are then exfoliated into the lumen.

the villus. The cells are then removed (digested) by macrophages.[16] It is now thought that apoptosis is a combination of these two mechanisms. Small intestinal epithelial cells express a wide range of receptors for cytokines such as TNF-α, IFN-γ, TGF-β and a variety of interleukins, enabling the modulation of proliferation, cell survival, changes in paracellular permeability and activation of cytokine expression.[17]

1.2.2 Crypt Cells

Enterocytes, goblet cells, Paneth cells and endocrine cells are derived from the clonogenic stem cells located at the base of the crypts. These cells have the ability to regenerate tissue after injury.[9] Proliferation studies in mice show that crypt stem cells divide daily and that the crypt is renewed in two days.[18] Villi are lined primarily by the absorptive enterocytes (~95%) [19] and goblet cells, whereas, the crypt comprises stem cells, poorly differentiated proliferative cells, and the longer living subset of Paneth cells (secretory cells).[20] Since the greatest proportion of the small intestine is relatively inaccessible in humans, most villus-crypt axis studies have been carried out in cell culture (*in vitro*) or in rodent models. The migration of enterocytes from the small intestinal crypt to the tip of the villus coincides with the appearance of specific phenotypes enabling cells to absorb nutrients,[12] where specific enzyme activities increase along the crypt-villus axis. These brush-border enzymes generally decrease in activity from the proximal to the distal regions of the small intestine.[21]

1.2.3 Cell Death

Apoptosis, initially termed shrinkage necrosis,[22] is the controlled process whereby cells are deleted.[1] It is an important mechanism for maintaining homeostasis and occurs throughout the body. Redundant cells are removed, tissue structures are maintained, and cells that have genetic damage, and have the potential to become neoplastic, are aborted. The small intestine has the capacity to regulate the removal of enterocytes and is essential for maintaining its function. Whilst the molecular control of apoptosis is not completely clear, the involvement of p53, the Bcl-2 family and caspases is known to be involved.[22,23] The Bcl-2 family of proteins contain members which signal for up- and down-regulating apoptosis, subsequently leading to the activation of caspases.[24] p53 is a nuclear phosphoprotein that regulates the expression of the previously described apoptotic proteins.[1]

1.3 CHEMOTHERAPY AND THE SMALL INTESTINE

Chemotherapy agents are used to treat malignancy and to eradicate cancer cells. It is well recognised that these agents have a narrow therapeutic index, resulting in a constellation of side-effects as these drugs are unable to distinguish between normal and neoplastic tissue.[2] This therefore, results in toxicity in all organs where there is a high cell turnover rate conferring a higher vulnerability in these tissues.[6] The gastrointestinal tract is a system which is affected.

Recently a 5-phase model of damage and repair for chemotherapy-induced damage to the oral mucosa has been applied to the intestine.[25] It has now been proposed that the level of damage is not limited to the mucosa. Damage extends to the submucosa, the muscularis externa, and their associated blood supply. The five phases described are: (1) initiation, (2) upregulation and message generation, (3) signalling and amplification, (4) ulceration and inflammation and (5) healing (Figure 1.2). The initiation phase incorporates the generation of reactive oxygen species (ROS) which lead to the damage of cells, tissues and blood vessels. This then leads to the activation of nuclear factor kappa B (NF-κB),[26] which initiates a cascade of upregulation of genes responsible for the production of adhesion molecules; cyclo-oxygenase-2 and pro-inflammatory cytokines (TNF-α, IL-1 and IL-6), causing apoptosis and tissue injury. Production of pro-inflammatory cytokines then signals back on NF-κB which upregulates these cytokines in a second phase. The tissue damage sustained from these first phases leads to ulceration, where bacteria colonise, and subsequently leads to inflammation due to a further increase in pro-inflammatory cytokines. It is in this phase that bacterial translocation poses a real problem which can ultimately lead to sepsis, placing the sufferer at a heightened risk of death. Healing finally occurs due to the renewal of the epithelium via cell proliferation and differentiation, and the re-establishment of the appropriate local microbiota.[25,27] However, it is important to note that the healed epithelium does not return to its original state once the healing process is completed.

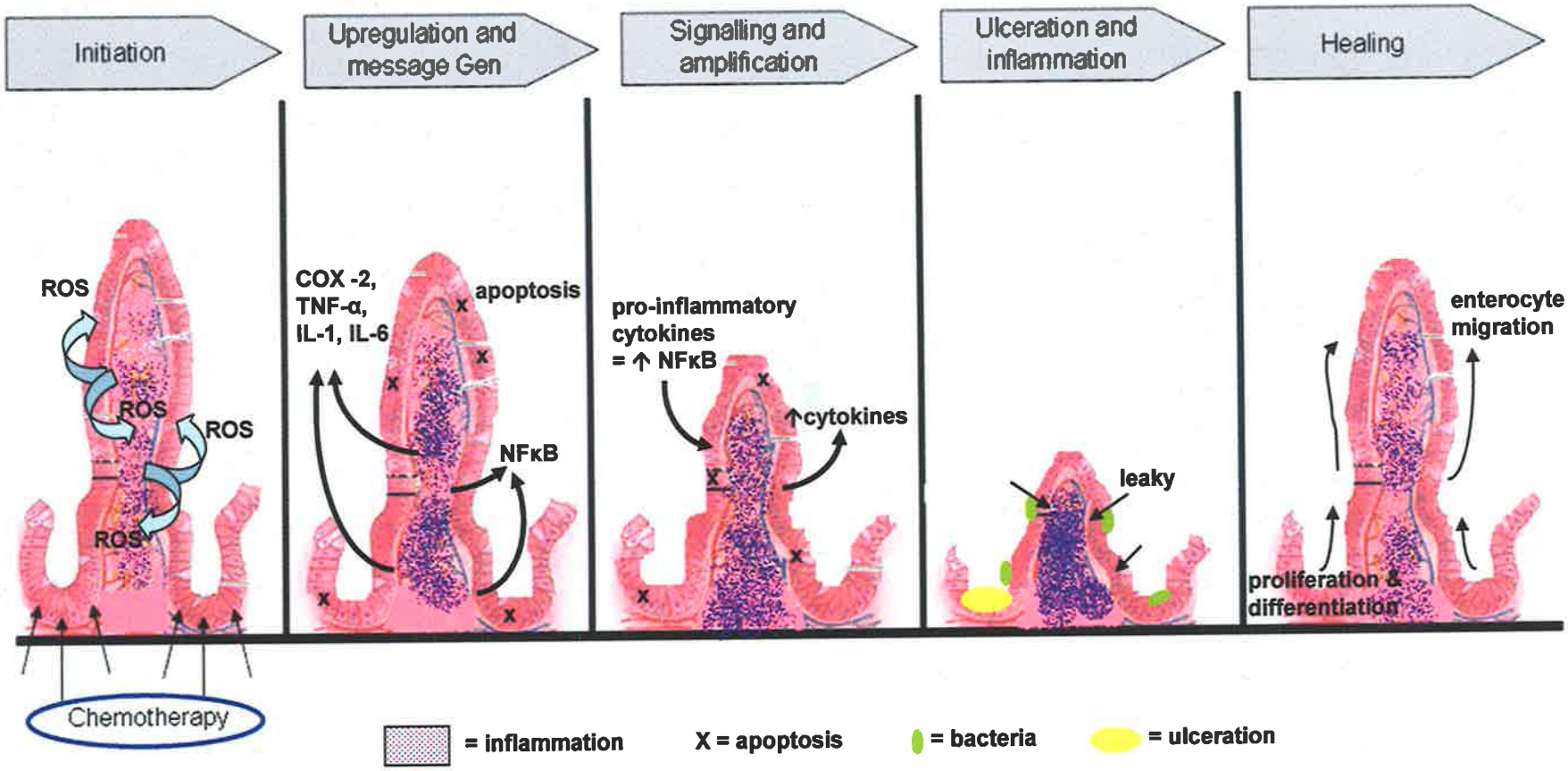

Figure 1.2: The five-phase mucositis model adapted for small intestinal epithelium. (1) Initiation: chemotherapy and the production of reactive oxygen species (ROS) damage epithelium; (2) Upregulation and message generation: ↑cytokines leads to inflammation and apoptosis; (3) Signalling and amplification; (4) Ulceration and inflammation; (5) Healing and repair. (Modified image of a villus from Sherwood, 1997).[28]

Taminiau *et al.* (1980)[13] first showed sucrase activity was significantly reduced from 72.6 U/g to 23.3 U/g 48 ($p < 0.005$) 48hrs after MTX administration. Keefe *et al.* (2000)[29] reported that the earliest effect caused by chemotherapy occurs one day after administration in adults, in whom a seven-fold increase in apoptosis in small intestinal cells was observed. This was followed by a reduction in crypt length, villus area and mitotic count. Similar results have been described by Xian *et al.* (2000)[5] finding villus shortening 5-6 days after chemotherapy administration to rats. An earlier study by Keefe *et al.* (1997),[6] revealed that patients undergoing chemotherapy had an altered small intestinal permeability where there was a decreased permeation to monosaccharides, and an increase in permeation of disaccharides. This indicated that chemotherapy drugs, especially in high-doses, resulted in a cascade of side-effects including impaired barrier function. Additionally, it has also been found that small intestinal function does not return to normal for several weeks,[29] as malabsorption and diminished enzyme activity still persist after structural repair of the gut.[30]

Cell turnover rate of the small intestinal epithelium is faster than that of the colonic or oral mucosa, and is therefore more susceptible to chemotherapy-induced damage. Whilst there are many studies outlining the effects of chemotherapy to the GIT, the comparative effect of chemotherapy-induced damage to different GIT mucosal surfaces has not been addressed in one combined study. Indeed, the well documented oral chemotherapy-induced mucositis is devastating and painful to the individual, which limits oral intake and reduces the capacity to ingest vital nutrients. It could be assumed that the development of mucositis would not be limited to the oral mucosa, such that there is likely to be small intestinal involvement or damage. Villus blunting and crypt shortening leads to decreased disaccharidase activities, resulting in a reduced absorptive

capacity. This induces the risk of complications, as the small intestine is the primary absorptive organ of the body. Not only is the absorptive organ of the body damaged, but its capacity to renew, repair and regenerate is hindered due to the decreased nutrient absorption. Nutrition is vital for the ongoing repair that is required when undergoing multiple regimens of chemotherapy, especially in paediatric patients who are also undergoing body growth. To the best of my knowledge no studies have been reported addressing long-term effects of growth in paediatric cancer patients, in relation to adequate nutrition, and small intestinal function.

The financial implications of the serious clinical consequences of oral mucositis have been reported only rarely [25] and the cost of GI mucositis has not been studied systematically. However, reductions in cure rates as a result of treatment interruptions or inadequate treatment, the cost of symptom management, and maintaining adequate nutrition are all short-term outcomes that need to be addressed. Chronic GI toxicity has been shown to occur in 70-90% of long-term cancer survivors undergoing radiotherapy [31] but less is known about the long-term effects of chemotherapy. It is possible that patients may be asymptomatic but still exhibit a variety of deleterious functional changes.

1.3.1 Anti-metabolite Chemotherapy Agents

Anti-metabolite chemotherapy agents exert their cytotoxic effect through an inhibition of folate metabolism by down-regulation of the enzyme dihydrofolate reductase resulting in an inhibition of DNA synthesis.[32] Methotrexate (MTX) and 5-Fluorouracil (5-FU) belong to this family of anti-cancer drugs. MTX is a chemotherapy agent that is

used widely for the treatment of leukaemia and solid tumours. Additionally, it is the most studied anti-cancer drug in experimental animal models of mucositis. Cancer patients receiving chemotherapy with drugs that further decrease folate levels are therefore at additional risk of cancer recurrence and gastrointestinal toxicity. The magnitude of folate depletion in patients receiving high dose MTX therapy is such that folate repletion is mandatory within a 24 hr period following chemotherapy, in order for DNA synthesis and repair to occur. Folinic acid is utilised as the source of folate in this scenario, in a life-saving procedure known as 'folinic acid rescue' or 'folinic acid salvage'.[33] Whilst the delivery of folinic acid rescues many tissues, due to its systemic delivery, MTX toxicity on the GIT remains high. Additionally, the handling or clearance of the drug can be highly variable from one patient to the next (genetic polymorphisms) and can alter the cytotoxic exposure to the tissue.[2] Methylenetetrahydrofolate reductase (MTHFR) is involved in the maintenance of folate and homocysteine levels. The $MTHFR_{677}$ gene has been implicated with increased MTX plasma toxicity, due to the reduction in MTHFR activity.[34,35] This decrease in MTHFR activity reduces the level of chromosomal damage, thus decreasing the efficacy of MTX. Higher doses of MTX may prove beneficial for cancer patients with polymorphisms of the MTHFR carrier, as they have a lower sensitivity to MTX, thus improving tumour kill and overall cancer treatment.

The predominant site of MTX-induced damage is in the small intestine, where apoptosis can be detected as early as six hours post administration in the dark agouti rat (DAR).[36] Apoptosis is located predominantly in the crypt. Due to this phenomenon, it diminishes the capacity of the epithelium to regenerate and renew itself. This leads to: blunted villi, irregular enterocytes, shallow and disrupted crypts. Inflammatory infiltrate and oedema

are common histological features of MTX-induced small intestinal mucositis. The irregular or damaged enterocytes that remain are immature, leading to decreases in brush-border enzymes such as sucrase, lactase, maltase and alkaline phosphatase. As indicated by the 5-phase damage and repair model,[25] abscesses can be located in crypts, and finally repair occurs due to the stem cells hyperproliferating to regenerate the epithelium. Table 1.1 illustrates some representative studies utilising the MTX model of chemotherapy-induced mucositis.

5-Fluorouracil (5-FU) is another anti-metabolite chemotherapy agent that exerts its cytotoxic effect by blocking the enzyme thymidylate synthetase, resulting in the inhibition of DNA synthesis.[37] 5-FU has been utilised in both oral [38] and small intestinal animal models of mucositis.[39] Forty-eight hours post treatment (single dose) a significant increase in apoptosis and a decrease in proliferation is evident, leading to a decrease in villus height and crypt depth. A moderate level of damage, as determined histologically, is induced, with a moderate decrease in the brush-border enzyme, sucrase.[39]

1.3.2 DNA Topoisomerase Inhibitors

DNA topoisomerase inhibitors, are a more recent class of chemotherapy agent, and are highly toxic. All mammalian cells contain DNA topoisomeraese (DNA enzymes) that are essential for resolving/fixing problems during replication and transcription.[40] DNA topoisomerase inhibitors interfere with the DNA breakage-rejoining reaction, resulting in a single- or double-strand DNA break.[41] Examples of such agents are Etoposide and Irinotecan. Etoposide is used extensively in clinical practice for the

Table 1.1: Summary of representative MTX-induced gastrointestinal mucosal damage in rodent models.

Study	Year	Species	Dose	Small Intestinal Damage Scientific Findings
Taminiau *et al* [13]	1980	Wistar rats (M)	30 mg/kg once (i.v.)	Reduced mitoses in crypts, villus atrophy and ↓ activity thymidine kinase 24-48 h after MTX. Small intestine hyperproliferative 96 h, ↓ disaccharidase, alkaline phosphatase and Na+/K+ ATPase activities.
Howarth *et al* [42]	1996	Sprague Dawley Rat (M)	2.5 mg/kg (s.c.) Daily, 3 days	↓ food intake, bodyweight and sucrase activity. Villus atrophy and crypt disruption and capillary dilatation. Increased bacterial translocation. Assessed efficacy of whey growth factor extract.
Howarth *et al* [43]	1998	Sprague Dawley Rat (M)	2.5 mg/kg (s.c.) Daily, 3 days	As previously described in above studies. Decrease in protein content and DNA content in the jejunum. Assessed efficacy of IGF-I treatment.
Nakamaru *et al* [44]	1998	ddY mice (M)	20 mg/kg (orally) Daily, 6 days	Changes to transcellular permeability, increases paracellular permeability
Xian *et al* [45]	1999	Sprague Dawley Rat (M)	2.5 mg/kg (s.c.) Daily, 3 days	Similar findings described above.TFF3 mRNA increased and cell population expression expanded.TFF3 aids in remodeling and repair.
Xian *et al* [5]	2000	Sprague Dawley Rat (M)	2.5 mg/kg (s.c.) Daily, 3 days	Similar to previous findings listed above. HGF and c-met protein/mRNA expression upregulated 4-7 post MTX, confined to crypt and lower villus.
Gibson *et al* [46]	2002	Dark Agouti Rat (DAR; F)	1.5 mg/kg (i.m.) Daily, 2 days + tumour	Increase in apoptosis (crypt) 6 h post 2nd MTX dose. Villus atrophy (2-4 days post 1st MTX). MTX reduced proliferation of tumour day 1, weight day 2 and increased apoptosis. Assessed efficacy of Interleukin-11
Xian *et al* [47]	2002	TGF-α wild-type and -/- mice	300 mg/kg (s.c.) once	TGF-α aids in the modulation of apoptosis and enhance repair and proliferation.
Gibson *et al* [48]	2002	DAR (F)	1.5 mg/kg (i.m.) Daily, 2 days + tumour	Similar to previous findings listed above. Assessed efficacy of KGF.
Tran *et al* [49]	2003	Sprague Dawley Rat (M)	2.5 mg/kg (s.c.) Daily, 3 days	Moderate-severe damage in duodenum and jejunum 5 days after 1st injection, damage ↓ by day 7. Altered 51Cr-EDTA levels 5 and 7 days post MTX. Assessed efficacy of zinc as treatment

Continued...

Beck *et al* [50]	2004	C57/B6 and Sv129/C57/B6 mice	150 mg/kg (i.p.) once	Reduction in goblet cells, and intestinal trefoil factor mRNA and promoter activity
Carneiro-Filho *et al* [51]	2004	Adult Wistar rats (M)	1.5, 2.5 or 3.5 mg/kg (s.c.) Daily, 3 days	Similar to original key findings. Increased Na+ and K+ secretion and mannitol/lactulose permeability
Miyazono *et al* [52]	2004	Adult Wistar rats (M)	20 mg/kg (i.v.) once	Induced oxidative stress. ROS preceded MPO increase. Assessed efficacy of NAC and tungsten.
Van't Land *et al* [53]	2004	Wag/Rij rats (M)	20 mg/kg (i.v.) Daily, 2 days	Reduction in small intestinal surface area, increased permeability, increase in GLP-2 receptor expression. Assessed the efficacy of Lactoferrin.
Clarke *et al* [54]	2006	DAR (F)	1.5 mg/kg (i.m.) Daily, 2 days	Similar to original key findings. Non-invasive SBT time-course reflective of *in vitro* damage. Assessed efficacy of folinate.
Tooley *et al* [55]	2006	DAR (F)	1.5 mg/kg (i.m.) Daily, 2 days	Similar to Clarke *et al* decrease in thymus weight, decrease in sucrase and MPO activity, SBT significantly correlated. Assessed the efficacy of *Streptococcus thermophilus*.

treatment of solid tumours and is known to elicit a severe mucositis, especially when administered in a regimen in combination with the drugs vincristine and cyclophosphamide. Relatively few reports of etoposide for the induction of mucositis in rodent models have appeared in the literature. A small study conducted by Johansson and colleagues (2001)[56] in male Lewis rats, reported a significant increase in intestinal permeability, as measured by ^{51}Cr-EDTA absorption, 4 and 48 hours after a single 100 mg/kg dose of etoposide. Additionally, a reduction of the adenylate pool (AMP, ADP and ATP) was evident 4 h post-etoposide administration, and remained at 48 h but had recovered at 72 h. The treatment induced diarrhoea, leucopaenia and increased the incidence of mortality. Interestingly, this study did not conduct a dose-response trial to ascertain the optimal etoposide dosing, nor did it make reference to any former research optimising etoposide dosing in this rat model. An extensive literature search revealed that no former studies had been conducted for etoposide dose optimisation. More recently, etoposide has been administered (100 mg/kg) in a Sprague-Dawley rat model of small intestinal damage.[57] The methods utilised in this study were invasive (implementing ligated intestinal loops), and reported that microvascular permeability increased by 4 h, and by 48 h induced a significant haematological toxicity, induced small intestinal permeability and inhibited stem cell proliferation.

Irinotecan hydrochloride, or CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin), is a semi-synthetic derivative of camptothecin and is a relatively new chemotherapy agent that is commonly used to treat colo-rectal cancer.[58] It has a wide spectrum of anti-tumour activity through inhibition of topoisomerase 1, a DNA enzyme. Its most potent symptom-related toxicity is diarrhoea, which is thought to be a two-phase phenomenon, in which the first phase is secretory

due to a cholinergic response, but the latter phase remains to be elucidated. Irinotecan-treated mice (100 mg/kg daily for three days) have been shown to have vacuolation in the ileum, associated with an increase in apoptosis and goblet cell hyperplasia, with a preferential differentiation for sulfomucin (caecum), six days after the initial injection.[4] More recently Gibson *et al* (2003)[36] described irinotecan-induced intestinal damage in a tumour-bearing (dark agouti specific mammary adenocarcinoma; DAMA) time-course in a DAR model. Irinotecan was found to increase villus atrophy and hypoplasia of the crypt in the small intestine, mirrored in the large intestine with the addition of crypt dilatation and mucus secretion. These changes in the crypt were observed at 6 and 24 h post administration with both 100 and 150 mg/kg (two daily doses) irinotecan. Reactive rebound hyperplasia in this repair phase was observed 72 and 96 h post treatment. A larger irinotecan dose of 200 mg/kg induced crypt damage which was more pronounced, with death occurring in all DAR by 96 h due to a perforated duodenum which led to peritonitis. These findings were further supported when anti-mucositis agents were assessed in an irinotecan-damage DAR model.[59,60]

1.3.3 Anthracycline Chemotherapy Agents

Anthraclycines are chemotherapy agents that interact with topoisomerase II. This alters the breakage-reunion process of the DNA sequence for this protein by stabilising topoisomerase II-DNA cleavage complex,[61] and are otherwise known as antineoplastic antibiotics. Examples of these agents are doxorubicin and bleomycin. There are a number of animal models that utilise the administration of doxorubicin to induce small intestinal mucositis.[61-65] Doxorubicin hydrocholoride (Andriamycin),[64] is used to treat solid tumours and often leads to the development of mucositis, which can be dose-

limiting and fatal.[64] Doxorubicin administration in rats and mice induces weight loss,[64] and apoptosis in the small intestine which is predominantly located in the crypts,[62] Sun and colleagues (1998)[61] confirmed these apoptotic changes, and described apoptosis as epithelial shrinkage, nuclear condensation around the nuclear membrane, organelle structure disappearance and vacuolised mitochondria. A trial conducted by Kimura *et al* (2001)[65] utilised a tumour-bearing mouse model (sarcoma), in which doxorubicin significantly reduced the tumour weight, small intestinal weight and small intestinal sucrase activity.

1.3.4 Alkylating Agents

Alkylating chemotherapy agents are derivatives of nitrogen mustard, and when administered are incorporated into the tissue permanently via the formation of covalent bonds. These drugs are also non-specific and induce damage in all rapidly dividing cells by transferring its alkyl group to the DNA, resulting in DNA strand breakage, leading to cell cycle arrest and apoptosis.[66] Examples of alkylating agents are cyclophosphamide and melphalan. Cyclophosphamide has been assessed in several animal model studies of mucositis. Cyclophosphamide suppresses the immune system,[67] increases the likelihood of bacterial translocation and sepsis,[68] induces mild-moderate mucositis; consisting of an increase in apoptotic bodies in the crypts.[69] Melphalan is a chemotherapeutic agent commonly used in bone marrow transplant patients. It also induces severe small intestinal mucosal damage in mouse models. Damage manifests as crypt cell irregularity, crypt disruption, villus blunting and necrosis, and often leads to mortality.[70]

1.4 METHODS FOR ASSESSING SMALL INTESTINAL FUNCTION

Since the small intestine is an extensive organ with inaccessible regions, determining its status of health has proved difficult.[71] This in turn has limited the development and evaluation of the efficacy of therapeutic interventions. Common techniques for assessing small intestinal function range from surgery, endoscopy or colonoscopy, small bowel biopsy, x-rays and the barium swallow. Whilst endoscopy and colonoscopy procedures are used regularly in clinical practice for diagnosis of gastrointestinal complaints, only information about the more proximal portions of the small intestine can be routinely assessed, posing a real problem in determining the true functionality of an individual's "entire" small intestine. The current "gold" standard technique for assessing small intestinal function remains the small bowel biopsy. This technique is inadequate for various reasons:[72] a) it is invasive; b) only assesses the proximal small intestine; c) requires sedation; d) is painful; e) is expensive and f) only reflects the function of the biopsied fraction of the small intestine.[73-75] Thus, an adequate technique that assesses small intestinal function of the entire SI, and is ultimately non-invasive, should be implemented to improve current clinical practice.

In cancer patients it is apparent that gut function is greatly compromised. Unfortunately, these patients not only develop mucositis but also low platelet and white blood cell counts. This highlights the increased risk of small bowel biopsy in these patients, resulting in GI bleeding and bacterial translocation, potentially leading to sepsis.[29] This again highlights the importance to develop a non-invasive bio-marker for small intestinal function.

1.4.1 Small Intestinal Permeability

Small intestinal permeability (SIP) tests have been developed to non-invasively determine barrier function.[76] Traditionally, intestinal function in many diseased states has been measured by absorption of xylose, which is passively absorbed in the jejunum.[77-80] However, this test has been shown to be quite variable and has not been adopted for routine assessment of small intestinal function.[78] Further advancements have led to the combination of disaccharide/monosaccharide sugar permeability tests in which man-made sugar probes are utilised. These are not metabolised by intestinal mammalian cells, only bacteria. Current literature describes methods utilising monosaccharides such as L-rhamnose[6,76,78,81-83] and mannitol,[84-87] and disaccharides such as lactulose,[6,76,78,85,87] and more recently, sucrose.[85,87-90] In general, sugar probes utilised in permeability tests are safe, reproducible, well tolerated and cost effective.

In the healthy gut there are two routes available for passive permeation across the intestinal epithelium: across the enterocyte (transcellular) or between enterocytes (paracellular). The monosaccharide rhamnose, and the disaccharide lactulose are non-metabolisable sugars. A reduced urinary rhamnose is thought to be indicative of an altered small intestinal surface area, whilst elevated urinary lactulose levels represents a loss of tight junctions between enterocytes (Figure 1.3). Current literature describes the utilisation of dual-sugar permeability tests in different disease settings such as inflammatory-induced enteropathy,[91] non-steroidal anti-inflammatory drugs (NSAIDs),[92-94] diarrhoeal disease [83] and chemotherapy-induced mucositis.[6,95,96]

The first reported dual-permeability test was performed by Menzies *et al.* (1979)[76], who examined abnormal sugar permeability in patients with villus atrophy and diagnosed coeliac disease. Five-hour urinary excretion of lactulose and L-rhamnose was determined after an overnight fast. These investigators found that urinary L-rhamnose excretion significantly decreased (40%, $p < 0.02$), which was suggested as a reflection of a decreased surface area in the small intestine. Lactulose excretion increased (340% $p < 0.01$) possibly due to the small intestinal mucosa becoming "leaky" (more permeable) to larger probe molecules. It was also found that the median value of lactulose/rhamnose (L/R) ratio was seven times higher in patients with coeliac disease compared to normals.

Impairment of gut function and small intestinal barrier integrity has previously been described using small intestinal permeability tests in patients with chemotherapy-induced mucositis.[6,95-97] These tests used a combined monosaccharide and disaccharide sugar drink to determine enteropathy and permeable tight junctions, respectively. Keefe *et al* (1997)[6] highlighted that adults undergoing high-dose chemotherapy have a significantly altered permeability seven days post-chemotherapy. Whilst this test is useful in the assessment of barrier function, it does not necessarily give a clear or sensitive indication of the small intestine's absorptive capacity or the level of damage.

1.4.2 Oro-Caecal Transit Time

Oro-caecal transit time (OCTT) is determined by measuring the rise in hydrogen excretion (H_2) in breath after ingestion of lactulose and was first validated by Bond & Levitt.[98]. The technique is based on the principle that colonic bacteria ferment the

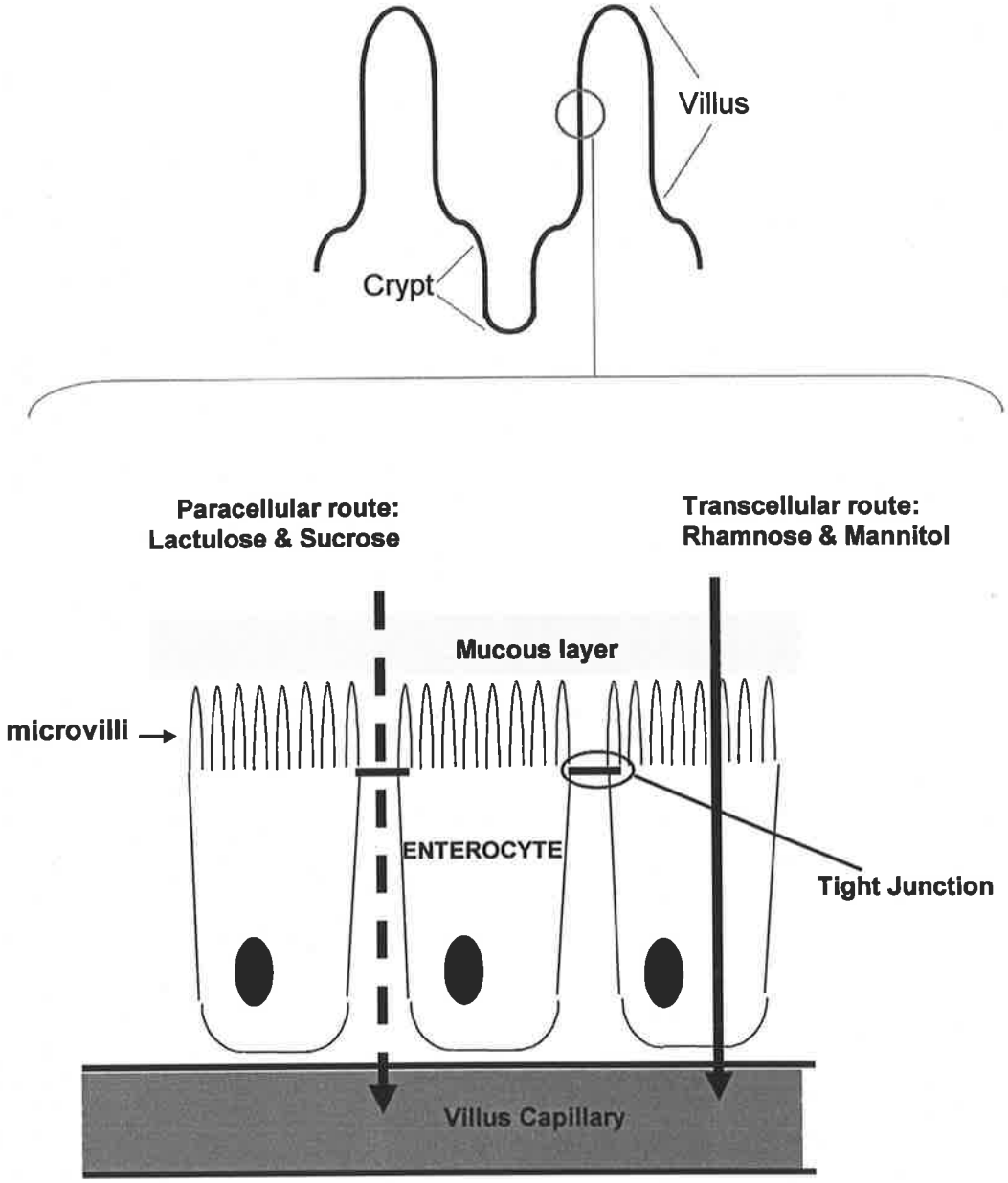

Figure 1.3: Depiction of the suggested transcellular (through the cell) and paracellular (in between cells) routes of permeation, where the transcellular route is determined by the small intestinal surface area, and tight junctions strictly regulate the paracellular pathway. The common substrates utilised for the assessment of transcellular routes are mannitol and rhamnose; paracellular: lactulose and sucrose.

synthetic sugar substrate to produce H_2, which is then expired in the breath. Lactulose is a synthetic disaccharide, comprising fructose and galactose, which is not digested by small intestinal brush-border enzymes.[99] Therefore the time between ingesting the substrate and the rise in H_2 is representative of oro-caecal transit time. In paediatric cancer patients to my knowledge OCTT has not previously been assessed.

1.4.3 Hydrogen Breath Test

The Hydrogen (H_2) Breath Test is the current non-invasive technique employed for detecting gastrointestinal damage via carbohydrate malabsorption. Here, clinicians observe the patient's ability to digest and/or absorb mono- and di- saccharides. The ingested sugar substrate, whether it be lactose, sucrose or fructose (common sugars assessed), in a healthy individual is digested and/or absorbed. Individuals who do not have the necessary enzyme present in the small intestine malabsorb the macro-nutrient. This is subsequently propelled towards the large intestine. The substrate, once in the colon, is metabolised to form H_2 due to the presence of H_2-producing bacteria. This gas then enters the bloodstream and is transported to the lungs where it is expired. Whilst this breath test is the test of choice for the malabsorption of sugars for clinicians, it does not give a clear representation of small intestinal damage, and secondly it relies solely on the presence of H_2-producing bacteria residing in the colon.[100] Approximately 20% of the population do not have these bacteria as part of their colonic micro-biota, and the growing use of antibiotics lessens the sensitivity of this test.[100]

1.4.4 ^{13}C-Sucrose Breath Test

A more direct method of assessing gastrointestinal damage than the H_2 breath test is via evaluating disaccharidase activity directly, as it has been shown in biopsies that mucosal damage is commonly accompanied by decreased brush-border enzyme activities.[75] The non-invasive detection of low-intestinal lactase activity in children was studied by Koetse *et al.* (1999), with the aid of a combined ^{13}C-Lactose $^{13}CO_2/H_2$ breath test. This study found that the combined $^{13}CO_2/H_2$ (lactose) breath test was preferable for diagnosis of gastrointestinal damage than the H_2 breath test alone. This suggested that in order to ensure reliable results in the future the combined $^{13}CO_2/H_2$ BT would be a more accurate and direct method for determining the digestive capability of the small intestine. However 80% of non-Caucasians exhibit an age-related low lactase activity,[100] indicating that the ^{13}C-lactose breath test is not a suitable marker of small intestinal damage. In comparison, sucrase stays relatively constant throughout life,[101] and only 0.2% of the population present with a genetic sucrase deficiency.[102] Thus, a breath test utilising ^{13}C-sucrose would be a more reliable and superior prognostic indicator of mucosal damage.

In the healthy individual, sucrose is a disaccharide broken down by sucrase, a brush-border enzyme, into its constituent monosaccharides, fructose and glucose. Subsequent hepatic metabolism of these products leads to the production of CO_2, which is excreted in the breath (Figure 1.4). Sucrose derived from cane sugar is naturally enriched with ^{13}C, therefore the resultant $^{13}CO_2$ can be detected and measured using Isotope Ratio Mass Spectrometer (IRMS) analysis.[100,103,104] ^{13}C-sucrose derived from cane sugar is naturally occurring, the level of ^{13}C enrichment is "selective", as not all carbon ions in the carbohydrate molecule are labelled with ^{13}C. Due to this the level of ^{13}C-enrichment

needs to be determined via ^{13}C-combustion prior to SBT use as this is vital for recovered ^{13}C % dose calcuations.[105] The level of $^{13}CO_2$ is detected by measuring the relative enrichment of ^{13}C to ^{12}C in the CO_2 expired after ingestion of ^{13}C-sucrose.[100] *In vivo* determinations of sucrase activity can be used as an indicator of not only digestive enzyme activity but also of brush border integrity and enterocyte differentiation, thus providing an indicator of small bowel function.[54,55,103]

As described previously, mucositis is associated with villus blunting and crypt disruption. This leads to a decrease in sucrase activity compared to the healthy small intestine.[54,55,103] The SBT was initially assessed in a Sprague-Dawley rat model of MTX-induced mucositis to determine the small intestinal digestive and absorptive capacity.[103] MTX-treated rats showed a significantly decreased cumulative output of $^{13}CO_2$ and diminished small intestinal sucrase activity when measured seven days after treatment compared to controls. There was a significant correlation between *in vitro* sucrase activity ($r^2 = 0.85$), and the degree of histological damage. In this initial model, gavage doses of 1 and 2 g/mL of ^{13}C-sucrose were assessed. This is a 100 or 200% saturated solution, which could induce a hyper-osmotic effect. However, lower doses and their ability to detect small intestinal damage remain to be assessed. Additionally, the original SBT studies [54,103] also utilised a crude form of data analysis, and have since been updated using ^{13}C gas analyses as described by Koetse *et al* (1999).[100] More recently the SBT has been used successfully to monitor the efficacy of oral folinic acid (in water)[54] and of *Streptococcus thermophilus*[55] as candidate anti-mucositis treatments in the DAR.

The SBT has recently been applied in a number of novel settings in our laboratory (All data in preparation for publication). These preliminary studies have assessed: healthy individuals and healthy young adults in a model of sucrase-isomaltase deficiency using the drug acarbose (a known sucrase-isomaltase inhibitor). The initial study in healthy individuals demonstrated that a dose of 20 g of ^{13}C-sucrose dissolved in 100 mL of water was a suitable dose for humans, as opposed to doses of 40 and 60 g.[106] These latter doses appeared to be saturating and malabsorption occurred as determined by the H_2 breath test. When incremental doses of acarbose® were used to non-competitively inhibit the digestion of sucrose, a dose-dependent SBT result was observed (manuscript in preparation).[107] However, its application in patients with cancer undergoing chemotherapy is yet to be assessed.

In clinical practice it is known that certain chemotherapy agents are likely to cause mucositis, however, there are occasions when patients develop mucositis in a cycle of chemotherapy when it is not anticipated. This is most likely due to the repeated administration of chemotherapy and/or heightened sensitivity of individual patients. However, due to the lack of a sensitive, reliable and non-invasive marker of small intestinal function, the occurrence of this phenomenon can only be discussed retrospectively. The non-invasive SBT would allow the easy and cost-effective monitoring of small intestinal function in oncology patients to improve clinical management. This effectively would then provide more detailed information on the aetiology of mucositis, and allow documentation of the small intestine's response to chemotherapeutic insult over time.

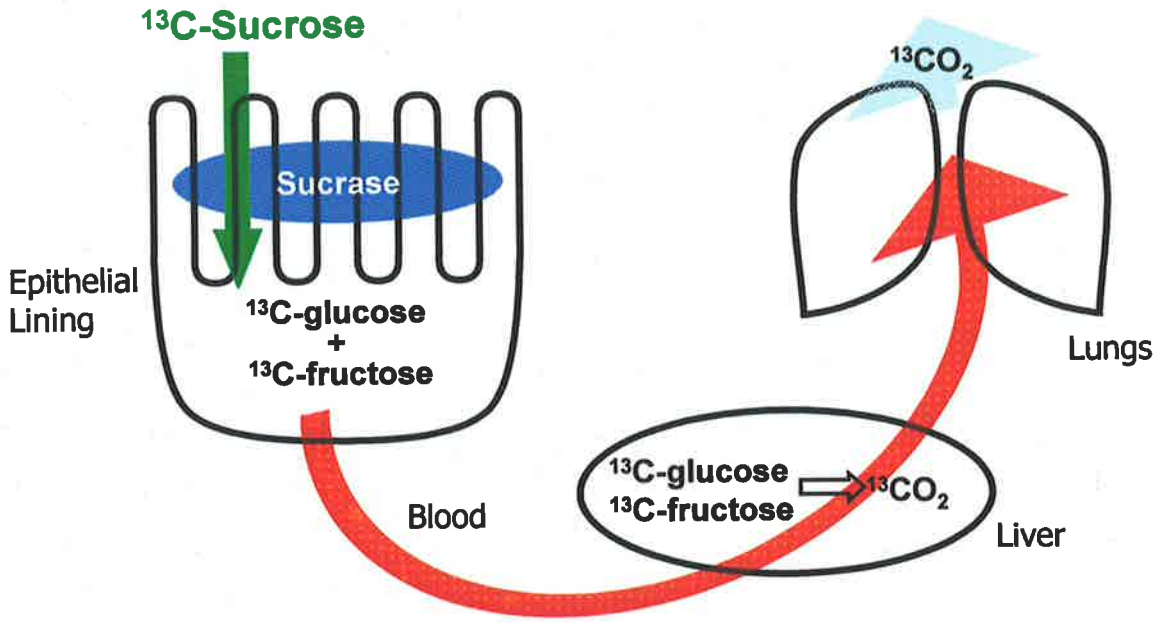

Figure 1.4: Illustration of the basic principles of the SBT in a healthy individual. The ingested ^{13}C-sucrose is broken down into glucose and fructose by the brush-border enzyme sucrase, these sugars are then transported to the liver, metabolised and the resultant $^{13}CO_2$ expired.

1.5 ANTI-MUCOSITIS THERAPIES

Whilst mucositis does not often lead to mortality, the associated symptoms are uncomfortable and painful, and often impair food intake, communication, sleep and mental status. The inability to swallow food, or indeed sometimes liquids, can result in dehydration, malnutrition and in many cases anorexia, where more drastic forms of energy delivery must be utilised such as naso-gastric feeds, or parenteral nutrition. In combination these measures lead to increased hospital stays, and affect quality of life. In the United States it has been estimated that each day a cancer patient is admitted for an unplanned hospital visit, it will cost on average US$5000/day. Despite this, an agent that ameliorates the development of chemotherapy-induced mucositis does not currently exist in clinical practice.

Research on the efficacy of treatment for mucositis has largely been confined to assessment of mucositis in the oral mucosa. As one means to reduce the severity of oral mucositis, basic oral care is adhered to in oncological practice, to reduce the level of oral bacteria flora, thus preventing infections and alleviate oral pain and bleeding.[108] Basic oral care includes: brushing, rinsing, flossing and the use of mouth moisturisers.[109] Cryotherapy has proved to be effective in the oral mucosa in patients receiving 5-Fluorouracil (5-FU). This phenomenon is thought to be due to the induction of vasoconstriction by cold temperature, thus reducing the capacity for the chemotherapeutic agent to induce damage to the oral mucosa.[110] Other alternative or natural treatments have been assessed in cancer patients, including: glutamine,[111-113] sucralfate,[114,115] and amifostine.[116]

More recently, the effects of mucositis in the small intestine have been researched more extensively. Interventions, either nutritional, such as by vitamin supplements[117] or pharmaceutical, such as by growth factors[118,119], are being assessed for efficacy in prevention or amelioration of mucositis. The proposal for the SBT to be employed as an index to monitor and optimise intervention in individual patients, and also to test the efficacy of candidate adjunctive therapies for the development of small intestinal mucositis, could prove highly beneficial for the patient. Potential gut mucositis treatments should be investigated in a tumour bearing animal model of mucositis to rule out the possibilities of enhanced tumour survival, before their implementation in phase II trials. [48]

Since the aetiology of small intestinal mucositis has become enhanced due to the emergence of the 5-phase model of mucositis, it is becoming increasingly apparent that to combat the development of mucositis, it is likely to require the administration of several agents acting through different mechanisms. Indeed, due to the multi-factorial damage (ROS, cytokines, inflammation) that occurs due to chemotherapy, it is thought that not just one agent will be the "magic bullet" to prevent the development of mucositis. It is likely that it will require several agents with different modes of action, such as the induction of growth, anti-inflammation and repair. However, it is important to remember that since the concept of the 5-phase model of mucositis occurs according to a specific time-line, that the administration of anti-mucositis agents will also need to follow this time-line. By doing this it is more likely to ensure maximal protection or indeed prevention for the small intestine from chemotherapy toxicity.

1.5.1 Growth Factors and Mucositis

In the healthy individual, growth factors and cytokines are produced and released by cells to communicate within the individual cell or between cells. When bound to a receptor located on the outer membrane of the target cell the growth factor triggers a variety of internal changes, such as initiating signalling pathways and protein generation. Growth factors are proteins that usually induce proliferation and growth in a multi-cellular capacity. It must be noted however that some growth factors, such as TGF-β, actually inhibit epithelial proliferation. Over the past ten years, research on many classes of growth factors and the amelioration of chemotherapy-induced small intestinal mucositis have identified growth factors including: keratinocyte growth factor (Palifermin™, Amgen),[48,60,118,120,121] epidermal growth factor[119] and whey growth factor extract.[38,42,49]

1.5.1.1 Keratinocyte Growth Factor

Keratinocyte growth factor (KGF) has become a high profile treatment for the amelioration of chemotherapy-induced mucositis in selected haematological malignancies. This growth factor is a member of the fibroblast growth factor family and is secreted by mesenchymal cells. This super-family of growth factors has the ability to induce proliferation in almost all non-inflammatory cells of the mucosa. Specifically, it has been suggested that KGF is important for epithelial cell signalling, and the stimulation of proliferation to epithelial cells.[120] These early studies have shown KGF to increase villus height and crypt depth and overall crypt cell survival (increasing mucosal thickness).[120,122] It has also been demonstrated that KGF reduces the loss in bodyweight

and decreases the incidence of mortality, while importantly maintaining tumour kill in mice.[122]

Meropol and colleagues [123] utilised KGF in a randomised phase I trial in patients with metastatic colorectal cancer and demonstrated that KGF was well tolerated when administered intravenously and that it lowered the incidence of grade 2 – 4 mucositis. Subsequently, a placebo-controlled, double-blind, phase III trial was performed in which KGF was administered for three days prior to treatment in patients undergoing autologous haematopoietic stem-cell transplantation.[124] This study showed that KGF was effective in reducing the duration and severity of oral mucositis in this specific group of patients with haematological cancer, who were receiving high dose chemotherapy and total body radiation therapy.[123] In light of these findings, a recommendation was given for its exclusive use in patients with haematological malignancies for clinical practice.[108]It is important to note here that this recommendation was made for oral mucositis only, and that its efficacy in the small intestine has not yet been investigated, presumably due to the inability to monitor the severity of mucositis in this region.

Whilst many studies show KGF to be a promising treatment in the amelioration of chemotherapy-induced small intestinal mucositis,[118,120-122,125] some publications have described heightened damage by KGF in the small intestine.[48,60] One study showed that while KGF, when administered at 3 mg/kg daily for 5 days, increased small intestinal weights in the DAR, yet increased apoptosis in the intestinal crypts and in the tumour itself contradicting epithelial healing.[48] Importantly, these findings suggest that for an increase in the tumour apoptotic index to occur, the tumour was likely to be expressing

the KGF receptor, however this was not discussed. This highlights a major potential problem of the promotion of tumour growth. In a further study KGF was administered to mice prior to radiotherapy and no positive effects were observed to the GIT epithelium.[126] However, a recent study by Gibson *et al* (2005) [60] demonstrated that when a single bolus of KGF was administered three days prior to the chemotherapy agent irinotecan in the tumour-bearing DAR, it reduced the second phase (96 h) of apoptosis and reduced diarrhoea and mortality. This study highlights the importance of the timing and duration of KGF delivery with respect to chemotherapy administration, and its ability to reduce the severity of small intestinal complications.

1.5.1.2 Insulin-Like Growth Factor-1

Insulin-like growth factor-1 (IGF-1) has been shown to be a potent mitogen for the gut *in vivo*.[127] The GIT has been documented to be one of the most sensitive sites for IGF-1 and its induced trophic properties in healthy animals.[128] IGF-1 has also been linked to trophic effects in rats with short bowel syndrome.[129] In rodents with MTX-induced mucosal damage, IGF-1 has been shown to stimulate repair and recovery, specifically in the distal small intestine, but did not prevent damage in the proximal small intestine which is the site of maximal toxicity.[43] A recent study performed by Cool *et al* (2005)[39] showed IGF-1 minimised the level of damage via changes in apoptosis, villus height and crypt depth, leading to a decrease in small intestinal length and weight 48 h after 5-FU treatment. However, IGF-1 was not able to prevent small intestinal damage associated with 5-FU administration. Moreover, there are indicators that administration of IGF-I coincident with chemotherapy could actually exacerbate small intestinal damage.

1.5.1.3 Epidermal Growth Factor

Epidermal growth factor (EGF) is a potent peptide that enhances proliferation and differentiation of epithelial cells. EGF is present in body fluids, such as colostrum and milk.[94,130-132] Few studies have addressed the effects of EGF alone in the gut. One study has reported that the administration of EGF in neonatal rats reduced the development of necrotising enterocolitis.[133] Only one study has described the effects of EGF in the small intestine of a transgenic mouse model that did not express EGF receptors.[119] These investigators found that EGF or the respective EGF receptor ligands, appeared to contribute to the prevention of repair and intestinal damage associated with chemotherapy damage. Similarly, a study by Xian and colleagues (2002)[47] demonstrated in TGF-α knock-out mice that TGF-α and its related ligands, an EGF-related ligand, play an essential role in the repair responses associated with MTX-induced mucosal damage. No further studies have assessed the efficacy of EGF and its role in chemotherapy-induced mucositis models.

1.5.1.4 Whey-Derived Growth Factor Extracts

Milk is a rich and natural source of growth factors with the potential to enhance growth and repair in the gut. Bovine milk has been shown to contain high concentrations of IGF (I and II). Milk contains an array of growth factors that can be extracted by cation-exchange chromatography from an extract of bovine cheese whey, a by-product of the cheese-making process. This whey growth factor extract, or WGFE as it is commonly known, is a biologically active extract containing naturally occurring whey proteins such as: TGF-β, fibroblast growth factor, insulin-like growth factor and platelet-derived growth factor. The combination and high levels of these naturally occurring proteins has

shown promise in the amelioration of chemotherapy-induced mucositis.[42] Administration of WGFE to rats with MTX-induced small intestinal damage, increased villus length in the jejunum and ileum and also increased crypt depth in the jejunum and the incidence of bacterial translocation was greatly diminished.[42] However, WGFE did not prevent the loss of jejunal sucrase activity nor did it completely prevent villus atrophy in the duodenum and jejunum. Moreover, in a hamster model of chemotherapy-induced oral mucositis, WGFE applied topically reduced the severity of oral mucositis but did not prevent the development of the ulcerative lesions.[38]

1.5.2 Glutamine and Mucositis

To sustain the rapid proliferating nature of the gastrointestinal epithelial cells a large source of energy is required. Glutamine, is the major fuel source for the GIT, and is absorbed from both the basal and luminal side of the epithelium. Nearly all luminal glutamine is absorbed and metabolised, and circulating glutamine is also extracted and used.[134] Orally administered glutamine, versus enterally fed glutamine, has been shown to be effective at reducing mucosal damage and subsequent bacterial translocation associated with MTX-induced enterocolitis.[135] In recent times this amino acid has been a popular treatment modality in patients undergoing bone marrow transplantation. These patients commonly require parenteral feeds, or enteral feeds through naso-gastric tubes or by the more invasive method of a percutaneous endoscopic gastrostomy (PEG).[136] Incorporation of glutamine via these methods of delivery, allows easy administration to the small intestinal luminal environment. Glutamine administered topically, by swishing the solution around the mouth followed by swallowing the solution, has reduced the duration and severity of oral mucositis caused by chemotherapy or radiation

therapy.[111,113] Other studies have revealed that parenteral glutamine supplementation improved lymphocyte recovery and decreased the severity score of oral mucositis in both adults[112] and children.[137]

1.5.3 Zinc and Mucositis

Oral zinc supplementation has been shown to reduce the duration and severity of diarrhoea in children living in third world countries[138] and also to reduce intestinal permeability.[139] A single study has assessed the efficacy of zinc in relation to MTX-induced mucositis and it was performed in combination with WGFE treatment in male Sprague-Dawley rat.[49] Tran and colleagues[49] demonstrated that oral administration of zinc alone significantly reduced intestinal permeability on days 3 and 6. In contrast, when zinc was administered in concert with WGFE it reduced damage on day 5 and enhanced mucosal recovery and repair. However, prevention of an increased intestinal permeability did not occur.

1.5.4 Folinic Acid (Folinate)

Folate represents an important vitamin, required in many metabolic pathways, especially purine and pyrimidine biosynthesis (DNA and RNA). On average, milk and dairy products provide 10% to 15% of the daily folate intake in many Western countries, especially among the younger population. A dietary intake of 400μg folate/day has now been suggested as optimal for healthy individuals.[140] MTX and 5-FU, belong to a family of anti-cancer drugs known as the anti-metabolites. These drugs exert their cytotoxic effect through an inhibition of folate metabolism by a down-

regulatory effect on the enzyme dihydrofolate reductase resulting in an inhibition of DNA synthesis.[32] The magnitude of folate depletion in patients receiving these agents is such that folate repletion is mandatory within a 24-hour period following chemotherapy, in order for DNA synthesis to be reactivated. Folinic acid is utilised as the source of folate in this scenario, in a life-saving procedure known as 'folinic acid rescue' or 'folinic acid salvage'.[33] This only minimally ameliorates intestinal toxicity as the folinate is not delivered specifically to the site of need. Unfortunately, systemic administration of folate during the folinic rescue process also has the undesirable effect of restoring DNA synthesis, re-establishing cell proliferation in malignant tissue. Recently, an experimental study of folinic acid administered orally to dark agouti rats (DAR) with MTX-induced small intestinal mucositis was conducted.[54] This study demonstrated that immediate access to folinic acid administered orally *ad libitum* in the rodent's drinking water after the first MTX injection protected the small intestine from damage as assessed by the SBT and this was supported by histological analysis and total sucrase activity in mucosal tissue. This study demonstrated that the SBT could non-invasively determine altered function in the small intestine and could be used as a biomarker to determine the efficacy of candidate treatments.[54]

1.6 EMERGING ANTI-MUCOSITIS THERAPIES

1.6.1 Probiotics

Recently it has become more apparent that commensal bacteria play an important role in maintaining intestinal barrier function[141,142] and probiotics are emerging as viable

alternatives to the use of synthetic substances in nutrition and medicine. Probiotic bacteria, such as *lactobacilli* and *bifidobacteria* have been demonstrated to enhance intestinal epithelial barrier function and to have preventative or therapeutic potential in human disease conditions usually associated with pathogenic invasion.[143] Although preventing adherence of pathogens to the mucosal surface is an important characteristic of many probiotics,[144] a broad range of other physiological effects have been described. These include enhanced phagocytosis, modulation of proliferative activity, non-specific immune stimulation following induction of pro-inflammatory cytokines, in addition to specific immune responses including IgA responses.[141] Evidence is accumulating for an active role for probiotics in prevention of antibiotic-induced diarrhoea with the concomitant administration of either *Lactobacillus GG* or *Saccharomyces boulardii*.[144] Preliminary studies in our laboratory have revealed improvements in sucrase activity and intestinal permeability in rats with chemotherapy-induced small bowel mucositis following ingestion of commercially available yoghurts containing probiotics (Manuscript in preparation). In this study MTX-treated rats receiving yoghurt derived from sheep milk showed a significant decrease in the lactulose/mannitol ratio (small intestinal permeability) on Day +3 (59%) and Day +6 (52%), and minimised the loss of sucrase and lactase activity compared to MTX-treated rats.

In more recent times, *Streptococcus thermophilus* has been assessed for its ability to ameliorate damage in various gastrointestinal diseased settings. Whilst *S. thermophilus* is not yet considered a probiotic, studies have demonstrated promising results in its ability to attenuate the severity of small intestinal damage. Specifically *S. thermophilus* has also shown to interact with epithelial cells *in vitro* to protect from pathogenic bacteria and to improve barrier function.[145] *S. thermophilus* has been shown to decrease

NFκB levels, increase anti-inflammatory properties (TNF-α), reinforce barrier function and stimulate a Th1 immune response.[146,147] In clinical studies *S. thermophilus* administered in combination with other designated probiotics, such as the previously reported VSL#3 containing four Lactobacilli, three Bifidobacteria and a Streptococcus salivarius species, as a treatment for gastrointestinal diseases. *S. thermophilus* has demonstrated capabilities of (1) reducing the incidence and severity of diarrhoea in infants when combined with *Bifidobacterium breve*;[148,149] (2) protecting the GIT epithelium from *Escherichia coli* infection when combined with *Lactobacillus acidophilus*;[145] and (3) as a preventative treatment for the post-operative occurrence of ulcerative colitis.[150] *S. thermophilus* has also shown promise in alleviating inflammation in gut cells *in vitro*.[147,151]

There are relatively few reports describing the ability of probiotic bacteria to synthesise folate. Milk is not a rich source of dietary folate. However, many dairy products are processed using microbial fermentation in which folate can be synthesised. A recent *in vitro* study by Crittenden *et al*[152] has described how bacterial strains used in fermented dairy products can affect folate levels. It was also reported that candidate combinations of organisms (*B. animalis* CSCC 1941 and *S. thermophilus* CSCC 2000) provided the greatest increase in folate levels during the fermentation process. Of the organisms examined, the *Streptococcus thermophilus* isolates increased the folate levels in skim milk approximately four-fold. Probiotic *Bifidobacterium* isolates all synthesised folate during growth in skim milk with *Bifidobacterium breve* 5181 produced the greatest increase in folate levels with *B. animalis* and *B. lactis* isolates doubling folate concentration. Folate production by the bacteria in mixed culture fermentations appeared to be additive with a mixed culture fermentation using two folate-producing

organisms (*B. animalis* CSCC 1941 and *S. thermophilus* CSCC 2000) increasing folate concentration more than six fold.

1.6.2 Lyprinol

It is well known that mucosal inflammation plays a significant role in the development of mucositis, as outlined in the five-phase model of damage and repair.[27] Leukotriene B4 and prostaglandin E2 are important inflammatory mediators that act to amplify signalling cascades, induce apoptosis and cause further tissue damage due to the production of reactive oxygen species.[153-155] Long-chain omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have been shown to exert beneficial effects in acute and chronic inflammatory conditions. Specifically, ω-3 PUFAs act to reduce inflammation by decreasing the production of inflammatory eicosanoids, cytokines, reactive oxygen species and the expression of adhesion molecules.[156] It has been demonstrated that ω-3 PUFAs occur naturally at high levels in marine oils.[157] Lipid extracts from the New Zealand green-lipped mussel (*Perna canaliculus*), commercially known as Lyprinol®, are rich in eicosapentaenoic acid (EPA), docosahexanoic acid (DHA) and ω-3 PUFAs. This extract has been shown to down-regulate inflammation by the inhibition of the 5'-lipoxygenase and cyclo-oxygenase pathways.[158]

Initially, the New Zealand green-lipped mussel demonstrated capabilities of reducing chronic inflammation associated with arthritis.[159-161]. More recently Lyprinol has been linked to having beneficial effects in: (1) patients with atopic asthma,[156] (2) a rat model of dysmenhorrea,[162] and (3) a mouse model of inflammatory bowel disease.[163] Moreover, Lyprinol does not induce any deleterious inflammatory processes in the

healthy individual.[164] Its application in chemotherapy-induced mucositis however, has not been assessed. Currently, the effects of Lyprinol are being assessed in our laboratory since chemotherapy induces a high degree of acute inflammation (manuscript in preparation).[153]

1.7 SUMMARY

Whilst many advances have been made in the effort to treat chemotherapy-induced small intestinal mucositis, applying these treatments in a clinical setting has been hampered by the lack of a clinical biomarker that sensitively assesses intestinal damage and indeed the efficacy of a proposed product. It is in these instances that the SBT could potentially be applied. The studies reported in this thesis aimed to delineate models of small intestinal damage in rodents using a cross-section of clinically relevant chemotherapy agents. The project further sought to assess a potential natural anti-mucositis treatment, *S. thermophilus*, utilising the non-invasive SBT as a biomarker for assessing small intestinal function. A number of clinical trials have been conducted to assess the efficacy of anti-mucositis agents. However, these studies have largely been confined to protection of the oral mucosa, primarily because of the unavailability of a suitable and reliable endpoint marker.

This project has ventured further into understanding the aetiology of mucositis by assessing small intestinal function in paediatric cancer patients in one, or multiple cycle(s) of chemotherapy. It is anticipated that the SBT will be successfully applied to paediatric cancer patients, to assess chemotherapy-induced small intestinal damage. The SBT could be used as the primary endpoint to determine the efficacy of potential anti-mucositis agents. This project was conducted to further mucositis research, with the intention of enhancing treatment protocols of cancer patients undergoing regular chemotherapy treatment.

1.8 PhD Study Aims:

Assessment of the Sucrose Breath Test in rats

1. To establish an appropriate ^{13}C-sucrose dose for the SBT in rats.
2. To establish the time-course of small intestinal damage and repair in rats using the SBT.
3. To develop new chemotherapy-induced mucositis models in the DAR utilising the SBT as the small intestinal bio-marker.
4. To assess the suitability of the SBT for the screening of potential anti-mucositis agents.
5. To assess the potential for *Streptococcus thermophilus* to attenuate MTX-induced mucositis in the rat.
6. To assess the potential for *Streptococcus thermophilus* to attenuate MTX-induced mucositis in the tumour-bearing rat.

Assessment of the Sucrose Breath Test in paediatric cancer patients

7. To utilise the SBT in a cohort of paediatric cancer patients to determine its potential to detect small intestinal mucositis within a cycle of chemotherapy.
8. To monitor small intestinal function longitudinally in paediatric cancer patients undergoing multiple regimens of chemotherapy.

PART II: NON-INVASIVE ASSESSMENT OF SMALL INTESTINAL FUNCTION IN RATS

CHAPTER 2: DETERMINATION OF THE OPTIMAL DOSE OF SUCROSE FOR APPLICATION OF THE SBT TO THE DARK AGOUTI RAT

2.1 INTRODUCTION

The aim of this study was to re-establish the correct dosing of ^{13}C-sucrose for the SBT utilising appropriate analytical techniques in female dark agouti rats (DAR) with MTX-induced mucositis. The ^{13}C-sucrose breath test was recently developed in our laboratory as a means of assessing the overall functional and absorptive capacity of the small intestine.[54,103] Pelton *et al* (2004)[103] demonstrated that rats treated with MTX had a significantly depressed SBT result when compared to controls seven days post treatment, which significantly correlated with *in vitro* sucrase activity (r^2 = 0.85). However, in the original breath test design, rats were gavaged with 1g/mL of sucrose solution, and further preliminary studies investigated a 2.0 g/mL sucrose solution, resulting in the gavage suspension being 100 or 200 % saturated with sucrose. Such a dose could have resulted in a hyperosmotic load to the small intestine, whereby the concentration of sucrose would accelerate luminal motility and the substrate would reach the colon without allowing the sugar to be in contact with the enzyme for a sufficient period of time. The aim of this study was to assess doses of sucrose below 1 g/mL for the ^{13}C-sucrose breath test in female dark agouti rats (DAR).

2.2 MATERIALS & METHODS

The following study was approved by the Animal Ethics Committee of the Women's and Children's Hospital and the University of Adelaide, Australia, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (1997).

Dose response study trial design

Female DAR (n = 20), mean weight 144 ± 1.5g, were acquired from the Institute of Medical and Veterinary Sciences (IMVS), Gilles Plains, Adelaide, Australia. Animals were grouped and housed in standard rat cages with an environmental temperature of 25°C with a 12 h light:dark cycle for the duration of the study. When animals entered the final phase of testing they were injected with either saline or MTX, each animal was individually housed in Tecniplast® metabolism cages for the remaining duration of the study. Upon arrival Rats were allowed 24 h for acclimatisation before being placed on a daily diet of 18 % Casein[127] with free access to water for the duration of the protocol. Additionally, when rats were moved to Tecniplast™ metabolism cages, a further 24 h was provided for acclimatisation. It has been noted previously that sucrase may be an inducible enzyme in rodents. For this reason, rats had continual access to a casein-based diet containing sucrose as soon as the acclimatisation period was completed as regular rat chow does not contain the ingredient sucrose. Rats were required to have consumed 18 % casein diet[127] for a minimum of four days prior to any SBT being performed.

PHASE 1: Initially four DAR were used to determine if the previously used sucrose dose of 1g/ml of water was optimal for the SBT. It was thought that this dose was too high as it equated to a hundred percent of equalled water volume, i.e. saturating, thus potentially causing variable results. In phase 1, doses of 0.1, 0.25, 0.50, 0.75 and 1.0 g/ml were examined. The SBT was performed every second day. To ensure that a lower sucrose dose could detect MTX-induced damage, the same four rats were injected intramuscularly with MTX (1.5 mg/kg) at time-point 0 and 24 h, and killed 96 h after the first injection,[46] where rats were gavaged with a dose of 0.25 g/mL of sucrose for the SBT. The SBT was performed at -24, 24 and 96 h after the initial MTX injection. The doses observed in phase 1 warranted further investigation as it illustrated that lower doses of sucrose would be optimal for SBT (Figures 2.3 and 2.4).

PHASE 2: A larger and more comprehensive dose-response study was performed using 16 female rats. Each rat was assessed receiving doses of 0.1, 0.25, 0.75, 1.0 and 1.5 g/ml of sucrose for the SBT. Breath tests were performed at least three days apart to ensure no carry-over of high dose sucrose to the next performed SBT. Rats were not sacrificed, and were used for phase 3 studies.

PHASE 3: The former 16 rats performed reproducibility studies at ^{13}C-sucrose doses of 0.25 and 0.75 g/ml. Rats performed three SBTs with four days between each test. To determine which dose was more sensitive at detecting MTX-induced small intestinal damage, these animals were subjected to further testing. Rats were placed in metabolism cages™. Seven days after completion of the second phase dose-response study, the rats received 1.5 mg/kg MTX intramuscularly (i.m.) at 0 h and 24 h, approximately 1130 h to induce mucositis as described previously.[46] Small intestinal function was

monitored/assessed non-invasively using the SBT at –24, 24 and 96 h. Weights, fluid and diet intake, and faecal and urine output were recorded daily. Rats were sacrificed 96 h after the first MTX injection.

^{13}C-Sucrose breath test

Following an overnight fast, rats were gavaged with 0.1, 0.25, 0.5, 0.75, 1.0 or 1.5 g/mL of sucrose dissolved in deionised water. In the initial stage of the dose-response study, the SBT was performed every two to three days, with the assessment of sucrose doses of 0.1, 0.25, 0.5, 0.75 and 1.0 g/mL. In the second phase (n = 16), the SBT was performed every three to five days, with sucrose doses of 0.1, 0.25, 0.75, 1.0 and 1.5 g/mL being assessed.

Breath collection was performed by placing rats in a sealed 600 mL Perspex container, allowing breath to accumulate for 2 min, drawing 20 – 30 mL into a syringe attached to a two-way tap on the lid of the breath chamber (Figure 2.1). Breath collection is simple and cost-effective, and multiple rodents can be assessed simultaneously (Figure 2.2). Breath samples were then injected into 2 x 10 mL pre-evacuated glass tubes (Exetainer, Labco, High Wycombe, England) for $^{13}CO_2$ analysis. The chamber was then opened to allow the return of room air. Immediately thereafter, rats were oro-gastrically gavaged with 1 mL of the respective dose of sucrose (AnalaR, BDH, MERCK, Pty. Ltd., Victoria, Australia) solution.[103]

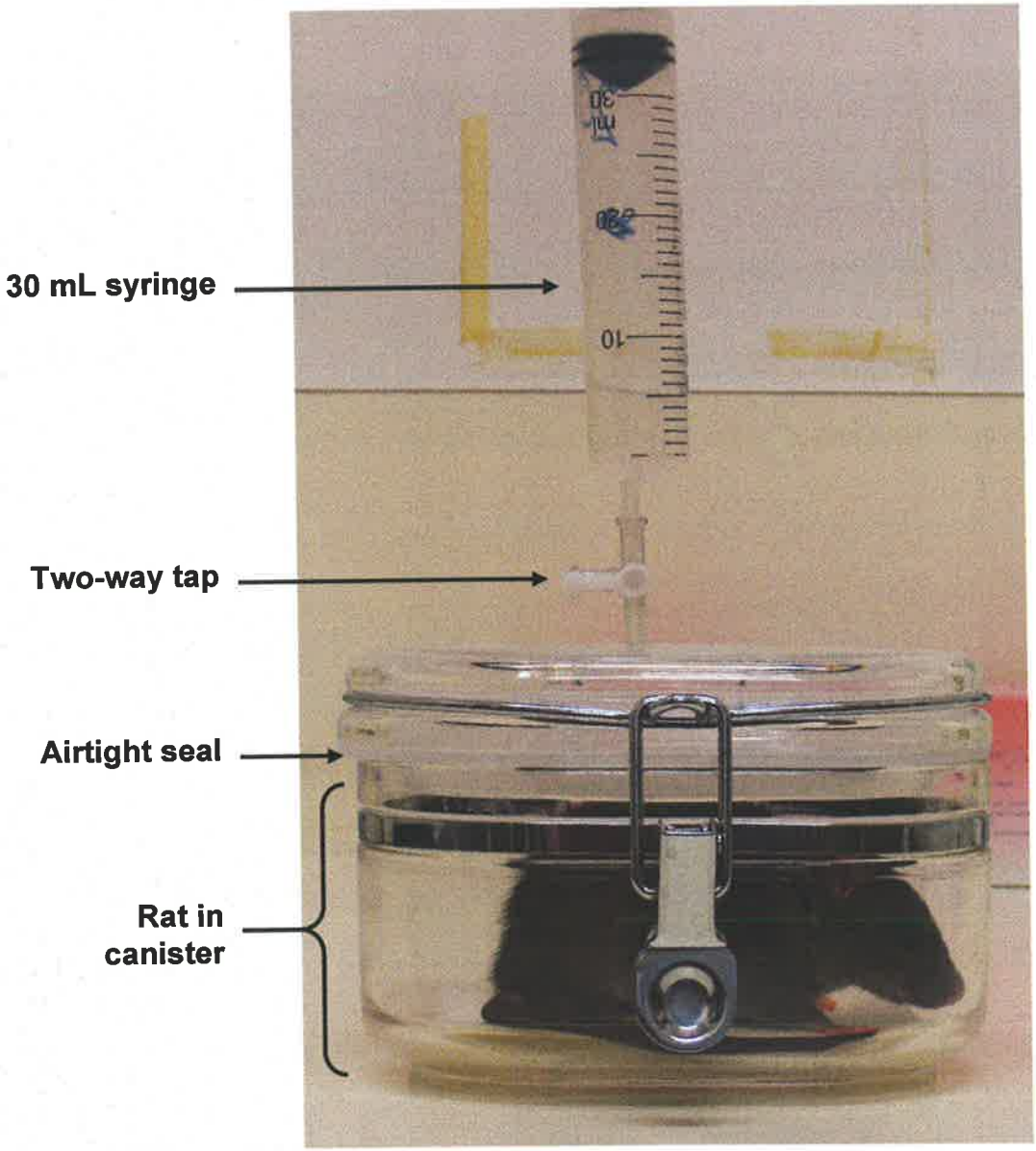

Figure 2.1: SBT breath collection for rats. Apparatus set-up for single rat, including a 600 mL Perspex chamber (sealable when clasp fastened), a two-way tap inserted into the chamber lid with the attachment of a 30 mL syringe for breath extraction.

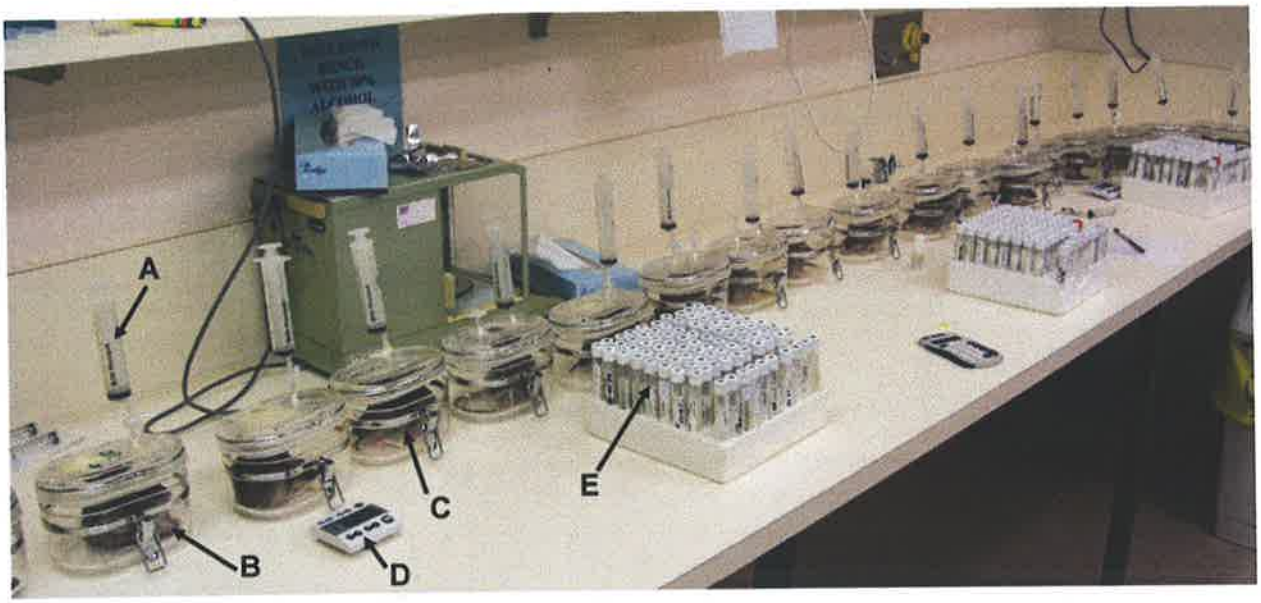

Figure 2.2: SBT apparatus set up for breath collection of multiple rats. Note (A) syringe, (B) perspex container, (C) rat, (D) timer and (E) evacuated 10 mL glass tubes.

Breath samples were collected at 15 min time intervals (as described above) for the following 120 min. Breath $^{13}CO_2$ samples (10 mL) were analysed using an isotope ratio mass spectrometer (IRMS; Europa Scientific, ABCA 20/20, Crewe, UK) equipped with a V410 data collection system. Results from the IRMS were expressed as a delta value, representative of the $^{13}C/^{12}C$ ratio in the sample relative to the internal standard of calcium carbonate (Pee Dee Belemnite Limestone, South Carolina, USA). Raw data was expressed as parts per thousand.

$^{13}CO_2$ data was previously expressed as delta over baseline, or area under the curve.[103] Recent literature for ^{13}C breath tests expresses $^{13}CO_2$ data as the % ^{13}C dose/hour recovered or the percentage cumulative dose of ^{13}C (%CD; see below for formulae).[105] Percentage Cumulative Dose of ^{13}C is defined as follows:

$$\textbf{\% }^{13}\textbf{C cumulative dose}_{ti+1} = \%\ ^{13}C \text{ cumul. Dose}_{ti} + \left(\frac{\%\ ^{13}C \text{ dose}_{ti} + \%\ ^{13}C \text{ dose}_{ti+1}}{2}\right) \text{ x } 1/n$$

n = number of samples/hr

ti = time i

Complete $^{13}CO_2$ analysis (not all formulae shown) considers the contribution of body weight, the amount of ^{13}C dose recovered over 90 min,[165] and CO_2 production rate which was approximated as 17 mL/kg/min for an adult female DAR.[166] Small intestinal sucrase activity, or villus damage, of the entire small intestine was assessed, therefore the cumulative breath $^{13}CO_2$ analysis up to 90 min was used for the SBT since this represented a time point when small intestinal transit was complete ($\%CD_{90}$).

Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) as all data displayed a normal Gaussian distribution (Kolmogorov-Smirnov test). A one-way analysis of variance (ANOVA) with a Tukey's *post-hoc* test was used for the comparison of different doses of ^{13}C-Sucrose for the SBT. The coefficient of variation (CV) was calculated for each dose to determine the intra- and inter-individual variability. A one-way ANOVA with repeated measures was used in order to evaluate reproducibility. $P < 0.05$ was considered significant. All data and statistical analyses were performed using GraphPad Prism version 3.00 for windows® (GraphPad Software, San Diego, CA, USA) or Microsoft Office 2003 Excel® for Microsoft WindowsXP.

2.3 RESULTS

Phase 1 dose-response

Naturally enriched ^{13}C-sucrose doses of 0.1 and 0.25 g/mL had reached their peak excretion and $^{13}CO_2$ levels (expressed as % ^{13}C dose/hr) were returning to baseline, however not completed at t = 120 min. In contrast, $^{13}CO_2$ levels at sucrose doses of 0.5, 0.75 and 1.0 g/mL were continuing to increase at $t \geq 90$ min, indicating that these doses could have been too saturating, thus affecting small intestinal transit/motility (Figure 2.3A). When $\%CD_{90}$ data were assessed (Figure 2.3B) a sucrose dose of 0.1 g/mL was significantly higher compared to all other doses ($p < 0.05$), and 0.25 g/mL was significantly higher compared to 0.75 and 1.0 g/mL ($p < 0.01$). No other significant differences were evident between sucrose doses. In a MTX model of mucositis, 0.25

g/mL of naturally enriched ^{13}C-sucrose was capable of detecting alterations in sucrase activity that has previously been associated with MTX-induced small intestinal mucositis (Figure 2.4). Twenty-four hours after the initial MTX injection DAR had a significantly elevated SBT ($p < 0.01$) compared to baseline levels (-24 h), and by 96 h had a significantly decreased ($p < 0.01$) SBT result compared to baseline (Figure 2.4)

Phase 2 dose-response trial

The percentage ^{13}C cumulative dose recovered is presented in Figure 2.5A. Even though a larger number of rats were assessed, the sucrose doses of 0.1 and 0.25 g/mL had reached their peak excretion and were returning towards baseline after 90 minutes had elapsed (Figures 2.5B and 2.5C). Sucrose doses of 0.75, 1.0 and 1.5 g/mL $^{13}CO_2$ levels were still rising after 90 min (Figures 2.5B and 2.5C). The average $\%CD_{90}$ was 13.73 ± 1.11, 14.14 ± 0.72, 6.28 ± 0.29, 3.38 ± 0.21, and 2.57 ± 0.17 for 0.1, 0.25, 0.75, 1.0 and 1.5 g/mL, respectively, and the coefficient of variation for the respective doses was: 32%, 20%, 19%, 25% and 26% (Figure 2.6). $\%CD_{90}$ levels for sucrose doses 0.1 and 0.25 g/mL were significantly ($p < 0.001$) higher compared to sucrose doses of 0.75, 1.0 and 1.5 g/mL. Additionally, 1.0 and 1.5 g/mL sucrose were significantly ($p < 0.05$) lower compared to 0.75 g/mL.

Phase 3: SBT reproducibility and detection of mucositis

SBT reproducibility of three tests performed four days apart showed that a dose of 0.25 g/mL was not significantly different compared to each other (Figure 2.7). In contrast, test 1 and test 2 SBTs were not significantly different for a sucrose dose of 0.75 g/mL, but test 3 was significantly ($p < 0.05$) higher compared to test 1.

Rats receiving a sucrose dose of 0.25 or 0.75 g/mL saline treated animals did not have significantly different at 96 h compared to baseline and 24 h post-MTX ($p > 0.05$). Ninety-six hours post MTX-injection rats had a significantly lower SBT compared to baseline and 24 h ($p < 0.05$) when a sucrose dose of 0.25 g/mL was utilised. MTX-treated rats receiving 0.75 g/mL sucrose for the SBT were significantly higher at 24 h compared to baseline levels ($p < 0.001$). Additionally, at 96 h post MTX rats receiving 0.75 g/mL sucrose were significantly lower compared to both baseline and 24 h SBT levels ($p < 0.001$).

A sucrose dose of 0.25 or 0.75 g/mL at the 96 h time-point yielded a significantly lower SBT result (48% and 61 % decrease, respectively) compared to their respective saline control ($p < 0.02$).

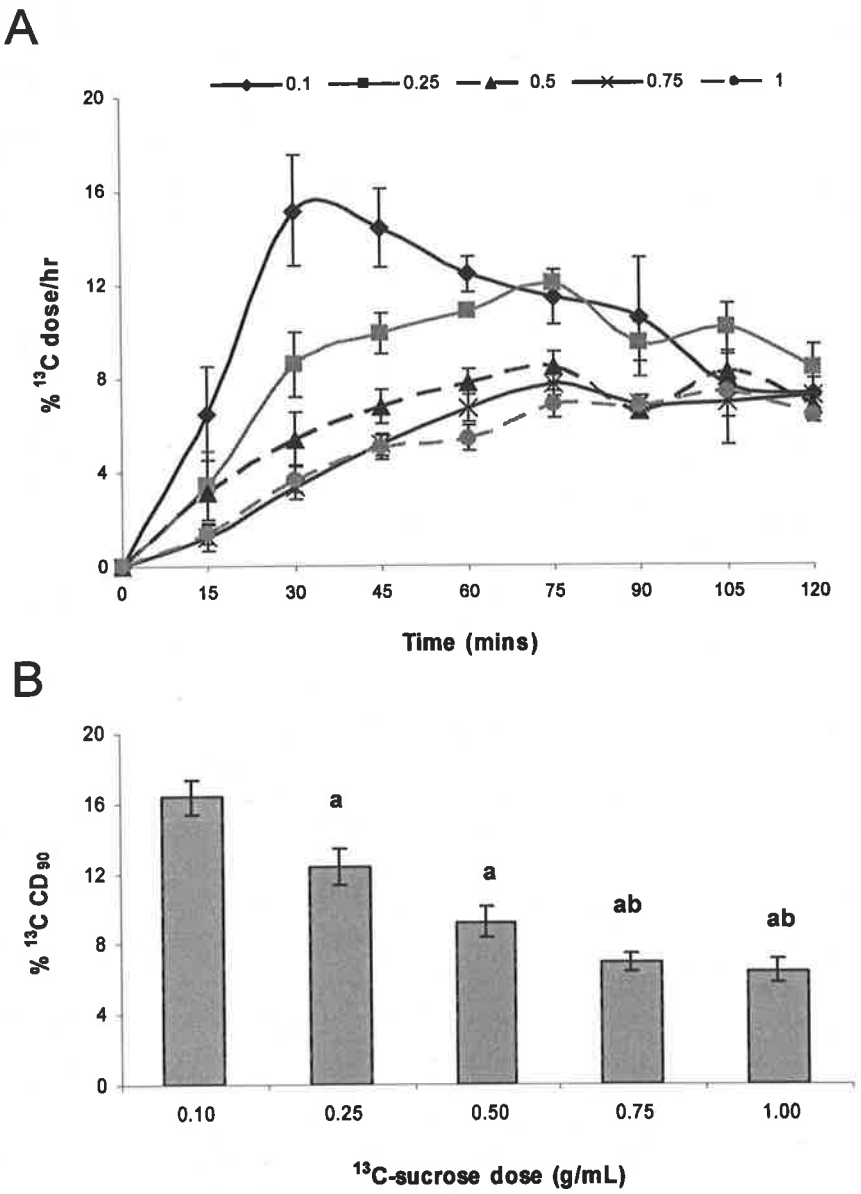

Figure 2.3: Phase 1 dose-response trial in rats (n = 4). Data expressed as (A) ^{13}C dose/hr recovered over 120min. Differing ^{13}C-sucrose gavage doses: 0.1 (black solid line - diamond); 0.25 (grey solid line - square); 0.50 (black broken line - triangle); 0.75 (black solid line - cross); and 1.0 g/mL (grey broken line - circle). (B) ^{13}C CD_{90} SBT data for increasing doses of sucrose. All data expressed as mean ± SEM. Where [a] denotes significance compared to 0.1 g/mL ($p < 0.05$); [b] denotes significance compared to 0.25 g/ mL ($p < 0.05$)

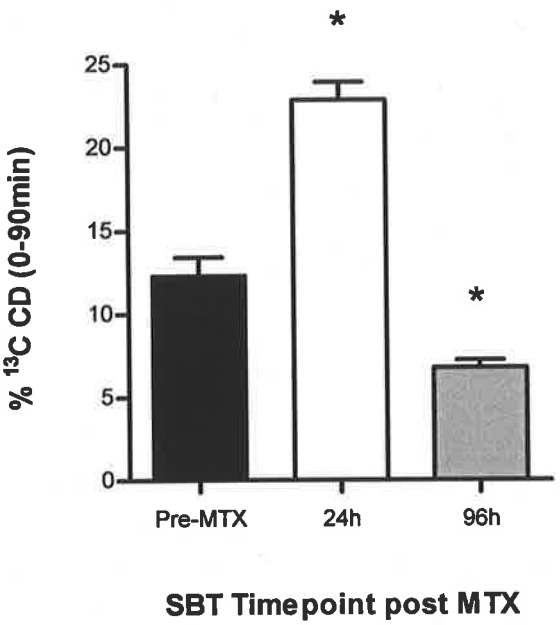

Figure 2.4: Phase 1 SBT utilising 0.25 g/mL ^{13}C-sucrose in rats (n = 4) with MTX-induced mucositis at three time-points: Pre-MTX (green bar); 24 h post MTX (blue bar); and 96 h (kill) post MTX (pink bar). Data expressed as mean ± SEM of % ^{13}C CD_{90}. Significance denoted by *, where $p < 0.01$ compared to pre-MTX animals.

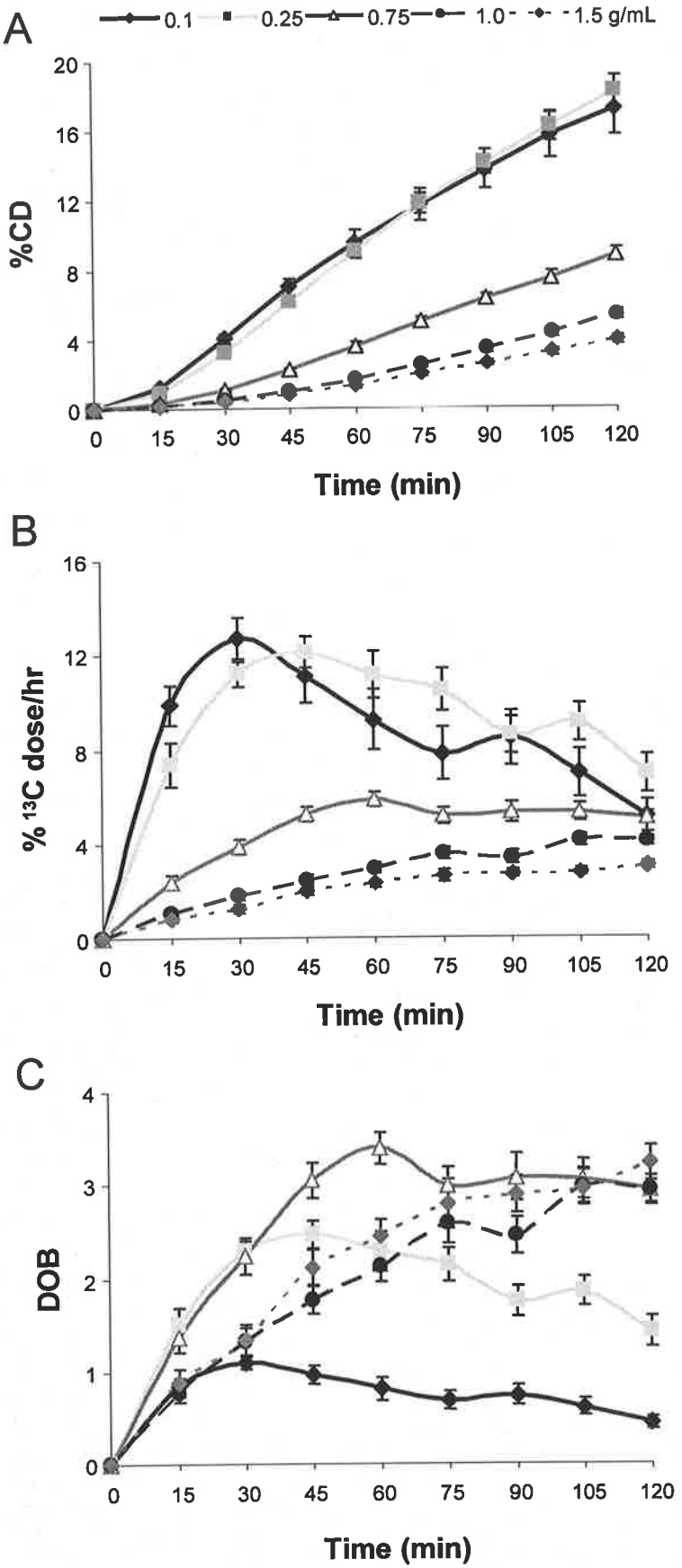

Figure 2.5: Phase 2 dose-response study (n = 16) for increasing doses of ^{13}C-sucrose (g/mL). All data expressed as mean ± SEM. (A) %CD time-course (0 - 120min); (B) % ^{13}C dose/hr recovered for increasing doses of ^{13}C-sucrose; (C) Change from baseline (DOB) derived from $^{13}C/^{12}C$ ratio. Sucrose doses for all parameters: 0.1 (black diamond); 0.25 (grey square); 0.75 (grey unfilled triangle); 1.0 (black circle; dashed line); and 1.5 g/ml (grey diamond; dashed line).

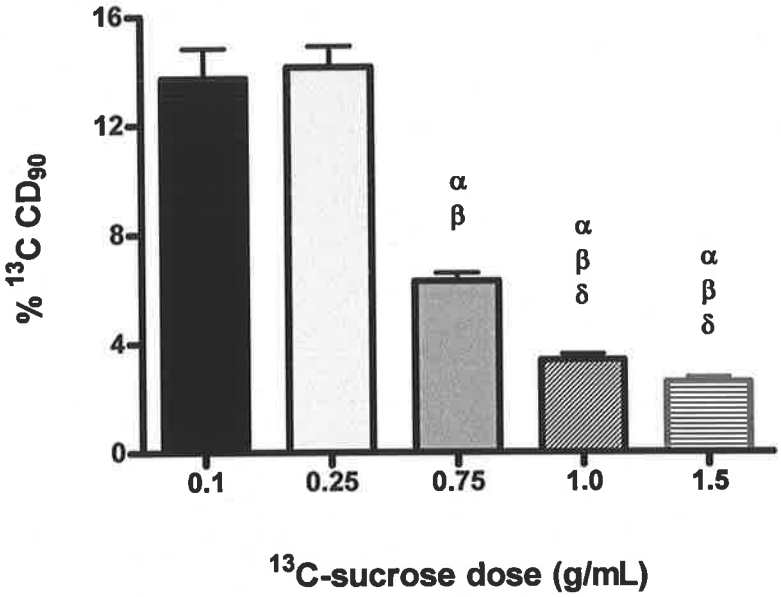

Figure 2.6: Phase 2 dose response of increasing doses (g/mL) of ^{13}C-sucrose for the SBT (%^{13}C CD_{90}) in rats (n = 16). Data expressed as mean ± SEM. Significance denoted by α, where $p < 0.001$ compared to 0.1 g/mL sucrose dose; β where $p < 0.001$ compared to 0.25 g/mL; and δ where $p < 0.05$ compared to 0.75 g/mL dose.

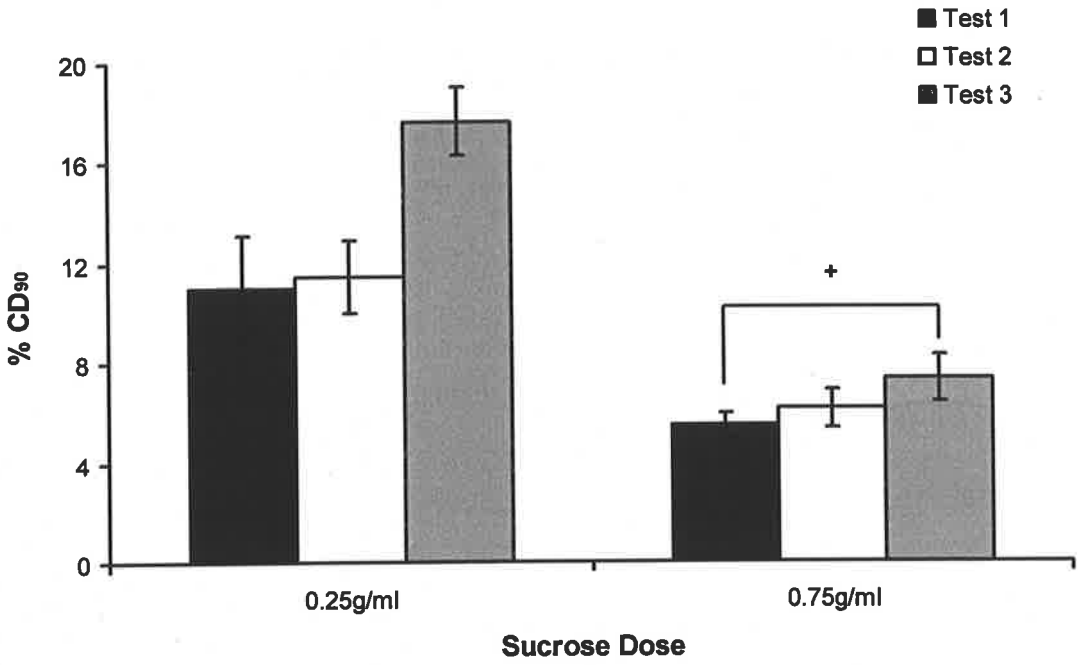

Figure 2.7: SBT reproducibility utilising ^{13}C-sucrose doses of 0.25 (n = 8) and 0.75 (n = 8) g/mL. Breath tests performed four days apart: test 1 (black), test 2 (white) and test 3 (grey) Data expressed as mean ± SEM. Significance difference between tests for 0.75 g/mL denoted by $^{+}$, where $p < 0.05$.

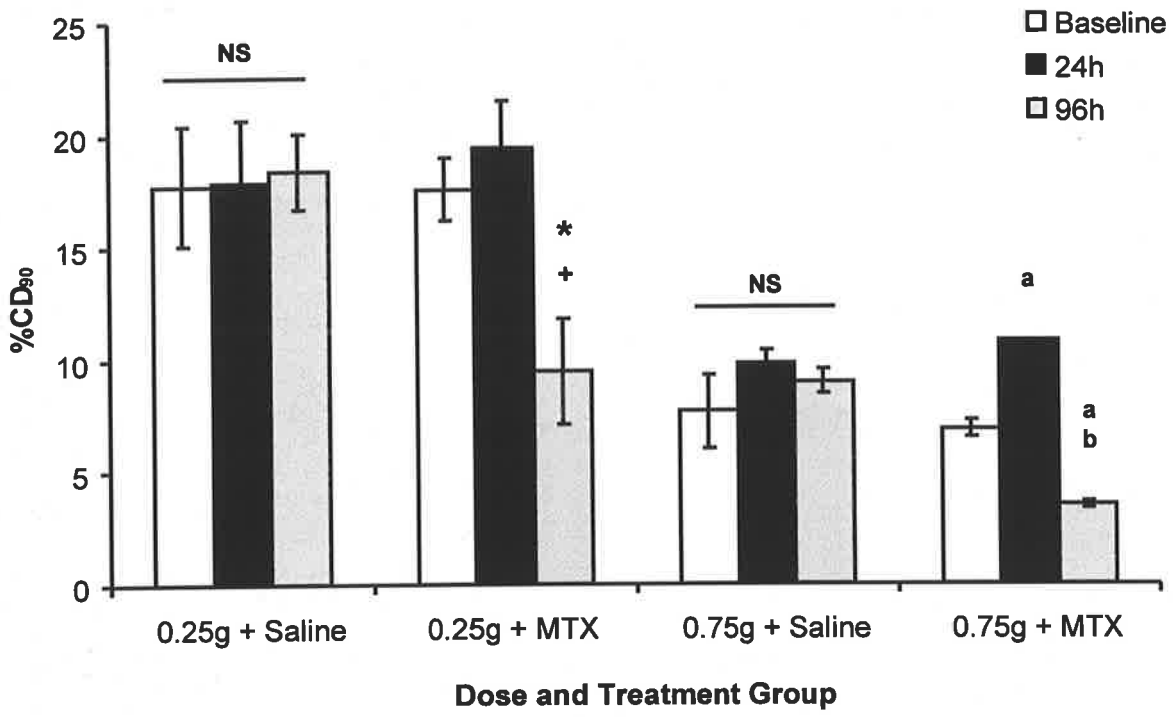

Figure 2.8: SBT time-course of animals receiving saline or MTX, utilising 0.25 or 0.75 g/mL of sucrose for the SBT. SBTs were performed at baseline, 24 and 96 h post MTX/saline. Data expressed as mean ± SEM. Significance between 0.25 g/mL + MTX at 96 h compared to baseline and 24 h ($p < 0.05$) denoted by * and $^{+}$ respectively. Significance between 0.75 g/mL + MTX: compared to baseline, denoted by ^{a}, where $p < 0.001$; compared to 24 h, denoted by ^{b}, where $p < 0.001$.

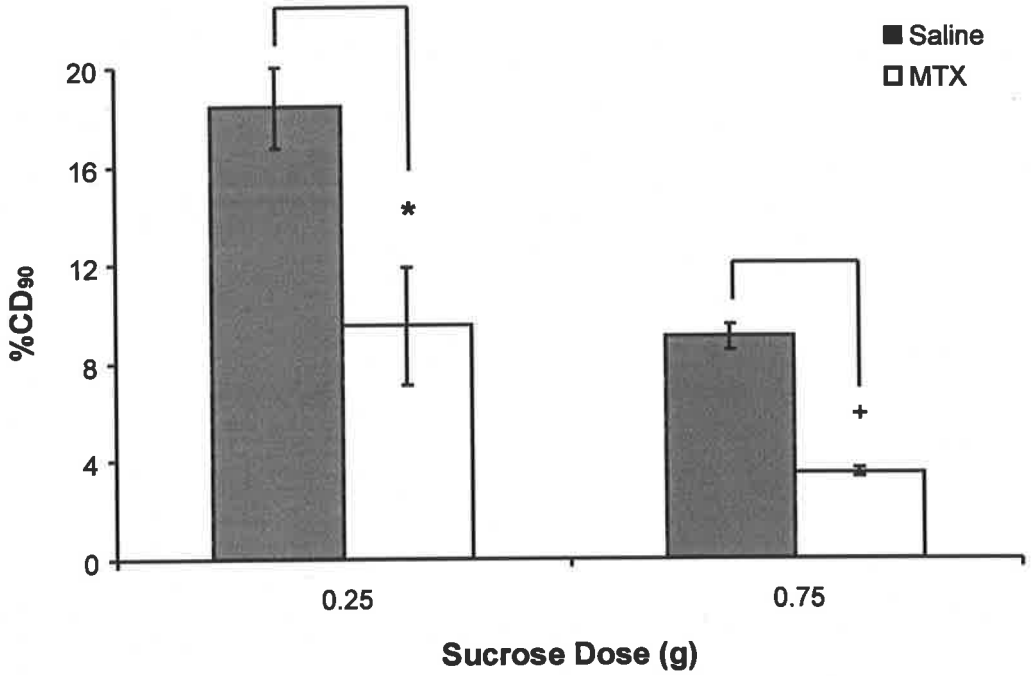

Figure 2.9: SBT levels 96 h post-MTX or its post-saline counterpart. Rats received 0.25 or 0.75 g/mL of sucrose. Data expressed as mean ± SEM. Significance between 0.25g/mL denoted by *, where p = 0.02. Significance between 0.75 g/mL denoted by $^{+}$, where p = 0.0002.

2.4 DISCUSSION

Although there have been reports in which the non-invasive ^{13}C-sucrose breath test has been implemented to assess the severity of chemotherapy-induced mucositis,[39,54,55,103] this represents the first comprehensive study to assess the dose-response of naturally enriched ^{13}C-sucrose utilising the correct analytical methodology. This study demonstrated that the previously determined and implemented sucrose dose of 1.0 g/mL was indeed saturating. Specifically, this study demonstrated that doses of 0.25 g/mL and 0.75 g/mL were capable of detecting MTX-induced mucositis more sensitively.

It is known that the brush-border enzyme sucrase is inducible in the rodent.[167-169] This phenomenon is vitally important when designing studies utilising the SBT as a biomarker of small intestinal damage, as rodents need to be consuming a diet containing sucrose to induce the appropriate expression of sucrase in the small intestine. Additionally, it has also expected that there is a time-lag with the subsequent up-regulation of the sucrase-isomaltase mRNA (sucrase) found on the villus.[170] Ferraris and colleagues[171] have suggested that this was due to the inability of mature enterocytes to reprogram the density of the enzyme sucrase and glucose transporters. Instead, the presence of luminal sucrose signals to crypt cells to reprogram the expression of the respective mRNA, and the ongoing presence of luminal carbohydrate ensures the appropriate enzymes and transporters to be expressed as the cell migrates to the tip of the villus.[171-173] Specifically, this illustrates the need for rodents to be consuming a diet containing sucrose for 72 - 96 h prior to undergoing SBT procedures to ensure villus sucrase activity. Hence rats were allowed to consume a 18% casein-based diet[127] containing sucrose, versus the non-sucrose containing regular rat chow. Additionally,

rats consumed casein diet for a minimum of four days to ensure brush-border sucrase expression.

The initial pilot study (phase 1) of increasing doses of sucrose, revealed that the original dose of 1.0 g/mL was not a suitable dose for the SBT and that lower doses of sucrose appeared to have undergone the bulk of digestion and metabolism before the expected small intestinal transit time-point of 90 mins, and thus warranted further investigation. This pilot study also illustrated that a 0.25 g/mL dose of sucrose had the capability of detecting MTX-induced small intestinal damage.

A more extensive sucrose dose-response study utilising sixteen rats revealed similar SBT results to those observed in the initial pilot study. Specifically, a sucrose dose of 0.1 g/mL reached its peak excretion level 30 min post-gavage and the ^{13}C signal rapidly declined thereafter. This indicates that the substrate, sucrose, may have been digested rapidly in the proximal small intestine, thus not reflecting the health of the entire small intestine. ^{13}C ($\%CD_{90}$) signals in rats receiving 0.25 g/mL of sucrose reached maximal excretion by 75 min and gradually declined thereafter. Thus giving a truer reflection of small intestinal health as it is expected that small intestinal transit time is approximately 90 min.[54,103] Thereafter, any ^{13}C signal is thought to consist of a mixed signal from the digestion of the substrate by both small intestinal enterocytes and bacteria residing in the large intestine. In contrast, ^{13}C signals obtained from sucrose doses of 0.75, and specifically 1.0 and 1.5 g/mL of sucrose, continued to rise past 90 min. Collection of delta over baseline (change from baseline) SBT data in response to 1.0 g/mL sucrose gavages revealed similar results to previously reported SBT studies.[39,54,103]

Reproducibility studies of 0.25 and 0.75 g/mL sucrose revealed that 0.25 g/mL was not significantly variable, compared to the dose of 0.75 g/mL. Interestingly, test 1 and test 2 of 0.25 g/mL reproducibility studies SBT results were considerably lower compared to previously determined values. The time between each SBT, where escalating doses of sucrose was received, was at least 3 – 5 days apart. However, a wash-out period of only seven days from the completion of the dose-response study and the commencement of reproducibility studies was utilised. Thus the saturating gavage dose of 1.5 g/mL from the final dose response studies could have altered the level of sucrase activity, indicating that the wash-out period of 7 days was not sufficient. A change from incremental increases in sucrose doses and then a significantly lower dose produced a great degree of variation. This suggested that a longer wash-out period from high levels of sucrose was required, allowing sucrase expression to be regulated by diet alone.

The time-course of SBT results following MTX treatment revealed that both sucrose doses 0.25 and 0.75 g/mL were capable of detecting MTX-induced damage as previously seen at 96 h post MTX injection.[54] Twenty-four hours following MTX injection a subtle form of hyperproliferation was detected by the SBT from rats receiving a dose of 0.75 g/mL sucrose. Additionally, MTX-treated rats had significantly damaged small intestines as measured by the SBT for both sucrose doses of 0.25 and 0.75 g/mL.

It is imperative when handling animals that measures are taken and adhered to throughout animal studies to ensure the low occurrence of injury of both parties. It was

noted by all animal handlers that larger doses of sucrose were difficult to gavage due to the viscosity of the fluid. Previous SBT studies in rats utilising a sucrose dose of 1.0 g/mL sucrose required the collection of breath samples for a minimum of 3 h.[39,54,103] Since both designated doses in this present study were capable of sensitively detecting chemotherapy-induced mucositis after 90 min, a sucrose dose of 0.25 g/mL was proposed as the dose of choice for future rat SBT studies.

For future studies utilising the non-invasive SBT as a means of monitoring small intestinal function in the rat it was concluded that a sucrose dose of 0.25 g/mL would be suitable. Specifically, this dose is sensitive enough to detect chemotherapy-induced damage 96 h post MTX administration. Alternatively a sucrose dose of 0.75 g/mL may prove useful in detecting early changes 24 h post-MTX, should an early damage model be desired. The SBT provides a simple non-invasive biomarker for the assessment of small intestinal function and its utilisation in future studies assessing potential treatments for mucositis and other gastrointestinal diseases could prove highly beneficial.

CHAPTER 3: TIME-COURSE OF THE SMALL INTESTINAL RESPONSE AFTER MTX IN THE RAT USING THE SBT

3.1 INTRODUCTION

The former study by Clarke and colleagues[54] outlined a time-course of MTX-induced mucositis in the female DAR. In this study a sucrose dose of 1.0 g/mL and delta over baseline (DOB) data analyses were utilised. As described in Chapter 2 the appropriate dose for the SBT was determined to be 0.25 g/mL. Understanding the time-course of methotrexate-induced small intestinal damage and repair using the SBT in the rat would be beneficial for future studies, as anti-mucositis agents are likely to be developed to ameliorate the damage targeting a specific phase of the proposed five-phase mucositis model.[25,153]

The aim of this study was to establish the SBT time-course and its correlation to *in vitro* sucrase analyses of MTX-induced small intestinal damage in the rat using the newly established ^{13}C-sucrose dosing and the appropriate breath testing calculations as determined by Ghoos et al (1993).[105]

3.2 MATERIALS & METHODS

Animals

Female DAR (n = 15) with a starting weight of 136 ± 2 g were acquired from the IMVS, Gilles Plains, Adelaide. Each animal was individually housed in a Tecniplast® metabolism cage with an environmental temperature of 25°C with a 12 h light:dark cycle in the Animal Care Facility of the Children, Youth and Women's Health Service. Approval was obtained by the Animal Ethics Committee of the Children, Youth and Women's Health Service and the University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2004).

Rats had access to 18% casein-based diet[127] and were allocated to 2 groups: group 1: -24 to 96 h time-course (n = 7) and group 2: -24 to 144 h time-course (n = 8). Daily indices of weight, food intake, fluid intake and faecal and urine output were recorded. Rats were injected with MTX as previously outlined in Chapter 2. All time-course data was compared to saline-treated, weight-matched controls that had been utilised in a previous trial as a means of reference and therefore minimising animal numbers. In the short time-course group SBTs were performed at -24 (baseline), 24 and 96 h following the initial MTX injection and were sacrificed immediately after the cessation of the 96 h SBT. The extended time-course DAR group underwent SBTs at -24, 72 and 144 h after the initial MTX injection and were sacrificed at 144 h.

Kill procedure

Rats were killed by CO_2 overdose and cervical dislocation at the respective time-point. The abdomen was opened via midline incision and the stomach, duodenum, small and large intestines were removed. The jejunum was separated from the duodenum by cutting the ligament of Treitz.[39,43] Liver, heart, lungs, kidney spleen and thymus were also excised and weighed (expressed as weight g/kg bodyweight). Gut contents were removed and placed on an ice-cold slab and mesenteries were carefully separated.[43] The stomach, duodenum, jejunum, ileum and colon were then separated and their contents gently manually emptied and then weighed. Small intestine (duodenum and jejuno-ileum) and colon lengths were measured un-stretched. Five sites of the jejuno-ileum were determined and corresponded to 10%, 25%, 50%, 75%, and 90% of the SI length, moving in a distal direction from the ligament of Trietz. At each site 2 cm and 4 cm sections were removed for histology and biochemical analyses, respectively. Histological samples were immediately placed in formalin. Four centimetre intestinal segments were placed into pre-weighed 5 mL sterile tubes, weighed, rapidly frozen in liquid nitrogen and then stored at -70°C until thawed and homogenised.

^{13}C-Sucrose breath test

Rats were fasted overnight and breath collection was performed as described in Chapter 2. Rats were gavaged with 1 mL of 0.25 g/mL sucrose solution (mixed in water), as previously determined in the dose-response SBT study (Chapter 2). Breath tests were performed at: a) -24, 24 and 96 h (short time-course); and b) -24, 72 and 144 h (long time-course), using a dose of 0.25 g/mL of selectively enriched ^{13}C-sucrose as outlined in Chapter 2, and data expressed as ^{13}C $\%CD_{90}$. Selective enrichment of sucrose with ^{13}C is defined as the percentage of ^{13}C molecules determined in 1 g of sucrose derived

from cane-sugar in comparison to the percentage of ^{12}C molecules. It is selective as only a small percentage of the naturally occurring ^{13}C molecules have been determined in sucrose derived from cane sugar.

In vitro sucrase activity

Tissue samples (4 cm) of the duodenum, proximal jejunum (10%) and distal ileum (90%) were thawed on ice in 1.5 mL 10 mM PBS (pH 6.1), homogenised (mechanical), aliquoted into 200 µL samples and stored at -70 °C until sucrase activity analysis was performed by a modification of the assay described by Dahlqvist.[174] Briefly, this assay involved diluting the homogenates between 1/100 and 1/10 with 50 mM phosphate buffer containing 0.02% Triton-X. The addition of the substrate, 50 µl of 0.2 M sucrose, was added to 50 µl of each tissue sample containing an unknown level of sucrase and incubated at 37 °C for 30 minutes. The sucrose substrate is cleaved to its constituents, glucose and fructose by sucrase in the homogenates. Glucose liberation was then detected colorimetrically by the addition of horseradish peroxidase and glucose oxidase solution, and then measuring the optical density of samples at 490 nm using a Dynatech MR7000 plate reader set at 490 nm after 30 min. Sucrase concentrations were determined using the program TableCurve 2D windows (version 4.06) from a glucose standard curve. Sucrase activity was expressed as nmol glucose/min/cm liberated from the duodenum, proximal jejunum and distal ileum homogenates. An average of the three analysed sections was calculated, reflective of the total sucrase activity of the small intestine. In addition, since sucrase activity is predominantly found in the proximal small intestine, an average of the duodenum and jejunum homogenates was also calculated.

Data and statistical analyses

All data were expressed as mean ± SEM. Previously determined data in weight-matched saline treated DAR were used as a reference for normal data of DAR at the same age. Daily data indices were analysed using a repeated measure one-way ANOVA with a Tukey's *post-hoc* test. A two-way ANOVA was not performed to determine significance across the time-points as designated fasting time-points for SBT were different for the short versus long time-course. All other data were analysed using a one-way ANOVA with a Tukey's *post-hoc* test. Significance was determined when $p < 0.05$. All data and statistical analyses were performed using GraphPad Prism version 4.01 for windows® (GraphPad Software, San Diego, CA, USA) or Microsoft Office 2003 Excel® for Microsoft WindowsXP.

3.3 RESULTS

Effect of MTX on daily indices in the DAR

The short and long time-course effects of MTX injection on bodyweight are illustrated in Figure 3.1A and highlight the time-points of fasting for each group. Short duration time-course (0 - 96 h) rats had significantly lower bodyweights 48, 72 and 96 h post MTX injection compared to their baseline weight ($p < 0.01$). In the longer time-course (0 – 144 h) rats had significantly lower bodyweights 72, 96, 120 and 144 h post MTX compared to baseline weights ($p < 0.001$). The greatest impact of MTX on food intake, coupled with the effects of fasting required for the SBT, was observed at all time-points after 0 h in the short time-course ($p < 0.01$), where food intake was significantly lower

compared to baseline weights (g). Rats from the long duration time-course had maximal effects observed 48 – 144 h post MTX compared to baseline ($p < 0.001$; Figure 3.1B). No significant differences were observed for fluid intake at all time-points for both groups (Figure 3.1C).

Lung weight [weight (g)/bodyweight (kg)] was significantly elevated in the animals undergoing the extended time-course compared to weight-matched saline controls and DAR at 96 h post-MTX ($p < 0.05$; Table 3.1). Thymus weights from animals undergoing the short duration time-course were significantly lower compared to saline weight-matched controls ($p < 0.05$). The left and right kidney, liver and spleen were all significantly lower 144 h post-MTX compared to saline control rats at 96 h post-MTX ($p < 0.05$).

No significant differences were observed in total gut weight [weight (g)/bodyweight (kg)], stomach weight or total small intestine (duodenum, jejunum and ileum) weight or colon length ($p > 0.05$; Table 3.2) in all groups. A significant decrease in small intestinal length was observed in rats 144 h post-MTX compared to both saline controls and rats 96 h post-MTX ($p < 0.05$). Additionally at 144 h, rats had a significantly lower colon weight compared to saline controls and 96 h post-MTX ($p < 0.05$).

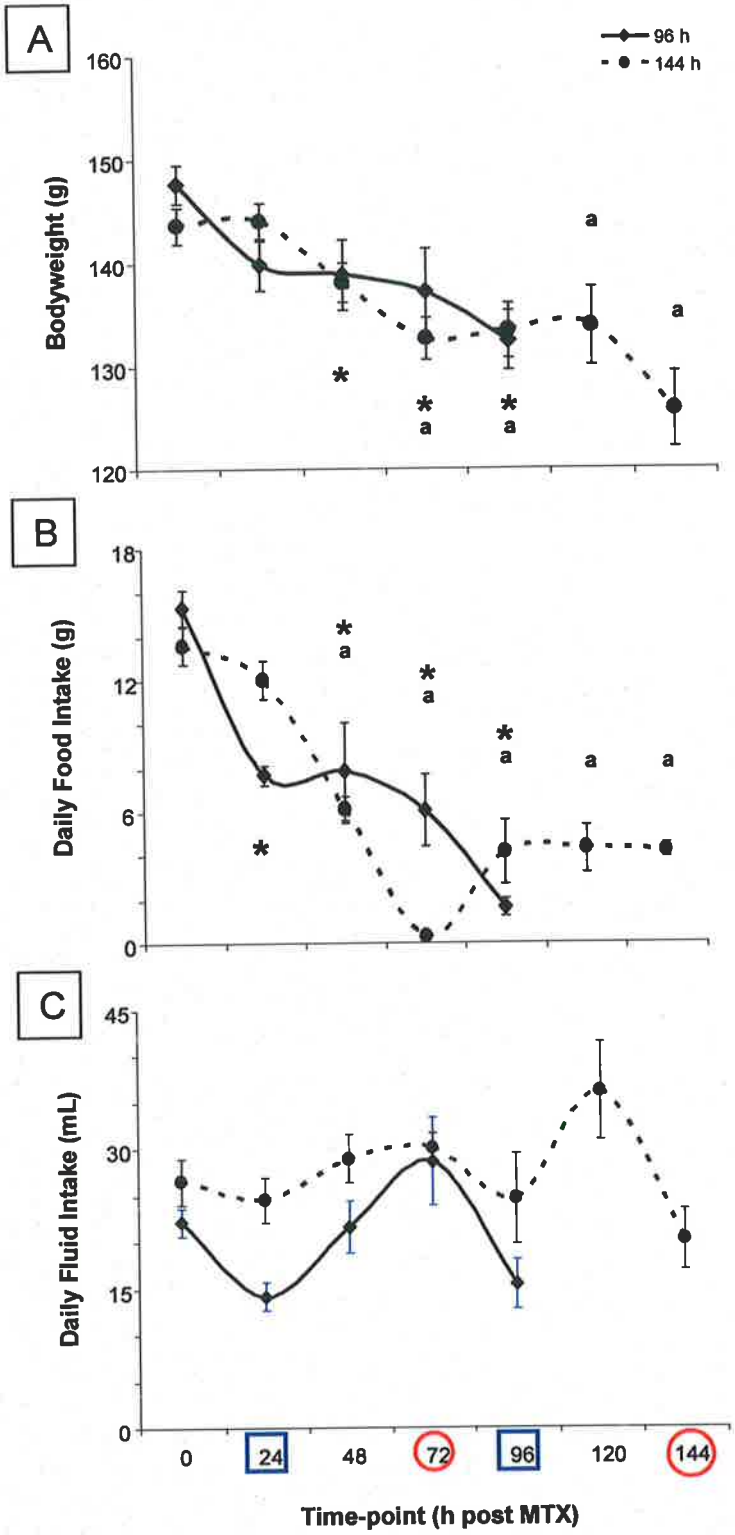

Figure 3.1: Daily data indices over the time-course. Rats are grouped according to length of time-course assessed: short time-course (0 - 96 h; n = 7) and long time-course (0 – 144 h; n = 8); (A) Bodyweight (g); (B) Food Intake (g); and (C) Fluid Intake (mL). Over-night fasts for short and long time-course denoted by blue squares and red circles, respectively. Data expressed as mean ± SEM. Significant differences in the short time-course compared to baseline is denoted by *, where $p < 0.01$. Significant differences in the long time-course compared to baseline is denoted by [a], where $p < 0.001$.

Organ Weights (g wt/kg bwt)	Controls	96 h	144 h
Heart	4.28 ± 0.13	4.22 ± 0.09	4.30 ± 0.11
Lungs	6.17 ± 0.16	6.58 ± 0.23	7.67 ± 0.31 [a,b]
Thymus	1.70 ± 0.16	0.97 ± 0.08 [a]	1.24 ± 0.13
Left Kidney	3.87 ± 0.08	3.99 ± 0.05	4.28 ± 0.08 [a,b]
Right Kidney	4.13 ± 0.12	4.05 ± 0.03	4.50 ± 0.11 [a,b]
Liver	29.7 ± 1.0	31.4 ± 0.8	35.8 ± 1.0 [a,b]
Spleen	2.06 ± 0.06	1.91 ± 0.05	2.23 ± 0.04 [a,b]

Table 3.1: Fractional weights (g weight/ kg bodyweight) of visceral organs from rats 96 and 144 h post-MTX compared to weight-matched saline-treated controls. Data are expressed as mean ± SEM; where [a] denotes significance compared to controls ($p < 0.05$); [b] denotes significance compared to 96 h ($p < 0.05$).

Effects of MTX on SBT time-course in DAR

Figure 3.2 illustrates the time-course of the SBT in rats after MTX injection. Twenty-four hours after the initial MTX injection all rodents had a significantly elevated SBT level compared to baseline levels (39% increase; $p < 0.05$). SBT levels were significantly lower in rats 72 h ($p < 0.001$) and 96 h ($p < 0.01$) post-MTX compared to baseline levels, where there was a 54% and 33% decrease, respectively. SBT levels 144 h after the initial MTX injection were not significantly different compared to baseline ($p > 0.05$).

Effects of MTX on biochemically determined sucrase activity time-course

Duodenal sucrase activity was significantly reduced at both 96 h (100% decrease) and 144 h (84% decrease) post MTX ($p < 0.001$; Figure 3.3) compared to saline weight-matched controls. In contrast, proximal jejunal sucrase activity was significantly lower in animals at 96 h compared to saline controls and 144 h post-MTX ($p < 0.001$). Sucrase activity in rats 144 h post-MTX was not significantly different compared to saline controls in proximal jejunum homogenates ($p > 0.05$; Figure 3.3). Rats 144 h post-MTX injection had significantly elevated ileal sucrase activity (approximately 280%) compared to both saline controls and rats sacrificed 96 h after MTX injection ($p < 0.001$). Average sucrase activity from pooled small intestinal homogenates was significantly lower in animals 96 h post-MTX compared to both saline controls and 144 h post-MTX ($p < 0.001$). Similarly, duodenal-jejunal sucrase activity average was significantly decreased in rats 96 h post-MTX compared to both saline controls and rats

Gut Tissue	Controls	96 h	144 h
Total gut wt (wt g/kg bwt)	44.78 ± 0.95	47.87 ± 2.40	49.56 ± 1.93
Stomach wt (wt g/kg bwt)	6.95 ± 0.18	7.58 ± 0.64	7.42 ± 0.19
SI wt (wt g/kg bwt)	30.68 ± 1.20	33.33 ± 2.04	36.73 ± 1.75
SI length (cm)	75.62 ± 1.04	74.74 ± 1.43	91.08 ± 0.93 [a,b]
Colon wt (wt g/kg bwt)	7.15 ± 0.34	6.95 ± 0.60	5.41 ± 0.19 [a,b]
Colon length (cm)	13.52 ± 0.35	11.41 ± 0.47	12.96 ± 0.24

Table 3.2: Fractional weights (wt g/kg bwt) and lengths (cm) of intestinal regions in rats 96 and 144 h post MTX, compared to weight-matched saline treated controls. Data are expressed as mean ± SEM; where [a] denotes significance compared to controls ($p < 0.05$) and [b] denotes significance compared to 96 h ($p < 0.05$).

sacrificed 144 h post-MTX. Additionally, rats sacrificed 144 h post-MTX had significantly decreased duo-jej average compared to saline-treated matched controls (Figure 3.3).

SBT and biochemical sucrase activity correlations

Duodenal and jejunal sucrase activity correlated significantly with SBT levels ($r = 0.74$ and $r = 0.71$, respectively). Ileal biochemical sucrase activity did not correlate significantly with SBT levels (Data not shown). Sucrase activity is predominantly found in the proximal regions of the small intestine. Combinations of different small intestinal regions were assessed to better determine if an optimal correlation of the parameters for SBT and *in vitro* sucrase activity could be found.[169] Thus, calculated averages of sucrase activity were determined from all individual small intestinal segments: (1) total small intestinal sucrase activity average, and (2) duodenum-jejunum average were calculated. When these calculations were made a better correlation was found. Average small intestinal and duo-jej sucrase activity correlated significantly to SBT levels, where $r = 0.83$ and $r = 0.83$, respectively (Figure 3.4).

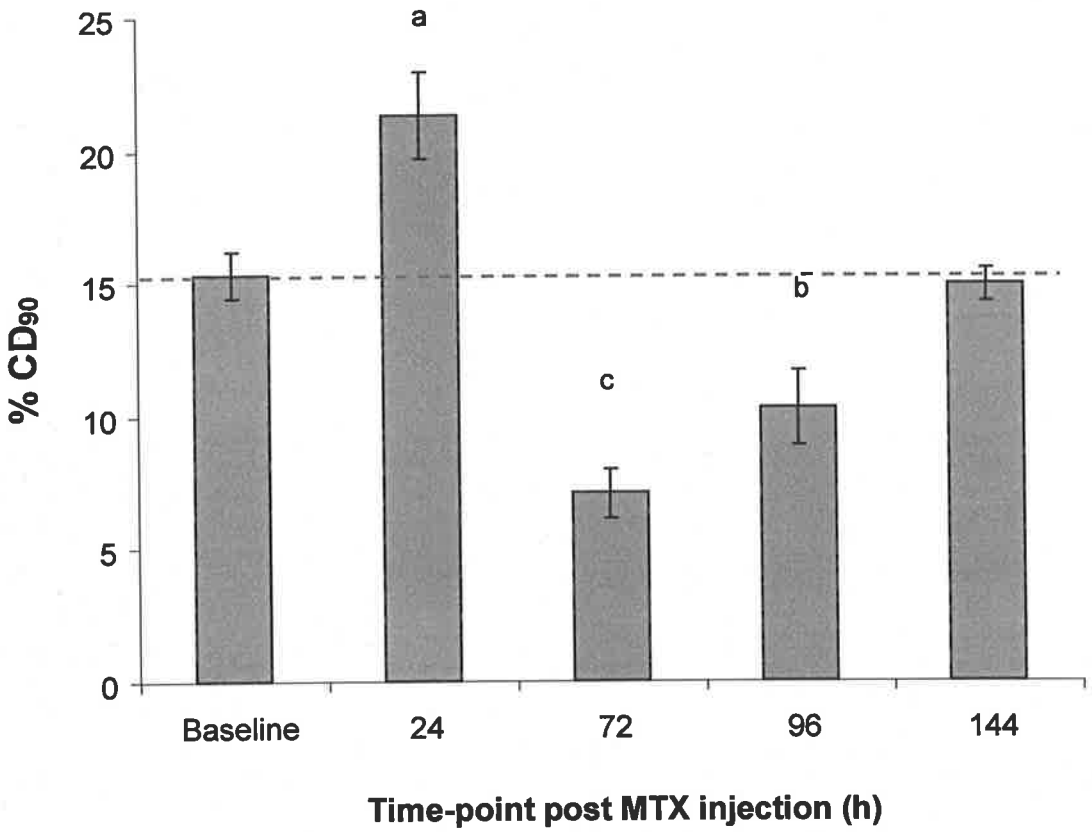

Figure 3.2: SBT (percentage ^{13}C cumulative dose 0-90min; $\%CD_{90}$) time-course of rats injected with MTX (expressed as h post MTX injection). Data are expressed as mean ± SEM; [a, b] and [c] denotes significant difference compared to baseline SBT, where $p < 0.05$, 0.01 and 0.001, respectively.

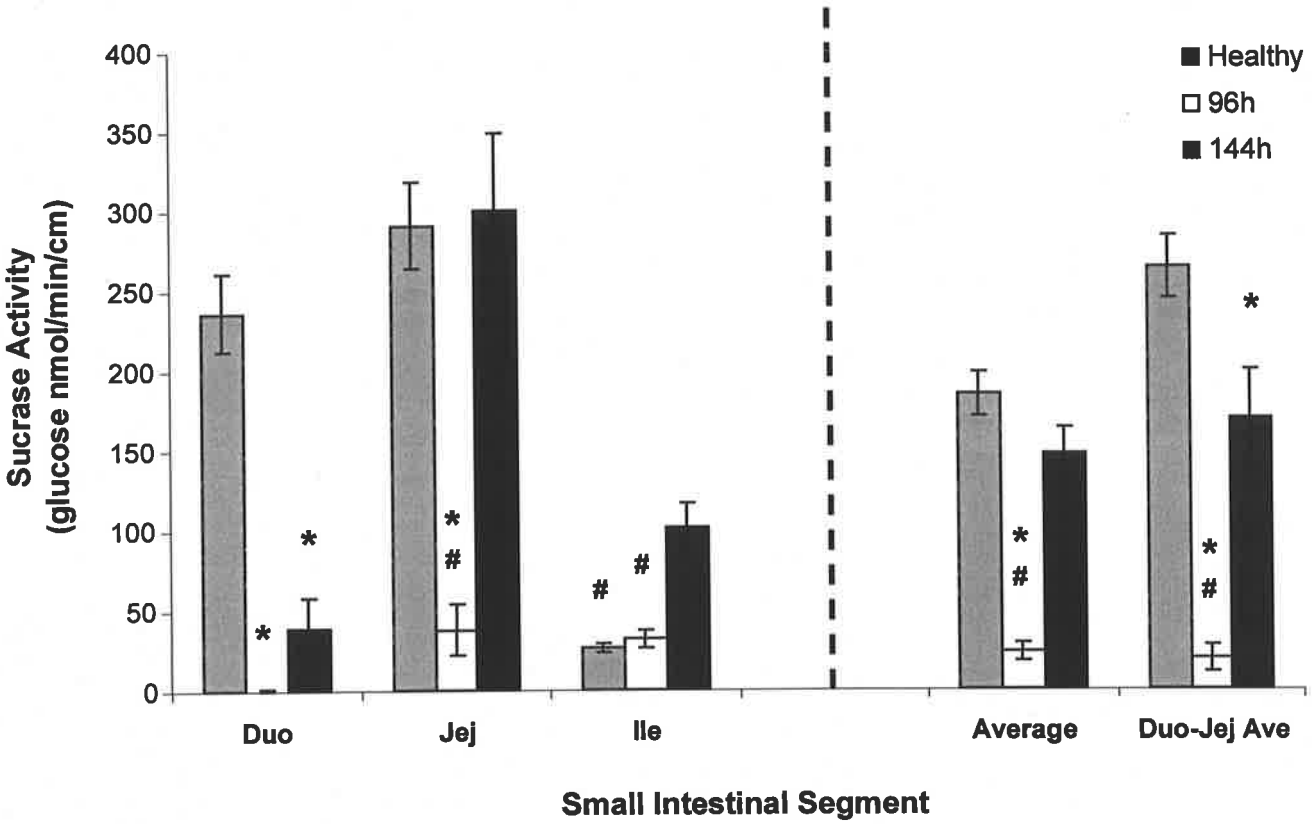

Figure 3.3: Biochemical determinations of sucrase activity (glucose released nmol/min/cm) of rats 96 and 144 h post MTX injection in the duodenum, proximal jejunum (10%) and distal ileum (90%), compared to saline-matched controls. Additionally sucrase activity represented as: the average of the three combined segments (Average); duodenum-jejunum average (Duo-jej Ave). Data are expressed as mean ± SEM; * denotes significance between saline-matched controls for each respective segment ($p < 0.001$); # denotes significance compared to 144 h ($p < 0.001$).

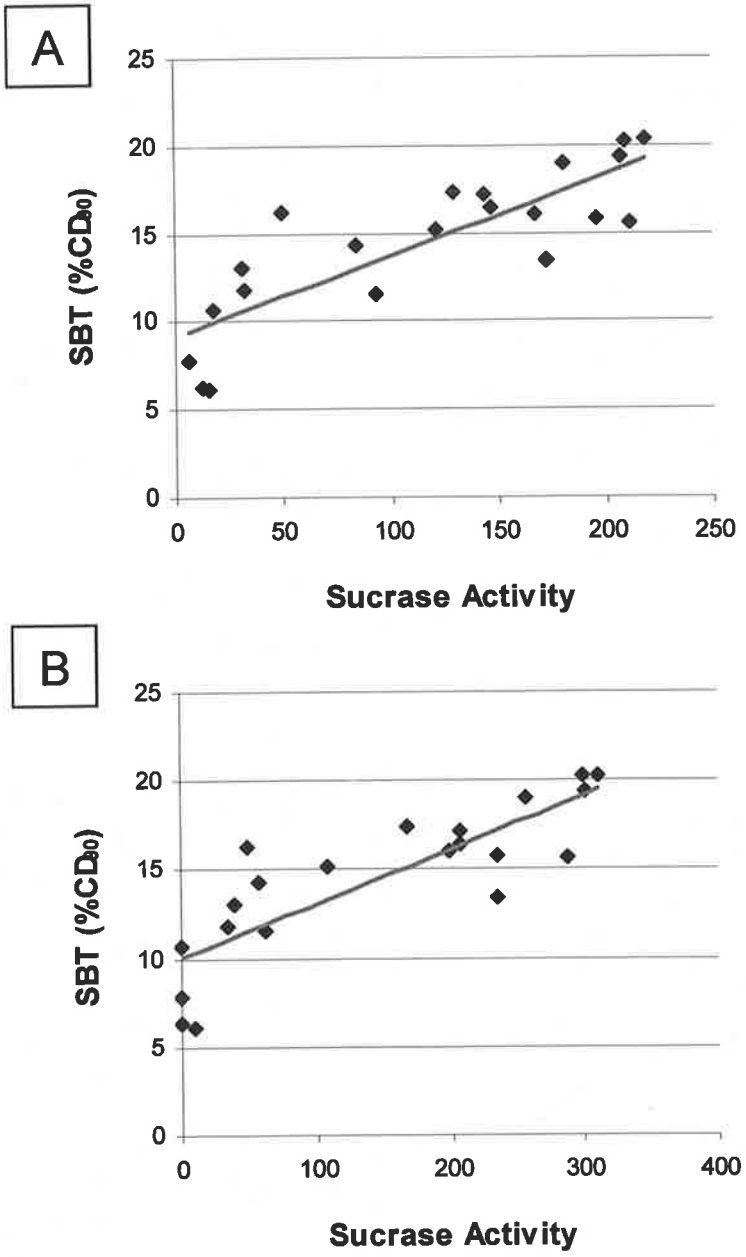

Figure 3.4: Correlation between SBT ($\%CD_{90}$) and (A) total small intestinal sucrase activity (r = 0.83) and (B) duodenal-jejunal sucrase activity (r = 0.83), determined biochemically (nmol glucose/min/cm), 96 and 144 h after administration of MTX to rats.

3.4 DISCUSSION

Whilst the production of agents to prevent or ameliorate the effects of chemotherapy-induced mucositis has been assessed extensively, these studies have been performed in animals with the potential extrapolation to cancer patients undergoing chemotherapy. One obstacle of adopting the administration of these products to cancer patients would be the inability to assess the efficacy of the new treatment modality. As with all gastrointestinal disorders, the shift from animal models to human application eliminates the ability of the clinicians/scientists to assess the direct effect to the entire small intestine, especially over multiple time-points, using the currently available invasive methods.

The aim of this study was to determine the SBT (non-invasive) time-course of a two injection model of MTX-induced mucositis in the rat, incorporating the appropriate analytical methodology. The recently reported study by Clarke and colleagues (2006)[54] describes the SBT time-course of this MTX-damage model utilising a saturating sucrose dose of 1 g/mL. The current study revealed that a sucrose dose of 0.25 g/mL for the SBT was capable of detecting small intestinal changes attributed to MTX damage over time.

The present study also demonstrated that the SBT together with biochemical sucrase measurement could, at specific time-points, identify an adaptation of the functional response to damage. The SBT measures the integrated response to small intestinal damage and as such is a useful functional marker. Used in its present form however, it

does not discriminate between regional damage and adaptive responses which occur to maintain absorptive homeostasis.

The SBT revealed that the time-point of maximal damage was approximately 72 h after the initial MTX injection. A significant increase in SBT may be indicative of alterations in differentiation and migration of mature absorptive cells 24 h post MTX. Additionally, the SBT was capable of detecting the early phase of mucosal repair 96 h post MTX.[54] The 24 h time-point for the two MTX injection model would be beneficial for the future assessment of potential anti-mucositis products. The SBT 144 h post MTX administration illustrated that the small intestine was undergoing repair. Further time-points beyond 144 h need to be assessed as the small intestine could be in a hyperproliferative state before returning to physiological homeostatic conditions.

The 24 h time-point did not include biochemical sucrase activity measures. It could be speculated that the raised SBT levels observed 24 h post-MTX were associated with an accumulation of more mature enterocytes on the villus, after damage to the more susceptible immature cells. This phenomenon could be termed villus adaptation. Induction of crypt stem cell apoptosis and the combination of any remaining proliferating cells located in the crypt, could differentiate and migrate quickly, while migration of the already mature enterocytes up the villus is retarded. The later time-point of 144 h after MTX illustrates the small intestine's capacity to adapt in response to chemotherapy damage sustained in the proximal small intestine. Whilst the SBT was unable to discriminate between the region of damage and adaptation 144 h post-MTX, it clearly indicated that villus health and its absorptive capacity, as indicated by sucrase

activity, was being restored. This second phase of adaptation can be termed as a regional adaptation.

Sucrase activity in rodents is found predominantly in the proximal small intestine and declines gradually along the small intestine to the lowest levels located in the distal ileum[169] and is known to reflect villus maturation and health. Sucrase expression is highly regulated by the presence of dietary sucrose in rodents,[172] such that the decreasing gradient in sucrase activity along the small intestine is thought to be due to the decline in luminal carbohydrate levels (glucose, fructose and sucrose) along a similar gradient. Biochemical sucrase analyses revealed that the return to baseline SBT levels 144 h after the initial MTX injection was indeed attributed to an increase in jejunal and ileal sucrase activity. Specifically, jejunal sucrase activity 144 h post-MTX injection had returned to normal levels. In contrast, duodenal sucrase activity was not restored and a compensatory increase in ileal sucrase activity was observed. To the best of my knowledge this phenomenon has not been previously reported. Since specific RNA and immunohistochemical analyses were not performed, it could only be hypothesised that the changes in regional expression of sucrase activity could be due to continued consumption of a diet containing sucrose (18% casein[127]), thus upregulating regional sucrase activity. Since damage to the proximal small intestine, specifically the duodenum, was sustained, the probability of undigested sucrose reaching the distal regions of the small intestine was increased. As outlined in Chapter 2, it is well documented that the brush-border enzyme sucrase is inducible in rodents.[167-169] Damage to the proximal small intestine alters the luminal carbohydrate concentration, such that the increased presence of sucrose in the ileum would lead to a compensatory expression

of sucrase activity[170] and its associated glucose transporter.[172,173] This phenomenon would be indicative of a small intestine region adaptive response.

In vitro biochemical sucrase activity and the non-invasive SBT data correlated very closely, with r = 0.83. Since the SBT is a good reflection of sucrase activity, this study has shown that the biochemical sucrase activity in the 144 h time-point confirms the cut-off time-point of SBT breath collection at 90 min, as the determination of *in vitro* sucrase activity of the ileum was assessed from its most distal portion. All other parameters assessed were similar to findings in previous studies.[42,43,45,46]

In conclusion, the SBT is capable of non-invasively monitoring small intestinal function over time after MTX administration, and is a good marker of *in vitro* sucrase activity. The assessment of the two MTX injection model in the rat highlights the time-points of the initial phase of repair (96 h) and healing (144 h) as determined by the SBT and *in vitro* determinations of sucrase activity. More importantly, this study has demonstrated that when sucrose is maintained in the diet after MTX administration, the ensuing proximal small intestinal damage results in a regional adaptive response by increasing the level of sucrase activity in the distal ileum.

CHAPTER 4: THE SBT AND DIFFERENT CLASSES OF CHEMOTHERAPY DRUGS

4.1 INTRODUCTION

Recently, our laboratory has developed the sucrose breath test (SBT) to address the current shortcomings in clinical management of cancer patients and to aid in the development of new chemotherapy drugs with reduced intestinal toxicity. We have described utility of the SBT in the detection of small intestinal damage induced by the antimetabolite chemotherapy drug, MTX in the rat.[103] The SBT has further been demonstrated to detect intestinal mucositis induced by a second antimetabolite, 5-Fluorouracil (5-FU).[39] To date, however, the potential for the SBT to detect and quantify intestinal damage induced by chemotherapy drugs and drug regimens other than the antimetabolites has not been described.

Chemotherapy drugs can be categorized on the basis of their differing mechanisms of action.[175,176] It was aimed to investigate the potential for the SBT to detect intestinal mucositis induced by a single drug from each category. Accordingly, we investigated drugs from the alkylating agent (Cyclophosphamide), anthracycline (Doxorubicin) and DNA topoisomerase inhibitor (Etoposide and Irinotecan) classifications. Specifically, it was sought to compare and correlate biochemical and histological indicators of

intestinal mucositis with the SBT in independent rat models of intestinal mucositis induced by these clinically-relevant drug regimens.

4.2 MATERIALS & METHODS

Animals and experimental design

Female DA Rats were maintained in Tecniplast metabolism cages for acclimatization and collection of baseline data. The environment of the animals was maintained at 25°C with a 12h light/dark cycle. Rats had continual access, unless otherwise indicated, to water and a standard casein-based diet.[127] Rats were randomly allocated into 5 treatment groups (n = 8 rats/group). Group 1: Saline vehicle (V); Group 2: Doxorubicin (Doxo); Group 3: Etoposide (Etop); Group 4: Irinotecan (Irin) and Group 5: Cyclophosphamide (Cy) and Etoposide in combination (Cy + Etop). This protocol followed the Australian Code of Practice for the Care and Use of Animals and was approved by the Animal Ethics Committee of the Children, Youth and Women's Health Service, Adelaide, South Australia.

Induction of Mucositis

When average bodyweight had reached 140 g, each animal was injected intraperitoneally (i.p) with either saline (V) or their respective chemotherapy drug. Chemotherapy drug details and doses were as follows:- Doxo [20 mg/kg (Mayne Pharma Pty Ltd, Mulgrave North, Melbourne, Victoria, Australia)]; Etop [80 mg/kg

(Bristol-Myers Squibb Company, Wallingford, Connecticut USA)]; Irin [80 mg/kg (Pharmacia Corporation, Peapack, New Jersey, USA)]; and the combination of Cy+Etop was 120 mg/kg (Bristol-Myers Squibb Company) and 40 mg/kg, respectively. All chemotherapy drugs were diluted in normal saline for injection. Drug doses required to produce an appropriate and ethically acceptable degree of mucositis were determined in preliminary studies on the basis of published findings in rodent model systems for Irinotecan,[58] Doxorubicin,[64] Etoposide[56] and Cyclophosphamide.[69] Food and water intake, weights, and urine and faeces output were measured daily. A loss in bodyweight of greater than 10% in a DAR on non-SBT experimental days resulted in culling.

Collection of gut tissues

Rats were injected with 50 mg/kg 5'-bromo-2'-deoxyuridine (BrdU, DAKO, Carpinteria, CA, USA) one hour prior to kill to label S-phase nuclei for studies of proliferation. BrdU is an analog of thymidine that is incorporated into DNA during the S phase of the cell cycle. Rats were killed by CO_2 overdose and cervical dislocation 72 hours after administration of chemotherapy. The abdomen was opened surgically as previously described in Chapter 3. Samples of duodenum, jejunum, and ileum were frozen at –80 °C for analysis of sucrase and myeloperoxidase (MPO) activity and fixed for 24 hours in 10% v/v formalin fixative for histological processing. Other organs including the spleen, liver, thymus, heart, kidneys and adrenals were weighed.

Sucrose Breath Test

Breath collection was performed as described in Chapter 2. Rats were gavaged with 1 mL of 0.25 g/mL sucrose solution (mixed in water), as previously determined in the

dose-response SBT study (Chapter 2). Breath tests were performed 24 hours prior to chemotherapy and 72 h post chemotherapy administration, immediately prior to kill. Data was expressed again as % cumulative dose at 90 minutes ($\%CD_{90}$). In a separate study, SBT analyses were conducted in 112 normal rats to provide information on the inherent variability of the test. In this study, the $\%CD_{90}$ was 15.3 ± 0.3% (mean ± SEM) with a coefficient of variation of 23.7%. In a previous study, a subset of normal animals was subjected to three sucrose breath tests over a 14 day-period, with no significant differences evident in SBT values (%CD90).[39]

Total small intestinal sucrase activity

Sucrase activity was measured in 200µL aliquots of pooled homogenates sampled from the entire residual small intestine (jejunum and ileum) by methods described previously in Chapter 3. Briefly, 4 cm sections from the proximal and distal jejunum, jejunal-ileal junction, and proximal and distal ileum were homogenised in 1.5 ml 10 mM phosphate buffer and pooled for sucrase activity of the small intestine. Homogenates from highly damaged intestinal tissues were commonly diluted 1/20 and 1/10 to achieve determination of sucrase activity, however many sample sucrase levels were undetectable.

Small Intestinal Myeloperoxidase (MPO) Activity

Homogenates of remnant small intestine, described above, were also used to determine myeloperoxidase (MPO) activity. MPO is an enzyme found in high levels in the primary granules of neutrophils, and is a marker for tissue activated neutrophil content, and hence, an indicator of inflammation. Tissue MPO levels were measured by a

modification of a previously published technique,[177] in which tissue samples were suspended in 0.5% hexadecyltrimethyl ammonium bromide (Sigma Chemical Co., St Louis, Mo., USA) pH 6.0 and homogenized for 30 seconds. Homogenates were made to a final concentration of 50 mg tissue per ml of buffer. Samples were then frozen and thawed and centrifuged at 13000 g for 2 min. MPO activity in the supernatant was measured spectrophotometrically. Aliquots were transferred to a 96-well plate with reagent containing o'dianisidine (Sigma Chemical Co., St Louis, Mo., USA) and 0.0005% hydrogen peroxide (BDH, Poole, Dorset, England), and the change in absorbance at 450 nm was measured using a microtitre plate scanner (Dynatech MR7000 microplate reader, Dynatech, Denkendorf, Germany).

Histological assessment

Segments of duodenum, jejunum, and ileum were placed in formalin fixative for 24 hours and then transferred to 70% ethanol. For histological examination, transverse sections of 4 μm were stained with haematoxylin and eosin, and examined with a light microscope (Olympus BH-2, Tokyo, Japan) and fitted with a digital camera (Sony, Tokyo, Japan) for the production of histological images. A semi-quantitative histological assessment of intestinal damage was utilised to obtain an overall score of damage severity.[42] Untreated rat intestinal tissue was used as a baseline reference to grade the histological criteria that included villus blunting, enterocyte disruption, crypt distortion, lymphoid cell infiltration and oedema, each graded from 0 - 3 to provide a maximal score of 33 (Table 4.1).[42]

Statistical analysis

Data and statistical comparisons were made using the Instat program V3.05 (Graph Pad, San Diego, CA, USA) and Excel for Microsoft XP. For the semi-quantitative scoring of intestinal damage, data are presented as medians and ranges and each region was compared statistically using the Kruskal-Wallis non-parametric analysis of variance (ANOVA), and where significance was identified ($p < 0.05$), the Dunn's *post hoc* multiple comparison test was used. Correlations between the SBT and biochemical sucrase activity were conducted by the method of Bland & Altman.[178,179] For all other measurements, data are presented as mean ± standard deviation of the mean (SD) and were analysed by a one-way ANOVA and when the significance level was $p < 0.05$ a *post hoc* analysis of groups was performed using a Tukey's test.

4.3 RESULTS

Effects of chemotherapy agents on disease activity indices

Doxo and the combination of Cy+Etop resulted in the most profound effects on food intake, with a 65% reduction over the 72-hour experimental period compared to saline-treated controls (Table 4.2). Etop and Irin, however, recorded only a 33% reduction in food intake (Table 4.2). Doxo treatment resulted in a 41% decrease in water intake whereas Irin and Etop actually increased water consumption compared to controls, whilst the combination of Cy+Etop had no significant effect (Table 4.3). The combined

Epithelium	Damage Severity Scoring Criteria	
Mucosa	villus fusion and stunting / villus:crypt ratio	Each 11 criterion scored 0 -3, with respect to damage severity, where 0 = normal 1 = mild 2 = moderate 3 = severe
	enterocyte disruption	
	reduction in goblet cell numbers	
	reduction in mitotic figures	
	crypt disruption	
	crypt cell disruption	
	crypt abscess formation	
	lymphocytic and polymorphonucleocyte infiltration	
	capillary and lymphatic dilatation	
Submucosa	thickening/oedema	
Muscularis Externa	thickening	
Total		**Score / 33**

Table 4.1: Histological scoring criterion for assessment of the mucosa, submucosa and muscularis externa. Comprising of 11 criteria, each scored 0 – 3 in regards to damage severity wtih a maximum score of 33.

effects of these factors resulted in a decrease in body weight over the experimental period that was greatest for Etop, and least for Irin (Table 4.2). Mild diarrhoea was apparent in the Etop and Cy+Etop treated rats.

Administration of Doxo, Etop or the combination of Cy+Etop resulted in significant decreases in small intestinal weight compared to saline-treated controls, with the latter two treatments inducing a coincident decrease in small intestinal length (Table 4.3). However, only Irin treatment affected stomach weight, recording a minor (9%) decrease compared to controls. This finding was accompanied by a somewhat unexpected increase in small intestinal weight following Irin administration (Table 4.3). Similarly, the weight and length of the duodenum was significantly decreased by Doxo, Etop or Cy+Etop but actually increased by Irin. Effects of all chemotherapy regimens on the colon were minimal, with only Irin treatment recording a significant reduction in weight, whilst no drug regimen significantly affected length of the colon compared to control. However, although all drug regimens, with the exception of Irin, significantly decreased total weight of the gut organs, statistical significance was lost when weights were expressed as a proportion of body weight (Table 4.3). This result implied that the decreased weights of the gastrointestinal organs following treatment with Doxo, Etop or Cy+Etop, were primarily the result of a decrease in body weight. Since body weight change had not been affected by Irin, the net result was an increase in fractional weight of the gut organs (Table 4.3).

	Chemotherapy Treatment				
	Saline	**Etop**	**Cy+Etop**	**Doxo**	**Irin**
Food Intake (g)	30.6 ± 4.4	20.6 ± 3.2$^{\#}$	10.7 ± 2.4$^{\#}$	10.8 ± 2.4$^{\#}$	20.1 ± 2.2$^{\#}$
Water Intake (ml)	101 ± 25	130 ± 25^{+}	111 ± 17	60 ± 11$^{\#}$	135 ± 19$^{\#}$
Body wt Δ (g)	-3.1 ± 4.9	-19.9 ± 2.2$^{\#}$	-10.5 ± 2.1$^{\#}$	-6.6 ± 1.7	-2.1 ± 1.8

Table 4.2: Effects of different regimens of chemotherapy on food and water intake and change in bodyweight 72 hours after administration of saline, Etop, Cy+Etop. Doxo or Irin in rats. Statistical significance compared to saline controls, where * denotes $p < 0.05$, $^{+}$ $p < 0.01$ and $^{\#}$ $p < 0.001$.

Gut Tissue	Chemotherapy Treatment				
	Saline	Etop	Cy+Etop	Doxo	Irin
Tot Gut Wt (g)	4.65 ± 0.27	4.25 ± 0.15*	4.26 ± 0.32*	4.21 ± 0.28^{+}	4.90 ± 0.35
Tot Gut Wt / kg Bwt	33.82 ± 2.40	32.91 ± 0.82	33.02 ± 3.41	32.87 ± 2.06	39.64 ± 2.56$^{\#}$
Stomach Wt (g)	0.79 ± 0.03	0.78 ± 0.04	0.81 ± 0.04	0.80 ± 0.08	0.72 ± 0.03^{+}
Colon L (cm)	11.9 ± 1.0	10.5 ± 1.0^{+}	9.9 ± 0.8$^{\#}$	11.2 ± 0.7	10.7 ± 0.9*
Colon Wt (g)	0.69 ± 0.08	0.73 ± 0.05	0.66 ± 0.09	0.69 ± 0.08	0.69 ± 0.07
Duodenum L (cm)	7.39 ± 0.38	6.03 ± 0.72$^{\#}$	6.41 ± 0.34$^{\#}$	6.02 ± 0.54$^{\#}$	7.13 ± 0.58
Duodenum Wt (g)	0.43 ± 0.03	0.37 ± 0.07*	0.36 ± 0.03*	0.36 ± 0.07^{+}	0.50 ± 0.04^{+}
SI L (cm)	83.1 ± 5.1	74.2 ± 2.4$^{\#}$	63.9 ± 3.6$^{\#}$	81.0 ± 6.2	81.4 ± 3.0
SI Wt (g)	3.21 ± 0.22	2.75 ± 0.12$^{\#}$	2.79 ± 0.29$^{\#}$	2.79 ± 0.19$^{\#}$	3.56 ± 0.17^{+}

Table 4.3: Effects of different regimens of chemotherapy on weights of the gastrointestinal organs 72 hours after administration of saline, Etop, Cy+Etop, Doxo or Irin in rats. Statistical significance compared to saline controls, where * denotes $p < 0.05$, $^{+}$ $p < 0.01$ and $^{\#}$ $p < 0.001$.

The effects of differing classes of chemotherapy on the SBT and sucrase activity

Etop and Cy+Etop resulted in the most profound effects on the SBT when assessed by the $\%CD_{90}$ (Figure 4.1), with values reduced to almost 30% of saline-control values. Doxo treatment decreased the SBT to 43% of control values whilst Irin was least toxic, to the intestine, as evidenced by SBT values that were 53% that of saline-treated controls. The degree of small intestinal damage induced by each of the drug regimens, as indicated by the SBT results, was also reflected by the biochemically-determined sucrase activity results (Figure 4.2) in which Cy+Etop, and Etop, decreased intestinal sucrase activity to only 2% and 14% of saline-treated controls, respectively. Doxo and Irin respectively decreased sucrase activity to 36% and 60% of control values. The SBT results and sucrase activity data revealed a strong concordance, recording an r value of 0.79 (Figure 4.3). This association was supported by the Bland and Altman plot that compares the ratios between the two techniques with the averages of the two techniques (Figure 4.4).

Different chemotherapy agents and histology and MPO

Typical histological features of intestinal mucositis in the proximal jejunum are depicted in Figure 4.5. Mucosal damage was represented by massive crypt disruption and lymphoid cell infiltration in the mucosa, combined with severe villus atrophy, in addition to a marked thickening of the muscularis externa. Histological findings from the distal ileum are depicted in Figure 4.6. Damage to the more distal regions of the small intestine were not as severe but still displayed a moderate degree of damage in

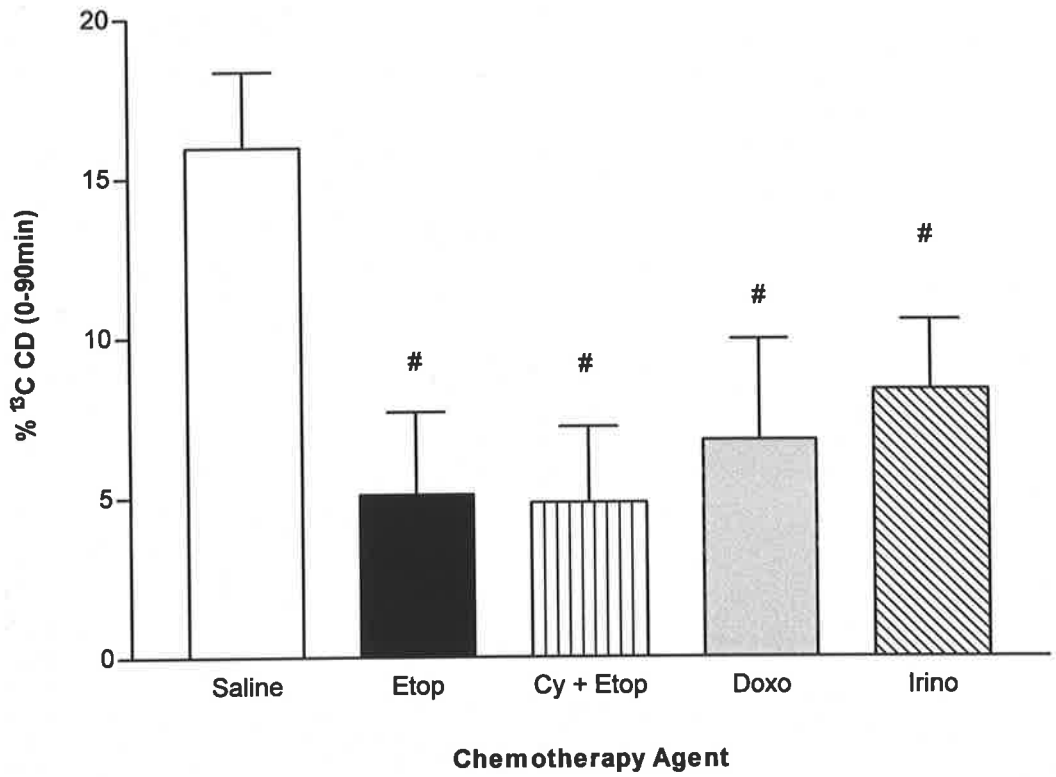

Figure 4.1: Effects of different chemotherapy drug regimens on the SBT (% Cumulative Dose at 90 minutes) when assessed 72 hours after intra-peritoneal drug administration. * denotes $p < 0.05$, $^{+}$ $p < 0.01$ and $^{\#}$ $p < 0.001$.

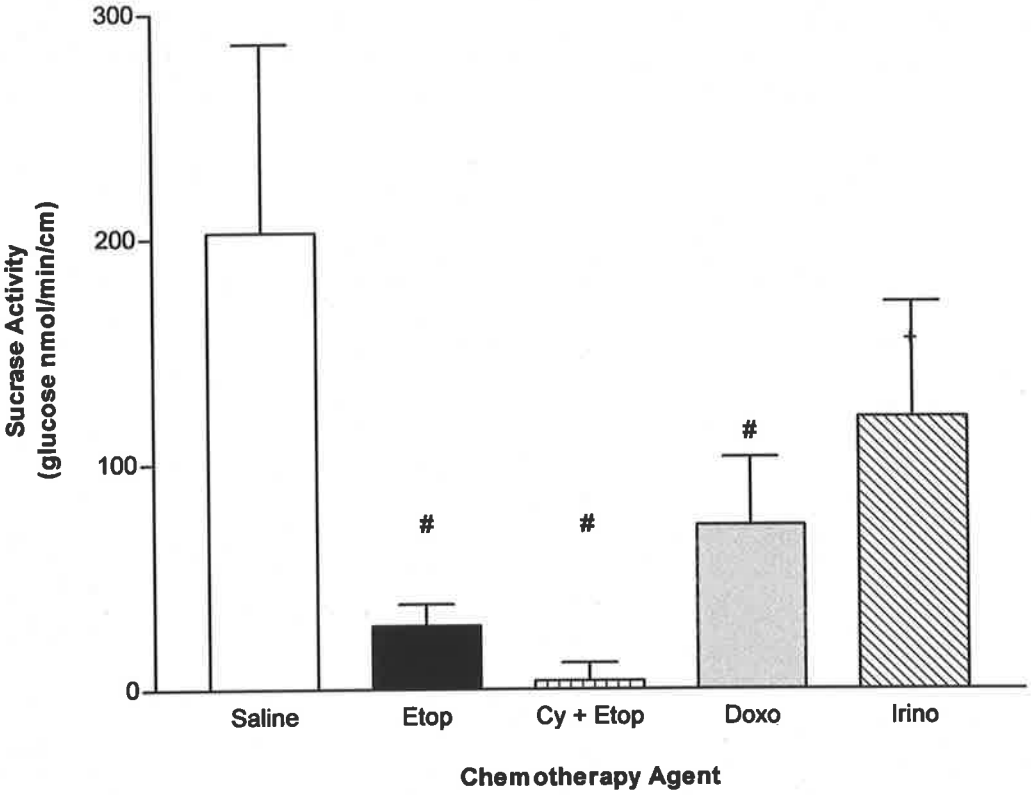

Figure 4.2: Effects of different chemotherapy drug regimens on total small intestinal sucrase activity (nmol glucose/min/cm), determined biochemically, when assessed 72 hours after intra-peritoneal drug administration. * denotes $p < 0.05$, $^{+}$ $p < 0.01$ and $^{\#}$ $p < 0.001$.

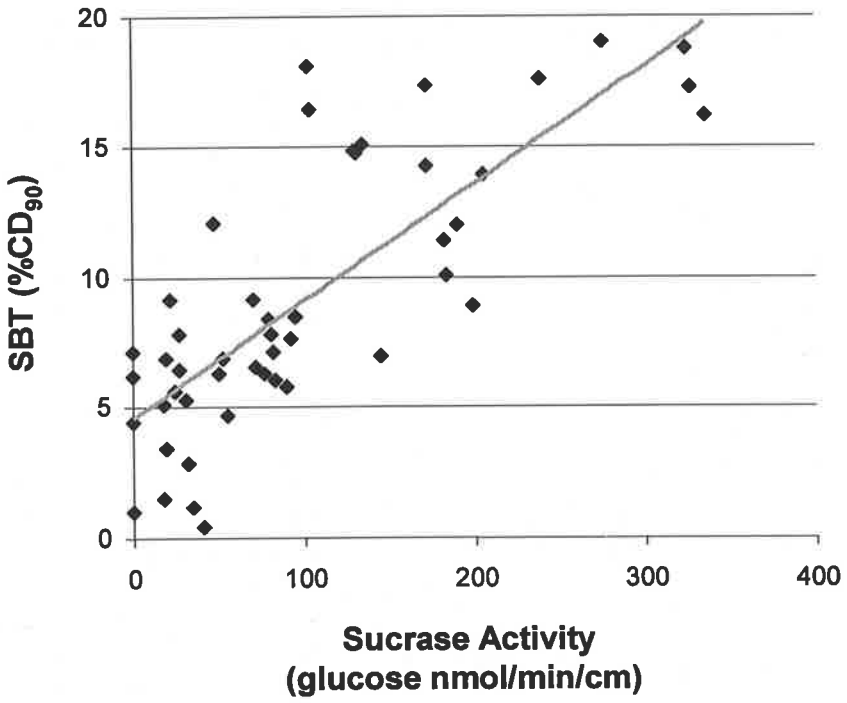

Figure 4.3: Correlation between SBT results (% CD 0-90min) and total small intestinal sucrase activity (nmol glucose/min/cm), determined biochemically, 72 hours after administration of different chemotherapy drug regimens to animals.

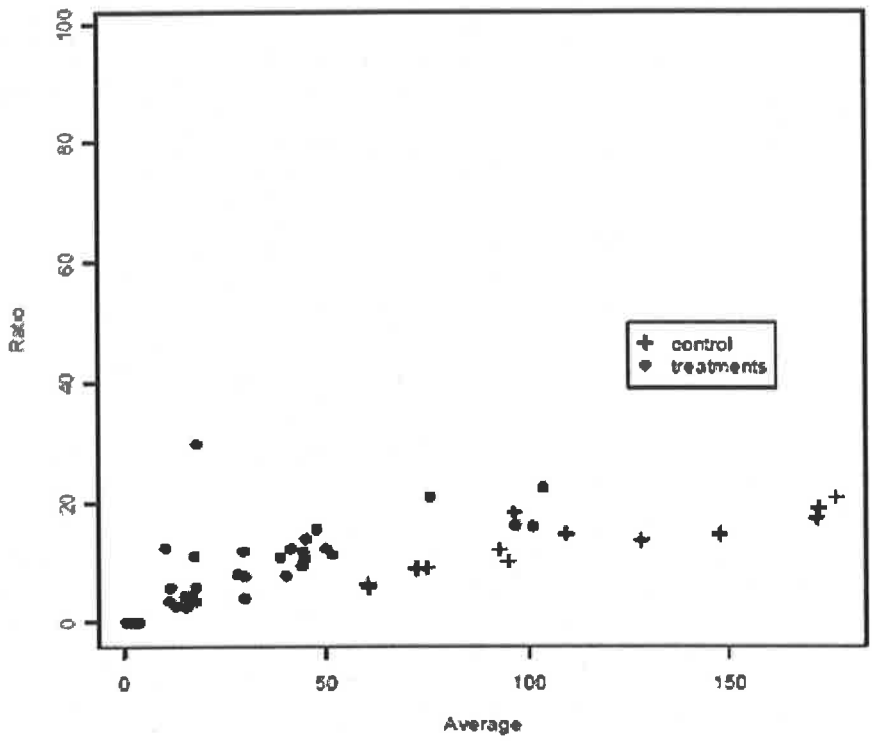

Figure 4.4: Bland & Altman plot [178,179] between SBT results and biochemically-determined intestinal sucrase activity by comparing the ratio of the SBT results and biochemical sucrase activity with the mean of the two measurements. No significant differences in the mean SBT/sucrase ratio were detected.

Gut Tissue	Chemotherapy Treatment				
	Saline	Etop	Cy+Etop	Doxo	Irin
Jejunum	0	28 (23 – 30)$^{\#}$	27 (18 – 31)$^{\#}$	19 (15 – 29)$^{\#}$	11 (4 – 16)
Ileum	0	17 (8 – 23)$^{\#}$	20 (14 – 26)$^{\#}$	14 (6 – 20)$^{+}$	11 (5 – 20)$^{+}$

Table 4.4: Semi-quantitative histological assessment of rats with mucositis induced by various chemotherapy agents in the proximal jejunum and distal ileum 72hrs after treatment. Values are the sum of scores for 11 independent histological criteria as described previously. Data are expressed as median (range). Statistical significance compared to saline controls, where * denotes $p < 0.05$, $^{+}$ $p < 0.01$ and $^{\#}$ $p < 0.001$.

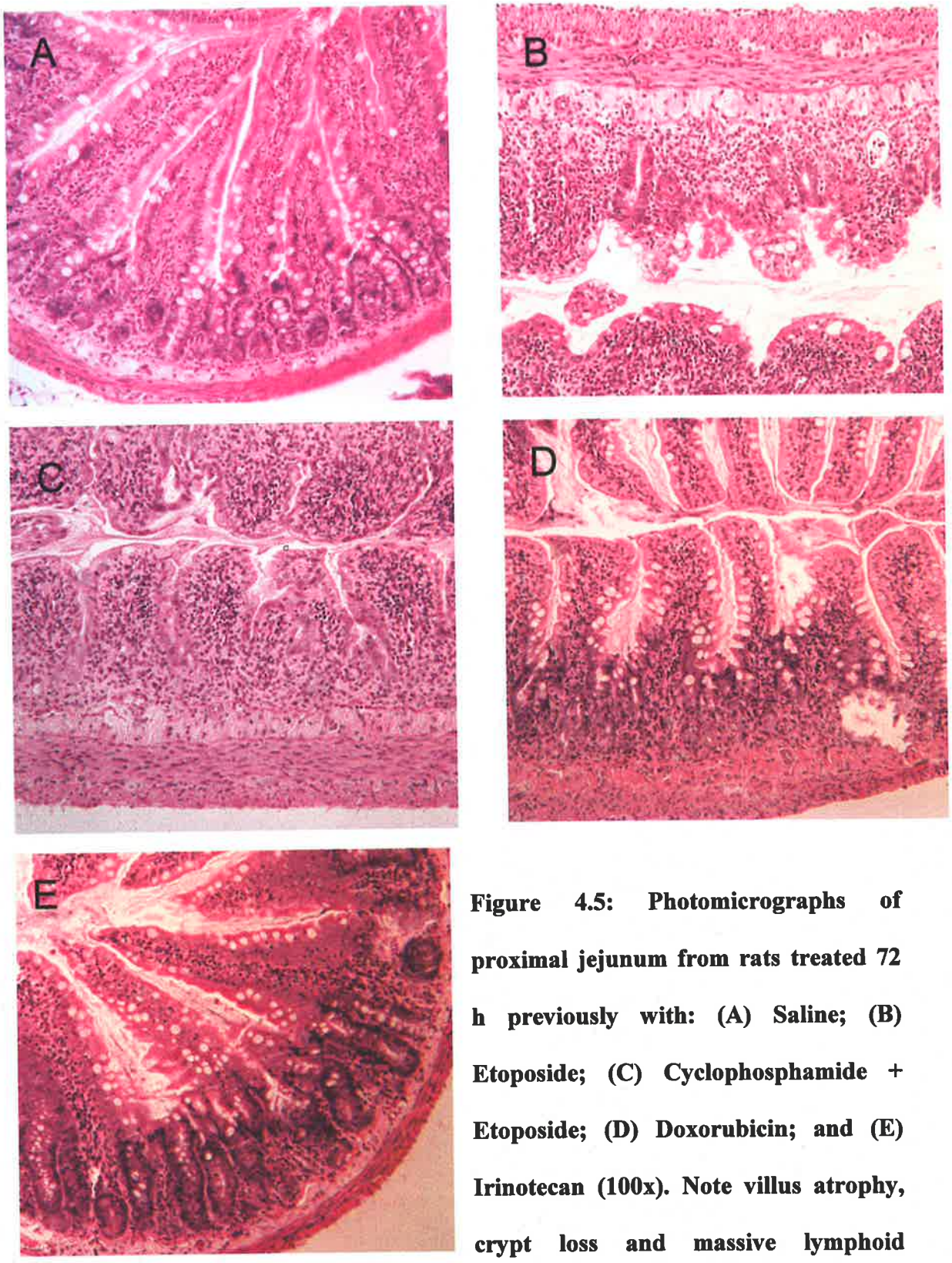

Figure 4.5: Photomicrographs of proximal jejunum from rats treated 72 h previously with: (A) Saline; (B) Etoposide; (C) Cyclophosphamide + Etoposide; (D) Doxorubicin; and (E) Irinotecan (100x). Note villus atrophy, crypt loss and massive lymphoid cellular infiltrate in the mucosa.

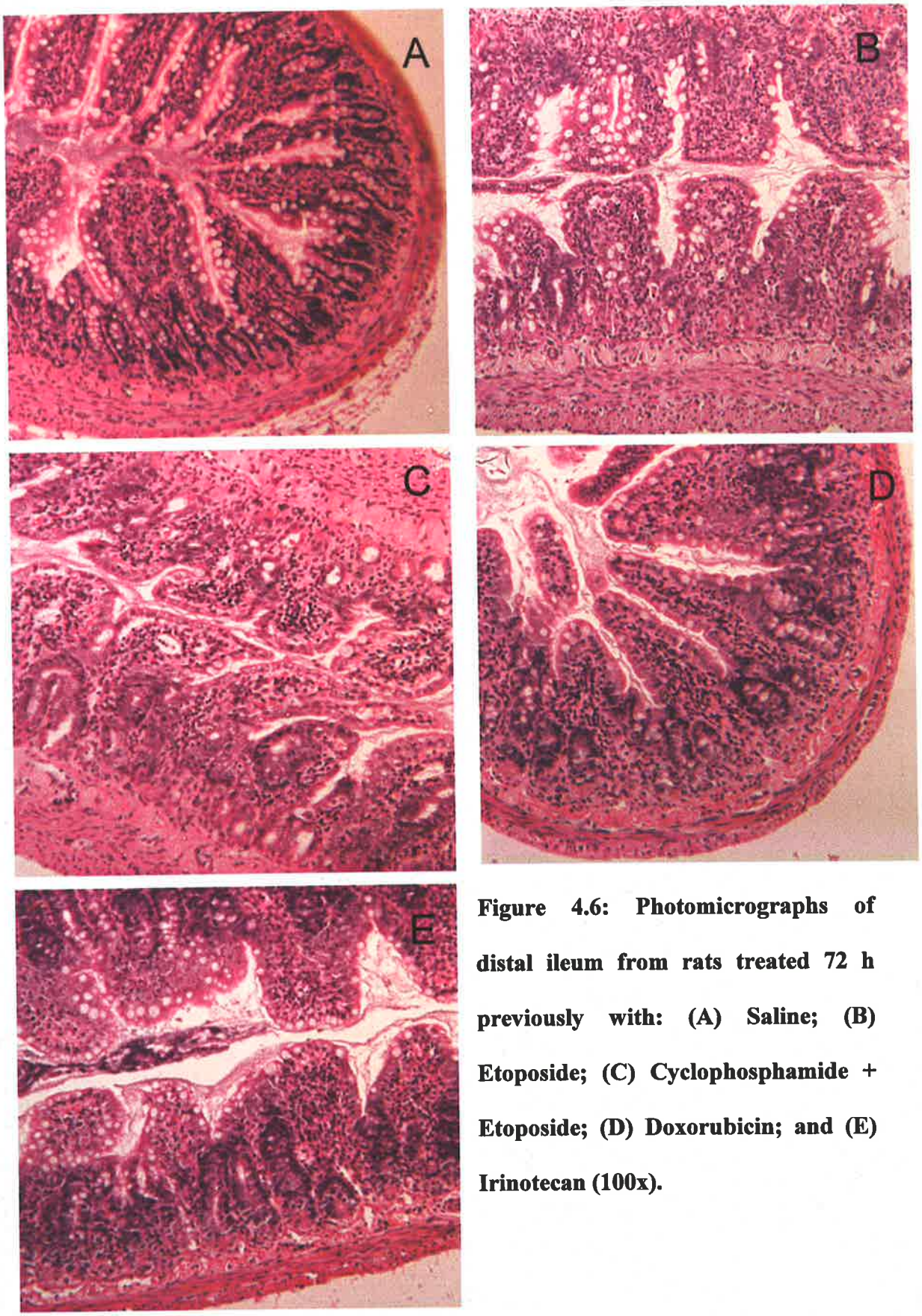

Figure 4.6: Photomicrographs of distal ileum from rats treated 72 h previously with: (A) Saline; (B) Etoposide; (C) Cyclophosphamide + Etoposide; (D) Doxorubicin; and (E) Irinotecan (100x).

relation to that observed more proximally. All chemotherapy regimens variably produced severe damage to the jejunum when assessed by the semi-quantitative histological severity score (Table 4.4). The features of mucositis were less severe in the ileum following treatment with all drug regimens. Consistent with the strong correlations observed between the SBT and total intestinal sucrase activity, there was a very good association between the SBT results and jejunal (Figure 4.7a) or ileal (Figure 4.7b) histological severity scores, attaining r value of 0.81 and 0.80, respectively.

Finally, MPO activity in the jejunum was greatest following administration of Etop, representing almost a 10-fold increase compared to saline treatment (Figure 4.8). Cy+Etop and Doxo administration produced a lesser increase in jejunal MPO activity, whereas Irin did not significantly alter MPO activity compared to normal, saline-injected, controls.

4.4 DISCUSSION

This study demonstrated that the sucrose breath test (SBT) provided a rapid, non-invasive marker of damage and dysfunction in the small intestine in a variety of rat models of small intestinal injury. The SBT has only recently been introduced as a simple technique to detect and monitor total small intestinal brush-border sucrase activity.[39,54,55,103] To date, in rodent model systems, the SBT has demonstrated the capacity to monitor the development of mucositis following administration of MTX.[103]

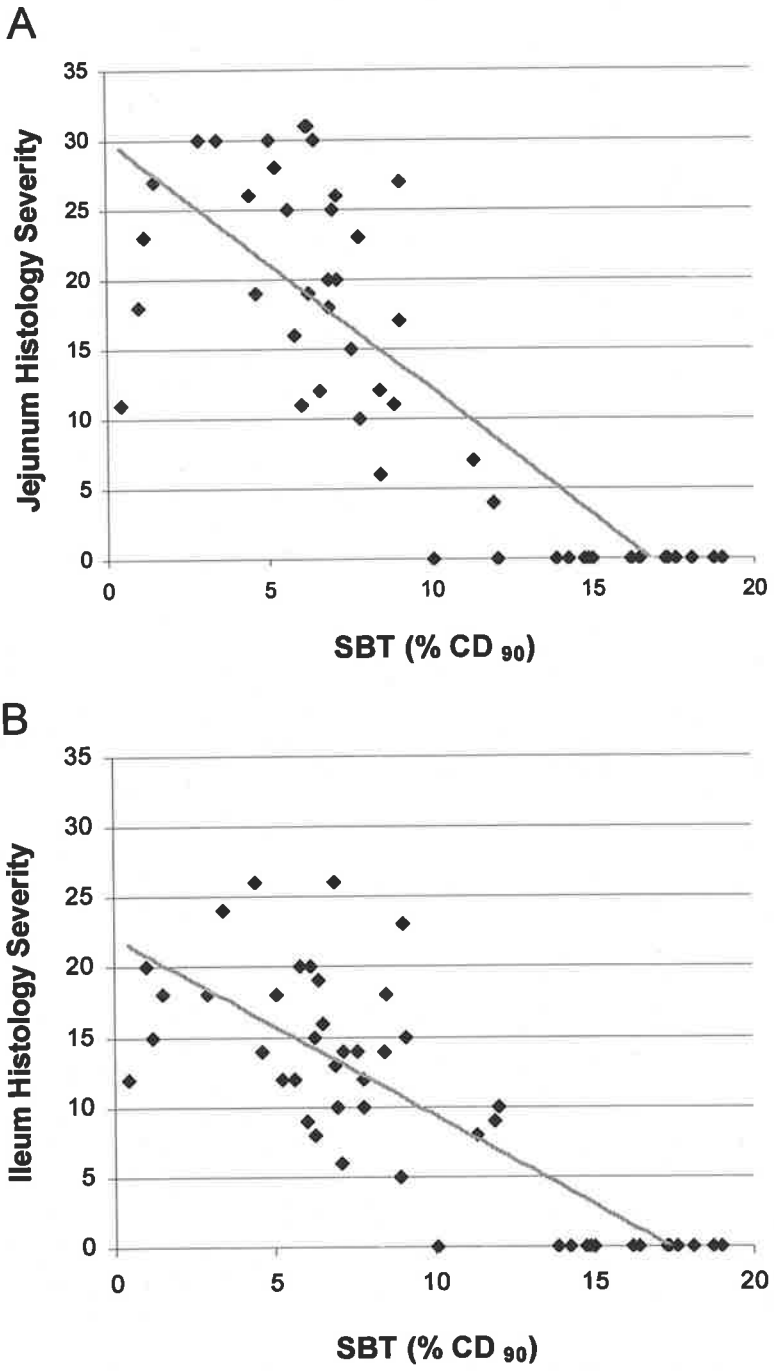

Figure 4.7: Correlation between SBT results (% Cumulative Dose at 90 minutes) and histological severity score in the (A) Jejunum and (B) Ileum when assessed 72 hours after administration of different chemotherapy drug regimens.

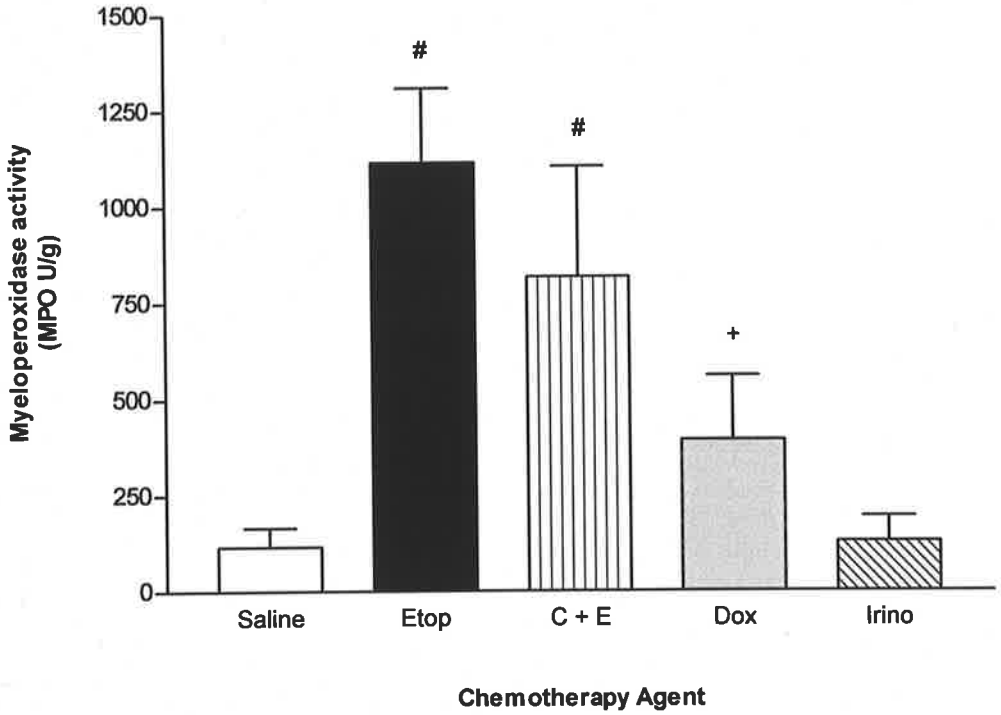

Figure 4.8: Effects of different chemotherapy drug regimens on small intestinal myeloperoxidase activity (MPO U/g), determined biochemically, when assessed 72 hours after chemotherapy. * denotes $p < 0.05$, $^{+}$ $p < 0.01$ and $^{\#}$ $p < 0.001$ compared to saline-treated controls.

Since the SBT has also been utilised to detect intestinal mucositis induced by the anti-metabolite, 5-FU,[39] it would appear reasonable to assume that the SBT would be applicable to the detection of intestinal injury induced by chemotherapy drugs other than anti-metabolites.

Whilst the anti-metabolites, MTX and 5-FU, are known to damage the proximal small intestine, preferentially, [39,54,55] [42,43] a systematic characterisation of damage along the length of the intestine has not been reported for the drug regimens investigated in the current study. Intestinal damage in representative sections of small intestine from all chemotherapy drug treatment groups in the current study reflected the body weight data, with maximal damage, following the combination treatment of Cyclophosphamide and Etoposide, with Irinotecan producing lesser damage, and Doxorubicin intermediate. In all chemotherapy regimens tested, ileal damage was less severe than in the jejunum. Biochemical indicators of mucosal injury including brush-border sucrase activity and MPO activity closely mirrored the severity of jejunal and ileal damage induced by each of the drug regimens studied, when assessed by histological means.

Each of the chemotherapy drug regimens employed in the current study variably resulted in adverse effects on body weight gain and food and water intake. Irinotecan recorded the least significant effects, whilst the most profound effects resulted from the combination of Etoposide and Cyclophosphamide. These parameters correlated closely with the more invasive biochemical and histological indicators of intestinal injury. Previous studies of anti-metabolite administration to rats have revealed similar effects on body weight and food intake to those obtained in the current study, with both

MTX[42,43] and 5-Fluorouracil[39] resulting in decreased body weight, largely due to a suppression of appetite.

In the current study, the DNA topoisomerase inhibitor, Etoposide, resulted in severe mucositis when assessed by histological assessment of both the jejunum and ileum. This was coupled with a marked decrease in brush-border sucrase activity. These effects were further exacerbated when Etoposide was combined with the commonly-prescribed alkylating agent, Cyclophosphamide. Importantly, the SBT was also able to detect and quantify these effects with a high degree of concordance with the more invasive indicators of mucositis, particularly the biochemical assessments of sucrase activity. Similarly, the anthracycline, Doxorubicin, which produced a more moderate degree of mucositis than Etoposide, resulted in an intermediate SBT result, further supporting the relationship between intestinal injury, sucrase activity, intestinal function and the SBT.

Homocamptothecins (hCPTs) represent a new generation of anti-tumour agents targeting DNA topoisomerase I.[180] Irinotecan, a member of the hCPT chemotherapy drug class is being used increasingly for the treatment of colo-rectal cancer.[181] However, its use at therapeutically effective doses is frequently associated with the development of severe mucositis.[36] Although the dose of Irinotecan employed in the current study was comparable to that used in previous studies of mucositis,[58] the preparation in saline yielded only a mild to moderate degree of mucositis. Irinotecan is known to be cytotoxic to both the small and large intestine. In this study only the small intestinal damage was assessed and this was not as severe as predicted. This may be due to an indirect effect derived from damage to the large intestine modifying the function of the small intestine. Alternatively, it may be due to a lower than expected cytotoxic dose

related to the preparation in saline as opposed to a sorbitol-lactic acid solution.[36] This provided an opportunity to investigate the sensitivity of the SBT with a less severe chemotherapy-induced mucositis model. The more invasive indicators of mucositis detected this mild-moderate severity of mucositis, which was mirrored by the SBT values in Irinotecan-treated rats. These values were less than half that of normal controls, indicating that the SBT is capable of detecting milder forms of small intestinal damage. Future studies should assess the effects of escalating doses of irinotecan using different routes and matrices for drug delivery and determining the relative effects on the small and large intestine damage and function.

Clinical trials of new treatment modalities for intestinal mucositis have been limited by the lack of availability of a simple test to quantify mucositis and the response to clinical intervention. A range of non-pharmacological approaches for the prevention of *oral* mucositis have been investigated, although few have been examined extensively for their potential to ameliorate *intestinal* mucositis. Chlorhexidine, amifostine, hematologic pentoxifylline, glutamine, probiotics and growth factor formulations are currently being investigated for their ability to prevent oral mucositis. Indeed, KGF[125] represents the first bioactive growth factor preparation to enter clinical trial for its potential to protect against mucositis, and this is confined to the oral manifestations. In clinical trials, the SBT will provide an important tool to define efficacy of novel bioactive factors including KGF, insulin-like growth factor-I [39,43] glucagon-like peptide-2 [182,183], and whey-derived growth factors.[38,42,49,184] or transforming growth factor-beta, glutamine, and short chain fatty acids, [185] which now will be able to be assessed in a variety of drug damage models. Indeed, this will allow easier extrapolation to cancer patients coupled with the knowledge that the SBT also has the capacity to detect

intestinal mucositis induced by representative drugs from the anti-metabolite, anthracycline, alkylating agent and DNA topoisomerase classes of chemotherapy drug. Importantly, the SBT not only reflects biochemical sucrase activity but it also is indicative of overall villus health, and indeed the level of maturation, as previously described in Chapter 3.

Referencing the drugs employed in the current study as comparators, the SBT could be further utilised to detect and monitor intestinal mucositis induced by other members of these drug categories in addition to other classifications such as the histone deacetylase inhibitors. Moreover, since the SBT detects sucrase activity specifically, it is unlikely to be affected by injury to intestinal regions in which sucrase activity is not present, such as in the colon. Indeed, preliminary indications from as yet unpublished experimental studies, that the SBT is unaffected by damage to the distal ileum induced by the non-steroidal anti-inflammatory drug, indomethacin, and also unaffected by the florid colonic inflammation induced by dextran sulphate sodium. The SBT in its current form may therefore not detect damage to the distal ileum, specifically when the proximal small intestine is unaffected, or when an adaptive response has occurred as observed in Chapter 3.

In conclusion the SBT has the potential to non-invasively detect and monitor the development of intestinal mucositis induced by different classes of chemotherapeutic agents. Utilization of the SBT in rodent model systems provides an effective means to predict the toxic effects of newly-developed pharmaceutical or bioactive agents on the small intestine.

CHAPTER 5: ORAL INGESTION OF *STREPTOCOCCUS THERMOPHILUS* AND ITS EFFECTS ON MTX-INDUCED SMALL INTESTINAL MUCOSITIS

5.1 INTRODUCTION

Currently no effective treatment regimens exist for mucositis, however there is a clear need to develop new agents to protect the small intestine. Commensal bacteria are now known to play an important role in maintaining intestinal barrier function and health,[141,142] and probiotics have emerged as viable alternatives to the use of synthetic substances in nutrition and medicine.[186] Probiotic bacteria, such as *lactobacilli* and *bifidobacteria* have been demonstrated to enhance intestinal epithelial barrier function, and have demonstrated preventative or treatment potential in human disease conditions usually associated with pathogenic invasion.[143]

Although preventing adherence of pathogens to the mucosal surface is an important characteristic of many probiotics,[144] a broad range of other physiological effects have been described including enhanced phagocytosis, modulation of proliferative activity, non-specific immune stimulation following induction of pro-inflammatory cytokines, in addition to specific immune responses including IgA responses.[141] Moreover, when candidate probiotic strains are combined with milk, adhesion and survival is enhanced.[187] *Streptococcus thermophilus* has not generally been designated as a probiotic. However, it has been shown to protect the gastrointestinal epithelium,[145]

induce better growth in infants,[188] and reduce the severity and incidence of acute diarrhoea in young infants,[148,149] thus exhibiting probiotic properties. However, its effects on the development of chemotherapy-induced mucositis have not been investigated. Thus, the aim of the following study was to determine the effects of the bacterium *Streptococcus thermophilus* (TH-4) on MTX-induced intestinal mucositis in rats using the SBT as the primary biomarker to monitor small intestinal damage and absorptive function.[103]

5.2 Materials & Methods

Animals

Twenty-seven female DAR, with an initial bodyweight of 148.0 ± 1.0g, were acquired from the IMVS, Gilles Plains, Adelaide. Each animal was individually housed in Tecniplast® metabolism cages with an environmental temperature of 25°C with a 12 h light:dark cycle in the Animal Care Facility of the Children, Youth and Women's Health Service. Approval was obtained by the Animal Ethics Committee of the Children's, Youth and Women's Health Service and the University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2004).

Streptococcus thermophilus (TH-4) inoculum

Streptococcus thermophilus (TH-4) was kindly donated by Chr.-Hansen (Chr. Hansen Aust Pty. Ltd, Bayswater, Victoria, Australia) in the form of TH-4, a freeze dried yoghurt culture skim milk mix. TH-4 was grown on De Mann/Rogosa/Sharpe (MRS; Oxoid, Ltd., Basingstoke, England) agar and also MRS (Oxoid) broth, and was incubated at 37 °C with continual 5% CO_2 feed for 48 h.[152] A stock culture of this TH-4 concentration was kept at -70 °C for later use.

Reconstituted Bonlac® skim milk, kindly donated by Impak Foods Inc. (Royal Park, Adelaide, Australia), was used as the gavage vehicle. The skim milk was reconstituted to 10% w/v and 1% w/v glucose. After a viable count, two doses of TH-4, 10^9 cfu/mL and 10^8cfu/mL, were used for treatment of MTX-induced mucositis.

S. thermophilus (TH-4) administration to MTX-treated rats

Rats were allocated to four groups (see Figure 1). Group 1 (n = 7) received saline (sodium chloride injection for BP, 1.5mmol NaCl; Astra Zeneca, North Ryde, New South Wales, Australia) via intra-muscular (i.m) injection and skim milk (vehicle) gavage; group 2 received 1.5 mg/kg (i.m.) MTX (Pharmacia Corporation, Peapack, New Jersey, USA) and vehicle (n = 8); group 3 (n = 8) received MTX and high TH-4 (10^9 cfu/mL); and group 4 (n = 4) MTX and the lower TH-4 dose (10^8 cfu/mL). Rats were placed in metabolism cages for 24 h to allow acclimatization before the experimental procedure commenced. At approximately 1130 h rats received either 1.5 mg/kg MTX or saline (i.m.) at 0 h and 24 h to induce mucositis as described in Gibson et al (2002) [48]. Rats were fed an 18% casein-based diet[127] and were allowed water *ad*

libitum for the duration of the 7 day protocol. Rats were gavaged with 1 mL of TH-4 or skim milk solution at -48, -24, 0, 12, 24, 48 and 72 h in relation to time of first MTX or saline injection (Figure 5.1). Body weights, fluid and diet intake, and faecal and urine output measurements were collected daily. From the MTX time-course results outlined in Chapter 3, rats were sacrificed 96 h post the first MTX or saline injection, as a time-point of mucosal repair was desired.

^{13}C-sucrose breath test (SBT)

The SBT was applied to determine small intestinal function non-invasively, thus minimizing animal distress. The SBT was performed on all rats at approximately 0900 h at -24 h, 24 h and 96 h. Following the protocol as outlined in Chapter 2 and a ^{13}C-sucrose dosing of 0.25g/mL. Data was expressed as $\%CD_{90}$ as previously described.

Kill procedure and tissue collection

Rats were injected with 50 mg/kg 5'-bromo-2'-deoxyuridine (BrdU, DAKO, Carpinteria, CA, USA) one hour prior to kill to determine numbers of S-phase cells for proliferation analysis. Ninety-six hours after the first MTX/saline injection rats were sacrificed via CO_2 anaesthesia and cervical dislocation. A blood sample was collected into heparinised tubes via a cardiac puncture and plasma was separated by centrifugation at 3000 g for 10 min and frozen at -70 °C. Collection of organs was performed as previously outlined in Chapter 3. For each intestinal segment, samples for cryostat sectioning (optimal cutting temperature (OCT) added; Tissue-Tek®, Sakura Finetek, CA, USA) (1 cm), histological analysis (2 cm) and frozen sections (4 cm) were collected. Frozen specimens were immediately placed in liquid nitrogen and stored at

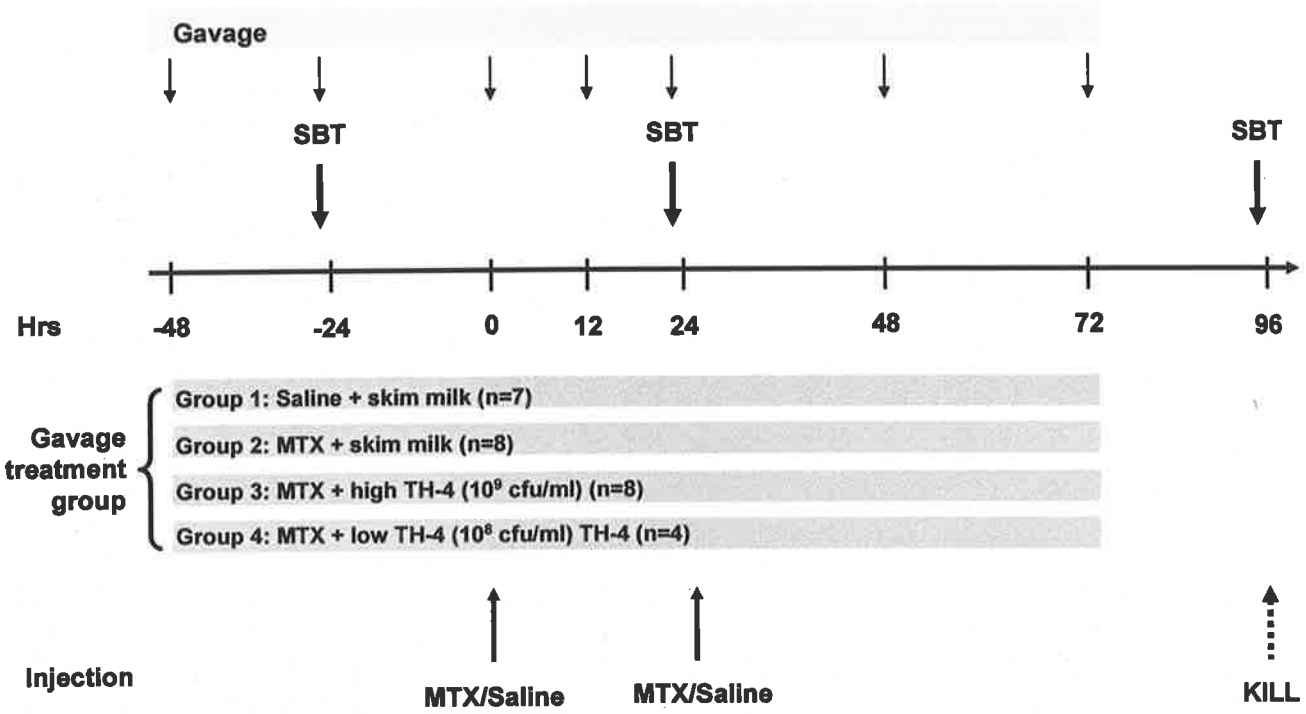

Figure 5.1: Time-course of MTX or saline injected rats (0 h) with daily treatment of vehicle (skim milk) or TH-4, at a dose of 10^8 or 10^9 cfu/mL, -48, -24, 0, 12, 24, 48, and 72 h in relation to administration of MTX or saline. The SBT was conducted -24, 24 and 96 h post-MTX.

-70°C and segments for histological analysis placed in formalin for 24 h and then transferred to 70% ethanol.

Histological assessment

Sections (4 μm) from the duodenum, proximal jejunum and distal ileum were fixed for histological assessment, stained with H&E and examined for damage as previously described in Chapter 4 using the 11 scoring criteria for intestinal damage.[42] Histological images were acquired using a a light microscope (Olympus, BH-2, Tokyo, Japan) and a digital camera (Sony, Tokyo, Japan). The sections were assessed by a blinded, trained investigator.

In vitro assay of sucrase activity

Tissue samples (4 cm) of the duodenum, proximal jejunum and distal ileum were thawed on ice in 1.5 mL 10 mM PBS (pH 6.1), homogenized (mechanical), aliquoted into 200 μL samples and stored at -70°C until sucrase activity analysis was performed as described in Chapter 3. Data was expressed as sucrase activity (glucose nmol/cm/min)

Myeloperoxidase (MPO) activity assay

MPO was determined using a modification of the assay described by Krawisz,[177] as outlined in Chapter 4. Homogenates from the duodenum, proximal jejunum (10%), distal ileum (90%) and total small intestine (a pooled sample using six homogenised small intestinal sections in total: duodenum, proximal and distal jejunum, jejunal/ileal

junction and proximal and distal ileum), were examined. MPO was expressed as U/g tissue.

Statistical analysis

Organ weights were corrected for animal bodyweight and expressed as weight (g) / bodyweight (kg). A Kruskal-Wallis ANOVA with a Dunn's *post hoc* test was used to determine significance for intestinal semi-quantitative histology scoring, where data for each small intestinal section was compared to saline controls. Data were expressed as median with ranges. Remaining data have been expressed as mean ± SEM. A one-way ANOVA, in conjunction with a Tukey's *post-hoc* test, was used for other analyses. All correlations were determined using a Pearson's product moment test and expressed as an r value. Statistical significance was considered if $p < 0.05$. All data and statistical analyses were performed using GraphPad Prism version 3.00 for windows® (GraphPad Software, San Diego, CA, USA) or Microsoft Office 2003 Excel® for Microsoft WindowsXP.

5.3 RESULTS

Effects of TH-4 on rats with MTX-induced mucositis

A time-course of the effects of MTX injection on bodyweight in rats gavaged with either TH-4 or skim milk is illustrated in Figure 5.2. Independent of overnight fasts imposed for the purposes of the SBT, the bodyweights of MTX-treated control rats were

significantly lower ($p < 0.05$) than saline controls at t = 48, 72 and 96h post-MTX. The bodyweights of MTX-treated rats receiving the low (10^8 cfu/mL) dose of TH-4 were significantly lower ($p < 0.05$) than those of normal controls at t = 96 h. In contrast, treatment with the high TH-4 dose resulted in bodyweight not differing significantly from saline controls. However, no significant effect on bodyweight was obtained for either TH-4 dose compared to MTX-treated controls at any time-point. Total loss of bodyweight over the experimental period (see Table 5.1) reflected these results ($p < 0.05$).

The greatest impact of mucositis on food intake was observed in the MTX-treated control rats 96h post MTX, with a 31% decrease in food intake compared to saline-injected controls ($p < 0.001$, Table 5.1). However, administration of low- and high-dose TH-4 to MTX-treated rats partially attenuated this effect with reduced intakes of 22% and 20%, respectively ($p < 0.05$), compared to normal controls. No statistical significance was attained for either TH-4 dose on bodyweight compared to MTX-treated controls. MTX injection had no significant effect on fluid intake compared to normal controls and there were no significant effects of either TH-4 dose on water intake throughout the experimental period ($p>0.05$, Table 5.1).

Thymus weight [weight (g) / bodyweight (kg)] in MTX-treated control rats was 42% lower than normal controls, $p < 0.001$ (Table 5.2). MTX-treated rats receiving the low, or high, doses of TH-4 also recorded significant reductions in thymus weights of 37% ($p < 0.01$) and 23% ($p < 0.05$) respectively, compared to normal controls, although neither TH-4 dose was statistically significant compared to MTX-treated control rats. The weights of all other non-gastrointestinal organs in MTX-treated controls and MTX-

Daily parameters	Gavage Treatment			
	Saline Control	MTX Control	MTX + High TH-4	MTX + Low TH-4
Fluid Intake (mL)	101 ± 5	100 ± 8	94 ± 3	123 ± 8
Food Intake (g)	55 ± 4	38 ± 2#	44 ± 1*	43 ± 2*
Body wt Δ (g)	-6 ± 2	-12 ± 2#	-10 ± 1	-14 ± 2*

Table 5.1: Effects of TH-4 (10^8 or 10^9 cfu/mL) treatment on food and water intake and change in bodyweight 96 h after administration of saline or MTX to rats. Data are expressed as Mean ± SEM. Statistical significance compared to saline controls, where * denotes $p < 0.05$ and # $p < 0.001$.

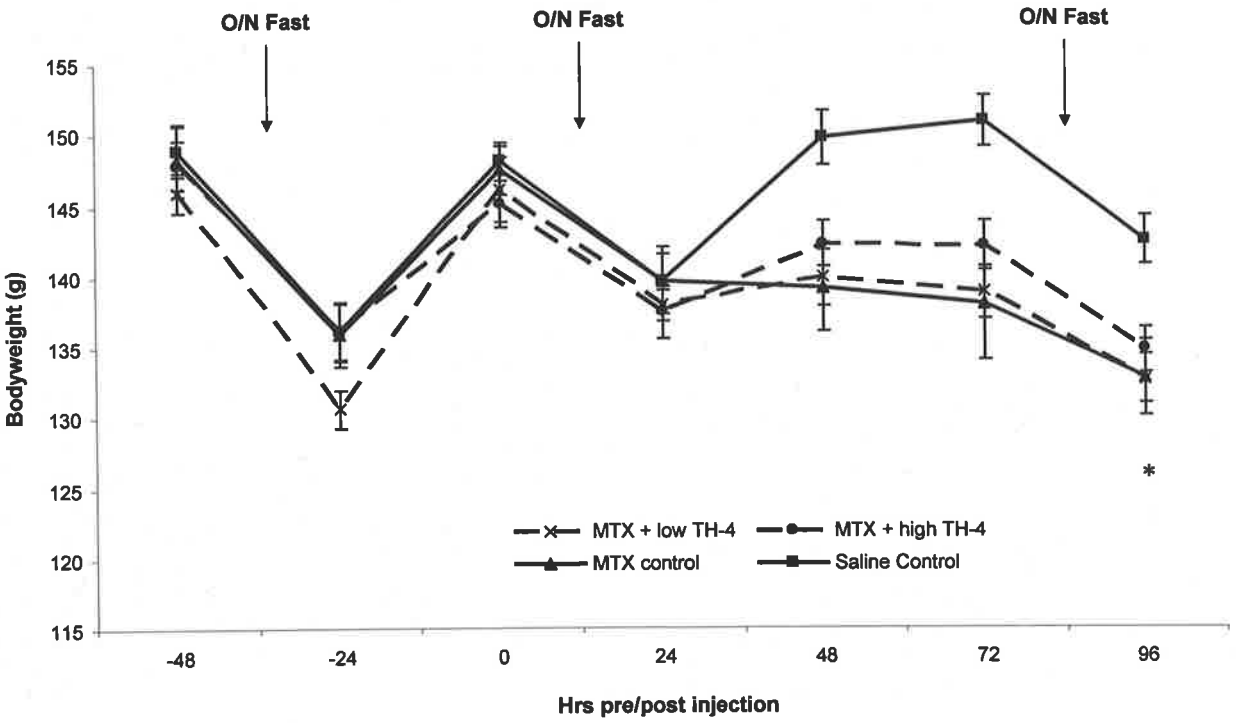

Figure 5.2: Daily body weight changes in rats (148±1g) with respect to MTX or saline injection, where overnight (O/N) fasts are indicated. Treatment groups as indicated above. Data expressed as mean ± SEM. * denotes significance ($p < 0.05$) of low dose TH-4 and MTX control compared to saline controls.

Weights (wt g/kg bwt)	Gavage Treatment			
	Saline Control	MTX Control	MTX + High TH-4	MTX + Low TH-4
Small Intestine	30.2 ± 1.1	33.4 ± 1.8	30.4 ± 0.9	30.9 ± 1.3
Colon	8.0 ± 0.8	5.8 ± 0.2	6.8 ± 0.5	7.0 ± 0.3
Stomach	7.0 ± 0.2	6.9 ± 0.2	7.6 ± 0.6	7.1 ± 0.2
Heart	4.3 ± 0.1	4.2 ± 0.1	4.6 ± 0.1	4.3 ± 0.5
Lungs	6.3 ± 0.2	6.7 ± 0.2	6.4 ± 0.2	7.0 ± 0.5
Thymus	1.7 ± 0.1	1.0 ± 0.1 #	1.3 ± 0.1 +	1.1 ± 0.2 *
Spleen	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.1
Liver	29.7 ± 0.9	31.6 ± 0.7	30.1 ± 0.4	30.3 ± 3.5
Left Kidney	3.9 ± 0.1	4.0 ± 0.1	4.1 ± 0.1	4.1 ± 0.1
Right Kidney	4.1 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	3.9 ± 0.1

Table 5.2: Fractional weights of visceral organs in rats injected in rats. Data are expressed as Mean ± SEM. # denotes significance between saline controls and MTX controls ($p < 0.001$); +denotes significance between controls and MTX + high TH-4 ($p < 0.05$); *denotes significance between controls and MTX + low TH-4 ($p < 0.01$).

treated rats receiving TH-4 did not differ significantly compared to normal animals (Table 5.2).

Compared to saline controls (Table 5.3) the length of the colon was decreased following MTX treatment ($p < 0.05$). In contrast, colon length following administration of either TH-4 dose did not differ significantly from saline or MTX-treated controls. However, colon weight in rats receiving the high TH-4 dose was significantly lower than in those receiving the low TH-4 dose ($p < 0.05$). Shortening of the duodenum was not pronounced in MTX-treated controls compared to saline controls, nor was high or low dose TH-4. However, the high TH-4 dose resulted in a significant lengthening of the duodenum ($p < 0.05$) compared to MTX-treated control rats (Table 5.3).

Small intestinal function: SBT results and in vitro sucrase activity

Figure 5.3 shows the time-course of $^{13}CO_2$ production in rats from each treatment group 96 h after MTX injection. The SBT remained normal for MTX-treated rats receiving skim milk, high TH-4 or low TH-4, 24 h pre- and 24 h post-MTX, compared to controls (Figure 5.4A). MTX-treated control rats receiving skim milk (48% decrease) or the low dose of TH-4 (41% decrease) had a significantly lower SBT result compared to saline-injected controls at 96 h, where $p < 0.001$ and $p < 0.01$, respectively (Figure 5.4B). In contrast, at 96 h, SBT values in MTX-treated rats receiving the high TH-4 dose did not differ significantly from those of saline-treated controls. In addition, SBT values following administration of the high TH-4 dose were significantly higher than both MTX-treated controls and rats receiving the low TH-4 dose ($p < 0.01$ and $p < 0.05$ respectively).

Gut Tissue	Gavage Treatment			
	Saline Control	MTX Control	MTX + High TH-4	MTX + Low TH-4
Duodenum				
Weight (g)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Length (cm)	7.2 ± 0.2	6.4 ± 0.3	7.5 ± 0.2 *	6.6 ± 0.2
Jejunum/Ileum				
Weight (g)	3.8 ± 0.2	4.0 ± 0.3	3.6 ± 0.1	3.6 ± 0.2
Length (cm)	68.6 ± 1.0	69.1 ± 1.3	72.5 ± 1.1	67.8 ± 3.1
Colon				
Weight (g)	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1 #	1.1 ± 0.1
Length (cm)	13.5 ± 0.3	11.6 ± 0.4 +	12.9 ± 0.3	12.8 ± 1.0

Table 5.3: Weights and lengths of small intestinal regions for all treatment groups. Data are expressed as Mean ± SEM. * denotes significance between MTX control and MTX + high TH-4, where $p < 0.05$; # denotes significance between MTX + high TH-4 and Low TH-4 + MTX, where $p < 0.05$; + denotes significance between MTX controls and saline controls, where $p < 0.05$.

The degree of small intestinal damage induced by MTX, as indicated by the SBT results, reflected the *in vitro* sucrase activity determinations of small intestinal homogenates. Duodenal sucrase activity was significantly decreased by 99% in MTX-treated control rats compared to saline controls (2 ± 1 vs. 229 ± 22 nmol glucose/min/cm tissue, $p < 0.001$), with a similar decrease evident in rats receiving the low TH-4 dose (Figure 5.5). Duodenal sucrase activity in rats receiving the high dose of TH-4 was significantly lower compared to saline controls ($p < 0.001$), but was significantly higher compared to MTX–injected controls ($p < 0.05$).

Jejunal sucrase activity in MTX-treated controls was significantly lower (85%, $p < 0.001$) compared to saline controls, (42 ± 15 vs. 279 ± 26 nmol glucose/min/cm tissue) whilst rats receiving the low TH-4 dose recorded a similar decrease (41 ± 19 nmol glucose/min/cm tissue, $p < 0.001$). Jejunal sucrase activity following treatment with the high TH-4 dose was significantly higher (268 ± 36 nmol glucose/min/cm tissue) than both MTX-treated controls and rats receiving the low dose of TH-4 ($p < 0.001$), and importantly, was not significantly different from saline-treated control values.

No significant difference was observed in ileal sucrase activity between MTX-treated control rats and normal saline controls, nor did treatment with TH-4 at either dose evoke a change ($p > 0.05$). The average sucrase activity in the three intestinal regions is illustrated in Figure 5.5, where MTX-treated control rats had a significant decrease of 87% compared to saline controls ($p < 0.001$). Similarly, the low dose of TH-4 resulted in a 87% decrease in sucrase activity compared to saline controls, and high TH-4 by 34% ($p < 0.001$ and $p < 0.01$, respectively). The high dose of TH-4 resulted in a

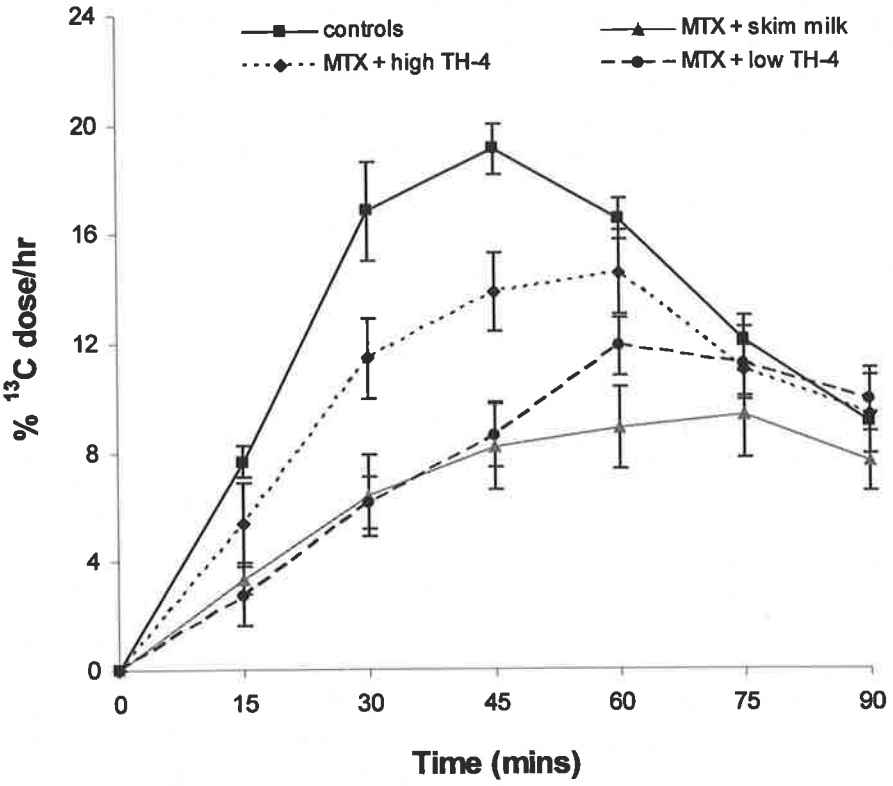

Figure 5.3: Breath $^{13}CO_2$ levels (expressed as % ^{13}C dose excreted per hour) following sucrose gavage in rats at 96 h receiving either MTX + skim milk (n = 8, MTX controls), MTX + high TH-4 (n = 7) or MTX + low TH-4 (n = 4), and rats receiving saline + skim milk (saline controls, n = 7). Data expressed as mean ± SEM.

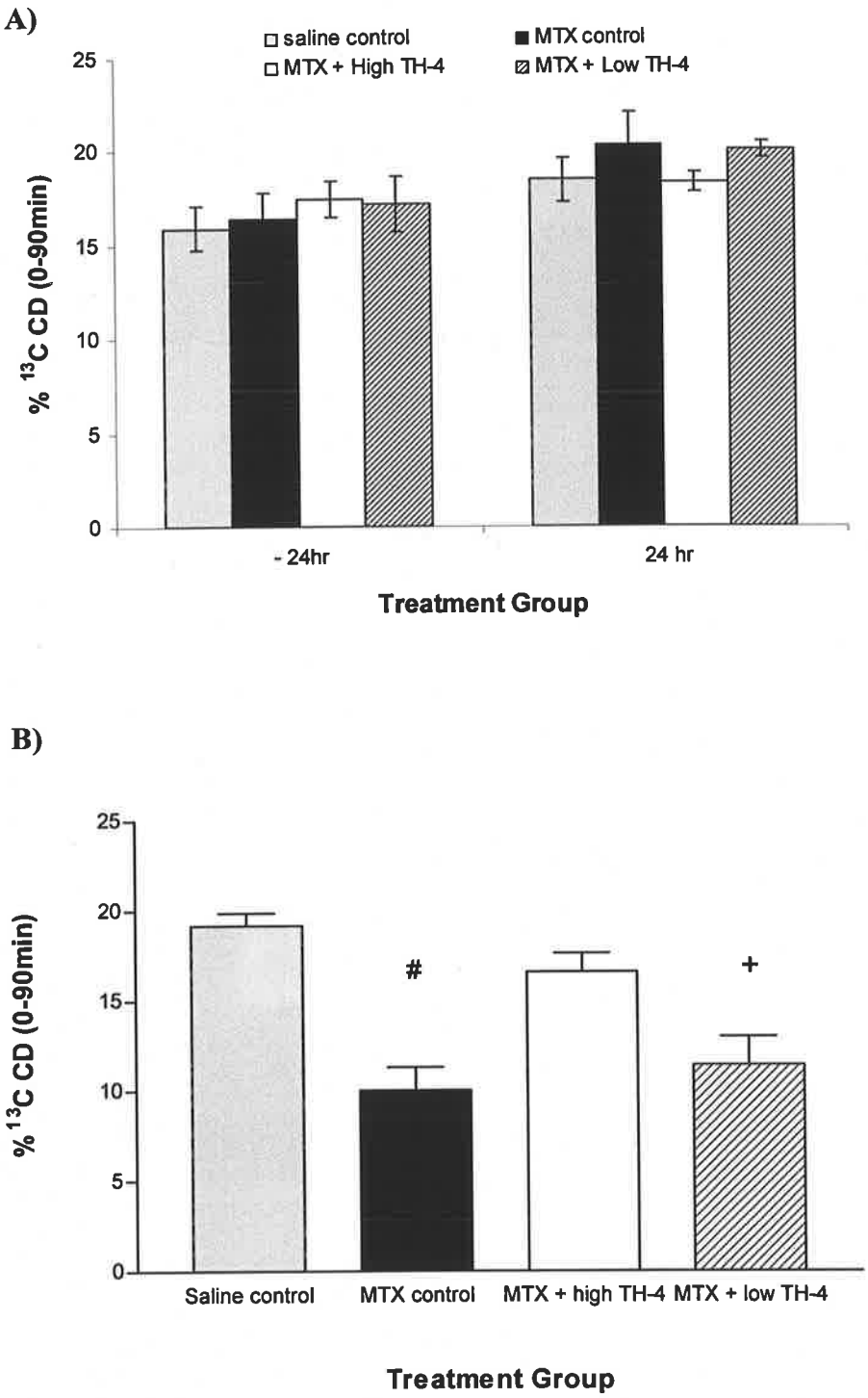

Figure 5.4: (A) SBT ($\%CD_{90}$) 24 h prior to, and 24 h post, MTX injection in rats. Treatment groups as indicated. NS. (B) The effects of high or low dose of TH-4 on the SBT 96 h post MTX compared to saline and MTX controls in rodents. Data expressed as % cumulative dose (%CD), mean ± SEM. Significance denoted by $^{+}$ ($p < 0.01$) and $^{\#}$ ($p < 0.001$) compared to controls.

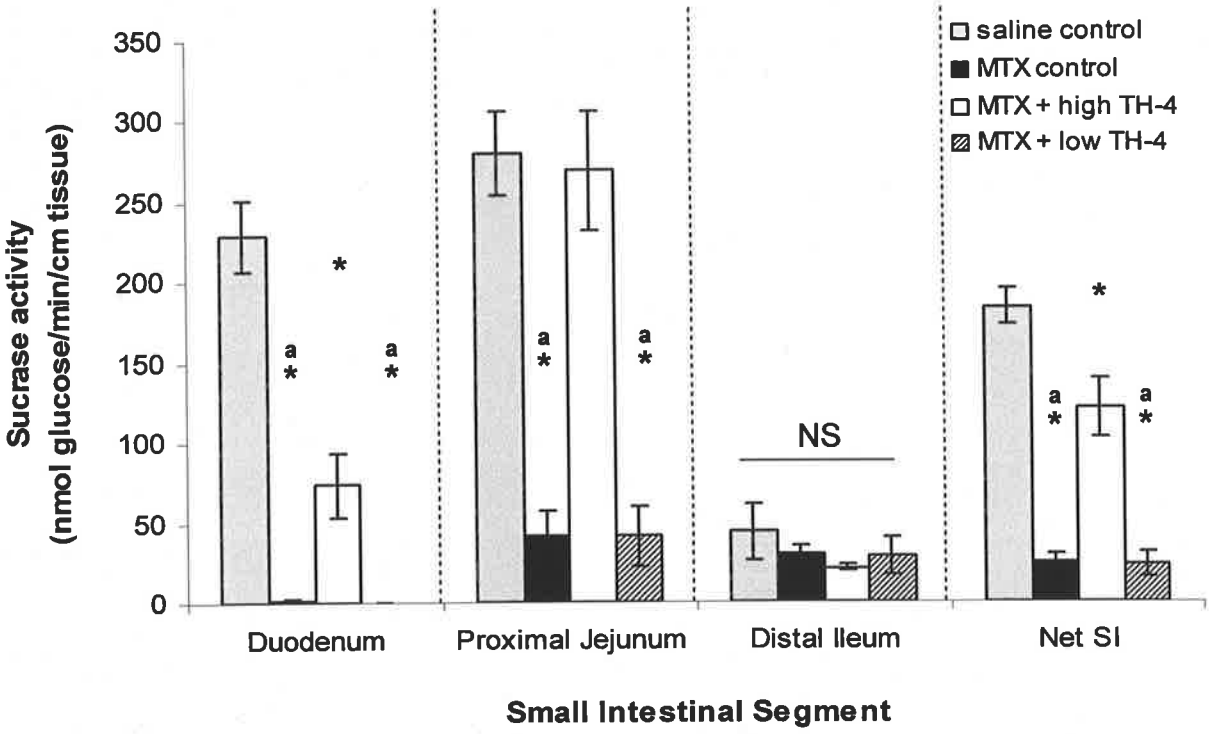

Figure 5.5: In vitro sucrase activity in the duodenum, proximal jejunum, distal ileum and the average of the sections [Average small intestine (SI)], expressed as nmol glucose/min/cm tissue, mean ± SEM. * denotes significant difference ($p < 0.05$) compared to controls and [a] denotes significance to MTX + TH-4 ($p < 0.05$).

significantly higher sucrase activity compared to MTX-treated control rats and those receiving the low TH-4 dose, by 48% and 34% respectively ($p < 0.001$).

Since the SBT is a measure of sucrase activity of the entire small intestine, an average of the small intestinal activity in the three sampled intestinal segments (duodenum, proximal jejunum and distal ileum) was calculated to allow a more appropriate correlation with the SBT, where an r value of 0.91 was evident (Figure 5.6).

Inflammation and intestinal damage

MTX-injection resulted in significant damage to the duodenum, proximal jejunum and distal ileum when assessed by the semi-quantitative histological severity score ($p < 0.05$; Table 5.4), although features of mucositis were less severe in the ileum. Typical histological features of proximal jejunum in rats treated with MTX ± TH-4 are depicted in Figure 5.7. Tissue damage in MTX-treated control rats consisted of moderate villus blunting, irregular enterocytes, shallow and disrupted crypts, increased neutrophil infiltration, oedema and thickening of the muscularis externa. Similarly, these histological findings were also seen in rats receiving low dose TH-4.

In contrast, in rats receiving high dose TH-4, the epithelial architecture in the jejunum was protected and mirrored histology as seen in saline control rats. The observed villi lengths are consistent for a state of epithelial regeneration and repair, which supports earlier findings in Chapter 3 and the desired time-point for treatment efficacy. Rats receiving the low TH-4 dose reflected similar histological results to MTX-treated

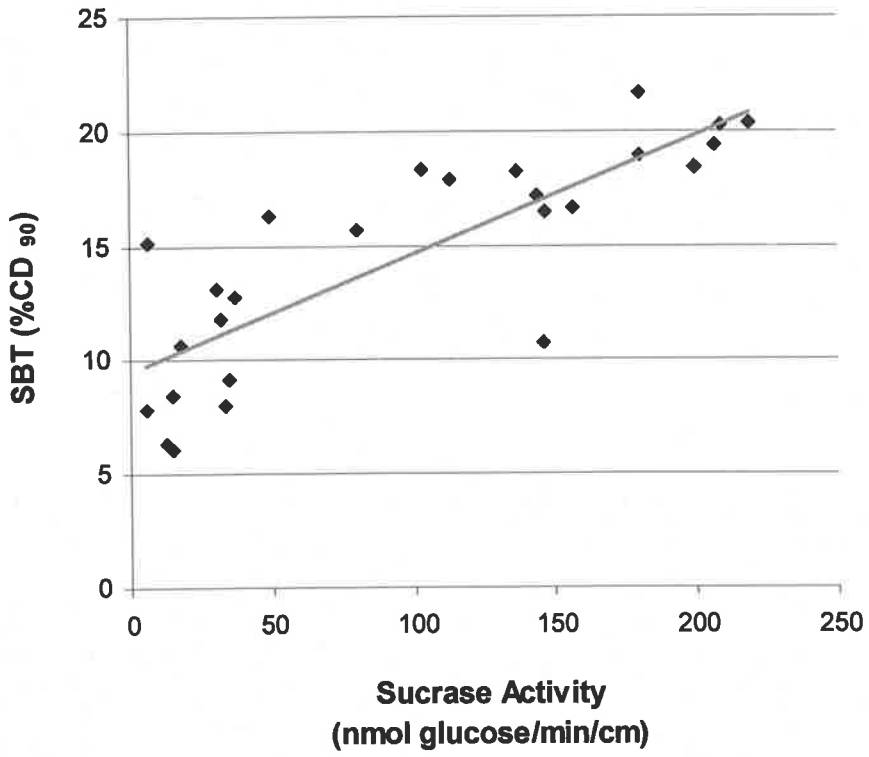

Figure 5.6: Correlation between SBT (% ^{13}C Cumulative Dose at 90 minutes) and total small intestinal sucrase activity (nmol glucose/min/cm), determined biochemically, 96 h after administration of MTX in female DAR, where r = 0.91

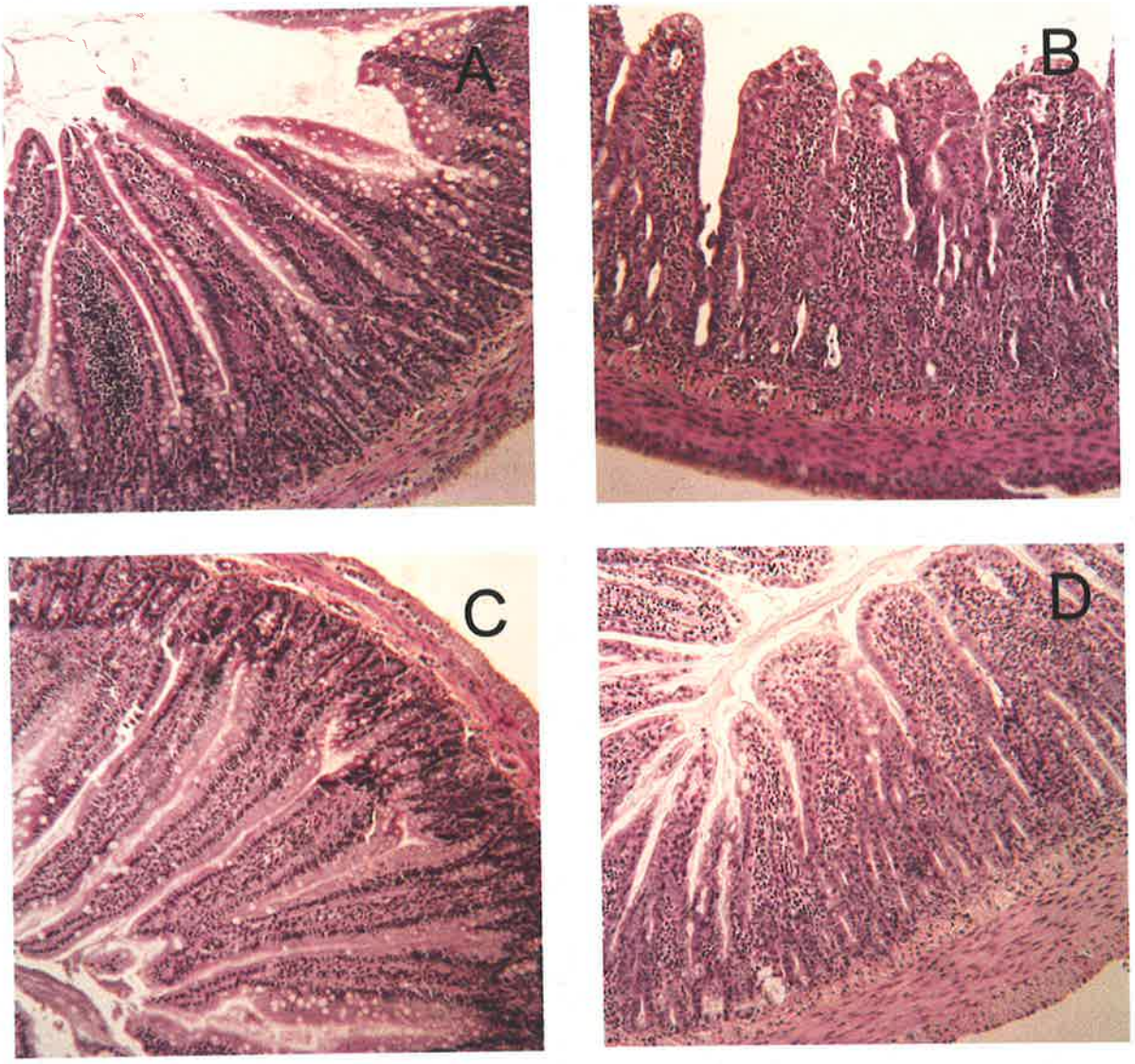

Figure 5.7: Photomicrographs of proximal jejunum histological sections with H&E staining, in rats 96 h post initial MTX/Saline injection: (A) Saline controls; (B) MTX controls; (C) High dose TH-4; and (D) Low dose TH-4. Note the loss of epithelial architecture in the crypt and villus, with neutrophil infiltration and oedema in the MTX controls and low dose TH-4 groups. A high dose of TH-4 protects the jejunum architecture as seen when compared to saline controls.

Gut Tissue	Gavage Treatment			
	Saline Control	MTX Control	MTX + High TH-4	MTX + Low TH-4
Duodenum	0	18 (10 – 23) $^{\#}$	10 (3 – 12)	15 (5 – 22) *
Jejunum (10%)	0	16 (11 – 24) $^{\#}$	11 (8 – 14)	17 (10 – 23) $^{+}$
Ileum (90%)	0	13 (9 – 20)$^{+}$	9 (3 – 13)	16 (9 – 16) *

Table 5.4: Semi-quantitative histological assessment of intestinal tissues from rats 96 h after MTX injection. Values are the sum for 11 independent histological criteria.[42] Data are expressed as median (range). Statistical significance compared to saline controls, where * denotes $p < 0.05$, $^{+}$ $p < 0.01$ and $^{\#}$ $p < 0.001$.

control rats for each respective region, whilst histological features in rats receiving the high TH-4 dose did not differ significantly from saline-treated controls or other treatment groups (Table 5.4). Correlations between SBT results and histological severity score were as follows: Duodenum: $r = 0.87$; proximal jejunum: $r = 0.85$; and distal ileum: $r = 0.84$.

Myeloperoxidase (MPO) levels (Figure 5.8) in duodenal specimens were increased in MTX-treated controls compared to saline-treated animals ($p < 0.05$). MPO levels in rats receiving the low TH-4 dose were also significantly increased compared to saline controls, whilst MPO levels in the rats receiving the high dose of TH-4 did not differ significantly from saline controls. Proximal jejunal MPO levels were not as marked compared to the duodenum, however, MTX-injected control rats continued to have an elevated MPO activity compared to saline controls ($p < 0.05$). Irrespective of TH-4 dose, duodenal MPO activity was not significantly different compared to saline-treated controls. In the distal ileum, MPO activity in MTX-injected controls was not significantly different compared to saline-treated controls, and the high dose of TH-4 produced similar results. However, MPO values following the low TH-4 dose were significantly increased compared to saline controls ($p < 0.05$). When small intestinal homogenates from the duodenum, proximal and distal jejunum, jejunal/ileal junction and proximal and distal ileum were pooled (data not shown), MPO activity in MTX-injected rats was significantly elevated compared to saline controls ($p < 0.05$). In contrast, pooled MPO levels in rats treated with TH-4 at either dose, were not significantly different compared to saline controls ($p > 0.05$) or MTX-treated controls.

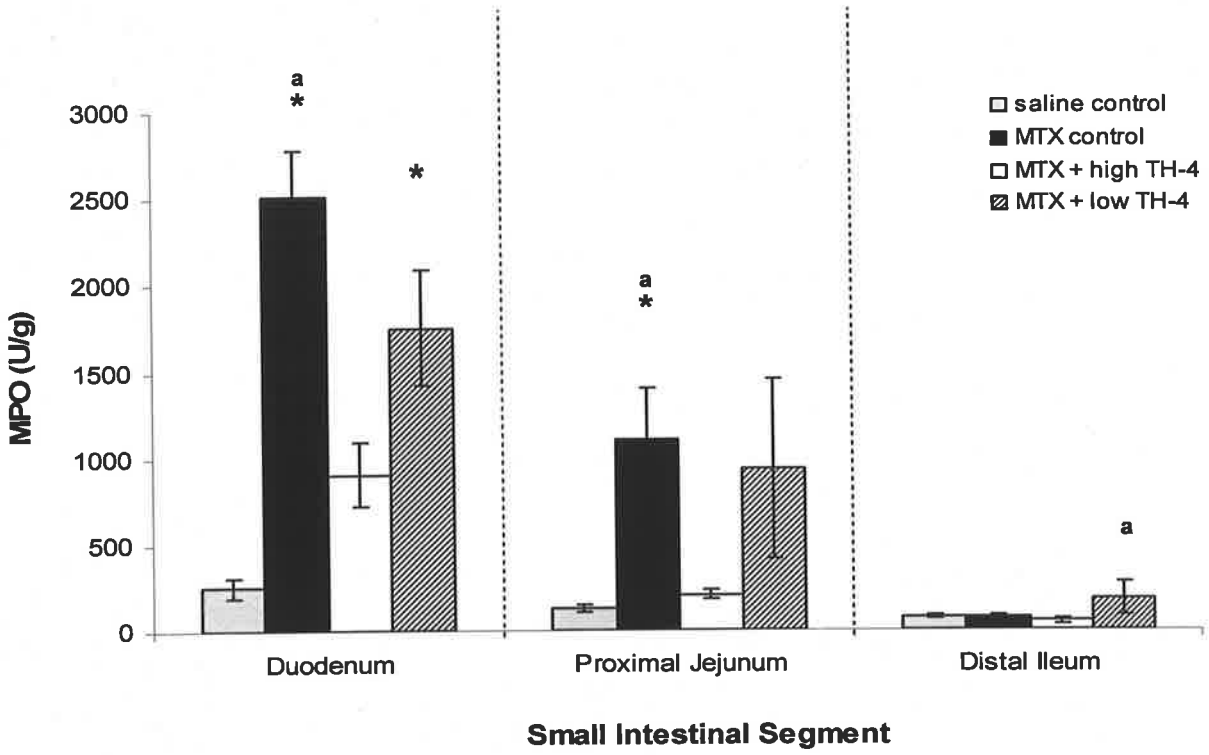

Figure 5.8: Effects of orally administered TH-4 on myeloperoxidase activity (MPO U/g) on the duodenum, proximal jejunum and distal ileum, determined biochemically, assessed 96 h after MTX-injection in rodents. Data expressed as mean ± SEM, and * denotes $p < 0.05$ compared to controls.

5.4 DISCUSSION

Numerous studies have been conducted to assess potential therapies for the amelioration of chemotherapy-induced mucositis all with varying efficacy. More recently, these studies have largely been confined to KGF[48,122,125], IGF-1,[43] glutamine supplementation,[112,137] and whey derived growth factors,[42] when administered orally, and where endpoints of efficacy have relied heavily upon patient symptoms and the development of oral complications. However, it has become increasingly apparent that epithelium of the oral cavity does not necessarily reflect small intestinal health. Moreover, to determine the efficacy of anti-mucositis therapeutics in rodents, previously described endpoints have required rodents to be sacrificed. In recent times, a new non-invasive breath test, the SBT, has become available to assess small intestinal damage and functional absorptive capacity in chemotherapy-treated animals,[39,54,103] allowing longitudinal monitoring of the timing and severity of damage in individual animals. In the current study, the oral administration of *Streptococcus thermophilus* (TH-4), a potential new treatment modality, was assessed for its capacity to decrease the severity of chemotherapy-induced mucositis utilizing the SBT as a non-invasive indicator.

The current study showed that orally ingested *S. thermophilus* (TH-4) at a dose of 10^9 cfu/mL, partially attenuated mucositis. Importantly, this damage was measurable using the SBT. Previous studies of MTX administration to DAR have induced similar effects on food intake and bodyweight irrespective of the fasting required to perform the SBT.[42,43,49,103] The administration of TH-4 at two differing doses produced differing results. TH-4 at a dose of 10^8 cfu/mL offered no protection, producing a similar SBT

and biochemical sucrase activity, MPO level and histological severity to that seen in MTX + vehicle rats. In contrast to MTX-treated controls a ten-fold increase in the level of TH-4 reduced the level of damage induced by MTX. TH-4 at the higher dose partially prevented the loss of bodyweight and the decrease in food intake. The high TH-4 dose also normalized the SBT and total small intestinal sucrase activity, and improved food intake. This is an important finding since malnutrition, or short-term fasting, has been associated with apoptosis and proliferation rates of enterocytes and a decrease of small intestinal disaccharidases.[189]

The higher dose (10^9 cfu/mL) of TH-4 partially protected the duodenum with respect to biochemical sucrase activity, but protected the proximal jejunum completely, although the mechanism of protection of TH-4 in the MTX-mucositis model is not known. To our knowledge, this is the first study to assess TH-4 in this model of MTX-induced small intestinal damage. Previous studies have shown *S. thermophilus* to be a transient colonizer of the gut, where its concentration dramatically decreased below measurable limits in patient's faeces only six days after the cessation of a 10 day probiotic yoghurt treatment.[190] This characteristic is favourable in the setting of chemotherapy-induced mucositis as treatment is desired for only a defined period of time. Persistent colonisation could increase the likelihood of bacterial translocation, leading to a heightened risk of septicaemia.

S. thermophilus is currently not classified as a probiotic when administered on its own, therefore, gastrointestinal disorders, in which *S. thermophilus* has been clinically assessed, have usually been combined with at least one known probiotic. *S. thermophilus* has been shown to protect the epithelium of the gastrointestinal tract from

an enteroinvasive *Eescherichia coli* infection when combined with *Lactobacillus acidophilus*,[145] and has reduced the incidence and severity of acute diarrhoea in infants when combined with either *Bifidobacterium breve* C50 or *B. bifidum*.[148,149] VSL # 3 is a well documented probiotic combination, which contains *S. thermophilus,* demonstrating usefulness in protecting the bowel and ameliorating colonic inflammation in rats with experimentally-induced inflammatory bowel disease.[191] Moreover, VSL#3 has minimized the rates of relapse in patients with colitis,[192] and pouchitis[150], as well as the prevention of post-operative recurrence of Crohn's Disease.[150] Furthermore, extracted DNA from VSL#3 has been shown to protect experimental colitis induced by dextran sulphate sodium, where the mechanism was linked to an inhibitory effect on apoptosis and inflammation.[193] In mice with colitis, a combination of *Bifidobacterium breve* and *S. thermophilus* has been reported to enhance Th1 immune responses and intestinal barrier function.[147]

Only two studies, to our knowledge, have addressed the efficacy of probiotics in an animal mucositis model. It has been shown that *Lactobacillus plantarum* improved the food intake and bodyweight in rats receiving 5-Fluorouracil (5-FU). This study demonstrated that *L. plantarum* could only reduce some of the side-effects and complications associated with 5-FU.[194] More recently a study by Mauger and colleagues (2007)[195] demonstrated that *L. fermentum BR12*, *L rhamnosus GG* or *Lactis BB12* at doses of 10^6 cfu/mL had no positive effects on the small intestine in 5-FU treated rats. Unpublished data from our laboratory has indicated that rats receiving *Lactobacillus johnsonii* decreased small intestinal permeability in MTX-treated rats. Generally speaking, *lactobacilli* and *bifidobacteria* have been shown to enhance intestinal

epithelial barrier function and a preventative or therapeutic potential in human disease that are associated with pathogenic invasion.[143]

In conclusion, *Streptococcus thermophilus* (TH-4) administered to female DAR at a dose of 10^9cfu/mL partially attenuated MTX-induced mucositis, which could be detected and monitored by the SBT. Alternatively, TH-4 at a dose of 10^8 cfu/mL failed to protect the small intestine. The SBT provides a simple and non-invasive means to assess the efficacy of novel anti-mucositis and other bioactive agents on the small intestine, where the SBT can be used for longitudinal assessment over time. *S. thermophilus* demonstrated some probiotic properties, however, its mechanism of action requires further investigation. Further studies could investigate the effectiveness of increased doses of TH-4, or as a combination with a proven probiotic or growth factor, in which the SBT can be applied to monitor decreases in the severity of chemotherapy-induced mucositis.

CHAPTER 6: *STREPTOCOCCUS THERMOPHILUS* IN A TUMOUR-BEARING DAR MODEL WITH MTX TREATMENT

6.1 INTRODUCTION

It is important that putative treatments for mucositis do not promote tumour growth or compromise apoptosis and tumour cell death during chemotherapy.[46] In the previous chapter it was demonstrated that administration of TH-4 partially attenuated MTX-induced mucositis, with maximal protection in the proximal jejunum. The effects of TH-4 on tumour growth have not been assessed. In our laboratory we have used a rat model with mammary adenocarcinoma,[46,196] based on the inoculation of specific mammary adenocarcinoma cells to the DAR.[48] Indices of tumour establishment and progression were monitored in rats following TH-4 treatment to determine TH-4 affected growth of the tumour. Simultaneously the efficacy of TH-4 on ameliorating MTX-induced mucositis in a tumour-bearing DAR was assessed.

6.2 MATERIALS & METHODS

Animals

Thirty-six female DAR (initial bodyweight: 138.8 ± 1.2 g) were acquired from the IMVS Gilles Plains, Adelaide. Rats were individually housed in single rat cages for the entire study and not metabolism cages, as used previously. This caging was for comfort of the animals, as expected large tumour growth on their rear flanks hampers their ability to reach their food through the narrow gate. To allow the monitoring of food consumption, temporary plastic feed holders were designed and incorporated into each individual rat cage. Rodents were housed in the Animal Care Facility of the Children, Youth and Women's Health Service (Women's and Children's Hospital Campus) with an environmental temperature of 25°C with a 12 h light:dark cycle. Ethical approval was obtained from the Animal Ethics Committees of the Children, Youth and Women's Health Service and the University of Adelaide. All experimentation complied with the National Health and Medical Research Council (Australia) Code of Practice of Animal Care in Research and Training (2004).

Streptococcus thermophilus (TH-4) inoculum

The *S. thermophilus* inoculum was prepared as previously outlined in Chapter 5.[55] A dose of 10^9 cfu/mL TH-4 was produced and was confirmed by a viable count. A total volume of 150 mL of 10^9 cfu/mL TH-4 and skim milk (gavage vehicle) was prepared for administration to the rats. Treatments of TH-4 were administered to saline-control and MTX-treated rats with tumours.

Tumour passage protocol

The mammary adenocarcinoma used in this DAR (DAMA) model arose spontaneously in the 1970s.[196-198] This cancer cell line was kindly donated by Dr A. Rofe (IMVS, Adelaide, Australia) and Dr R Gibson (Hanson Institute, IMVS, Adelaide, Australia). The method to establish the tumour model was kindly shared by Gibson et al (2002).[46] The first tumour passage after a freeze-thaw process commonly produces a tumour that rapidly proliferates and has a high degree of necrosis. To overcome this, two tumour passages must occur before injection of MTX to rats with the mammary adenocarcinoma. Initially, one female donor DAR was injected in the rear flanks (s.c.) with 0.5 mL (2.0×10^7 cells/mL) of tumour inoculum and the subsequent tumours were resected 14 days later. The rat was sacrificed via CO_2 asphyxiation and cervical dislocation. Subcutaneous tumours were removed and placed into sterile phosphate buffer solution (PBS; 16% NaCl; 0.4% KCl; 0.4% KH_2PO_4; 2.3% Na_2HPO_4 anhydr) for subsequent passage.

Two DAR were then anaesthetised, shaved and injected with 0.2 mL (2×10^7 cells/mL) of the prepared breast cancer cells. Remnant tumour cells were aliquoted and stored in liquid nitrogen for future studies. The two DAR were culled and tumours removed for passage to n = 36 rats using the tumour passage method previously described.

Tumour inoculum preparation

Briefly, any necrotic tissue adjacent to the tumour was removed and the remaining tumour tissue diced into small pieces, manually homogenised in PBS and filtered

through sterile gauze.[46] The tumour cell suspension was centrifuged at 1100 rpm for 3 min. The supernatant was then removed and replaced with PBS after. This was repeated 4 times. A viable tumour cell count was then performed using 0.1% nigrosine.

Experimental design: TH-4 administration to MTX-treated DAMA

All Rats (n = 36) were injected (s.c.) in the rear flanks with 2 x 10^7 cells/mL of tumour cells -188 h / 7 days prior to MTX injection. Rats were allocated to four groups (Figure 1): Group 1 (n = 9) received saline (sodium chloride injection for BP, 1.5 mmol NaCl; Astra Zeneca, North Ryde, New South Wales, Australia) via intra-muscular (i.m.) injection and skim milk (vehicle) gavage; Group 2 (n = 9) received saline and 10^9 cfu/mL TH-4; Group 3 (n = 9) received 1.5 mg/kg (i.m.) MTX (Pharmacia Corporation, Peapack, New Jersey, USA) and skim milk (vehicle); and group 4 (n = 9) received MTX and 10^9 cfu/mL of TH-4. Rats were individually housed after receiving tumour cell injections, to ensure safety and well-being of rats. Mucositis was induced by administration of MTX as outlined in Chapters 2 and 3.

Standard rodent chow was removed from the rat cages three days prior to tumour inoculation. Rats were allowed access to an 18% casein-based diet[127] and to water for the duration of the protocol. Rats were gavaged with 1 mL of TH-4 or skim milk solution at -48, -24, 0, 12, 24, 48 and 72 h in relation to the time of the first MTX or saline injection (Figure 6.1). Bodyweights, fluid and food intakes were measured daily. Tumour growth in DAR was monitored regularly (1 - 2 days) to ensure animal health using a mathematic calculation previously determined by Dr A. Rofe.[196-198]

Additionally, tumours were measured before chemotherapy (0 h) and prior to sacrifice (96 h) using digital callipers to determine approximate tumour weight and tumour weight as a percentage of bodyweight. Rats were sacrificed 96 h after the initial MTX or saline injection.

^{13}C-sucrose breath test (SBT)

The SBT was applied to non-invasively determine small intestinal function throughout the tumour passage and MTX/saline treatment. The SBT was performed on all rats at approximately 0900 h at each designated time-point. The SBT was performed in rats at: (1) -188 h to determine small intestinal function in DAR prior to tumour inoculation; (2) -24 h to assess if the mammary adenocarcinoma had any effect on small intestinal function prior to chemotherapy; (3) at 96 h to assess small intestinal function after chemotherapy and administration of TH-4 (Figure 6.1). The protocol followed is outlined in Chapter 2, using a sucrose dose of 0.25 g/mL in water. Data was expressed as $\%CD_{90}$ as previously described.

Kill procedure and tissue collection

Ninety-six hours after the first MTX/saline injection, rats were sacrificed via CO_2 asphyxiation and cervical dislocation. Blood samples were collected from each rat via cardiac puncture and placed into heparinised tubes, centrifuged at 3000 g for 10 min, for separation of plasma. Aliquots of plasma were stored at -70°C for later analysis. All remaining tissue was collected as outlined in Chapter 3. For each intestinal segment collected (see Chapter 3 for the determination of segments), samples for histological analysis (2 cm) and frozen sections (4 cm) were collected. Samples for histological

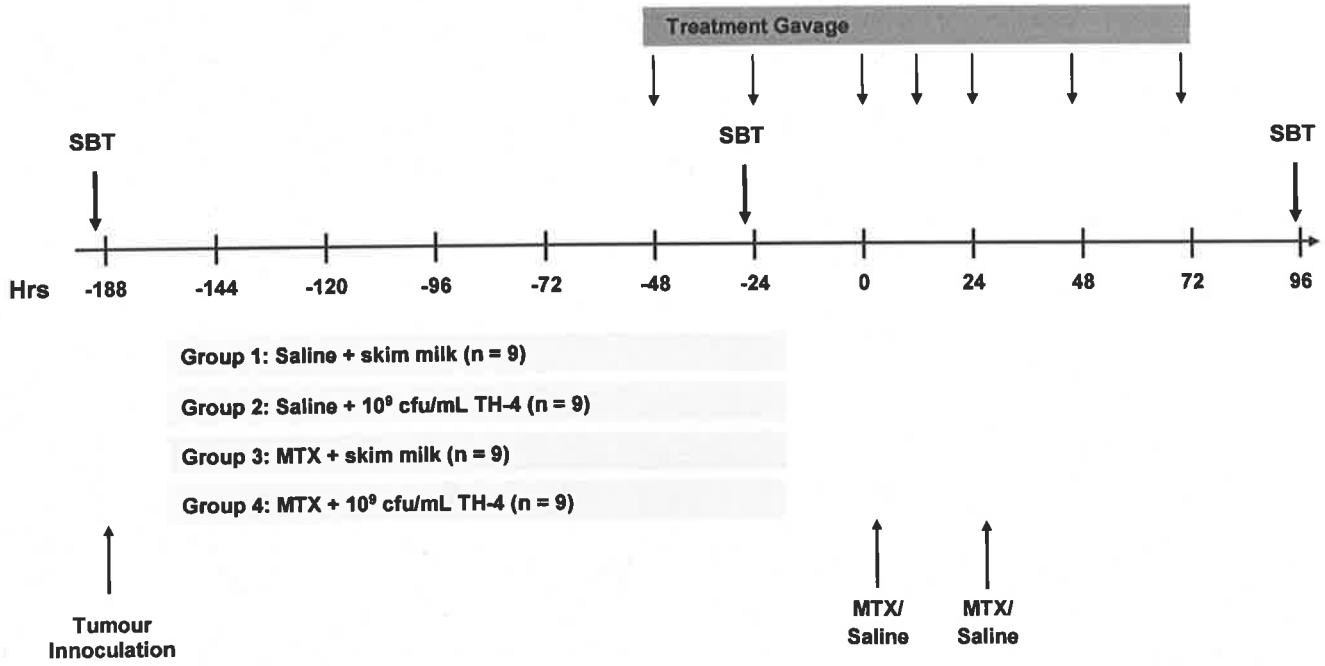

Figure 6.1: Time-course of MTX or saline injected rats with DA-specific mammary adenocarcinoma (0 and 24 h) with daily treatment of skim milk (vehicle) or TH-4 (10^9 cfu/mL) -48, -24, 0, 12, 24, 48 and 72 h in relation to administration of MTX or saline. The SBT was conducted at -188 (pre-tumour), -24 (pre-MTX) and 96 h (post-MTX). Note, DAR were inoculated with tumour cells at -188 h pre-MTX.

analysis were placed in formalin for 24 h and transferred to 70% EtOH. Sections for biochemical analyses (4 cm) were weighed and immediately placed in liquid nitrogen and stored at -70°C for later analysis.

Biochemical and histological analyses

Sucrase activity, MPO activity and small intestinal morphometry analyses were carried out as previously described in Chapters 3 and 4. Briefly, homogenate tissue samples (4 cm) of the duodenum, jejunum (proximal and distal jejunum 4 cm segments pooled) and ileum (proximal and distal ileum 4 cm segments pooled) were assessed for sucrase and MPO activity, where sucrase activity was expressed as nmol glucose/min/cm and MPO expressed as U/g tissue. Additionally, sucrase activity data from each small intestinal segment was averaged to determine a "Net SI" to reflect total SI sucrase activity. Proximal jejunum and distal ileum segments were fixed, stained with H&E and examined for histological damage as outlined in Chapter 4.

Statistical analyses

All data were expressed as mean ± SEM. Histological parameters were analysed using a non-paramatric one-way ANOVA (Mann-Whitney) with a Dunn's *post-hoc* test. All remaining data were analysed using a one-way ANOVA with a Tukey's *post-hoc* test. Significance was determined when $p < 0.05$. All data and statistical analyses were performed using GraphPad Prism version 3.1 for Windows® (GraphPad Software, San Diego, CA, USA).

6.3 RESULTS

Effect of TH-4 in the rat with DA-specific mammary adenocarcinoma

The effects of MTX on daily indices in rats with mammary adenocarcinoma are illustrated in Table 6.1. Regardless of TH-4 treatment, rats receiving MTX had a significantly larger ($p < 0.001$) decrease in bodyweight (g) compared to both saline control rats (receiving saline and skim milk) and TH-4 control rats (receiving saline and skim milk). Importantly, no changes in bodyweight were evident between TH-4 control rats and saline control rats. MTX control rats did not have a significantly altered food intake compared to saline and TH-4 control rats. In contrast, MTX-treated animals receiving TH-4 had a significantly decreased food intake over the course of the trial compared to both saline and TH-4 control groups ($p < 0.001$; Table 6.1). TH-4 administration in saline-treated animals did not affect food intake compared to its skim milk counterpart. No significant changes in fluid intake were seen between any of the groups (Table 6.1).

Thymus weights were significantly decreased by 50% in MTX control rats and MTX+TH-4 treated rats compared to both saline and TH-4 control rats ($p < 0.05$; Table 6.2). Spleen and heart weights were also significantly decreased in both MTX treated groups (regardless of TH-4 treatment) compared to saline and TH-4 control groups ($p < 0.05$; Table 6.2). Liver weights in MTX-treated control rats were significantly elevated compared to both saline and TH-4 control rats ($p < 0.05$). In contrast, MTX+TH-4 treated animals did not have a significantly different liver weight compared to saline-

treated control groups. TH-4 control animals were not significantly different compared to their saline treated counterparts for all visceral organs fractional weights.

Regardless of TH-4 treatment, duodenal lengths, and colon lengths and weights in animals injected with MTX were significantly shortened ($p < 0.05$) compared to both saline control and TH-4 control rats (Table 6.3). Small intestinal (jejunum + ileum) lengths were significantly decreased in rats receiving MTX or MTX+TH-4 compared to TH-4 control rats only. The treatment of TH-4 in rats administered saline did not induce any significant changes compared to saline-treated controls (Table 6.3).

The effect of TH-4 on mammary adenocarcinoma growth

Manual measurement of tumour size and weight using digital calipers showed that, prior to the initial MTX injection, no significant differences were observed between the four treatment groups (Table 6.4). In relation to the manual measurement (mathematical determination), animals treated with MTX or MTX+TH-4 had significantly reduced tumour weights compared to both saline and TH-4 controls ($p < 0.05$). Additionally, upon excision of the tumours at sacrifice (96 h), similar weights were measured compared to those determined via mathematical equation (Table 6.4). The degree of correlation between the two forms of methodology is depicted in Figure 6.2, illustrating a strong concordance between the mathematically-derived weight and the WET weight of the excised tumour, where $r^2 = 0.94$ ($r = 0.97$; $p < 0.05$). In addition, when expressed as the percentage of bodyweight (Table 6.4) again, animals treated with MTX or MTX + TH-4 had significantly smaller tumours vs. saline-treated controls and TH-4 treated controls.

Daily Parameters	Gavage Treatment			
	Control	TH-4	MTX	MTX + TH-4
Body wt Δ (g)	2 ± 1	0 ± 1	-16 ± 1[ab]	-19 ± 1[ab]
Food Intake (g)	42 ± 3	41 ± 4	32 ± 4	23 ± 1[ab]
Fluid Intake (mL)	112 ± 7	119 ± 5	97 ± 10	109 ± 7

Table 6.1: Effects of TH-4 on change in bodyweight, food intake and water intake 96 h after administration of saline or MTX to rats with mammary adenocarcinoma (-48 – +96 h). Data are expressed as mean ± SEM. Saline control group received saline + skim milk, n = 9 (control); TH-4 control group received saline + TH-4, n = 9 (TH-4); MTX control group received MTX + skim milk n = 9 (MTX); treatment group received MTX + TH-4, n = 9 (TH-4 + MTX). Statistical significance compared to saline controls (control), where [a] denotes $p < 0.001$; compared to TH-4 controls (TH-4), where [b] denotes $p < 0.001$.

Weights (wt g/kg bwt)	Gavage Treatment			
	Control	TH-4	MTX	MTX + TH-4
Stomach	6.5 ± 0.2	6.8 ± 0.2	7.2 ± 0.2	6.9 ± 0.2
Heart	3.5 ± 0.1	3.6 ± 0.1	4.1 ± 0.1[ab]	4.2 ± 0.2[ab]
Lungs	5.2 ± 0.5	5.9 ± 0.2	5.1 ± 0.2	5.8 ± 0.2
Thymus	1.3 ± 0.1	1.2 ± 0.1	0.6 ± 0.1[ab]	0.6 ± 0.1[ab]
Spleen	2.1 ± 0.1	2.1 ± 0.1	1.4 ± 0.3[ab]	1.2 ± 0.1[ab]
Liver	33.2 ± 0.5	33.9 ± 0.7	36.9 ± 0.8[ab]	35.6 ± 0.8
Left Kidney	3.4 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.9 ± 0.1[a]
Right Kidney	3.6 ± 0.1	3.7 ± 0.1	3.9 ± 0.1[a]	3.9 ± 0.1

Table 6.2: Fractional weights of visceral organs in animals with mammary adenocarcinoma 96 h after injection of MTX/saline and treatment of TH-4 or skim milk. Data are expressed as mean ± SEM. Treatment groups are as defined previously. [a] denotes significant difference compared to saline controls ($p < 0.05$) and [b] denotes significant difference compared to TH-4 controls ($p < 0.05$).

Gut Tissue	Treatment Groups			
	Control	TH-4	MTX	MTX + TH-4
Duodenum				
Weight (wt g/kg bwt)	2.9 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.8 ± 0.2
Length (cm)	5.5 ± 0.1	5.4 ± 0.1	4.4 ± 0.1[ab]	4.5 ± 0.2[ab]
Jejunum + Ileum				
Weight (wt g/kg bwt)	20.5 ± 0.8	22.2 ± 0.6	20.3 ± 0.8	20.7 ± 0.4
Length (cm)	66.9 ± 2.6	69.4 ± 1.2	62.4 ± 1.2[b]	62.6 ± 1.1[b]
Colon				
Weight (wt g/kg bwt)	5.2 ± 0.2	4.7 ± 0.6	7.2 ± 0.5[ab]	6.5 ± 0.2[b]
Length (cm)	11.3 ± 0.4	11.2 ± 0.2	9.1 ± 0.4[ab]	9.7 ± 0.3[ab]

Table 6.3: Weights and lengths of small intestinal regions in rats with mammary adenocarcinoma, injected with saline or MTX, receiving skim milk or TH-4 as treatment. Data are expressed as mean ± SEM. Treatment groups are as defined previously. [a] denotes significant difference compared to saline controls ($p < 0.05$) and [b] denotes significant difference compared to TH-4 controls ($p < 0.05$).

Assessment of small intestinal function: SBT and biochemical analyses

Time-course SBT data for rats with mammary adenocarcinoma is illustrated in Figure 6.3. Prior to tumour inoculation (Figure 6.3A) no significant differences were evident between the four treatment groups. Additionally, prior to MTX administration (-24 h; Figure 6.3B) no significant differences were evident between the treatment groups ($p > 0.05$). Ninety-six hours after the initial MTX injection, irrespective of TH-4 administration, SBT levels were significantly depressed compared to both saline and TH-4 control rats ($p < 0.001$; Figure 6.3C). Additionally, reproducibility studies of the SBT for saline and TH-4 control rats were not significantly different over the three time-points. As expected, all animals receiving MTX had significantly lower SBT levels compared to their previously assessed SBT time-points ($p < 0.001$).

The degree of damage as measured by the biomarker SBT was reflected by biochemical sucrase activity (Figure 6.4) from small intestinal homogenates. Duodenal sucrase activity was significantly decreased by 100% in MTX control rats and MTX+TH-4 treated rats ($p < 0.001$) compared to both saline and TH-4 control rats. Jejunal *in vitro* sucrase activity reflected similar findings to duodenal sucrase activity, where MTX-control and MTX+TH-4 rats were significantly lower compared to saline and TH-4 controls ($p < 0.001$). A significant decrease was observed between MTX control and TH-4 control rats ($p < 0.001$). In contrast, *in vitro* sucrase activity was significantly lower in MTX+TH-4 rats compared to both saline-treated control and TH-4-treated controls (Figure 6.4; $p < 0.001$). No significant differences were observed when saline and TH-4 control rats were compared. A significant decrease (96%) in both MTX and MTX + TH-4 rats was evident when compared to saline control and TH-4 control DAR (Figure 6.4; $p < 0.001$) for "Net" small intestinal sucrase activity (average).

Tumour Weight	Treatment Groups			
	Control	TH-4	MTX	MTX + TH-4
Measured				
0 h	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.1
96 h	8.4 ± 1.1	7.1 ± 0.4	0.5 ± 0.2[ab]	0.5 ± 0.3[ab]
Weighed (g)				
96 h	6.3 ± 0.4	6.7 ± 0.5	0.3 ± 0.1[ab]	0.5 ± 0.1[ab]
% Bodyweight				
96 h	4.3 ± 0.3	4.6 ± 0.3	0.2 ± 0.1[ab]	0.4 ± 0.1[ab]

Table 6.4: Tumour weights determined via manual measurement (mathematical approximation) and weighed (excised at sacrifice) from MTX treated rats. Treatment groups are as defined previously. [a] denotes significant difference compared to saline controls ($p < 0.05$) and [b] denotes significant difference compared to TH-4 controls ($p < 0.05$).

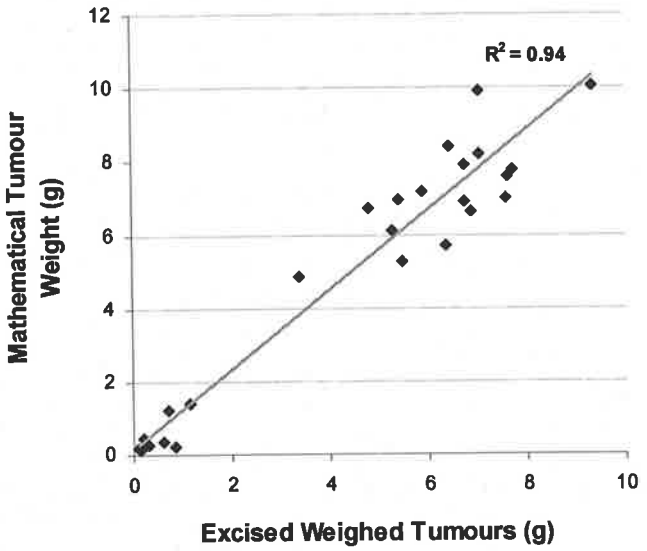

Figure 6.2: Correlation between excised weighed tumours (g) vs. manually measured/mathematically calculated tumour weights (g) using calipers at 96 h post-MTX injection. Significant correlation: $r^2 = 0.94$.

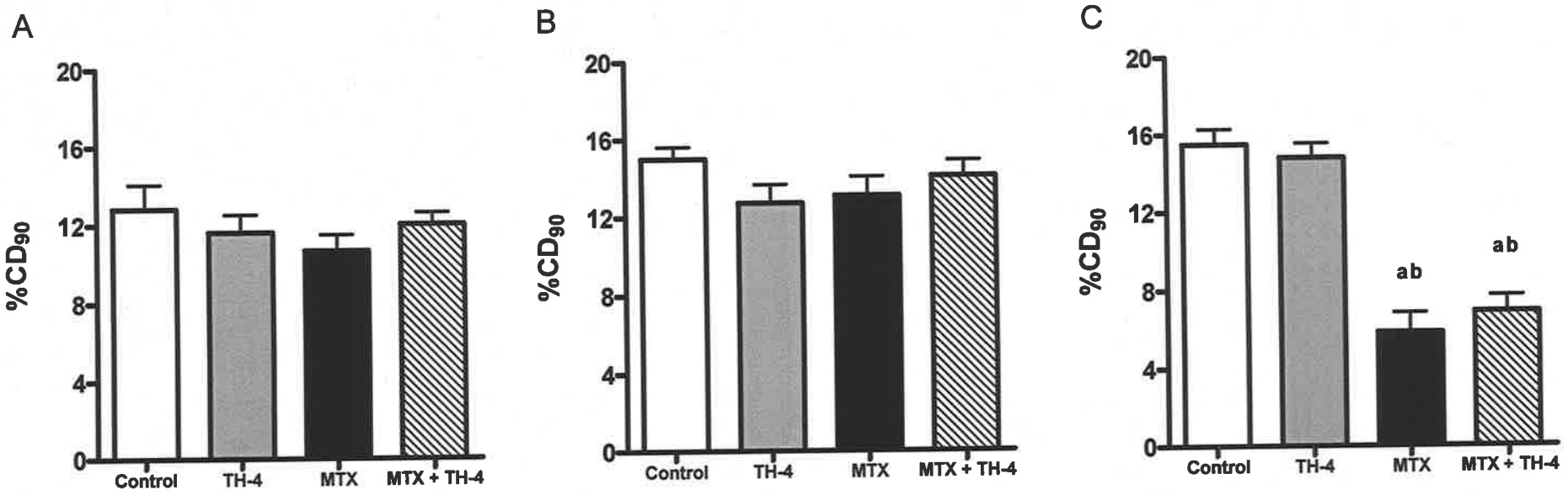

Figure 6.3: Small intestinal sucrase activity as determined by the non-invasive SBT ($\%CD_{90}$) in DAR with mammary adenocarcinoma, with/without TH-4 treatment: (A) SBT prior to tumour inoculation; (B) SBT 24 hours pre-MTX injection; and (C) SBT 96 h after initial MTX injection. White bar: saline controls; grey bar: TH-4 controls; black bar: MTX controls; hatched bar: MTX+TH-4. Data are expressed as mean ± SEM. Significant difference compared to saline control and TH-4 control at 96 h denoted by [a] ($p < 0.001$) and [b] ($p < 0.001$), respectively.

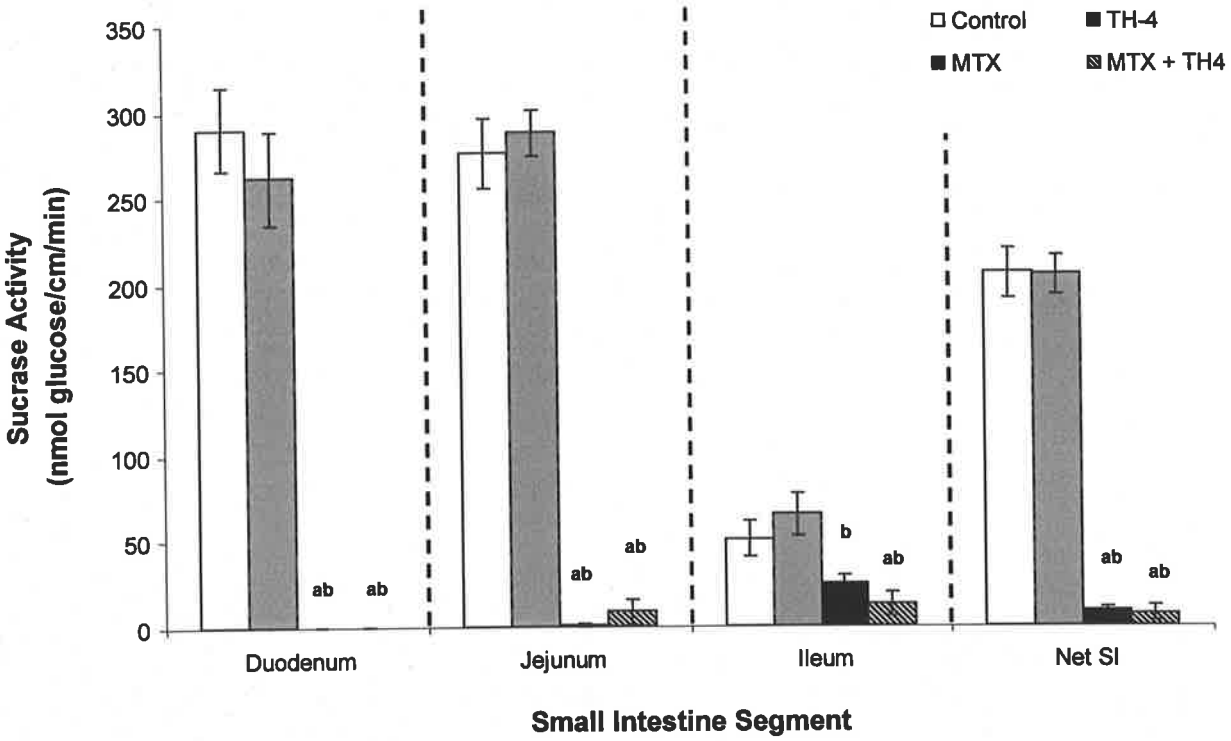

Figure 6.4: *In vitro* sucrase activity of intestinal segments from rats with mammary adenocarcinoma 96 h post-MTX injection, with or without TH-4 treatment. Segments: duodenum, jejunum (proximal and distal combined: 10% and 25% regions of SI), ileum (proximal and distal combined; 75% and 90% regions of SI) and the average of the sections [Net small intestine (SI)], expressed as nmol glucose/min/cm tissue, mean ± SEM. [a] denotes significant difference ($p < 0.001$) compared to saline controls, and [b] denotes significant difference ($p < 0.001$) compared to TH-4 controls.

Intestinal damage and inflammation

Typical histological features of the proximal jejunum in animals treated with MTX or saline, ± TH-4 are depicted in Figure 6.5. The level of damage evident 96 h post MTX are more severe in rats with mammary adenocarcinoma, where there was significant villus atrophy, shallow crypts, a higher decree of inflammation, and the presence of abscesses in the crypt, are more prominent. These features were common in all rodents receiving MTX (Figure 6.5C) regardless of TH-4 treatment (Figure 6.5D). Semi-quantitative small intestinal damage scores (Figure 6.5) revealed that rats administered MTX had significantly damaged jejunal (proximal) and ileal (distal) mucosa compared to saline and TH-4 controls ($p < 0.05$) irrespective of TH-4 treatment. No differences were observed between MTX controls and MTX-TH-4 treated animals. Damage was maximal in the proximal jejunum (Figure 6.6). No other significant differences were observed between treatment groups.

With respect to the level of inflammatory infiltrate (Figure 6.7), as measured by myeloperoxidase activity, animals receiving MTX or MTX + TH-4 had significantly elevated MPO levels compared to both saline controls and TH-4 controls in the duodenum ($p < 0.001$), jejunum ($p < 0.001$), ileum ($p < 0.01$), and an average of the small intestine ($p < 0.001$).

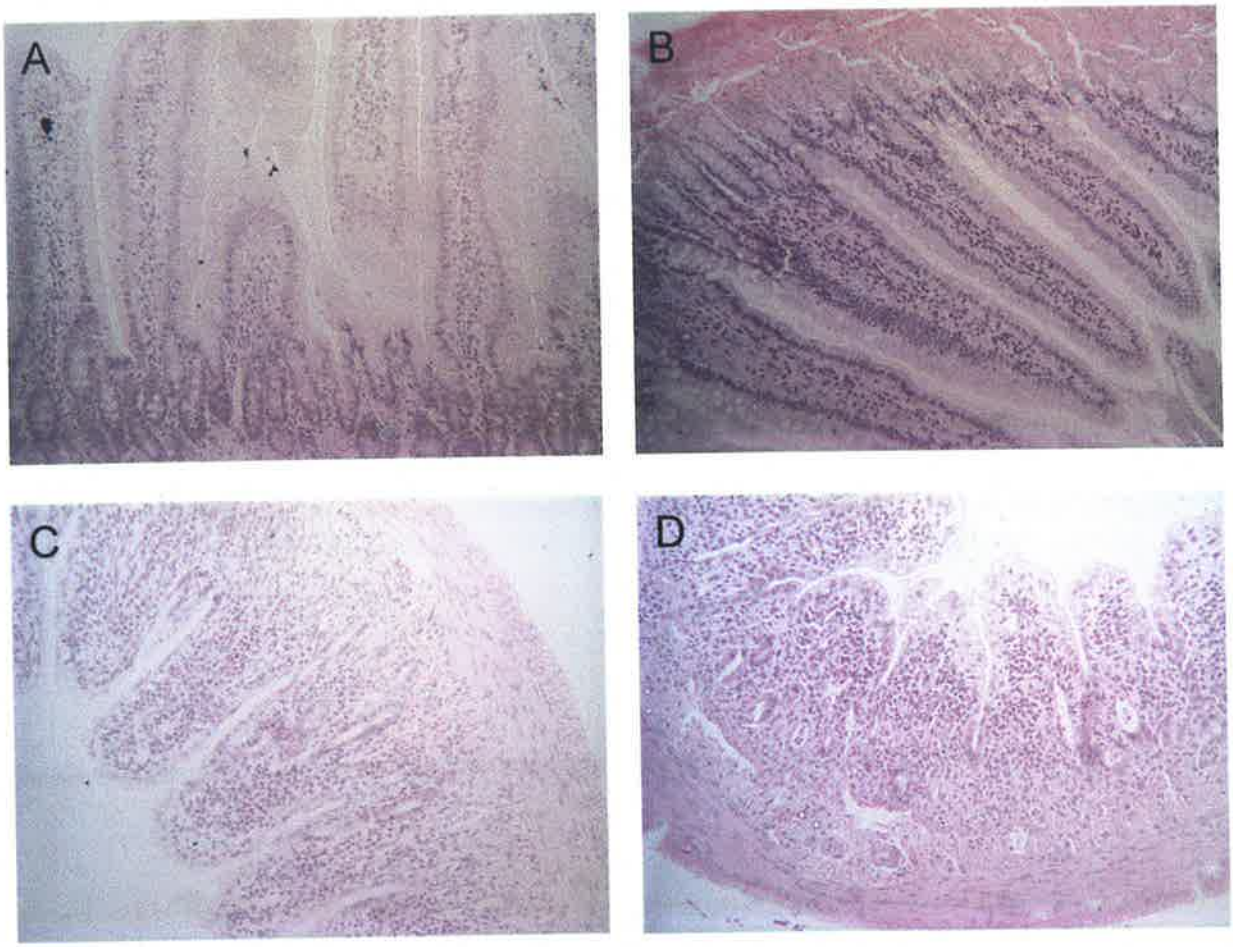

Figure 6.5: Photomicrographs of proximal jejunum in rats with mammary adenocarcinoma 96 h after initial MTX or saline administration. Treatment groups: (A) Saline control; (B) TH-4 control; (C) MTX control; (D) MTX+TH-4. Note villus atrophy, irregular architecture and shortened crypts in DAR receiving MTX or MTX+TH-4.

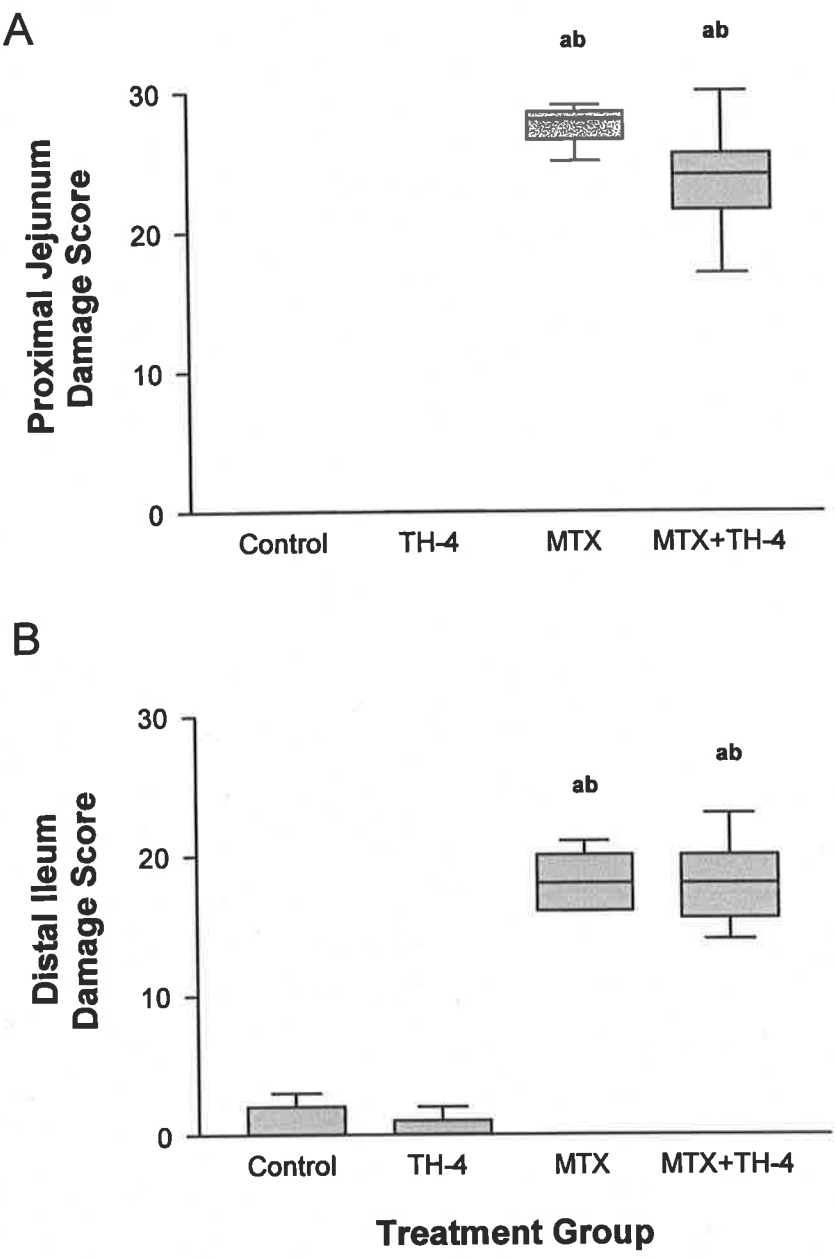

Figure 6.6: Semi-quantitative histological assessment of intestinal tissues from rats with mammary adenocarcinoma 96 h after initial MTX injection. Values are the sum for 11 independent histological criteria (maximum score 33). Data are expressed as Box and Whisker plot. Significance denoted by [a] and [b] where $p < 0.05$ compared to Control and TH-4 DAR, respectively.

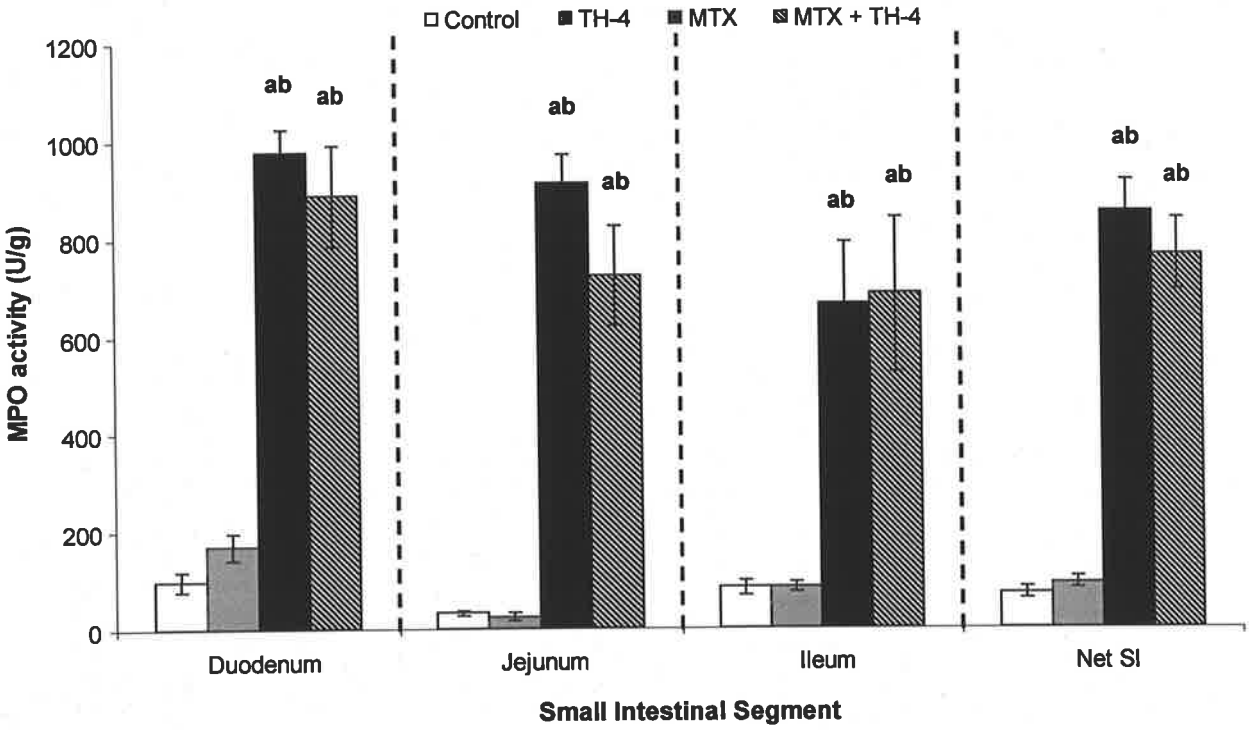

Figure 6.7: Effects of orally administered TH-4 96 h after MTX injection on biochemical myeloperoxidase activity (MPO; U/g) from rat intestinal homogenates with mammary adenocarcinoma: duodenum (A); jejunum (B); ileum (C); and Net SI (D). Data expressed as mean ± SEM. Significant difference compared to saline control DAR denoted by [a] $p < 0.001$; compared to TH-4 control DAR denoted by [b] $p < 0.001$.

6.4 DISCUSSION

In Chapter 5 *Streptococcus thermophilus* (TH-4) was shown to protect the small intestine, specifically the proximal jejunum, from MTX-induced mucositis.[55] Whilst this finding was significant, it was essential to assess the efficacy of TH-4 in a tumour-bearing rat model in which the mucositis is known to be more severe.[48] Additionally it is mandatory to determine that factors which ameliorate small intestinal mucositis do not promote cancer survival.[46,48]

The most important observations from this study were the following:

i) In contrast to the protective effect observed with TH-4 administration at a dose of 10^9 cfu/mL observed in a non-tumour bearing model (Chapter 5), this study illustrated that the same dose administered to rats receiving MTX with mammary adenocarcinoma yielded no protection.

ii) Food intake was significantly depressed in the MTX + TH-4 treated DAR compared with all other groups, and

iii) The tumour size was not significantly different in MTX + TH-4 rats compared to MTX control rats.

There are several possible explanations for these observations. Firstly, the degree of damage was more severe in a tumour-bearing animal,[48] which can be clearly seen when comparing SBT levels, biochemical sucrase activity, histological damage scores and MPO activity in this study compared to a non-tumour bearing rat (Chapter 5).[55] Other

reasons such as circulating cytokines and chemokines derived from or influenced by the tumour may impact on the functional status of the small intestine and sufficiently alter the effect and time-course of TH-4 protection. Cytokine levels have not previously been assessed in this cell line of the DAR mammary-specific adenocarcinoma, and would be useful to investigate in future studies.

Whilst the mechanism of protection by TH-4 to the small intestine seen in a non-tumour bearing animal is currently unknown, a recent *in vivo* study demonstrated that *Streptococcus thermophilus* was capable of increasing net folate levels in reconstituted milk.[152] Whilst the net levels of folate were in the picomolar range, it could be hypothesised that the effect of the folate-producing bacteria would be local. Currently, determination of folate levels in intestinal homogenates at this level is difficult and was not possible in these two studies. Clarke *et al* demonstrated that folinic acid (leucovorin^{TM}/folate) administered in drinking water prevented the development of MTX-induced mucositis entirely. However, the dose administered in this study has not been assessed with respect to any affects on tumour growth. It has been shown in humans that folate (leucovorin) administered intravenously has the potential to re-initiate tumour cell survival if given within 24hrs.[199,200] Sepehr *et al* (2003),[201] showed that caecal bacteria-synthesised folate is not absorbed or stored in substantial amounts in the liver, ie. systemic folate distribution is limited. If the mechanism of protection by TH-4 seen in non-tumour bearing animals is by delivering a micro dose of folate to the site of damage, then it may be that a dose of 10^9 cfu/mL was insufficient to diminish the mucositis in the presence of a tumour. Thus, a higher dose of TH-4 may have been needed in the tumour-bearing animal.

Of more concern in the tumour-bearing DAR, is the observation that TH-4 at a dose of 10^9 cfu/mL administered to MTX-treated rats significantly depressed food intake. Importantly, the tumour growth was not apparently altered and tumour kill was the same, as measured by tumour weight. The negative effect on food intake by TH-4 highlights the need to improve the understanding of the interaction of probiotics and gut function. In addition, the potential interactions with other micro-organisms (colonic micro-flora) and different kinds of tumours should be assessed in the future.

Whilst there were no differences seen in tumour growth between the respective treatment groups (ie. saline vs saline + TH-4) the tumour itself has been shown to have an exponential growth rate.[198] This specific tumour-cell line preferentially sequesters glucose, depriving peripheral (normal) tissues of this fuel to shift their metabolic profile from glucose (due to decreases in the blood pool) to ketone bodies.[196] In this setting it has been found that the DA-specific mammary adnocarcinoma will sequester four times more glucose than muscle.[197] The combination of rapid growth of the tumour, preferential glucose usage and the upregulated release of cytokines into the periphery from the tumour, may pose problems systemically, and to organs such as the gut. Potentially, this may lead to deleterious effects on other organs when combined with MTX treatment, whereby healing and repair of epithelial tissues could be hindered significantly. Due to this particular tumour cell line's tumour growth rate and its percentage of bodyweight, may not extrapolate to an equivalent tumour seen in a patient. Since the first three phases of mucositis[25] are probably driven primarily by pro-inflammatory cytokines, it would be important to assess circulating cytokine profiles and the link to mucosal cytokine profiles to better understand this rat model of mammary adenocarcinoma. It may be the tumour burden in this particular model is not

representative of human cancer, reducing the likelihood of observing efficacy of potential anti-mucositis treatments being assessed.

In conclusion, TH-4 administered at a dose of 10^9 cfu/mL to female DAR inoculated with a mammary adenocarcinoma yielded no protection to the small intestine when treated with MTX. Since this study has yielded contrasting results compared with the observations in Chapter 5, future studies should focus on ascertaining the level and type of cytokines in this animal model of cancer. Alternatively, increased doses of TH-4 should be assessed and whether live or attenuated TH-4 would be effective. Importantly, the non-invasive SBT was able to detect/monitor small intestinal changes associated with MTX and a potential anti-mucositis agent.

PART III: SMALL INTESTINAL FUNCTION IN PAEDIATRIC CANCER PATIENTS

CHAPTER 7: ASSESSING SMALL INTESTINAL DAMAGE IN CHILDREN WITH CANCER

7.1 INTRODUCTION

The treatment modalities for cancer have improved in recent years, however this has brought about an increase in treatment-related toxicity. Whilst most of the side-effects can be treated, mucositis remains elusive. This indiscriminate cyto-toxicity results in a cascade of side-effects where cells, particularly those with a rapid cell-turnover rate, are highly susceptible to damage. Consequently, mucositis leads to a diminished small intestinal functional absorptive capacity.[1,4,29]

In contrast to the relative ease of observing oral changes due to chemotherapy, defining the impact on gut function, particularly the small intestine, is difficult due to its inaccessibility and the lack of appropriate non-invasive techniques to assess the severity of damage. Since cancer patients are often neutropaenic and/or thombocytopaenic due to chemotherapy, the small bowel biopsy is physically hazardous and often ethically unacceptable.[6] A non-invasive technique that could assess small intestinal function would be beneficial and lead to improved clinical oncology practice and patient care.

Impairment of gut function and small intestinal barrier integrity has previously been described using small intestinal permeability (SIP) tests in patients with chemotherapy-induced mucositis.[6,95-97] Whilst this test is useful in the assessment of barrier function it does not necessarily give a clear or sensitive indication of the small intestine's absorptive capacity or the level of damage. The recently developed non-invasive SBT has been used to assess small intestinal digestive/absorptive function, [103] in a rat model of MTX-induced mucositis. Its application in a population of cancer patients undergoing chemotherapy is yet to be assessed.

The aims of this study were firstly to use the SBT to assess small intestinal mucosal status in children with cancer during chemotherapy and compare it to their own baseline and that of healthy controls. The second aim was to compare the SBT response at different stages after chemotherapy with loss of barrier function concurrently determined using the SIP.

7.2 PATIENTS & METHODS

Ethical approval

Informed written consent was obtained from all subjects and ethical clearance was granted from the Research Ethics Committee of the CYWHS, North Adelaide, Australia. The study was carried out in accordance with the Declaration of Helsinki.

Subject eligibility and study design

Forty-two eligible cancer patients were approached for the study at the Children, Youth and Women's Health Service (CYWHS), North Adelaide, Australia. Twenty-four patients declined to join the study and 22 patients (male n = 13, female n = 9) receiving HD-chemotherapy were recruited. Of the 22 patients enrolled, five withdrew due to patient/parent request, one was withdrawn due to non-compliance and one withdrew due to interstate relocation. The characteristics of the 15 remaining patients enrolled in the study are given in Table 7.1. All patients had received cycles of chemotherapy prior to enrolment, i.e. non-naïve. Chemotherapy regimens administered to the remaining patients for cycle 1 and cycle 2 can be seen in Table 7.2. Patients were asked to take part throughout one cycle of chemotherapy, and if possible, a second cycle. The SBT, SIP (including sucrose permeability) and oro-caecal transit time (OCTT), were combined and conducted throughout a cycle of chemotherapy on multiple test days (Figure 7.1). A baseline test was performed up to five days before administration of HD-chemotherapy (Test 1); day 1 test was performed within 24hs following administration of HD-chemotherapy (Test 2); 3-5 days after chemotherapy (Test 3); 6-9 days after chemotherapy (Test 4; Figure 7.2). Fifteen evaluable cancer patients contributed to the final assessment of 25 cycles of chemotherapy. Each cycle of chemotherapy was assessed individually as mucositis can develop in one cycle of chemotherapy independent of another. Thus for statistical analysis n = 25 cycles of chemotherapy were assessed rather than 15 patients.

Control subjects (n = 26, Table 7.1) refrained from ingestion of antibiotics, antihistamines and non-steroidal anti-inflammatory drugs for four weeks prior to testing. Control subjects carried out tests on two separate occasions within a minimum

of one week. The means of the SIP, OCTT and SBT were calculated for the two separate occasions (test 1 and test 2), as well as a combined mean of the two occasions (T_M).

Heights and weights were recorded on all subjects on the date of each test. Additionally, all subjects had no known history of gastrointestinal or liver disease and were non-diabetic.

Mucositis scoring

Patients were assessed for mucositis upon arrival by oncologists and/or oncology nurses using the WHO grading system for oral mucositis.[202] Additionally, patients were also asked for any abdominal and/or anal symptoms they were experiencing. Patients were graded 0 if they had no oral, abdominal or anal complaints (no mucositis); grade 1 (mild): soreness and erythaema; grade 2 (moderate) erythaema, ulcers and can eat solid food; grade 3 (moderate-severe) ulcers and tolerates only liquid diet; grade 4 (severe) no possible alimentation.[202] Patients experiencing purely gastrointestinal complaints were graded as not having developed mucositis as a validated intestinal mucositis grading system is not available. The development of mucositis in a cycle of chemotherapy was assessed independently by a clinical oncologist based primarily on oral symptoms (a modified WHO mucositis score). The patient group who did not develop clinical evidence of mucositis in a cycle of chemotherapy were labelled "no mucositis" and the group that developed mucositis labelled "mucositis".

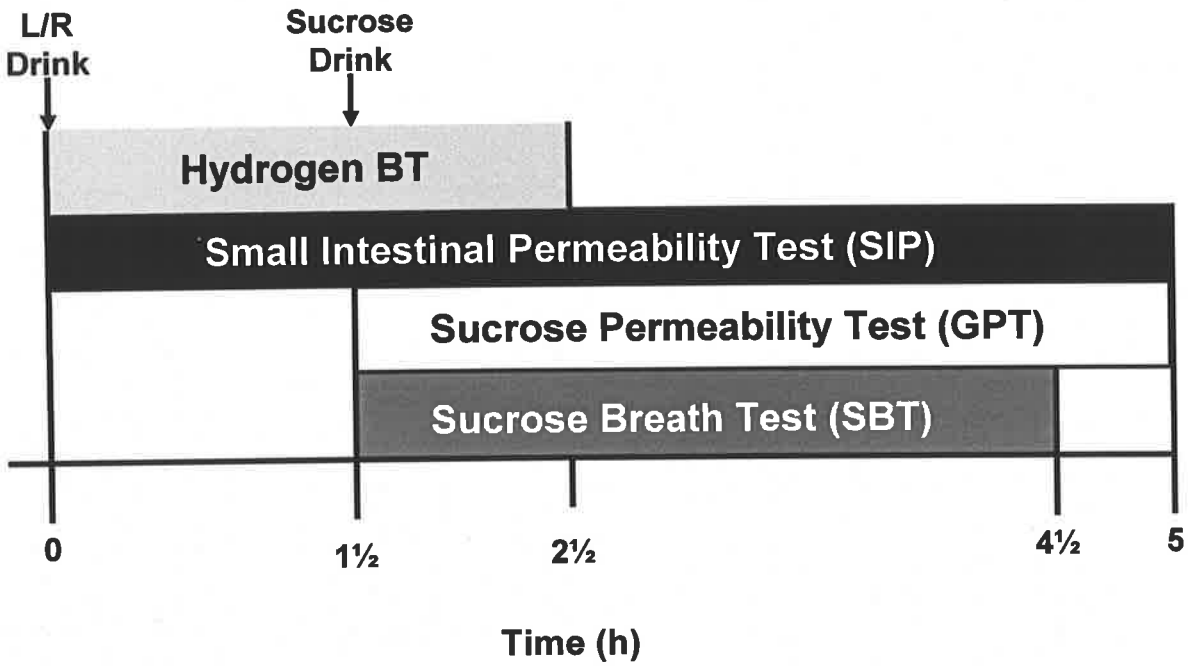

Figure 7.1: Timing procedure of the combined non-invasive tests.

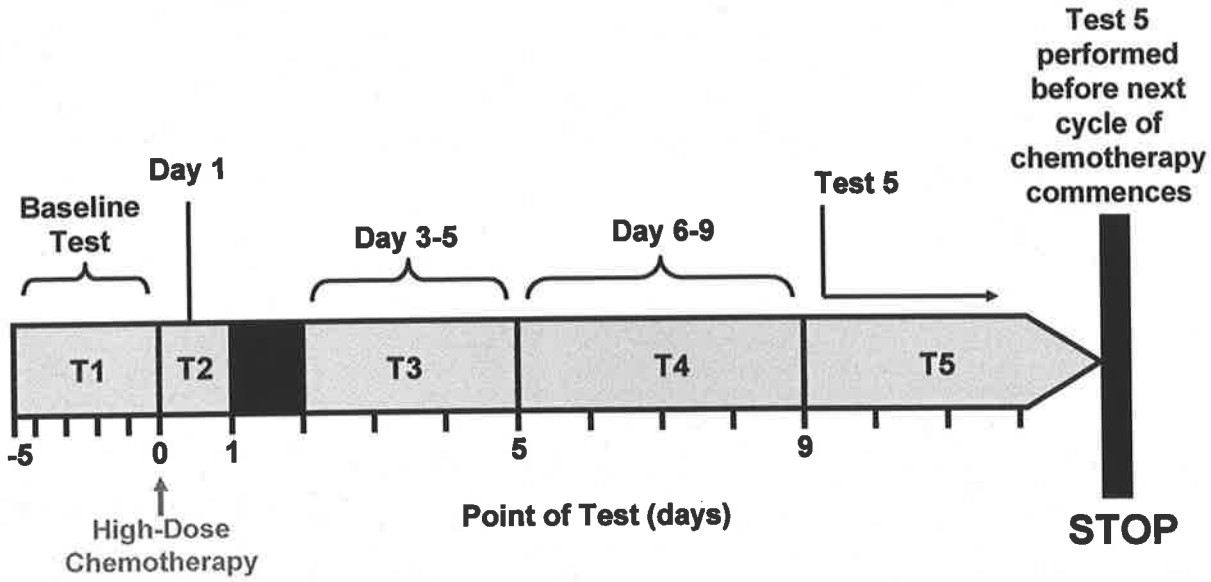

Figure 7.2: Time-course of tests to be performed in paediatric oncology patients undergoing chemotherapy. Day 0 represents the commencement of chemotherapy administration, followed by the respective testing days. Test 1, test 2, test 3, test 4 and test 5 are represented by T1,T2, T3, T4 and T5, respectively.

Subjects	
Controls	
Male	n = 11
Female	n = 16
Age (years)	11.2 ± 0.8
(range)	(5 – 17)
Height (cm)	150.7 ± 3.7
Weight (kg)	45.4 ± 3.2
	Cycles Assessed
Patients	
Male	13 (8 patients)
Female	12 (7 patients)
Age (years)	9.9 ± 1.0
(range)	(5 – 16)
Height (cm)	138.2 ± 5.8
Weight (kg)	40.5 ± 6.5
Disease	
Acute Lymphoblastic Leukemia (ALL)	13 (7 patients)
Acute Myeloid Leukemia	3 (2 patients)
Relapsed ALL	2 (1 patient)
Neuroblastoma	1
Non-Hodgkin's Lymphoma	1
Ewing's Sarcoma	5 (3 patients)
Total	25 (15 patients)

Table 7.1: Clinical data for patients and healthy subjects. All data is expressed as mean ± SEM. Number of cycles assessed with respect to cancer diagnosis is expressed as the number of cycles assessed (number of patients contributing to cycles assessed).

ID	CYCLE 1	CYCLE 2
1	VINC; CYCLO; DOX	VINC; CYCLOPHO; DACT
3	MTX	MTX; VINC
5	ME; CARB; ETOP	-
7	MTX; VINC	MTX
8	MTX; VINC; MERC	MTX
11	CYT; ID; ETOP	-
12	CYT	ME
13	VINC; CYCLO; DOX	ETOP; IFOS; CARB
14	MTX; MER	MTX
15	MTX; MER	MTX; VINC; DEX
16	MTX	-
18	MTX; CYT; ASP; VINC	MTX; CYT; ASP; VINC
20	MTX; CYCLO; DOX; VINC	-
21	MTX	DOX; VINC; DEX
22	ETOP; CYCLO; VINC	-

Table 7.2: Chemotherapy regimens administered to the 15 evaluable patients in cycle 1 and/or cycle 2 of testing. Chemotherapy agents: Vincristine (VINC); Cyclophosphamide (CYCLO); Doxorubicin (DOX); Methotrexate (MTX); Melphalan (ME); Carboplatin (CARB); Etoposide (ETOP); Dactinomycin (DACT); Cytarabine (CYT); E-Asparaginase; 6-Mercaptopurine (MERC); Dexamethasone (DEX); Ifosfamide (IFOS); Idarubicin (ID). Patients 5, 11, 16 and 22 did not perform cycle 2 testing.

Small Intestinal Permeability (SIP)

All subjects fasted overnight prior to testing and for a minimum of four hours during the test period. Small sips of water were permitted during testing and a small meal after 4 h. Physical activity was kept to a minimum. All subjects voided their bladder prior to commencement of testing. A lactulose (L)/rhamnose (R) drink, comprising 7.5 mL lactulose syrup (Dupholac, SOLVAY-DUPHOV, B.V., Holland) and 1.1 g L-rhamnose (SIGMA, Sigma-Aldrich, Germany) mixed with 92.5 mL water, was ingested (t = 0 h) and all subsequent urine voided over the next 5 h was collected. Additionally, since the test substrate sucrose was being administered for the SBT, sucrose (S) permeability, indicative of gastroduodenal permeability, was also able to be assessed in the collected urine (timed urine sample of 3.5 h). All subsequent urine was pooled for each subject on the respective test day, and stored in a container containing 0.1 mL of 10 g/L thiomersal as preservative. Urine volumes were measured then dispensed (12 mL) and stored at -20°C until analysis. L, R and S concentrations in urine were determined by high performance liquid chomatography (HPLC) (Dionex DX500 system; Dionex Corporation; Sunnyvale, California, USA) at the Royal Darwin Hospital, Darwin, Australia, as previously described.[81,83] Total percentage urinary excretion for L, R and S were calculated for each subject. Additionally, results were expressed as the percentage of L/R ratio (log_{10}) to eliminate confounding factors such as gastric emptying, intestinal transit and renal clearance.[81]

Oro-Caecal Transit Time (OCTT)

OCTT was assessed in all patients and healthy subjects concurrently with the SIP test. Prior to ingesting L/R drink all subjects exhaled into 4 x 10 mL Exetainer® glass tubes

(Exetainer, Labco Limited, High Wycombe, England) using a straw, ensuring that samples contained breath from end-expiration (OCTT baseline). Once the L/R drink was ingested breath samples were collected at t = 15, 30, 45, 60, 90, 120, 150 and 180 min as described above. A 20 mL sample of breath was required for analysis of Hydrogen (H_2) content by gas chromatography (Quintron, Model MicroLyzer, E.F. Brewer Company, Wisconsin, USA).[203] A rise in H_2 excretion (at least 10ppm above baseline) indicates that the test substrate, lactulose, has reached the large bowel.

Sucrose Breath Test (SBT)

At t = 1.5 h into the SIP test, subjects exhaled into 3 x 10 mL glass tubes using a straw (SBT baseline). Following the SBT baseline sample, subjects immediately ingested 20 g of selectively enriched ^{13}C-Sucrose (AnalaR, BDH, MERCK, Pty Ltd, Victoria, Australia) dissolved in 100 mL water. Triplicate breath samples were then collected every 15 min for 3 h. Breath $^{13}CO_2$ was analysed to determine small intestinal digestive/absorptive capacity.[103] 10 mL breath samples were analysed for $^{13}CO_2$ using an isotope ratio mass spectrometer (IRMS; Europa Scientific, ABCA 20/20, Crewe, United Kingdom) equipped with a V410 data collection system. Analysis of breath sample and subsequent data was carried out as previously described by Ghoos *et al* (1993).[105] $^{13}CO_2$ data was expressed as the percentage cumulative dose of ^{13}C (%CD) recovered over the first 90 min. The CO_2 production rate was calculated as 300 x body surface area (mmol CO_2/h) as described extensively by Ghoos *et al.*[105] The first 90 min of $^{13}CO_2$ excretion was used as a cut-off point for SBT analysis as this time-point is indicative of completion of small intestinal transit.

Statistical Analysis

A One-Way ANOVA (repeated measures and comparison of means) in conjunction with a Fisher-LSD *post-hoc* test, was used to determine significance for all analyses of SIP (non-parametric), OCTT (non-parametric), and SBT (parametric) between the group who did not develop mucositis in a cycle of chemotherapy (n = 18) and those who did (n = 7). A One-Way ANOVA in conjunction with a Fisher-LSD *post-hoc* test was also performed to determine significance between controls, the unaffected treatment cycles and the affected at baseline for SIP, OCTT and SBT. A Two-Way ANOVA was used to determine significance of sex and age in the control subjects with respect to test 1 and test 2 for SIP, OCTT and SBT. Statistical significance was considered if $p < 0.05$. All data have been expressed as mean ± standard error of the mean (SEM).

7.3 RESULTS

Patients developed mucositis in seven (28%) of the 25 cycles of chemotherapy and did not in 15 (78%). Using the WHO oral mucositis score incorporating some abdominal symptoms, in three cycles the mucositis was graded 1-2 (mild) and in four cycles the mucositis was graded 3 - 4 (severe), where hospitalization was required. Additionally, three patients who developed grade 3 - 4 mucositis in a cycle of chemotherapy were unable to complete test procedures at d6-9 (Test 4), due to the severity mucositis (n = 4). Overall, compliance was good, with subjects commenting on the ease of the test but also complaining of the length of time to complete the test.

Small Intestinal Permeability

There was no significant difference in mean L/R ($\log_{10}$) ratios, %L and %R for T1 and T2 of the 26 control subjects. The means for the L/R ratio in each group were 0.57 ± 0.02 vs. 0.56 ± 0.03, respectively. The %L and %R and for T1 and T2 were 0.27 ± 0.02 vs. 0.27 ± 0.03, and 7.1 ± 0.4 vs. 6.8 ± 0.4, respectively. Sex and age did not affect the outcome of all permeability parameters in healthy controls. The combined mean (T_M) average for L/R was 0.57 ± 0.02 and the range was 0.35 – 0.79 (± 2 standard deviations (SD)); for %L was 0.27 ± 0.02 (range 0.07 – 0.47); and for %R was 6.9 ± 0.3 (range 4.7 – 9.1). No significant difference was evident at baseline between the no mucositis and mucositis group compared to the controls (Table 7.3). Additionally no significance was detected between the no mucositis and mucositis group at every test point (baseline to d6-9) (Figure 7.3). When the percentage urinary L or R was assessed individually, again no significant differences were observed between the no mucositis and mucositis groups for all time-points assessed (Table 7.4).

Gastro-duodenal permeability (GPT)

No significant difference was observed between T1 and T2 for healthy controls (0.036 ± 0.008 and 0.034 ± 0.005, respectively. Again, sex and age did not cause any significant differences. The T_M average was 0.034 ± 0.006, and the range was 0.0 - 0.09% sucrose recovered. No significant difference was evident between the no mucositis and mucositis group at any time-point of testing (Figure 7.4).

	SIP L/R ratio (log_{10})	OCTT (min)	SBT (0-90min) %CD
Controls	0.57 ± 0.02	90.3 ± 5.6	8.49 ± 0.33
No Mucositis	0.59 ± 0.05	102.9 ± 11.5	8.91 ± 0.53
Mucositis	0.65 ± 0.10	141.4 ± 15.6*	5.29 ± 1.34$^{\alpha}$

Table 7.3: Assessment of small intestinal function of Healthy controls (n = 26), no mucositis (n = 18) and mucositis group (n = 7) at baseline for SIP, OCTT, and SBT. Data expressed as mean ± SEM, where * denotes significant difference between controls and mucositis ($p < 0.01$), and α denotes significance between mucositis group compared to controls and no mucositis ($p < 0.001$)

	Baseline	Day 1	Day 3-5	Day 6-9
% Rhamnose (4.7 – 9.1)				
No Mucositis	6.3 ± 0.7	7.4 ± 1.0	6.0 ± 1.0	5.3 ± 0.9
Mucositis	4.4 ± 0.9	6.0 ± 1.1	5.6 ± 1.1	5.0 ± 1.6
% Lactulose (0.07 – 0.47)				
No Mucositis	0.21 ± 0.02	0.36 ± 0.07	0.29 ± 0.05	0.24 ± 0.06
Mucositis	0.23 ± 0.26	0.26 ± 0.07	0.48 ± 0.14	0.42 ± 0.15

Table 7.4: Percentage urinary rhamnose and lactulose recovered in a 5 h urine collection in patients who did and did not develop mucositis in a cycle of chemotherapy. Patient data expressed as mean ± SEM and healthy controls expressed as a mean with the (range; ± 2 standard deviations of the mean). Not significantly different.

Oro-Caecal Transit Time

There was no significant difference in healthy controls for mean OCTT between T1 and T2 (88.8 ± 5.3 min and 92.0 ± 6.7 min, respectively). The combined mean (T_M) for OCTT was 90.3 ± 5.6min (range was 78.7 – 101.8 min, ± 95% confidence). OCTT in the mucositis group at baseline was significantly higher than in the controls ($p = 0.004$) (Table 7.3), however no difference was evident between the two patient groups. While the mucositis and no mucositis group had a longer OCTT from d1 compared to the reference range (mean ± 2SD), they were not significantly different from each other on all test days (Figure 5).

Sucrose Breath Test

There was no significant difference in mean %CD of ^{13}C-sucrose (0-90min), for T1 and T2 ($n = 26$) (8.48 ± 0.39 and 8.49 ± 0.43, respectively). Sex and age did not affect the outcome of %CD at either T1 or T2. The %CD of ^{13}C-sucrose of T_M was 8.48 ± 0.33 (range was 5.06 – 11.90; ± 2 SD). The SBT %CD was significantly lower in the mucositis group compared to controls and no mucositis groups ($p < 0.001$) at baseline (Table 3) and significance ($p < 0.05$) was also observed between the two patient groups at all time points tested (Figure 6). The mucositis group had a 41% ($p = 0.011$), 50% ($p = 0.01$), 63% ($p < 0.001$) and 52% ($p = 0.03$) decrease of excreted $^{13}CO_2$ (^{13}C-sucrose) compared to the non mucositis group at baseline, day 1, day 3-5 and day 6-9, respectively.

Correlations

No correlation was found between any of the parameters measured: L/R ratio, %L recovered, %R recovered, OCTT and SBT.

7.4 DISCUSSION

Mucositis is a complex process that involves all of the tissues and elements of the mucosa. Whilst oral mucositis has been the most studied adverse alimentary tract manifestation of chemotherapy and radiotherapy, gastrointestinal mucositis, which represents injury of the rest of the alimentary tract, most prominently in the small intestine, is increasingly being recognized as an equally important morbidity.[25]

The mucositis group showed significantly lower sucrose absorption throughout the study period and was always significantly different to the healthy controls. The patients who did not progress to mucositis showed a recovery in the % cumulative dose at day 3-5 after treatment and this was not significantly different at any time point thereafter when compared with healthy control levels. It needs to be emphasized that all the patients had received chemotherapy prior to enrolment and a high proportion of those who went on to develop mucositis showed a depressed SBT result just prior to administration of the chemotherapy. It is possible that an already damaged small intestine may have predisposed them to subsequent development of mucositis. A moderate degree of variability of damage to the epithelium was observed in the patients

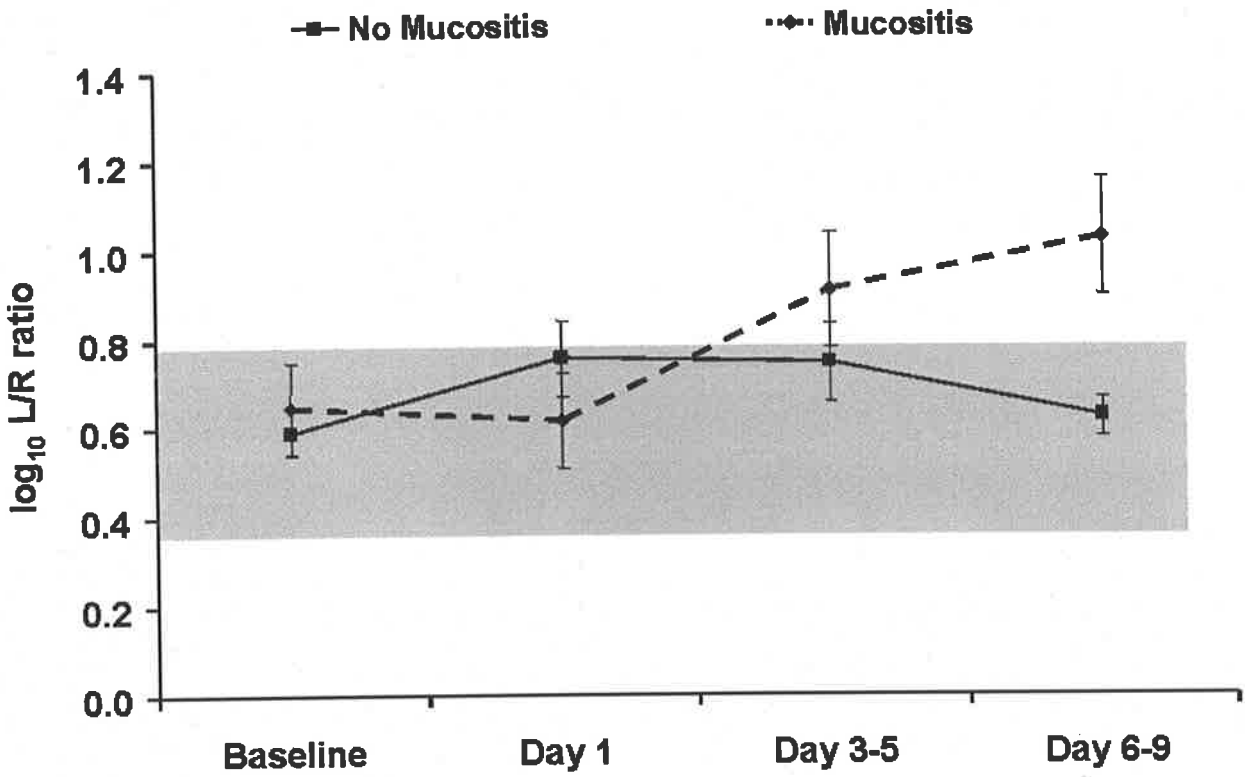

Figure 7.3: L/R ratios (log_{10}) in a timed 5 h urine collection in children who developed mucositis (♦) and those who did not (■) in a cycle of chemotherapy. Reference range for healthy individuals is represented by grey shading 0.57 ± 0.22 (±2 SD). NS.

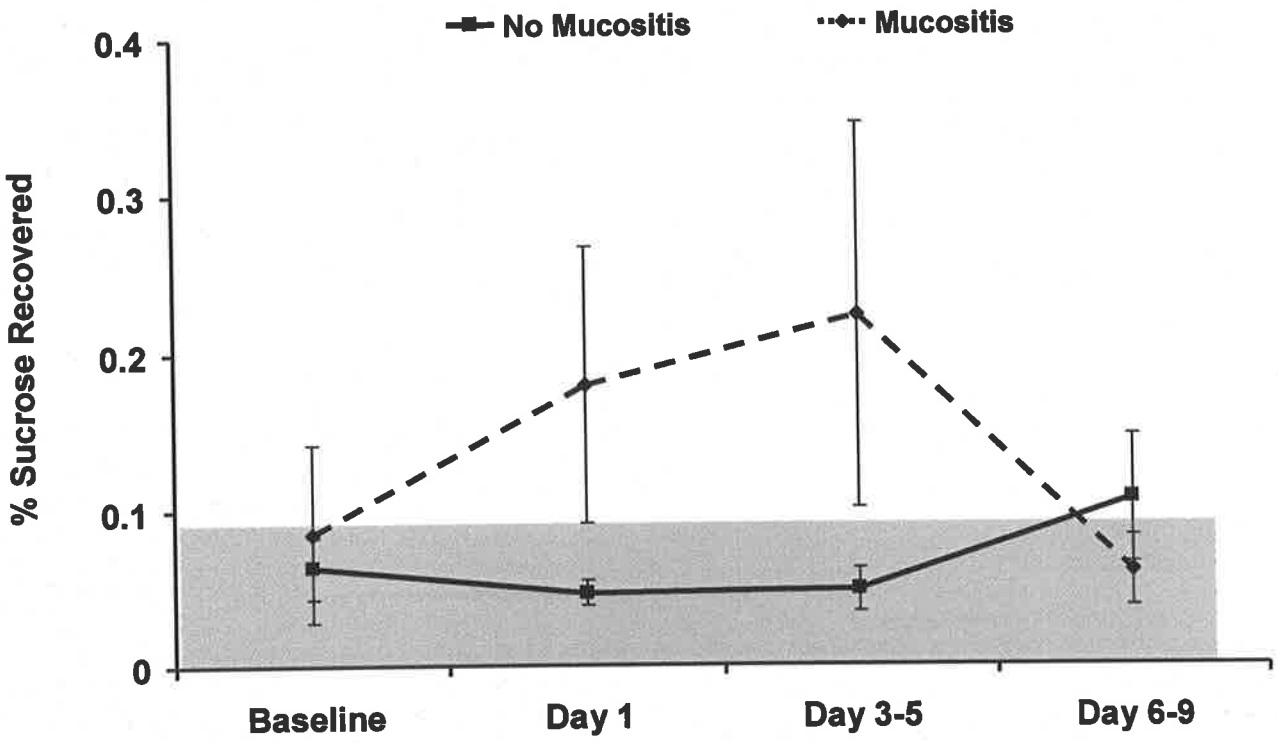

Figure 7.4: Time course of small intestinal absorptive capacity (SBT) represented by cumulative % dose (% $^{13}CO_2$), between the mucositis (broken line ♦) and no mucositis groups (filled line ■). NS. Reference range is 0.0 - 0.9 (mean ± 2SD) represented by grey shading.

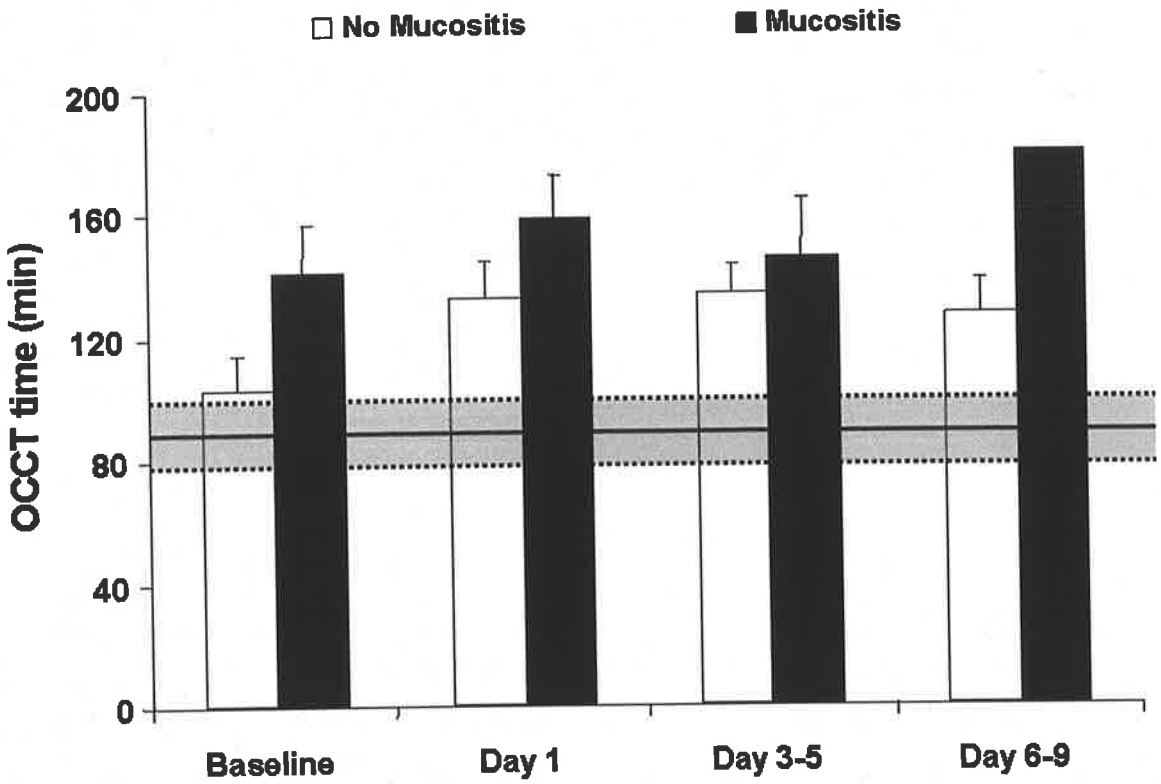

Figure 7.5: Time-course of OCCT between the no mucositis and mucositis groups. No significance was found between each group at each time-point. Grey shading represents healthy controls mean (90 min) ± 95% Confidence interval (± 11.3 min).

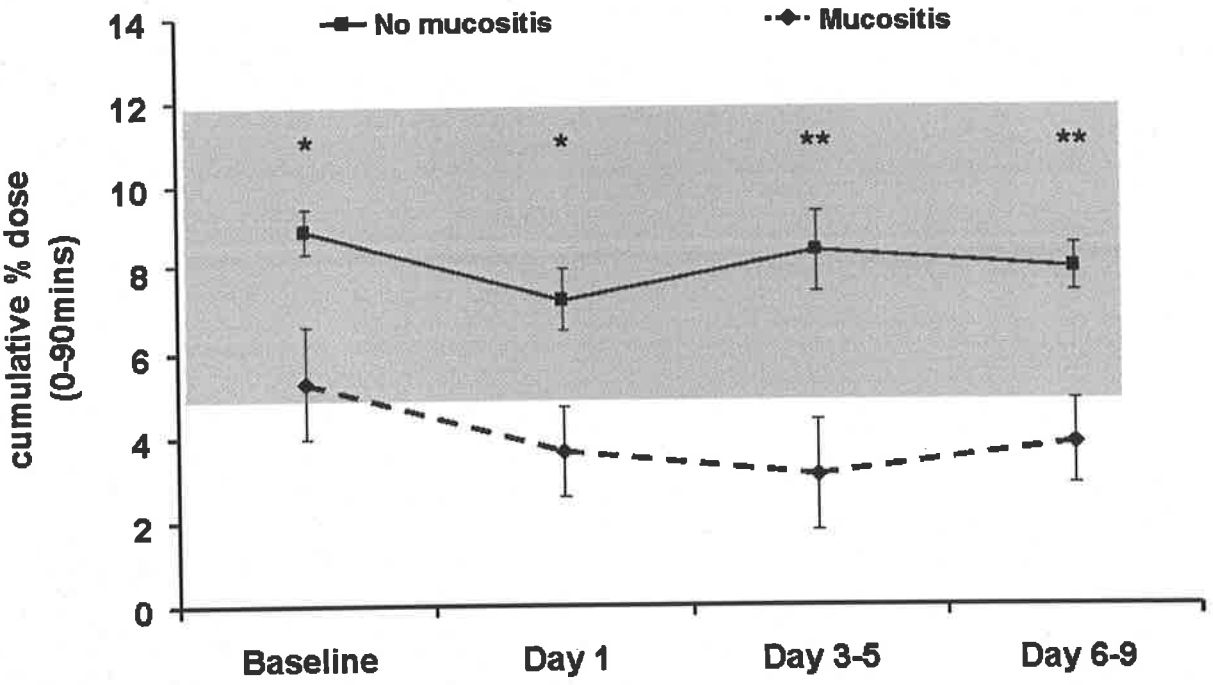

Figure 7.6: Time course of small intestinal absorptive capacity (SBT) represented by cumulative % dose (% CD_{90}), between the mucositis and no mucositis groups. Where * is $p < 0.01$ and ** is $p < 0.001$. Provisional range for healthy subjects is 5.08 – 11.90 %CD (mean = 8.49 ± 3.41 (2SD)) represented by grey shading.

who developed mucositis in a cycle of chemotherapy. This could be due to several factors, including duration of administration and/or doses, of the chemotherapeutic drugs in the individual and genetically determined handling of particular drugs. A further factor may be related to the time in the cycle of mucosal repair of an individual cancer patient. Keefe *et al* (2000)[29] have demonstrated that induction of apoptosis is an early event and with respect to the epithelial cells, occurs initially in the crypts, the engine for regeneration of the mature villus compartment. The extent of cell maturation in a patient's epithelium, the site in the small intestine, the type of chemotherapy, polymorphisms in drug handling, and the extent an entero-hepatic circulation, which might contribute to prolonged damage, are all potential contributing factors to the severity and time-frame of mucositis in the gastrointestinal tract.

This data clearly shows the SBT to be a superior marker of mucosal damage than the SIP as the latter failed to differentiate between the mucositis and no mucositis group. Additionally, no difference was seen in SIP between patient groups and controls. This result was unexpected but may be due to the small sample size or in fact reflect a real difference between intestinal permeability and different degrees of villus damage as measured by the SBT. Further studies are needed to clarify the relationship between the functional measure of the SBT and the way the small intestine responds to maintain rudimentary barrier function in patients who have undergone multiple chemotherapeutic insults.

Most studies have used changes in the oral cavity to monitor mucositis. It is now clear that whilst the mechanism(s) inducing staged damage and repair is similar in the oral cavity and in different parts of the intestine, the time of occurrence and the severity of

the insult is likely to be different and to occur earlier in the gut, particularly the small intestine.[25] This study demonstrated that the SBT is capable of non-invasively assessing small intestinal status in healthy children and small intestinal dysfunction in children with cancer undergoing chemotherapy.

It may also be that the mucosa exhibits a hyperplastic response in an attempt to hasten the repair and/or be colonised by bacteria with a resultant small bowel bacterial overgrowth. Nonetheless, patients who developed mucositis in a cycle of chemotherapy showed a significantly lower $^{13}CO_2$ output overall. This is seen at baseline and continues to be significantly depressed at day 6-9. The most common time frame for the appearance of mucositis is approximately 10 days after chemotherapy in these patients. It is possible that the depression observed at baseline could be indicative of a carry-over affect from the previous cycle of chemotherapy. This has been shown in a study by Keefe *et al* (2000),[29] where small intestinal morphometric severity scores, as measured by villus area, crypt length and mitotic count, in naive, adult cancer patients, did not return to pre-treatment values until 16 days after chemotherapy treatment.

In healthy individuals sucrose is catalysed by sucrase, a brush-border enzyme, in the small intestine into its constituent monosaccharides, fructose and glucose. Subsequent metabolism of these products in the liver leads to the production of CO_2, which is excreted in the breath. This can be detected and measured using isotope ratio mass spectrometry (IRMS).[104,107] It has been shown that sucrase activity is the rate-limiting factor in this technique, where the decreased level of sucrase activity, and the subsequent metabolism of its products, leads to a decrease in $^{13}CO_2$ excreted in the breath.[107] Previous studies have utilised the ^{13}C-lactose breath test as a marker of small

intestinal damage, however approximately 70 - 80% of non-Caucasians exhibit an age-related low lactase activity.[100] In comparison, only 0.2% of the population present with a genetic sucrase deficiency.[102] Thus, the ^{13}C-sucrose breath test is a reliable and superior prognostic enzyme for mucosal damage. The current study has shown that the SBT is a novel non-invasive biomarker, which can detect and monitor the presence of small intestinal damage after chemotherapy in a cohort of paediatric cancer patients undergoing chemotherapy.

The resultant decrease in $^{13}CO_2$, reflective of sucrase activity, has been supported previously, where a decreased brush-border hydrolase (maltase, lactase and sucrase) activity was observed in relation to the effects of chemotherapy on the small intestine.[5,13,107] Pelton *et al* (2002),[204] demonstrated that the SBT in a MTX-mucositis induced rat model caused a significant decrease in $^{13}CO_2$ output at d7, which correlated with intestinal sucrase activity as measured by a biochemical assay. It was also observed that MTX-treated rats exhibited a higher severity score, which was reflective of villus atrophy and crypt damage.

This is the first study to assess gastro-duodenal permeability in a cohort of paediatric cancer patients, or indeed in any cancer patients to the author's knowledge. Whilst the results were inconclusive, on day 3-5 of testing there was a trend for an increase in gastro-duodenal permeability, or gastritis. The level of % sucrose recovered observed was similar to results reported in previous studies of healthy and diseased patients.[85,88] Additionally, the permeation level of sucrose is minute (approximately 0.00 – 0.10 % recovery in healthy individuals), indicating that a low SBT is not confounded by an increase in gastro-duodenal permeability.

Oro-Caecal transit time (OCTT) was measured in the current study using a lactulose breath test. No significant differences were observed between the two patient groups, but a slower transit time was evident between healthy children and patients who developed mucositis. These findings are not surprising as in many cases pain-relief, such as morphine (commonly administered to patients who develop mucositis), and are known to slow transit time. Should OCTT studies be assessed in the future it may be necessary to shorten the sampling time-points (15 min) as the 30 min time interval used for sampling collection in this study did not detect differences between the two patient groups.

The development of mucositis relied solely on its detection by clinicians and the criteria previously defined in the methodology. It is important to note that when ethical approval was granted and the ensuing clinical trial was conducted, guidelines and criteria for the development of mucositis, including intestinal measures, had not yet been established. Since this trial, scoring criteria for some measures of intestinal mucositis has been incorporated with oral criterion for adults.[25,108] This study shows that even though mucositis is commonly clinically observed 7 -10 days after chemotherapy administration,[3,25] small intestinal changes occurred before this point. It also highlights that oral mucositis is a late marker of the development of small intestinal damage. This is most likely due to the fact that the current method of diagnosis, as documented by studies worldwide,[205] is primarily based on oral and anal assessment criteria, plus patient symptoms.

The SBT could be employed in the future to determine individualised safe dosing and regimens for anti-cancer drugs, and also to assess the gut toxicity of new anti-cancer

drugs in humans. Additionally, with the emergence of more potential candidate anti-mucositis products,[46,55,120,206-208] the SBT could also be employed as a marker/monitor to determine the best time to intervene for the individual patient, and also assess the efficacy in terms of gut repair and amelioration or prevention of symptoms.

In conclusion the non-invasive ^{13}C-sucrose breath test can be used to detect small intestinal changes with respect to chemotherapy-induced mucositis in children with cancer. In this pilot study the SBT appears to precede the development of oral mucositis and to be a better and earlier marker of gut damage/integrity compared with common clinical measures.

PART IV: FINAL CONCLUSIONS

CHAPTER 8: THESIS OUTCOMES, DISCUSSION AND FUTURE DIRECTIONS

8.1 INTRODUCTION

Defining physiological function of the small intestine is difficult due to its inaccessibility.[71,72] Because of this and the extensive nature of damage to all regions of the small intestine caused by chemotherapy-induced mucositis, understanding the pathogenesis of mucositis, and the development and implementation of therapeutic interventions have also been hampered.[55,209] Small bowel biopsy cannot be used in patients undergoing chemotherapy due to its potential compounding affects on platelet, white blood cell and red blood cell counts, adding high risk to the patient, to GI bleeding and infection.[29] Additionally, the biopsy does not accurately represent small intestinal function, as only the proximal region is accessible.[73-75] The implementation of a non-invasive test would be highly desirable in the clinical management of patients undergoing chemotherapy. Currently available non-invasive tests include the hydrogen breath test,[203] lactulose breath test (OCTT)[98] and small intestinal permeability (SIP).[6,76,210] However, these particular breath tests rely on the presence of H_2-producing colonic bacteria, which may be absent in up to 20% of patients.[100] Small intestinal permeability measures loss of barrier function but fails to describe the functional capacity of the small intestine.

Development of the non-invasive SBT has enabled monitoring of small intestinal function, and indeed the detection of small intestinal complications arising from chemotherapy. The SBT operates on the principle that, in the healthy individual, the ingested ^{13}C-Sucrose is digested by the brush-border enzyme sucrase into its monosaccharide constituents, fructose and glucose. After absorption hepatic metabolism of these products leads to the production of $^{13}CO_2$ which is excreted in the breath, which can be collected and analysed using IRMS.[100,104,105] The SBT in our hands, as outlined in this thesis, has been demonstrated to be (1) a biomarker of sucrase activity, a measure of villous health,[55,211] to reflect (2) the functional capacity of the small intestine to absorb nutrients, (3) the degree of maturation of the villus/crypt axis and (4) small intestinal adaptation in response to damage. The SBT has been applied successfully as a biomarker of small intestinal function in:

1. rodents, assessing different classes of chemotherapy agents ranging from mild to severe manifestations of mucositis; and
2. patients with different types of cancer undergoing diverse chemotherapy regimens.

8.2 Specific Outcomes

This thesis assessed and applied the non-invasive SBT to investigate mucositis induced by several different anti-cancer agents in both rodents and paediatric patients. Specifically, the outcomes of this thesis were:

1. Application of this novel non-invasive biomarker (SBT) to both animal and human studies of mucositis.
2. Optimisation of the SBT methodology in animal models.
3. Description of a time-course of MTX-induced intestinal damage and repair using the SBT.
4. Successful application of the SBT to several models of chemotherapy-induced mucositis utilising different classes of chemotherapy agents.
5. Utilisation of the SBT to assess partial amelioration of MTX-induced small intestinal damage using *Streptococcus thermophilus* (TH-4).
6. Demonstration that TH-4 was unable to ameliorate mucositis in a tumour-bearing model.
7. Demonstration that TH-4 did not potentiate tumour growth or hinder tumour kill.
8. Detection and monitoring of intestinal mucositis using the SBT in paediatric cancer patients, both between and within, cancer chemotherapy regimens.
9. Monitoring of other gut functional changes using non-invasive tests (SIP and OCTT).

8.3 DISCUSSION

Animal Studies

The initial aim of Chapter 2 was to determine the optimal dose of selectively enriched ^{13}C-sucrose required to conduct the non-invasive SBT in the DAR model. Indeed, the studies carried out in Chapter 2 illustrated that the previously published dose of 1.0 g/mL of sucrose[39,54,103] was saturating and that a significantly lower dose of 0.25 g/mL was found to be more suitable for assessing small intestinal function in the healthy rat. This study also illustrated that this sucrose dose was capable of detecting MTX-induced mucositis in the rat. It was further demonstrated that a wash-out period of greater than 1.5 weeks was required when large doses of sucrose were administered to the female DAR, whether by diet or gavage, to avoid unacceptable variability.

The newly defined dose of 0.25 g/mL was applied to a time-course study of MTX-induced mucositis (Chapter 3), where the non-invasive SBT was utilised to monitor gut function throughout treatment. This study demonstrated the ability of the SBT to non-invasively assess a time-course of damage and repair, highlighting specific time-points maximal damage (72 h), the initial phase of repair (96 h) and late phases of repair (144 h) in the MTX-induced rat model of mucositis. Interestingly, this study was the first to illustrate the capacity for the SBT to detect the adaptive response to MTX damage in the small intestine. The SBT in its present form cannot determine the specific region of adaptation when an integrated measure of the function of the whole small intestine is compared to biochemical analyses of sucrase activity.

Whilst the 48 h post-MTX time-point was not assessed in this study, the results from this time-course of damage can be applied to the 5-phase model of mucositis,[27] which outlines specific modalities of damage incurred by chemotherapy. Figure 8.1 illustrates the incorporation of the damage time-course of MTX-treated rats, as determined by the SBT, and the proposed 5-phases of mucositis. It is hypothesised that: (1) the initiation phase occurs from 0-20 h; (2) the phases of upregulation and message generation occurs at 21-35 h; (3) signalling and amplification overlaps with the previous phase (30-71 h post-MTX); (4) maximal damage due to inflammation and ulceration occurs from 72-95 h; and (5) healing has commenced by 96 h post-MTX (Figure 8.1) It is important to note that the final time-point (144 h post-MTX) assessed in this time-course illustrated that the small intestine was still undergoing healing and restitution and should be extrapolated further to ascertain this time-point. The application of the SBT enabled easy and safe monitoring of small intestinal damage induced by chemotherapy. Application of the SBT as a biomarker of small intestinal function could be applied to assess and monitor new cancer drugs or anti-mucositis agents.

The non-invasive SBT has been applied successfully in models of mucositis in which rats received the anti-metabolite MTX or 5-FU. However its ability to detect changes attributed to other classes of chemotherapy agents has not previously been assessed. The studies outlined in Chapter 4 assessed chemotherapeutic agents from the following drug classes: DNA topoisomerase inhibitors (Etoposide[56] and Irinotecan[58]), anthracyclines (Doxorubicin[62]) and alkylators (Cyclophosphamide[67]). Assessment of small intestinal function using the non-invasive SBT proved efficacious in this study and was able to detect the level of corresponding damage as obtained from biochemical sucrase activity,

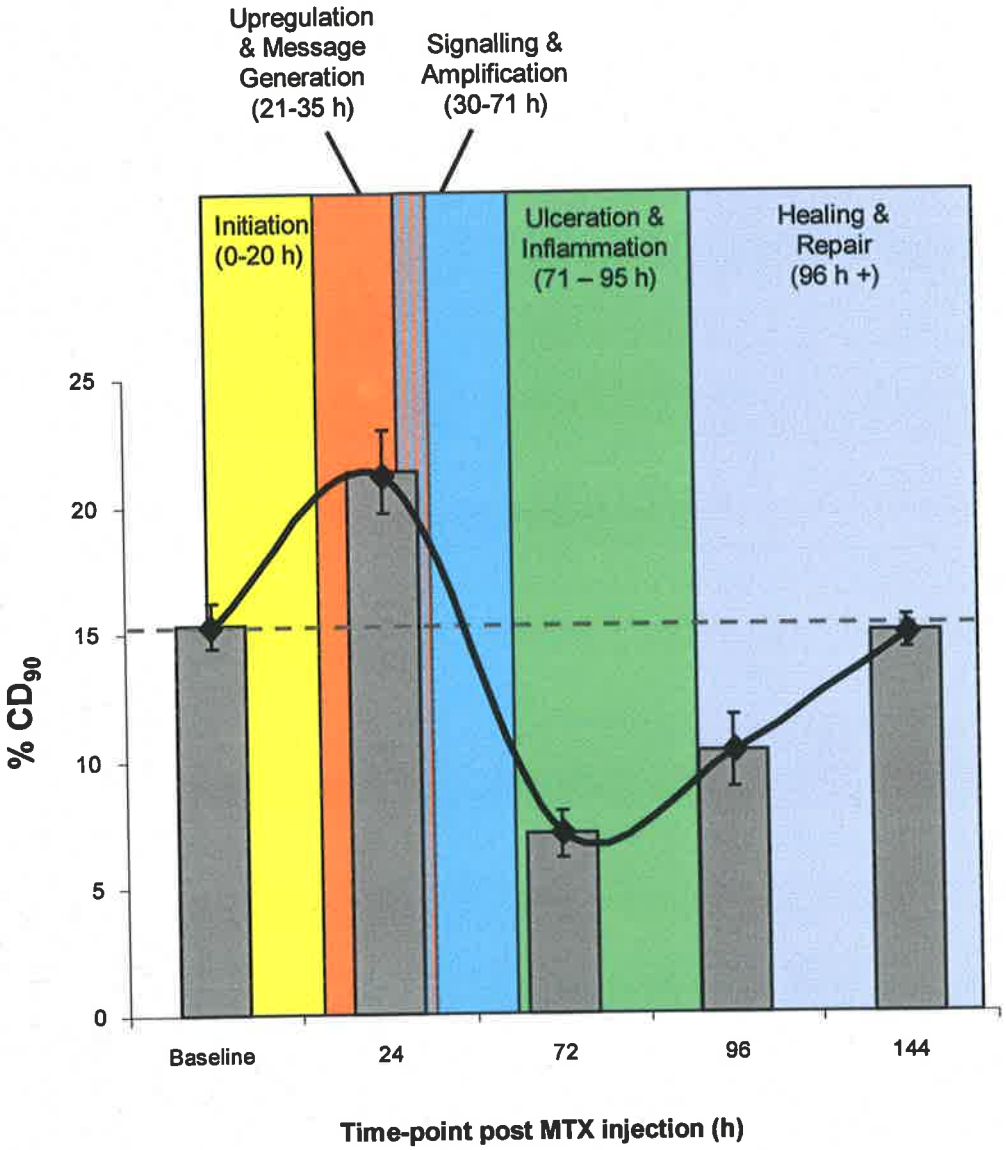

Figure 8.1: Representation of the 5-phase mucositis model incorporated with the SBT time-course in MTX-treated rats (outlined in Chapter 3).

histology and MPO levels. Thus the SBT can be applied easily to multiple animal models in which different classes of chemotherapy agents, old and new, could be assessed. Additionally, it is likely these current studies could be expanded to assess the efficacy of potential anti-mucositis treatments.

Chapter 5 aimed to assess the potential efficacy of *Streptococcus thermophilus* (TH-4) in a model of MTX-induced mucositis in the rat. This study showed that whilst it did not prevent or totally ameliorate the development of MTX-induced damage, it protected the proximal small intestine from severe mucositis that is commonly seen 96 h after MTX administration. Importantly this protection was detected by the SBT, which correlated with biochemical sucrase activity, histological and myeloperoxidase analyses. However, the mechanism of protection by TH-4 on the proximal small intestine remains unclear. Future studies should assess villus length and crypt depth, the number of mucin producing cells and the type of mucin produced, effects on tight junctions and profiling of the presence or induction of anti-inflammatory cytokines. Additionally, the recent finding of TH-4 increasing net folate levels in milk,[152] commonly used in clinical practice to re-initiate cell cycling systemically, suggests that small intestinal folate levels should be quantified.

Since Chapter 5 outlined a positive effect of TH-4 (dose of 10^9 cfu/mL) in rats receiving MTX it was important to characterise whether (1) the same protection could be achieved in a tumour-bearing model of MTX-induced mucositis, and (2) to ascertain if TH-4 impacted the tumour. In contrast to the protective effects observed in Chapter 5, a TH-4 dose of 10^9 cfu/mL was unable to yield any protection to the small intestine in the presence of the DAR specific mammary adenocarcinoma. Importantly, the tumour kill

was the same in TH-4 treated rats as demonstrated by tumour weights. Interestingly the level of intestinal damage induced by MTX in tumour-bearing DAR was significantly more severe compared to their non-tumour bearing counterparts (Chapter 5), This is illustrated in Figure 8.2 which demonstrates the differences in SBT levels in normal rats compared to tumour-bearing rats injected with MTX.

Perhaps more importantly the small intestinal function in tumour-bearing rats was significantly diminished, presumably due to the effects of the tumour. This could be related to altered food intake, although this did not appear to occur. Tissue segments for biochemical analyses (sucrase and MPO) were assessed differently in Chapter 5 compared to Chapter 6, such that direct comparisons can not be made.[55] It could be speculated that the changes observed in SBT and biochemical sucrase activity could be attributed to a population difference. However, when comparing histological damage scores between the two damage models the damage sustained to the tumour-bearing animals is significantly increased compared to their non-tumour bearing counterparts.

Specifically, histological scores from the duodenum and jejunum in tumour-bearing animals were 28 and 25, respectively, compared to damage scores of 18 and16, respectively, in non-tumour bearing animals. Pro-inflammatory cytokines could be affecting the mucosa or it may be related to a general change in metabolism induced by the tumour. These changes may also be associated with a shift in the time-course of MTX damage, such that the early phases of repair commonly observed 96 h post-MTX as outlined in Chapter 3 (Figure 8.1) have shifted and this time-point in a tumour-bearing rat represents maximal damage. Thus a time-course of MTX-induced damage in a tumour-bearing animal may need to be determined for future studies.

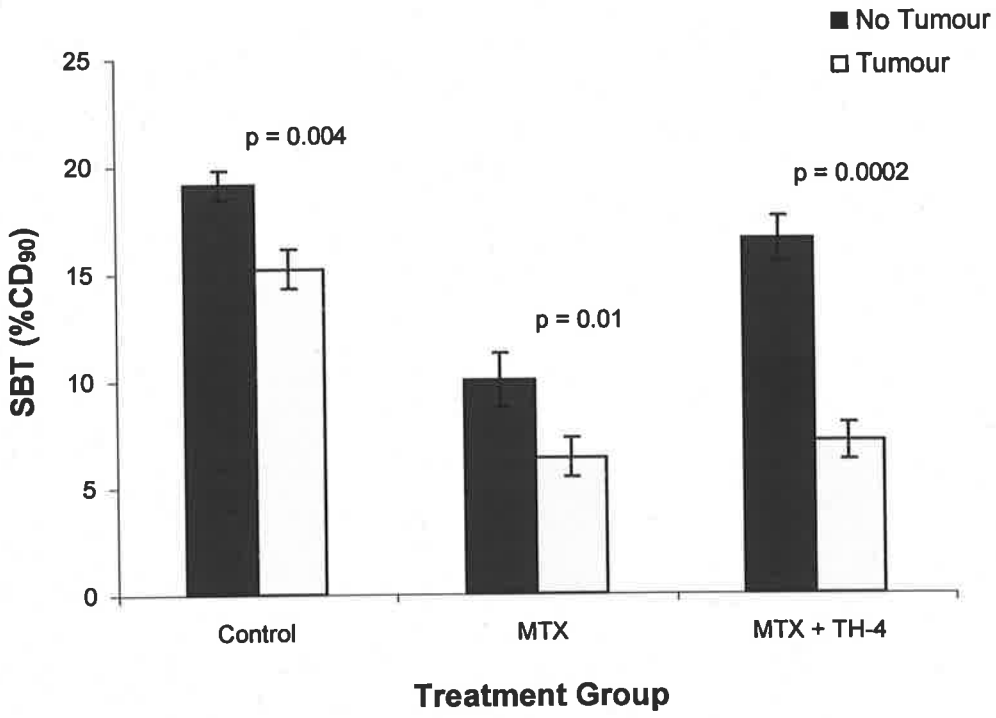

Figure 8.2: SBT results 96 h post-MTX in animals from studies outlined in Chapter 5 (no tumour; charcoal) and Chapter 6 (tumour-bearing; grey) for their respective treatment groups. Data are expressed as mean ± SEM. Significance denoted on graph, where no tumour treatment was compared to its respective tumour-bearing DAR group.

It is not clear why TH-4 administered at a dose of 10^9 cfu/mL could protect the proximal jejunum in non-tumour bearing versus tumour-burdened rats. Future studies need to address if protection can be achieved with increased doses of TH-4 in tumour burdened rats. Additionally the accelerated growth rate and preferential fuel characteristics associated with this particular DAR-specific mammary adenocarcinoma could induce increased pro-inflammatory cytokines. Thus, studies of systemic and small intestinal cytokine levels and profiles need to be carried out in tumour-bearing and non-tumour bearing animal models.

It was illustrated in Chapter 3 that the SBT was capable of detecting adaptive changes of the small intestine. The changes that were evident in this study were small and regionally different; suggesting that a more sensitive approach to the non-invasive test would be needed in the future. One way to achieve this would be the use of synthetically enriched ^{13}C-sucrose, which has a higher level of ^{13}C enrichment to improve the sensitivity of the SBT.

A small study was conducted (see Appendix 1) which reports on the use of synthetically enriched ^{13}C-sucrose for breath testing. This is the first study to address the effects of less severe chemotherapy-induced mucositis using a non-invasive breath test. It was demonstrated that 1.0 and 0.5 mg/150 g rat of synthetically-enriched sucrose increased the sensitivity of the SBT in a less severe model of damage induced by doxorubicin. To date, most animal studies have assessed small intestinal function in the more severe forms of mucositis.[36,42,43,55,60,211] In this pilot study, the addition of minute doses of synthetically enriched ^{13}C-sucrose to the formerly assessed sucrose had the ability to detect subtle changes in overall small intestinal function. The application of the

synthetic sucrose would be highly beneficial for paediatric cancer patients, as they are not only undergoing chemotherapy but are also in different stages of growth. It is here where nutrition is essential to sustain the body's needs. The application of the SBT utilising synthetically enriched ^{13}C-sucrose would be beneficial for future studies assessing more subtle small intestinal changes and evaluating effects on the absorptive compartment in patient nutrition.

Patient Studies

Until the use of the SBT, small intestinal health has only been determined using assays of intestinal permeability.[6,95-97] This biomarker only measures barrier function, which does not necessarily reflect the absorptive function or maturity of the gut, nor the different degrees of damage induced in patients undergoing chemotherapy. The foregoing animal studies suggested that the SBT should be applied to paediatric cancer patients as a biomarker of small intestinal health. The study outlined in Chapter 7 demonstrated that the SBT was capable of detecting small intestinal changes when mucositis was diagnosed clinically in paediatric cancer patients undergoing chemotherapy, compared to patients who did not develop mucositis in a cycle of chemotherapy.[209] However, due to the lack of a validated gut mucositis index, it is important to note that the categorisation of mucositis was based on the development of oral complications (WHO).[205] Additionally, other non-invasive methods were employed to assess gut function: (1) sucrose permeability, a measure of gastric-duodenal barrier function; (2) lactulose/rhamnose permeability, a measure of small intestinal barrier function; and (3) oro-caecal transit time. The current study was the first to assess gastric-duodenal barrier function in paediatric cancer patients. However, a larger sample size of patients is required to strengthen the data. Importantly, the SBT proved to be a

superior biomarker of small intestinal function compared to the previously published methodology of SIP.

More recently a novel test measuring serum citrulline concentrations has been implemented as a marker of chemotherapy-induced intestinal epithelial damage, as citrulline is metabolite of glutamine, the primary fuel for the gut.[212-214] This test measures decreases in citrulline concentrations as an indicator of small intestinal damage due to the decrease in surface area. However, its implementation has been confined to rodents[212] and patients[213,214] who have undergone total body irradiation or bone marrow transplant protocols; i.e. animals or patients with expected development of severe mucositis. Its implementation in less severe models of mucositis has not been addressed, which raises questions of the sensitivity of the test. Additionally, it requires more blood to be collected from the patient, which is an added burden to the patient. It could be argued that decreases in serum citrulline levels could indeed reflect the decreases in the patient's food intake, as reductions and withdrawal from food is commonly observed in bone marrow transplant patients.[136]

Clinicians have previously proposed that a cumulative effect of chemotherapy on the gut may lead to mucositis, as some patients have developed mucositis in low-risk cycles of chemotherapy. Thus, a small pilot study was carried out (see Appendix 2), to non-invasively assess small intestinal function using the SBT over multiple chemotherapy cycles to determine whether a cumulative damage was evident. Even though patient recruitment was slow and there were low numbers, this pilot study demonstrated that multiple chemotherapy cycles induced cumulative intestinal damage. Additionally, this study demonstrated that the application of the SBT at the time-points: (1) baseline (immediately prior to the commencement of a new cycle) and (2) d1 (within 24 h of

chemotherapy administration for each cycle) would be preferential for determining cumulative effects. Future directions would include: (1) the expansion of the sample size to ascertain the predictive/likelihood of mucositis; (2) application of SBT to monitor small intestinal function throughout chemotherapy with respect to selected cancers and therefore cancer regimens; and (3) application of synthetically enriched sucrose to increase sensitivity for the assessment of monitoring small intestinal health.

8.4 CONCLUDING REMARKS

Until now, methods for assessing small intestinal function have relied on information collected from small bowel biopsies. These require sedation, are invasive, costly and painful. Biopsies are usually restricted to the very proximal small intestine and therefore do not necessarily reflect the more remote regions, as it is only a marker of the biopsied fraction. This thesis has demonstrated that the non-invasive SBT can be implemented successfully as a biomarker of small intestinal (villus) health and maturation in both rat models of mucositis and patients undergoing chemotherapy. It has been the first to delineate an appropriate dose of selectively enriched ^{13}C-sucrose and its utilisation in a time-course of MTX-induced mucositis in the rat. Additionally, the SBT has been applied successfully and characterised in rat models of mucositis, in which different classes of chemotherapy agents were assessed. It was demonstrated that the SBT was a specific marker as it correlated highly with *in vitro* sucrase activity and histological damage. The SBT has also been validated as a biomarker of small intestinal health to assess potential anti-mucositis treatments in both non-tumour bearing and tumour-bearing rats. Most importantly, this thesis is the first to demonstrate application of the

non-invasive SBT as a biomarker of small intestinal function in a cohort of paediatric cancer patients undergoing chemotherapy. The SBT can be used to detect the development of small intestinal mucositis or as a biomarker for monitoring small intestinal function over multiple cycles of chemotherapy. With the utilisation of the SBT the future is promising for simple and non-invasive assessment of new chemotherapies with respect to gut toxicity, and also any potential anti-mucositis treatments in both animals and patients.

Future studies arising from this thesis include investigating possible mechanisms of protection elicited by TH-4 in MTX-treated animals, including mucin production and type, and cytokine profiling. Since TH-4 did not protect the small intestine in MTX-treated tumour-bearing animals, local and systemic cytokine profiling should be quantified. Additionally, increased doses of TH-4 should be assessed in a tumour-bearing model of mucositis. With respect to the application of the SBT in paediatric cancer patients, a larger sample size is required to strengthen the detection of intestinal changes associated with mucositis and also the cumulative effect of multiple chemotherapy regimens. It would also be of importance to assess the cumulative effects of chemotherapy in patients with specific cancer types and thus chemotherapy regimens thus reducing potential variability.

PART V: APPENDICES

APPENDIX 1: SYNTHETICALLY ENRICHED ^{13}C-SUCROSE AND THE SBT IN A MODEL OF MODERATE CHEMOTHERAPY-INDUCED DAMAGE; A PILOT STUDY

A1.1 INTRODUCTION

Studies thus far reporting on the SBT have described the administration of the selectively/naturally occurring ^{13}C-sucrose found in sugar cane [104] and its efficacy of detecting severe forms of chemotherapy-induced mucositis in rats.[54,55,103,211] Theoretically, the incorporation of 100% synthetically enriched ^{13}C-sucrose into the sucrose gavage used in the SBT for rodents, would allow an increased signal of $^{13}CO_2$ that could be detected in breath samples using an IRMS. Furthermore, the increase in sensitivity for the SBT would potentially allow milder/moderate intestinal damage to be detected. In Chapter 4 a number of SBT profiles for chemotherapy agents[211] were added to the existing known SBT profiles for MTX and 5-FU.

Recently, a small amount of synthetically enriched (100%) ^{13}C-sucrose became available through the Sugar Research Institute (SRI). The aims of this pilot study were to (1) investigate four doses of the synthetically enriched ^{13}C-sucrose to more accurately quantify sucrase activity and thus the small intestine's health and maturity by the SBT; and (2) apply the synthetically enriched sucrose to a moderate damage model of chemotherapy-induced mucositis in a female DAR.

A1.2 MATERIALS & METHODS

Synthetic ^{13}C-sucrose dosing for the SBT

Female DAR (minimum starting weight 150g) were used for this study and were acquired from the IMVS, Gilles Plains, Adelaide. Animals were grouped and housed in standard rat cages with an environmental temperature of 25°C with a 12h light:dark cycle for the duration of the study. Animals were housed in groups of two rats, having free access to water and 18% casein diet for the duration of the study.[127] Rat bodyweights were recorded on the day SBTs were performed. Synthetically-enriched ^{13}C-sucrose doses were combined with the 0.25g/ml of selectively enriched sucrose as the gavage vehicle. Approval was obtained by the Animal Ethics Committee of the Children's, Youth and Women's Health Service and the University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2004).

Initially rats (157 ± 3g) were fasted overnight. Baseline breath samples were collected as outlined in Chapter 2. DAR were gavaged with either 1 mL of normal laboratory grade sucrose selectively enriched with ^{13}C-sucrose (derived from cane sugar; n = 4; AnalaR, BDH, MERCK, Pty. Ltd., Victoria, Australia) or 1.0 (n = 7), 0.5 (n = 7), 0.25 (n = 6) or 0.125 (n = 6) g/ml/150g rat of 100% ^{13}C-labelled synthetically enriched sucrose (n = 30 DAR total) for the sucrose breath test. The remaining SBT breath testing procedures, once gavaging was completed, were unchanged from the described

method in Chapter 2. Rats were not sacrificed, and 26 were used for the next phase of the trial, but DAR synthetic ^{13}C-sucrose dose groupings were not kept the same.

Synthetic ^{13}C-sucrose in a moderate model of small intestinal damage

Rats (n = 26; 165 ± 3g) were grouped according to even-distribution of weight and all were injected with 20mg/kg doxorubicin (Mayne Pharma Pty Ltd, Mulgrave North, Melbourne, Victoria, Australia) i.p. on day 0. Doxo has been shown previously (Chapter 4) to produce a moderate level of small intestinal damage, characterised by crypt disruption and a moderate degree of villus atrophy and neutrophil infiltration. Rats performed SBTs on day zero (prior to injection) and day three. Rats received either 0.25g/mL of selectively enriched ^{13}C-sucrose (n = 10) or 1.0 (n = 4), 0.50 (n = 4), 0.25 (n = 4) and 0.125 (n = 4) g/ml/150g rat of synthetically enriched ^{13}C-sucrose. Synthetically enriched ^{13}C-sucrose can be defined as sucrose that is modified to comprise of 100% ^{13}C and 0% ^{12}C enrichment. Breath testing procedures were followed as described in Chapter 2 with the substitution of the appropriate dose of synthetically enriched ^{13}C-sucrose. Since the level of damage induced by doxorubicin has been previously described, and the focus of this study was primarily the SBT with different enrichment levels of ^{13}C-sucrose, DAR were not sacrificed and no intestinal weights and biochemical analyses were performed.

Statistical analyses

Data and statistical comparisons were made using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA) or Microsoft® Office Excel 2003 for WindowsXP. Comparison of selectively enriched sucrose to differing doses of

synthetic ^{13}C-sucrose was determined by a one-way ANOVA with a Tukey's *post-hoc* test. Significance between selectively enriched sucrose and synthetic ^{13}C-sucrose at different doses in a doxorubicin-induced damage model was determined using a one-way ANOVA with a Tukey's *post-hoc* test. SBT data from the baseline damage model phase was combined with the first phase SBT data without damage for data analyses between a damaged and non-damaged model, and termed "healthy". A one-tailed unpaired t-test was utilised to determine significance between "healthy" (control) rats and rats receiving doxorubicin at all doses of ^{13}C-sucrose. Data are expressed as median ± 25th and 75th quartile ranges (box and whisker plot) or mean ± SD. Significance was determined when $p < 0.05$.

A1.3 RESULTS

Synthetically-enriched ^{13}C-sucrose in healthy DAR

Rats gavaged with selectively enriched ^{13}C-sucrose (0.25g/mL) alone were not significantly different ($p > 0.05$) compared to each group gavaged with synthetically enriched ^{13}C-doses (Figure A1.1). In contrast, rats receiving 1 mg/mL of synthetic sucrose had a significantly lower ($p < 0.05$) SBT level compared to DAR receiving 0.5, 0.25 and 0.125 mg/mL of synthetically enriched sucrose.

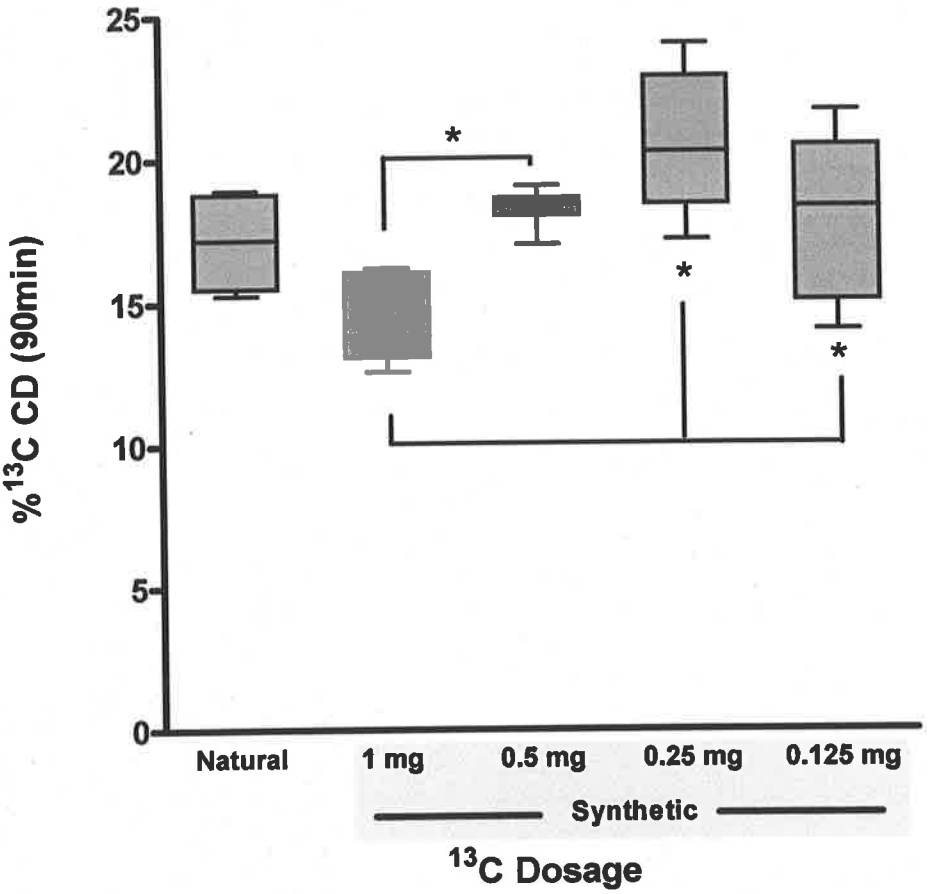

Figure A1.1: SBT with either selectively-enriched sucrose (0.25g/mL) or synthetically-enriched ^{13}C-sucrose at differing doses of: 1, 0.5, 0.25 and 0.125 mg/150g rat mixed in the 0.25g/mL vehicle solution. Data expressed as a Box and Whisker plot. Significance denoted by *, where $p < .05$ compared to DAR receiving the 1mg dose of ^{13}C.

Synthetically-enriched ^{13}C-sucrose in DAR with moderate intestinal damage

In rats with doxorubicin-induced small intestinal damage, rats receiving a dose of 1.0 mg/mL of synthetic sucrose were significantly lower (57% decrease; $p < 0.01$) compared to rats receiving selectively enriched sucrose (standard dose of 0.25 g/mL) three days post injection (Figure A1.2). In contrast, all other groups receiving doses of synthetically enriched ^{13}C-sucrose, SBT levels were not significantly different compared to selectively enriched ^{13}C-sucrose.

When comparing SBTs from healthy controls rats to rats with doxorubicin damage (Figure A1.3), selectively-enriched sucrose (0.25 g/mL), and 1.0, 0.5, 0.25 and 0.125 mg/ml doses of synthetically-enriched sucrose were all significantly lower compared to its healthy counterpart ($p < 0.001$), where there was a 52%, 75%, 75%, 76% and 66% decrease, respectively.

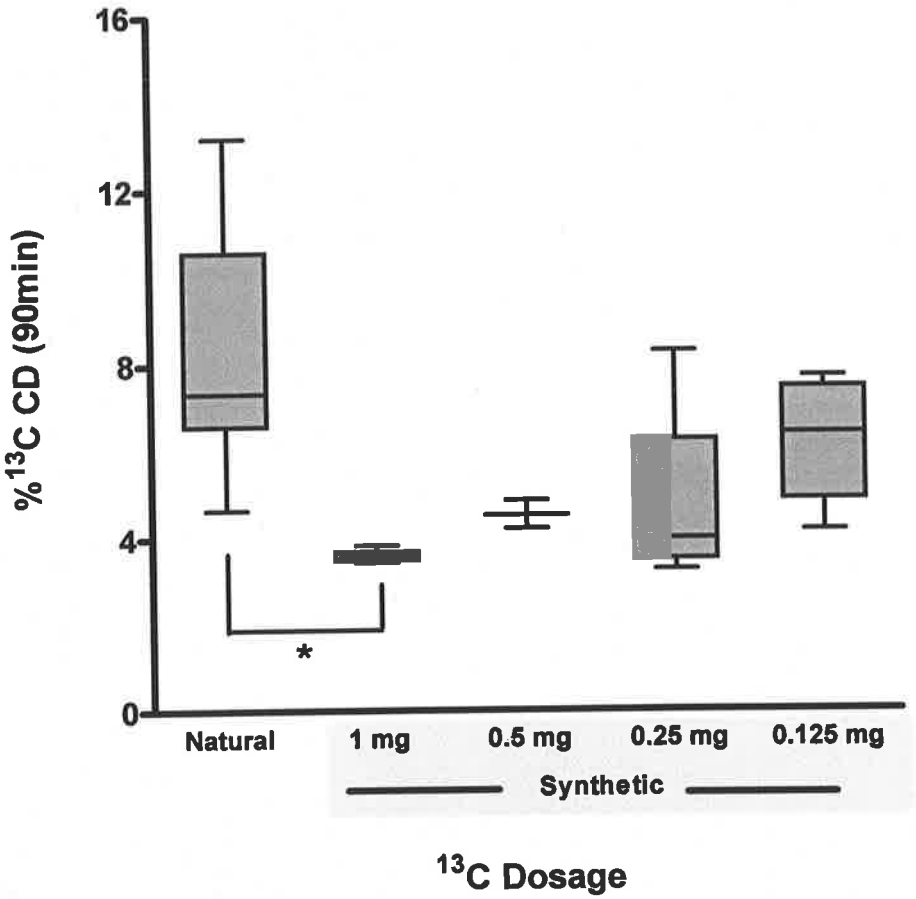

Figure A1.2: SBT using selectively-enriched ^{13}C-sucrose compared to differing dose of synthetically-enriched ^{13}C-sucrose in rats with doxorubicin-induced small intestinal damage. Data are expressed as a Box and Whisker plot. Significance denoted by *, where $p < 0.05$ compared to normal sucrose SBT.

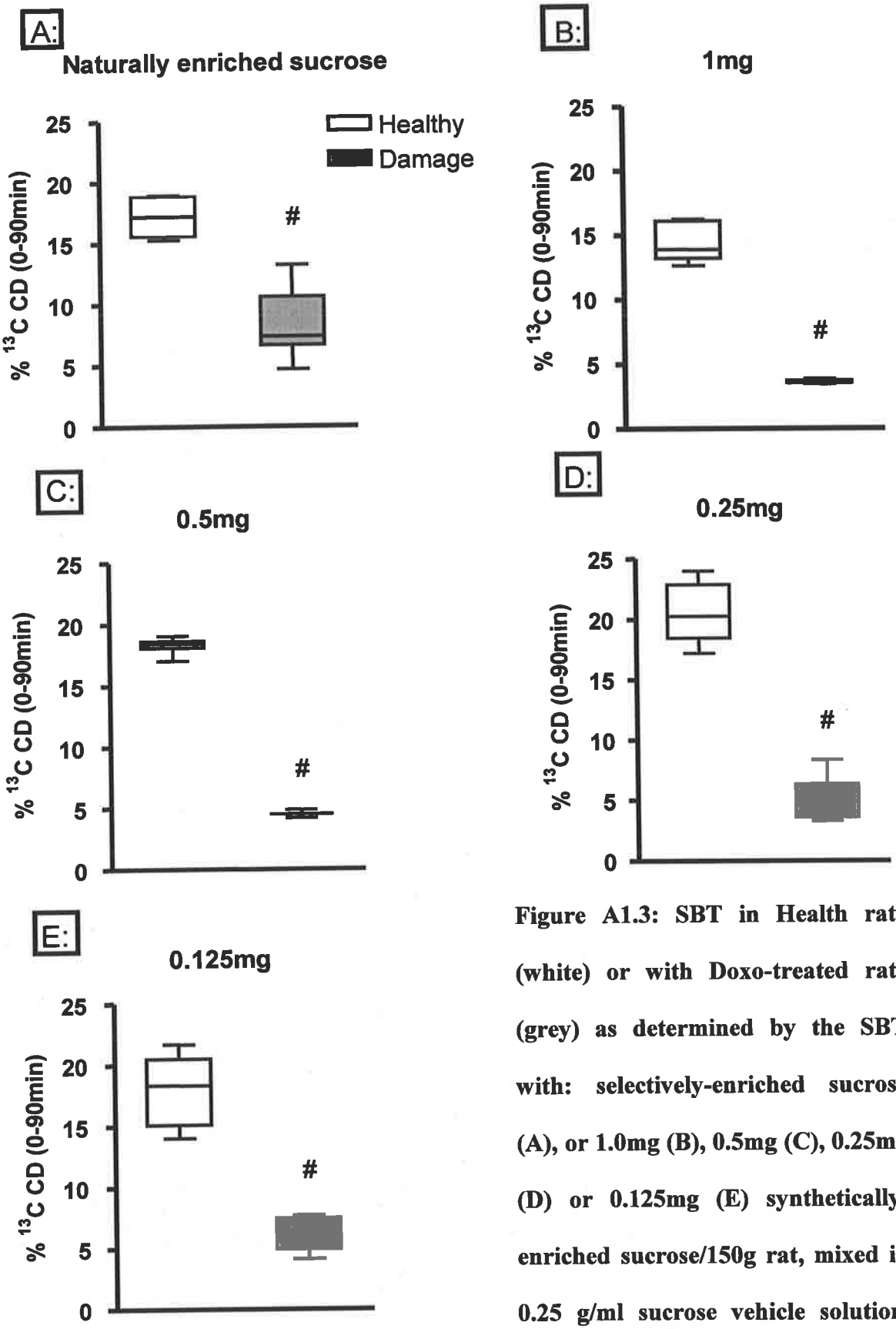

Figure A1.3: SBT in Health rats (white) or with Doxo-treated rats (grey) as determined by the SBT with: selectively-enriched sucrose (A), or 1.0mg (B), 0.5mg (C), 0.25mg (D) or 0.125mg (E) synthetically-enriched sucrose/150g rat, mixed in 0.25 g/ml sucrose vehicle solution. Data are expressed as a Box and Whisker plot. Significance denoted by # where p < 0.001 compared to healthy counterpart.

A1.4 CONCLUSIONS

Utilising previous results, as outlined in Chapter 4, from rats with different classes of chemotherapy agents, the drug doxorubicin was utilised to induce a moderate level of small intestinal damage.[211] This pilot study has shown that

1. Incorporation of synthetically-enriched sucrose for use in the SBT was applied successfully at different doses in healthy rat.

2. Rats receiving 1.0 mg/mL of synthetically-enriched ^{13}C-sucrose had a significantly lower SBT result compared to DAR receiving standard sucrose 72 h after doxorubicin administration.

3. Rats receiving 1.0, 0.5 or 0.25 mg/mL of synthetic sucrose had a more striking decrease in SBT levels compared to other doses of synthetically- or selectively-enriched sucrose.

APPENDIX 2: THE EFFECT OF MULTIPLE CHEMOTHERAPY REGIMENS ON SMALL INTESTINAL FUNCTION IN CHILDREN UNDERGOING CANCER TREATMENT

A2.1 INTRODUCTION

Whilst the ability to determine chemotherapy-induced small intestinal damage during a single cycle is helpful, for clinical management, prediction of impending mucositis and monitoring possible cumulative damage may prove to be more important. In Chapter 7 the SBT was shown to be capable of detecting small intestinal damage associated with the clinically diagnosed mucositis (primarily based on the development of oral complications).[209] The effect of chemotherapy throughout a regimen of cancer treatment has however not been assessed. Mucositis may develop in cancer patients unpredictably bearing no clear relationship to the dose and timing of the chemotherapy. Some believe there may be a carry-over effect from one cycle to the next, where the small intestine does not have sufficient time to renew and heal prior to the next cycle. A small pilot study was conducted to assess the effect of multiple chemotherapy regimens on small intestinal function over time using the SBT as the means for small intestinal function. The aim of this study was to assess if the SBT could monitor small intestinal function throughout cancer treatment in children undergoing chemotherapy.

A2.2 Materials and Methods

Patients and study design

A small pilot study was performed to assess small intestinal function throughout cancer treatment using the SBT as the biomarker. Nine paediatric cancer patients (aged 4 - 16 years) were recruited (five male and four female) from the Haematology/Oncology Department of the CYWHS (Women's and Children's Hospital Campus), North Adelaide, Australia. Recruited patients had a height of 150.4 ± 8.7 cm and a weight of 41.6 ± 5.6 kg. Patients were required to perform SBTs prior to the commencement of every cycle of chemotherapy (baseline), day 1 (within 24 h of chemotherapy administration) and day 3-5 after chemotherapy, with the assessment of four cycles as a minimum. Of these ten patients only six (five with ALL and one with Ewing's Sarcoma) were evaluable as they completed SBTs for four or more cycles. Whilst the development of mucositis was essential for the study carried out in Chapter 7, its development in this study was not the focus, thus it is not reported on in this pilot study.

Informed written consent was obtained from all subjects and ethical clearance was granted from the Research Ethics Committee of the CYWHS, North Adelaide, Australia. The study was carried out in accordance with the declaration of Helsinki.

SBT

Following an o/n fast, subjects exhaled into two 10 mL Exetainer glass tubes (Exetainer, Labco Limited, High Wycombe, England) as previously described in Chapter 7 for baseline $^{13}CO_2$ levels. Following the collection of the baseline sample patients ingested 20 g of selectively enriched ^{13}C-sucrose (AnalR, BDH, MERCK, Pty Ltd, Victoria, Australia) dissolved in 100 mL water. Thereafter two breath samples were collected every 15 min for 90 min. Breath samples were analysed for $^{13}CO_2$ content as described in Chapter 7. Data were expressed as $\%CD_{90}$.

Statistical analyses

SBT data were expressed as the percentage change from their initial baseline SBT as a means of monitoring overall gut function from their first test. An unpaired t-test was used to determine significance from their first test (n = 6) compared to the final cycle of collection, which was cycle 8 in this study (n = 3). Data were expressed as mean ± SEM. Significance was determined if $p < 0.05$.

A2.3 RESULTS

A total of six patients underwent SBTs for a minimum of four cycles, and numbers decreased for each respective cycle thereafter. Monitoring small intestinal function using the change in SBT levels from the respective patient's initial baseline compared with the baseline SBT for each subsequent cycle is shown in Figure A2.1. A general

decline in small intestinal function was evident over time, where there was a significant decrease in small intestinal function ($p = 0.03$) after undergoing eight regimens of chemotherapy. Comparison of percentage change in the level from the initial baseline to d1 for ongoing cycles is depicted in Figure A2.2, where a general decrease was seen over time. Comparison of cycle 7 (cycle 8 not assessed as patients failed to perform d1 tests for this cycle due to illness) d1 SBT tests versus the initial SBT (% change) illustrated that cumulatively there was a significantly decreased small intestinal function by cycle 7 ($p = 0.001$). When d3-5 SBT results were assessed as the percentage change from the initial baseline for each respective cycle, no significant changes (cumulative effect) were evident (Figure 9.3).

A2.4 CONCLUSIONS

This pilot study has demonstrated that the SBT is capable of monitoring small intestinal function over multiple cycles of chemotherapies. The preliminary evidence suggests that the SBT can measure a progressive decline in small intestinal function, using either baseline levels prior to each cycle or percentage difference of d1 levels after chemotherapy vs. baseline. Interestingly, a similar apparent cumulative effect was not seen 3-5 days after chemotherapy.

This is the first study to illustrate a putative cumulative effect of chemotherapy on gut function over multiple treatments. This data is very preliminary, with a small sample

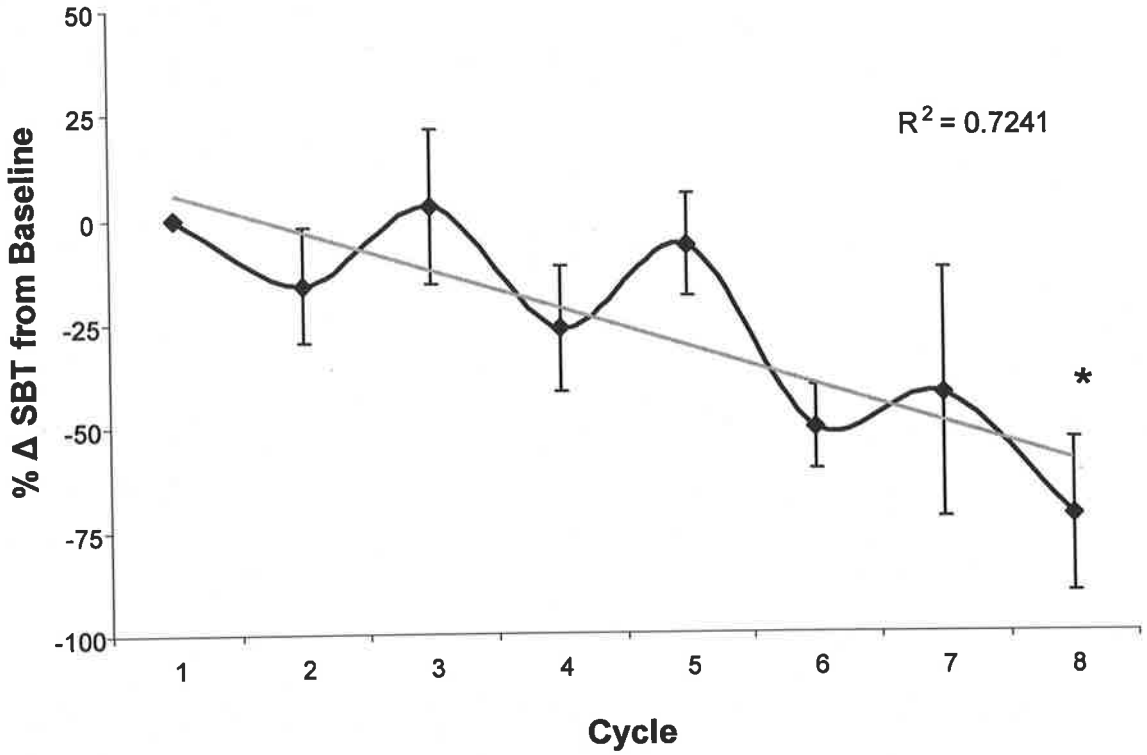

Figure A2.1: Monitoring of small intestinal function using the SBT comparing all baseline tests for each cycle compared to the initial SBT (percentage change from baseline SBT) in children with cancer undergoing multiple cycles of chemotherapy (n = 6). Data expressed as mean ± SEM. Cycle 8 compared to cycle 1 baseline SBT. Significance denoted by *, where p = 0.03. (Note: n = 6 at cycle 1, decreases to n =3 by cycle 8).

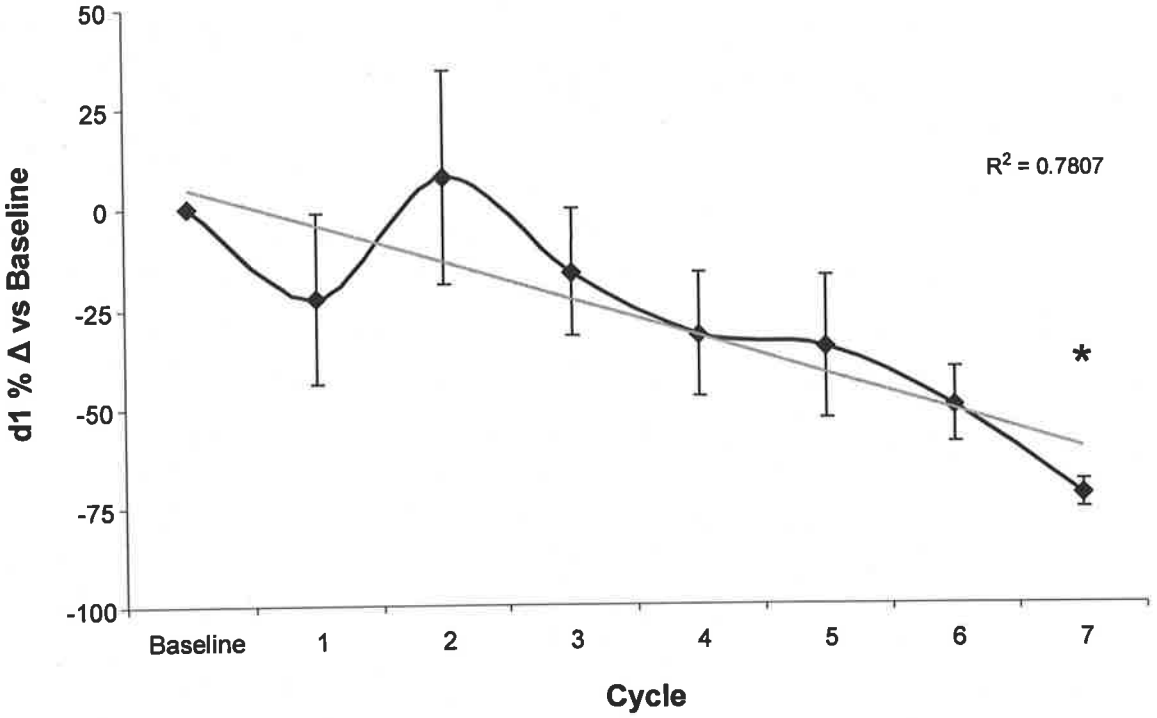

Figure A2.2: Monitoring of small intestinal function non-invasively using the SBT in children with cancer throughout multiple chemotherapy cycles. Data are expressed as percentage change for d1 SBT from the initial baseline SBT, mean ± SEM. Cycle 7 compared to initial baseline SBT. Significance denoted by *, where p = 0.001. Number of patients varied from cycle to cycle (initially n = 6)

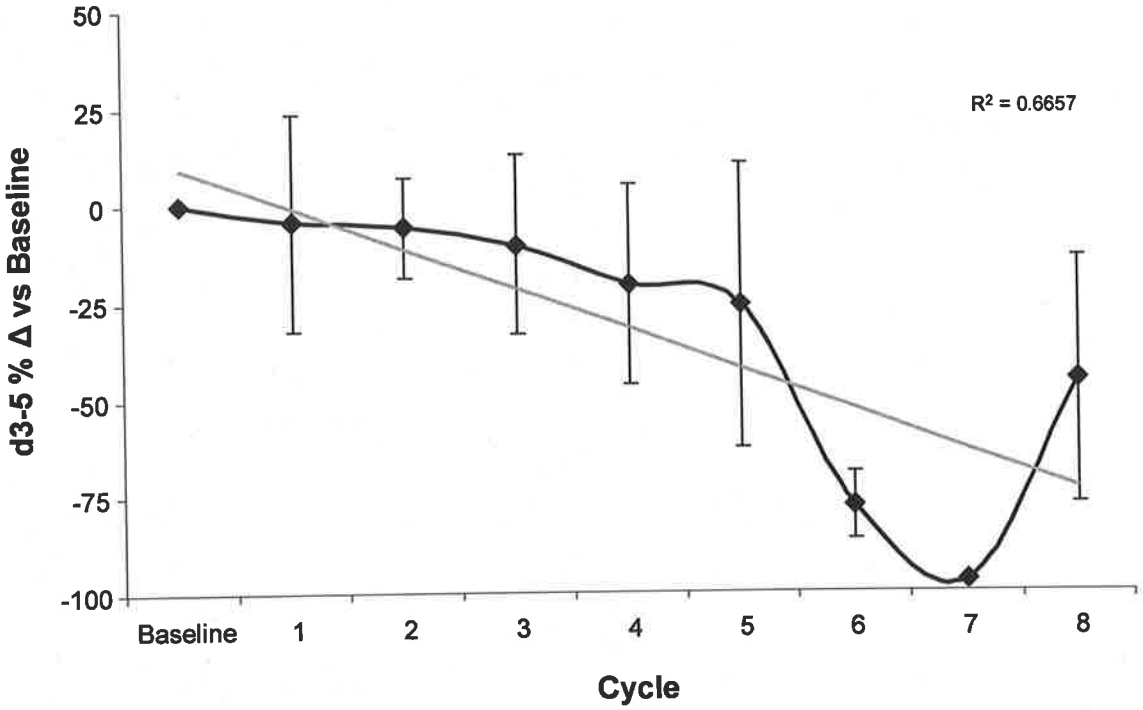

Figure A2.3: Monitoring of small intestinal function non-invasively using the SBT in children with cancer throughout multiple chemotherapy cycles. Data are expressed as the percentage change for d3-5 SBT from the initial baseline SBT, mean ± SEM. Cycle 8 not significantly different vs. baseline. Number of patients varied from cycle to cycle (initially n = 6)

size and serves only as the foundation for carrying out a more detailed study with other parameters, including assessment of oral mucositis.

The patients in this pilot study had different cancers, and were recruited at different phases of cancer treatment such that their individual chemotherapy regimens were varied. Clearly a more thorough analysis of cumulative effects of chemotherapy on the small intestine needs to be performed in a more standardised setting, preferably in patients with specific cancers.

In conclusion, a cumulative effect on the small intestine due to multiple chemotherapy cycles was apparent, when the non-invasive SBT was employed as the biomarker. It appears that for monitoring purposes an SBT performed at either baseline or d1 for each cycle would be likely to detect cumulative effects associated with multiple cycles of chemotherapy. However, a larger sample size is required for more definitive conclusions and assessment of possible predictivity.

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