Chemotherapy-induced mucositis: The role of gastrointestinal microflora and mucins in the luminal environment

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Andrea Marie Stringer

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Mucositis manifesting as diarrhoea is a common side effect of chemotherapy which remains poorly understood. It is one of a number of manifestations of alimentary mucositis, which affects the entire gastrointestinal tract. The exact number of patients that are affected by diarrhoea as a result of treatment is uncertain, although it is believed that approximately 10% of patients with advanced cancer will be afflicted. Despite advances in the understanding of oral and small intestinal mucositis over recent years, large intestinal mucositis, including diarrhoea, has not been well defined and the underlying mechanisms of the condition are yet to be established. The majority of the literature available concerning diarrhoea is based on clinical observations, with very little basic research existing. However, from the research conducted, it is likely that the intestinal microflora and mucins play a role in the development of chemotherapy-induced diarrhoea. This thesis will examine in detail what is known about the mechanisms of chemotherapy-induced diarrhoea (CID). Furthermore it will explore the potentially important relationship between intestinal microflora, the luminal environment and the subsequent development of chemotherapy-induced mucositis and diarrhoea.

5-Fluorouracil (5-FU) is a commonly used chemotherapy agent in clinical oncology practice. Two of its major side effects are mucositis and diarrhoea. The structure of mucins offers mucosal protection, and allows maintenance of intestinal flora by providing attachment sites and preventing bacterial overgrowth and/or penetration. Following treatment with 5-FU, we showed decreases in *Clostridium spp*., *Lactobacillus spp*. and *Streptococcus spp*., and an increase in Escherichia spp. in the jejunum. In the colon, 5-FU caused decreases in *Enterococcus spp*., *Lactobacillus spp*. and *Streptococcus spp*. Real time PCR of faecal samples showed decreasing trends in *Lactobacillus spp*. and *Bacteroides spp*., and an increasing trend in *E. coli*. Significant increases ($p<0.05$) were seen in *Clostridium spp*. and *Staphylococcus spp*. at 24 h. Goblet cell numbers decreased significantly in the jejunum from 24-72 h, with a significant increase in the percentage of cavitated goblet cells, suggesting 5-FU

treatment causes significant changes in intestinal flora and mucin secretion in rats. These changes could result in systemic effects, and in particular may contribute to the development of chemotherapy-induced mucositis.

Irinotecan causes cholinergic and delayed onset diarrhoea in patients, in which β-glucuronidase produced by gut bacteria is thought to be involved. Diarrhoea was observed in treated rats, as expected, following irinotecan treatment. β-glucuronidase expression increased in the jejunum and colon. Faecal flora changed quantitatively after treatment also, with increases in *E. coli*, *Staphylococcus spp.*, and *Clostridium spp.* (all β-glucuronidase producing), and decreases in *Lactobacillus spp.*, *Bifidobacterium spp.* (both beneficial bacteria), and *Bacteroides spp.* (β-glucuronidase producing, major component of intestinal flora), suggesting that irinotecan-induced diarrhoea may be caused by an increase in β-glucuronidase producing bacteria. However, the increase in bacteria may also be caused by irinotecan, further exaggerating the toxicity of the drug, and emphasising the need for these specific bacteria to be therapeutically targeted for successful treatment regimens to be accomplished.

Mucus production appears to be increased after irinotecan treatment, which may contribute to the development of diarrhoea. Goblet cells were demonstrated to decrease significantly after irinotecan treatment. However, mucin secretion increased. Mucin expression changed significantly after treatment. Muc2 and Muc4 decreased significantly in the villi of the jejunum after treatment, Muc2 and Muc4 decreased significantly in the crypts. Muc2 decreased significantly in the colon. This indicates that irinotecan causes an increase in mucin secretion and a net decrease in mucin-producing goblet cells, and the expression of Muc2 and Muc4 in the gastrointestinal tract is altered following treatment. Increased mucin secretion is likely to be related to altered mucin expression, and may contribute to chemotherapy-induced diarrhoea.

To determine if the changes to the intestinal microflora caused by chemotherapy could be translated to the clinic, a pilot clinical study was carried out. Sixteen patients experiencing CID were recruited to the study with two control subjects. A large proportion of patients (75%) demonstrated a reduced anaerobic component of their faecal microflora. A reduced diversity of species was also observed in patients. The majority of patients exhibited decreases in *Clostridium spp.*, *Lactobacillus spp.* and *Bifidobacterium spp.*, whilst all patients exhibited decreases in *Bacteroides spp.* and *Enterococcus spp.* Patients receiving antibiotics did not exhibit any marked differences to patients not receiving antibiotics. This indicates that the results observed in the animal studies are clinically relevant, and further research into this area should be undertaken. CID is associated with marked changes in the intestinal microflora. These changes may result in diminished bacterial functions within the gut, altering gut function and initiating intestinal damage, resulting in the onset of diarrhoea.

In conclusion, there is clear evidence demonstrating chemotherapy treatment results in changes to the intestinal microflora and mucin secretion, which may be responsible in part for the development of severe mucositis and diarrhoea. Irinotecan toxicity may be compounded by the increase in β-glucuronidase producing bacteria. The intestinal flora of cancer patients experiencing CID is also noticeably different to that of healthy subjects. Irinotecan causes changes to mucin secretion, and the specific expression of Muc2, Muc4 and Klf4, suggesting that secretory control by the enteric nervous system may also be affected by chemotherapy. This research has extended the understanding of chemotherapy-induced mucositis and diarrhoea, complex side effects of chemotherapy. However, new areas for future research have also been identified.

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Publications arising from this thesis

1. **Stringer AM,** Gibson RJ, Bowen JM, Logan RM, Yeoh, ASJ, Keefe DMK (2007). Chemotherapy-induced mucositis: the role of the gastrointestinal microflora and mucins. *J Support Oncol* **5**(6):259-267.
2. **Stringer AM**, Gibson RJ, Bowen JM, Logan RM, Burns J and Keefe DMK (2007). Chemotherapy-induced diarrhoea is associated with changes in the luminal environment in the DA rat. *Exp Biol Med* **232**(1):96-106.
3. **Stringer AM,** Gibson RJ, Logan RM, Bowen JM, Yeoh ASJ and Keefe DMK (2008). Faecal microflora and β-glucuronidase expression are altered in an irinotecan-induced diarrhoea model in rats. Accepted *Cancer Biol Ther* (September 8, 2008).
4. **Stringer AM,** Gibson RJ, Logan RM, Bowen JM, Laurence J and Keefe DM (2008). Irinotecan-induced mucositis is associated with changes in intestinal mucins. Accepted *Cancer Chemother Pharmacol* (October 11, 2008).

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1. **Stringer AM,** Gibson RJ, Logan RM, Bowen JM, Yeoh ASJ and Keefe DMK (2008). Gastrointestinal microflora and mucins play a role in the development of 5-Fluorouracil-induced gastrointestinal mucositis in rats. Submitted to *Exp Biol Med*.
2. **Stringer AM,** Gibson RJ, Bowen JM, Ashton K, Logan RM, Al-Dasooqi N, Yeoh ASJ and Keefe DMK (2008). Irinotecan-induced mucositis manifesting as diarrhoea corresponds with an amended intestinal flora and mucin profile. Submitted to *Int J Exp Pathol*.
3. **Stringer AM,** Gibson RJ, Yeoh ASJ and Keefe DMK (2008). Chemotherapy-induced diarrhoea is associated with changes to the microbiome in cancer patients. Submitted to *Int J Cancer.*

Professor Dorothy Keefe

Professor Keefe was my principal supervisor and therefore listed as a co-author on all publications arising from this thesis. She assisted in the development of my original research proposal and provided funding for the work that was completed during my candidature. In addition she read through many drafts of the individual papers as well as this thesis.

Dr Rachel Gibson

Dr Gibson was my co-supervisor and therefore listed as a co-author on all publications arising from this thesis. She assisted in the development of my original research proposal and provided funding for the work that was completed during my candidature. In addition she read through many drafts of the individual papers as well as this thesis.

Dr Richard Logan

Dr Logan is a member of the Mucositis Research Group. He assisted with all of the animal experiments undertaken in this study. He also read numerous drafts of the individual papers making up this thesis.

Dr Joanne Bowen

Dr Bowen is a member of the Mucositis Research Group. She assisted with all of the animal experiments undertaken in this study and provided advice on laboratory techniques. She also read numerous drafts of the individual papers that make up this thesis.

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Ms Yeoh is a member of the Mucositis Research Group. She assisted with all of the animal experiments undertaken in this study.

During my candidature, I was involved in several other studies, not presented in this thesis. These have resulted in co-authorship of several other manuscripts. I am first author on an invited review, and have been co-author on several other studies within the laboratory and reviews on a variety of mucositis-related topics.

1. **Stringer AM,** Gibson RJ, Bowen JM and Keefe DMK (2008). Chemotherapy-induced changes to microflora: Evidence and implications of change. *Curr Drug Metab* (invited review).

2. Logan RM, **Stringer AM**, Bowen JM, Gibson RJ, Sonis ST and Keefe DMK (2008). Serum levels of Serum levels of NFkappaB and pro-inflammatory cytokines following administration of mucotoxic drugs. *Cancer Biol Ther* 2008 **7**(7):1139-45.

3. Logan RM, **Stringer AM**, Bowen JM, Gibson RJ, Sonis ST and Keefe DM (2008). Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered? *Cancer Chemother Pharmacol* accepted March 20, 2008 *in press*.

4. Logan RM, Gibson, RJ, Bowen, JM, **Stringer, AM**, Sonis, ST, Keefe, DM (2007). Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: implications for the pathobiology of mucositis. *Cancer Chemother Pharmacol* **62**(1):33-41

5. Yeoh AS, Gibson RJ, Yeoh EE, Bowen JM, **Stringer AM**, Giam KA, Keefe DM (2007). A novel animal model to investigate fractionated radiotherapy-induced alimentary mucositis: the role of apoptosis, p53, nuclear factor-kappaB, COX-1, and COX-2, *Mol Cancer Ther* **6**(8):2319-27.

6. Bowen JM, Gibson RJ, Tsykin A, **Stringer AM**, Logan RM and Keefe DMK (2007). Gene expression analysis of multiple gastrointestinal regions following cytotoxic chemotherapy by oligonucleotide microarrays. *Int J Cancer* **121**(8):1847-56.

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9. Logan RM, **Stringer AM**, Bowen JM, Yeoh ASJ, Gibson RJ, Sonis ST and Keefe DMK (2007). The role of pro-inflammatory cytokines in cancer treatment-induced alimentary tract mucositis: Pathobiology, animal models and cytotoxic drugs. *Cancer Treat Rev* doi10.1016/j.ctv.2007.03.001..
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11. Bultzingslowen IV, Brennan MT, Spijkervet FJ, Logan R, **Stringer A**, Raber-Durlacher JE, Keefe D (2006). Growth Factors and Cytokines in the prevention and treatment of oral and gastrointestinal mucositis, *Supp Care Cancer* 14(6):519-27.
12. Yeoh A, Gibson R, Yeoh E, Bowen J, **Stringer A**, Giam K, Logan R, Keefe D (2006). Radiation therapy-induced mucositis: relationships between fractionated radiation, NF-kappaB, COX-1, and COX-2, *Cancer Treat Rev*. **32**(8):645-51.

This thesis is composed of eight chapters: literature review, six distinct research chapters, followed by general discussion. During the course of my candidature, four chapters were published, with a further three under review at various journals. Accordingly, each research chapter is written as a publication complete with introduction, materials and methods, results and discussions. Some minor editing of the chapters has been made to avoid significant repetition and to include relevant data omitted from the publications. Unavoidable repetition has occurred only as necessary due to the format of the papers.

The animal studies were approved by the Animal Ethics Committees of The Institute of Medical and Veterinary Sciences and of The University of Adelaide. They complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2004). Due to the potentially severe nature of the diarrhoea that can be induced by irinotecan, animals were monitored four times daily and if any animal showed certain criteria (as defined by the Animal Ethics Committee) they were euthanised. These criteria included a dull ruffled coat with accompanying dull and sunken eyes, coolness to touch with no spontaneous movement, and a hunched appearance.

The clinical study was approved by the Ethics of Human Research Committee of the Royal Adelaide Hospital and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrolment in the study.

1.0 Literature Review

1.1 Introduction

Mucositis is a major oncological problem, caused by the treatment of malignant disease with chemotherapeutic agents (Mitchell and Schein 1984; Cunningham *et al.* 1985; Keefe *et al.* 1997; Keefe *et al.* 2000; Keefe *et al.* 2004) and radiotherapy (Duncan and Grant 2003; Keefe *et al.* 2004). The entire gastrointestinal tract (GIT) is affected by mucositis, with recipients of radiotherapy, and/or chemotherapy exhibiting symptoms such as mouth pain, nausea, heartburn, ulceration, abdominal pain, bloating, vomiting, diarrhoea and constipation (Keefe *et al.* 1997; Keefe *et al.* 2000; Keefe *et al.* 2004). Major progress has been made in recent years in understanding the mechanisms of oral (Sonis 1998; Sonis 2004; Sonis 2004; Gibson *et al.* 2005) and small intestinal mucositis (Gibson *et al.* 2002; Gibson *et al.* 2003; Keefe *et al.* 2004; Bowen *et al.* 2005), which appears to be more prominent than colonic damage (Keefe *et al.* 2004). The large intestine becomes severely damaged following chemotherapy (Keefe 2004; Keefe *et al.* 2004), although further research into the mechanisms of large intestinal mucositis is still required. Recent studies have proposed that the underlying mechanisms for the development of mucositis should be similar throughout the alimentary tract, as embryologically it is formed from one structure (Sadler 2006). Therefore, research for both oral mucositis and gastrointestinal mucositis should be combined into 'alimentary mucositis' (Keefe 2004).

1.2 Mechanisms of Mucositis

Mucositis was thought originally to develop entirely as the consequence of epithelial injury (Sonis 2004). However, it is now known that the pathobiology is more intricate, and involves a series of steps, each of which entails complex signalling pathways (Sonis 2004). The steps include initiation, occurring promptly following chemotherapy/radiotherapy; primary damage response, initiated by DNA and non-DNA damage and reactive oxygen species (ROS); signal amplification, involving activation of a variety of genes and cytokine amplification of the primary signals; ulceration, resulting in painful lesions prone to bacterial colonisation and additional production of pro-inflammatory cytokines and damaging enzymes; and healing, the resolution once chemotherapy or radiotherapy has ceased (Sonis 2004; Sonis 2004).

Chemotherapy-induced diarrhoea (CID) is a well recognised side effect of cancer treatment (Saltz *et al.* 1996; Wadler *et al.* 1998; Gwede 2003; Viele 2003; Benson *et al.* 2004), and can be accompanied by blood, mucus and pain (Gibson and Keefe 2006). The absolute percentage of patients that have CID as a result of their treatment has yet to be defined, although of the total number of patients undergoing chemotherapy, approximately 20-40% experience severe diarrhoea (Takasuna *et al.* 2006). Very little research has been conducted into the causes of CID with the majority of information available based on clinical observations with very little basic science existing. However, the pathophysiology behind CID is extensive, complex and likely to be the result of a

number of mechanisms (Gwede 2003; Viele 2003). This literature review will examine in detail what is known about the relationship between CID and the gastrointestinal microflora.

The gastrointestinal microflora and mucins are affected by chemotherapy, and may be important factors in determining the severity of chemotherapy-induced diarrhoea. Unfortunately, it is not yet known how these mechanisms fit into the model for alimentary mucositis. A recent study by Yeoh and colleagues has demonstrated that the same genes are activated in the colon as in oral mucositis, suggesting that the whole alimentary tract may have the same underlying mechanisms for mucositis (Yeoh *et al.* 2005).

1.3 The Normal Functioning of the Gastrointestinal Tract

The gastrointestinal tract (GIT) is a hollow tube involved in breaking down food for absorption into the body (Burkitt *et al.* 1993). This occurs in five main phases: ingestion, fragmentation, digestion, absorption and elimination of waste (Burkitt *et al.* 1993). The entire GIT is composed of a lumen of varying diameter, surrounded by a layered structure composed of mucosa, submucosa, muscularis and serosa, and spans from the mouth to the anus (Burkitt *et al.* 1993).

1.3.1 Gastrointestinal Histology

Briefly, the GIT consists of layers; mucosa, submucosa, muscularis and serosa. The mucosa consists of an epithelial lining, a lamina propria with loose connective tissue rich in blood and lymphatic vessels and smooth muscle cells, and a muscularis mucosa layer consisting of a thin inner circular layer and an outer longitudinal layer of smooth muscle cells (Burkitt *et al.* 1993). There are four main types of epithelium present throughout the GIT: barrier, secretory, absorptive and fluid transport (Burkitt *et al.* 1993).

The stomach is composed of four regions; namely the cardia, fundus, body and pylorus, with the mucosa organised into longitudinal folds and the epithelial lining consisting of simple columnar cells, secreting mucous, acid and enzymes (Burkitt *et al.* 1993). The mucosa invaginates the lamina propria to form gastric pits, and branched tubular glands (gastric glands) open onto the luminal surface at these pits (Burkitt *et al.* 1993). The small intestine consists of three regions: duodenum, jejunum and ileum; and is the site of terminal food digestion, nutrient absorption and endocrine secretion (Burkitt *et al.* 1993). Digestion is the main function of the small intestine, the products of which are absorbed. The mucosa of the small intestine is arranged in villous architecture; the epithelium (spanning approximately 200m^2) consists of stem cells, absorptive cells, goblet cells, Paneth's cells and enteroendocrine cells. The surface design allows large quantities of fluid to pass bidirectionally daily, as well as the regulated transport of ions and nutrients (Burkitt *et al.* 1993; Neish 2002). The large intestine has the main functions of water

recovery from the contents of the small intestine, and the expulsion of faeces to the rectum prior to defaecation (Burkitt *et al*. 1993). Large intestinal mucosa consists of long intestinal glands (crypts). The crypts contain goblet cells, absorptive cells and a small number of enteroendocrine cells (Burkitt *et al*. 1993).

1.3.2 Cell Renewal

The epithelial layer of the GIT is a rapidly renewing tissue with a high cell turnover rate. Stem cells (specialised proliferating cells) located in the proliferative zones of crypts and glands divide, with the resulting daughter cells moving up the crypts (and onto the villi in the small intestine) (Potten *et al.* 2003). Growth and mitotic activity in adjacent crypts generates a force to drive undifferentiated cells upward along the villus walls (Westcarr *et al.* 1999). Eventually, the cells are sloughed off into the lumen. This rapid turnover of cells explains why the GIT is particularly susceptible to the effects of cytotoxic agents (Booth and Potten 2000). The DNA of these cells undergoes strand breaks (similar to the tumour target), resulting in direct cellular injury (Sonis 2004). The stem cells are capable of producing each cell lineage present in the GIT (Booth and Potten 2000).

The proliferative zone of the stomach is located in the neck of the glands and the adjacent portion of the pits. Proliferation in the small intestine is limited to the crypts, and in the large intestine is in the basal two-thirds of the crypts (Eastwood 1977). The rates of cell production and loss balance exactly and must be under extremely stringent controls

(Potten *et al.* 1997). Normal crypts contain 4-6 stem cells, and normal replacement of the epithelial lining takes 5-6 days in humans, and 2-3 days in rodent models (Westcarr *et al.* 1999). In situations of decreased proliferation or increased cell death, gastrointestinal integrity is lost (Potten *et al.* 1997).

Normal intestinal function is a balance between oral intake, secretions into the GIT, fluid reabsorption and metabolism (Gibson and Keefe 2006). The primary function of the stomach is to serve as a reservoir for meals, presenting food to the duodenum in small regulated amounts (Burkitt *et al.* 1993). The small intestinal epithelium has a brush border of enzymes on the luminal surface for metabolising ingesta and leading to absorption (Figure 1.1). Remaining fluid passes to the large intestine to be reabsorbed. The primary function of the large intestine is to compensate for large increases in fluid load by reabsorption (Gibson and Keefe 2006). Water reabsorption is a highly regulated process, with electrolytes and solutes playing a key role. Sodium is absorbed by epithelial cells, with chloride following due to the electrochemical gradient. An osmotic gradient is created, allowing water to follow the sodium and chloride into the cell. Normal bowel openings occur between three times daily and once every three days. Diarrhoea is associated with increased frequency and decreased consistency of bowel motions. Mucositis affects the absorptive capacity of the small and large intestines, resulting in increased solutes in the lumen. An increase in solutes in the intestinal lumen results in the

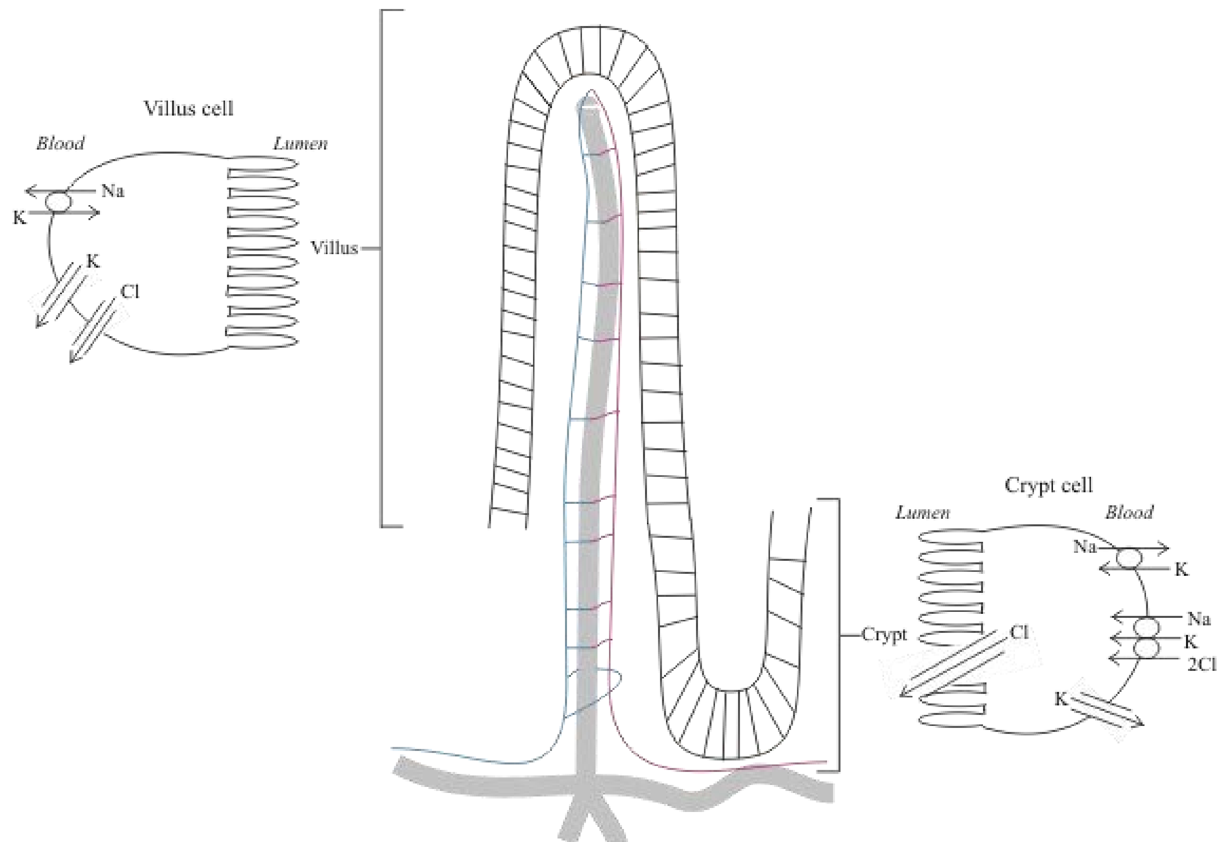


Figure 1.1 Fluid transport in the small intestine (Stringer *et al.* 2007).

osmotic movement of water into the lumen, resulting in diarrhoea (Gibson and Keefe 2006) (Figure 1.2).

1.4 Normal Gastrointestinal Flora

The gastrointestinal epithelium functions while in physical contact with a diverse ecosystem of prokaryotes. The largest, most diverse and most clinically relevant relationship in the gastrointestinal tract is that between eukaryotic and prokaryotic cells (Neish 2002). The biological systems involved in the intestines are exquisitely sensitive to the organisms and their products. The breakdown in the eukaryotic/prokaryotic relationship can be responsible for the manifestation of disease (Neish 2002).

The microflora of the GIT is a highly complex ecosystem consisting of both aerobic and anaerobic bacteria (Simon and Gorbach 1982) (Table 1.1, Figure 1.3). Intestinal flora varies between races, sex, age and diet within humans (Heller and Duchmann 2003). Furthermore, animal species used in experiments, such as rats, have their own unique flora (Simon and Gorbach 1986), making direct correlations between animal findings and human studies difficult. The normal microflora also differs between the stomach, small intestine and large intestine, both in organisms present and numbers of organisms (Gorbach 1971). The gastrointestinal microflora has a number of key functions including: protection, and metabolism of bilirubin, intestinal mucins, pancreatic enzymes, fatty acids,

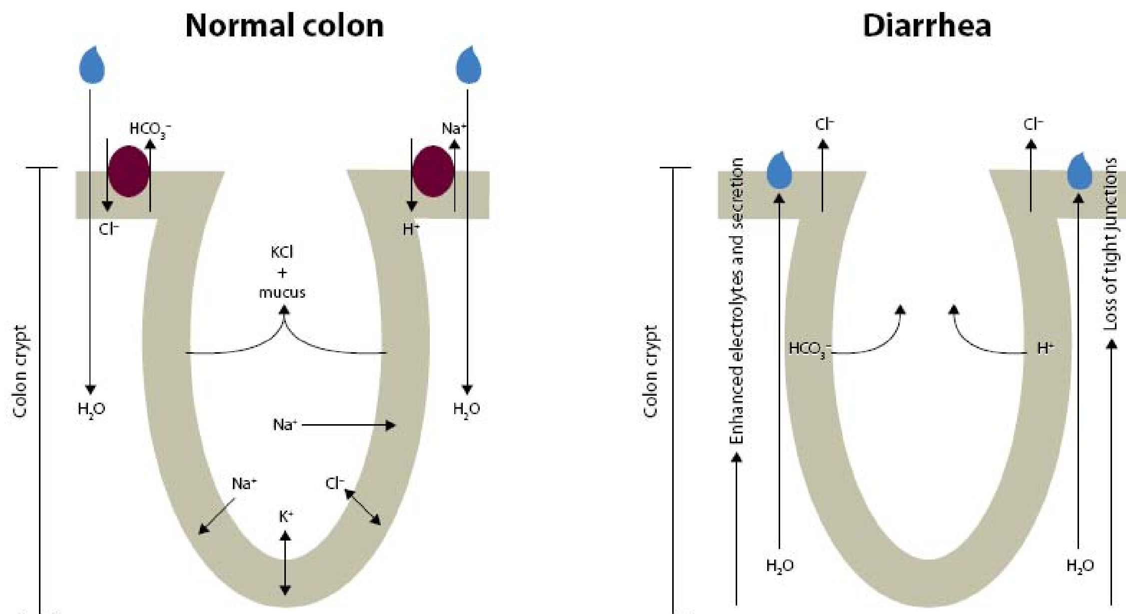


Figure 1.2 Fluid transport in the large intestine in the normal colon (left) and colon altered by diarrhoea (right) (Stringer et al. 2007).

Table 1.1: Composition of Intestinal Microflora

Organisms	Stomach	Small Intestine	Large Intestine	Faeces
Gram Positive Cocci	++	++	++	++
Aerobic	++	++	++	++
Anaerobic	+	+	+++	+++
Enterobacteriaceae	+	+	+++	+++

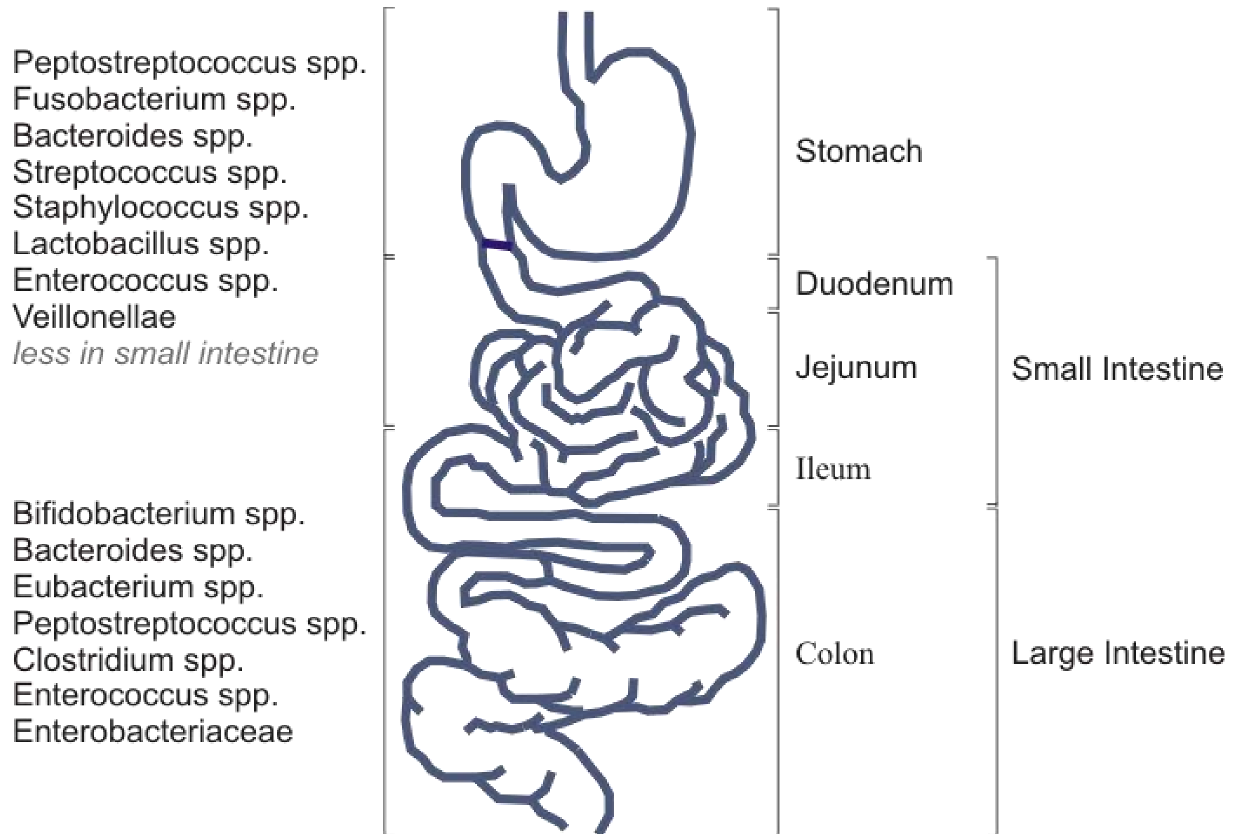


Figure 1.3 Human gastrointestinal tract showing distribution of microorganisms.

bile acids, cholesterol and steroid hormones (Umesaki *et al.* 1997). Other functions of gastrointestinal bacteria include nutrient processing, regulation of intestinal angiogenesis, and immune functions (Umesaki and Setoyama 2000). *Bifidobacterium spp.* is known to colonise the gastrointestinal tract soon after birth (McCartney *et al.* 1996), and is also known to play a pivotal role in maintaining the microbial balance in the healthy gastrointestinal tract (Masco *et al.* 2006). Alteration of the normal ecosystem can lead to either an overgrowth ofsome species of bacteria increasing the risk of infection, or a depletion of some species, with a subsequent loss of bacterial functions in the gastrointestinal tract (Neish 2002).

1.4.1 Stomach Microflora

The stomach microflora consists predominantly of microorganisms from the oral cavity which are washed down into the stomach with saliva and food particles. The bacterial populations of the stomach are regulated by gastric acidity, with numbers and complexity increasing as the pH increases (Simon and Gorbach 1984) leaving the organisms which are acid resistant. Oral anaerobes (*Peptostreptococcus spp.*, *Fusobacterium spp.*, and *Bacteroides spp.*) and *Streptococcus spp.*, *Staphylococcus spp.*, *Lactobacillus spp.*, Veillonellae, *Actinomyces spp.* and a variety of fungi are the main species' found in the stomach (Simon and Gorbach 1984). Coliforms, *Clostridium spp.* and *Bacteroides fragilis* are less common in the stomach (Simon and Gorbach 1986).

1.4.2 Small Intestinal Microflora

The small intestine is a transition zone between the stomach and colon, and contains far fewer organisms than the stomach or large intestine. The microflora found within the duodenum and jejunum are qualitatively similar to those of the stomach. Normal peristaltic motion and the high rate of motility are major host defences against bacterial overgrowth in the small intestine (Simon and Gorbach 1986). The ileum shows an increase in coliforms, *Bacteroides spp.*, *Bifidobacterium spp.*, *Fusobacterium spp.* and *Clostridium spp.*, with gram negative bacteria outnumbering gram positive (Simon and Gorbach 1982).

1.4.3 Large Intestinal Microflora

The number of anaerobes dramatically increases distal to the ileocaecal valve. Colonic flora consists of over 400 different species of bacteria (Simon and Gorbach 1986). These include large numbers of anaerobes (*Bifidobacterium spp.*, *Bacteroides spp.*, *Eubacterium spp.* (most common), *Peptotreptococcus spp.* and *Clostridium spp.*), enterococci and Enterobacteriaeceae (Simon and Gorbach 1984; Simon and Gorbach 1986)

1.4.4 Faecal Microflora

The faecal dry weight is approximately 33% bacteria (Simon and Gorbach 1982). Few studies have elucidated exactly which organisms are present in the faecal microflora. A

recent study by Stringer and colleagues determined the faecal microflora of the rat by culture methods. This study found the faeces to have a greater number of bacteria than was seen in the colon, jejunum or stomach. The most prominent cultured bacteria were Enterobacteriaeceae, and included *Lactobacillus spp.*, *Enterococcus spp.*, *Serratia spp.*, *Proteus spp.*, and *Staphylococcus spp.*). Anaerobes were present, but not in large numbers (Stringer *et al.* 2005). Another study by Matsuki et al showed human faeces to contain large amounts of *Clostridium spp.*, *Bacteroides spp.*, and *Bifidobacterium spp. (Matsuki et al. 2004).*

1.4.5 Regulation of Normal Microflora

Microbial interactions (particularly in the colon) are important in defining the microflora. There are several mechanisms where bacteria interact with each other to promote or prevent the growth of other bacteria (Simon and Gorbach 1984). These mechanisms include depleting substrate materials necessary for another species' growth (for example, coliforms compete for carbon), creating an environment to stimulate or inhibit growth (for example, facultative bacteria use available oxygen, maintaining a reduced environment, allowing the growth of strict anaerobes), and the by-products of bacterial metabolism (for example, short chain fatty acids inhibit bacterial proliferation) (Simon and Gorbach 1986).

1.5 Goblet cells

Goblet cells are unique highly polarised exocrine cells that synthesise and secrete mucins (Kurosumi *et al.* 1981; Specian and Oliver 1991; Robbe *et al.* 2004; Perez-Vilar *et al.* 2006), and are found in varying numbers throughout the lower GIT (Corfield *et al.* 2000). These cells arise during mitosis from multipotential stem cells at the base of crypts in the intestines, or from poorly differentiated cells in the lower crypt, known as oligomucous cells (Specian and Oliver 1991). Once propagated, goblet cells migrate (in between normal epithelial cells) from the base of the crypt to the villus tip (small intestine), where they are sloughed into the lumen. In the small intestine, goblet cell distribution in crypts and villi is consistent from the duodenum to the ileum. In the large intestine, the number of goblet cells increases from the caecum to the rectum (Specian and Oliver 1991). Goblet cells are not present in the stomach; instead, epithelial mucus cells in the neck of glands and surface of the stomach secrete mucus (Sheahan and Jervis 1976; Zarebska *et al.* 2004).

Immature goblet cells have a wide base. Mucin granules (and the organelles required for their synthesis) are found in the apical region of the cell (Specian and Oliver 1991). When the goblet cells have migrated to the mouth of the crypt, their shape tends to be the characteristic 'cup'. The apical region is distended and is packed with mucin granules. The basal region of the cells is narrow and contains the organelles and nucleus (Specian and Oliver 1991) (Figure 1.4). Goblet cell function has been shown to be dependent on a

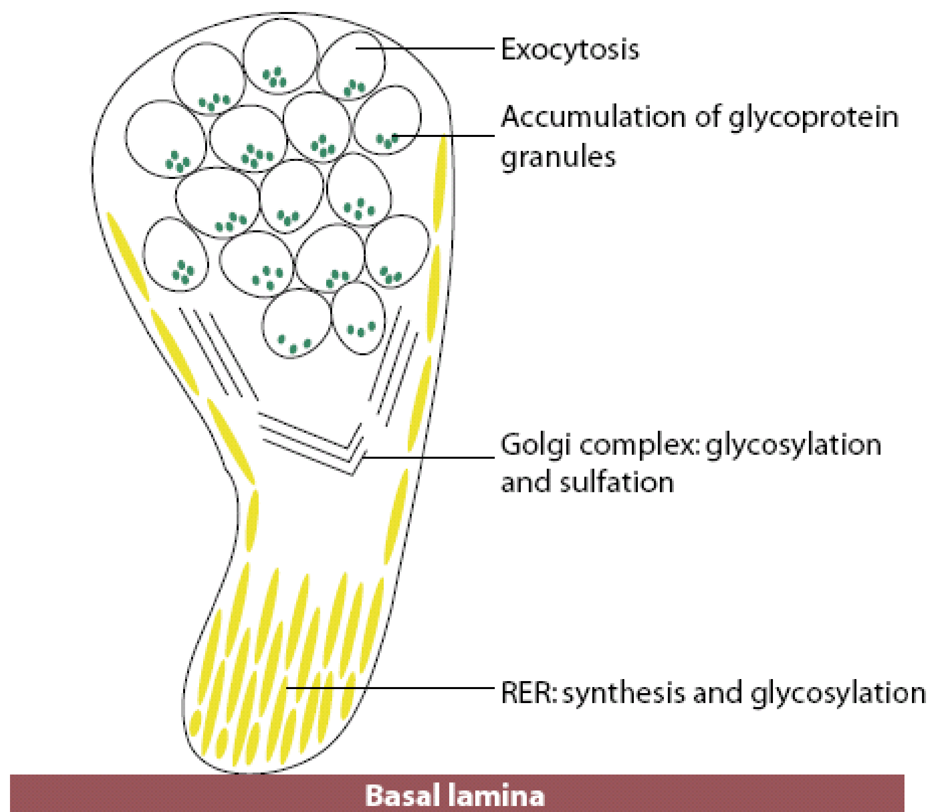


Figure 1.4 Structure of a goblet cell (RER = rough endoplasmic reticulum) (Stringer et al. 2007).

number of factors. Leptin (secreted in gastric juice before entering intestine) has been shown to increase mucus secretion, suggesting goblet cell function requires a presence of active leptin in the intestine (Plaisancie *et al.* 2006). Acetyl choline (ACh), vasoactive intestinal peptide (VIP), and neurotensin have also been shown to stimulate mucus secretion (Laboisse *et al.* 1996).

1.6 Mucins

Intestinal mucins are high molecular weight glycoproteins secreted by goblet cells (Specian and Oliver 1991), and consist of either linear or branched oligosaccharide chains (of varying length and degree of branching) attached via covalent linkage to a protein core (Specian and Oliver 1991; Jass and Walsh 2001). The biosynthesis of mucins involves the addition of monosaccharides by specific glycosyl transferases in the endoplasmic reticulum of goblet cells, before being further processed in the Golgi apparatus (Jass and Walsh 2001; Perez-Vilar and Mabolo 2007). The saccharides found in mucins include hexosamines (N-acetyl-D-galactosamine and N-acetyl-D-glucosamine), hexose (D-galactose), 6-deoxyhexose-L-fucose and neuraminic (sialic) acid derivatives. Sialic acid is limited to secretory mucins of the small and large intestine, and differs from the other sugars contributing to the oligosaccharide chain as it has an acidic carboxy group and a three

carbon sidearm (existing in a number of different forms). The sulphation of certain sugars contributes to the mucin's acidity (Jass and Walsh 2001).

Mucins are stored in granules until the proper regulatory signals trigger exocytosis (Perez-Vilar and Mabolo 2007). There is repeated debate as to the exact mechanisms behind mucus extrusion (Kurosumi *et al.* 1981). The baseline secretion of mucins consists of the periodic exocytosis of mucin granules, effecting the slow continual release of mucins, maintaining a mucous blanket (Specian and Oliver 1991). It is also thought that apocrine secretion may be involved in this process (Kurosumi et al. 1981). Once secreted, mucins hydrate and gel in the lumen and generate a protective mucous barrier overlying the epithelial surface, which protects the epithelium from mechanical and chemical stress (Robbe et al. 2004) and allows transport between the luminal contents and epithelium. Other components reside within the mucous layer, including water, electrolytes, sloughed epithelial cells and secreted immunoglobulins (Specian and Oliver 1991).

The structure of mucins allows the maintenance of the normal intestinal flora, by providing attachment sites for intestinal flora and pathogenic bacteria (Robbe *et al.* 2004), and simultaneously protecting the mucosa from bacterial overgrowth and/or penetration (Specian and Oliver 1991). The mucins also protect the epithelium from digestion by acting as substrates for the enzymes produced by the intestinal flora, such as α-galactosidase, β-N-acetylgalactosaminidase, sialidase, β-glucuronidase, blood group degrading enzymes and proteases (Specian and Oliver 1991). Sulphated mucins appear to

be less degradable by bacterial glycosidases and host proteases (Deplancke and Gaskins 2001). The metabolism of mucin may serve to regulate the microecology of the intestinal luminal environment (Specian and Oliver 1991). Acidic mucins have been suggested to protect against the translocation of bacteria (Deplancke and Gaskins 2001).

Different mucin types are produced within the goblet cells, and not all mucin types are secreted equally (Specian and Oliver 1991). Goblet cells rapidly discharge most or all of their stored intracellular mucin in response to a variety of stimuli, including cholinergic stimuli, intestinal anaphylaxis, and chemical or physical irritation (Specian and Oliver 1991). The differential secretion of mucins from surface epithelium and glands may provide a mechanism for the modulation of the protective mucous layer in response to acid secretion or presence of bacteria or noxious agents in the lumen (Robbe *et al.* 2004). Interleukin (IL)-1, IL-4, IL-6, IL-9 and tumour necrosis factor (TNF)-α have been shown to stimulate the rapid release of mucin from cells (Deplancke and Gaskins 2001). Other bioactive factors from intestinal bacteria or host immune cells may affect the goblet cell dynamics and mucous layer (Deplancke and Gaskins 2001).

1.7 Mucin Genes

Mucin genes (MUC1-17) are regulated by cytokines, bacterial products and growth factors (Smirnov *et al.* 2004). The biosynthesis of mucins is affected by conditions or agents affecting the differentiation of precursor cells into goblet cells, and those that uncouple the

process of protein synthesis (fasting or malnutrition) (Smirnov *et al.* 2004). The MUC gene family contains members which are dissimilar. There are two distinct subfamilies, both structurally and functionally: secreted gel-forming mucins and transmembrane mucins. Some of the products of MUC genes do not fit well into either subfamily, and some organs synthesise more than one type of mucin (Byrd and Bresalier 2004). Table 1.2 shows a summary of the structure, expression and chromosomal location of the MUC genes.

There are four genes encoding the secreted gel-forming mucins, MUC6, MUC2, MUC5AC and MUC5B, all of which are located respectively on chromosome 11. The genes encoding the transmembrane mucins are located on chromosomes 1 (MUC1), 3 (MUC4) and 7 (MUC3A, 3B, 11, 12, 17). MUC7, 8, 9, 13, 15 and 16 remain unclassified to date (Byrd and Bresalier 2004). MUC2 and MUC5AC have been of interest due to their altered expression in colon carcinomas (Allen *et al.* 1998). MUC2 is a major product of goblet cells in the colon and small intestine, but MUC5B and MUC6 are not largely expressed in the normal colon (Sylvester *et al.* 2001; Byrd and Bresalier 2004). Of the transmembrane mucins, MUC4 is expressed in normal stomach and colon (Choudhury *et al.* 2000), MUC3 is present in the columnar epithelial cells of the small and large intestine (rather that goblet cells) (Pratt *et al.* 2000), MUC17 is expressed in the duodenum and colon (Gum *et al.* 2002), and MUC11 and 12 are expressed in the colon, and down-regulated in colorectal cancers (Williams *et al.* 1999; Byrd and Bresalier 2004). Of the unclassified mucins, MUC13 is expressed highly in the columnar and goblet cells in the GIT

Table 1.2. Summary of MUC genes

MUC gene	Transmembrane/Secreted	Region of Expression	Chromosome
MUC1	Transmembrane	Whole GIT	1
MUC2	Secreted	Small intestine, colon	11
MUC3A/3B	Transmembrane	Small intestine, colon (epithelium)	7
MUC4	Transmembrane	Stomach, colon	3
MUC5AC	Secreted	Stomach, colon	11
MUC5B	Secreted	Stomach	11
MUC6	Secreted	Stomach	11
MUC7	Unclassified	Unknown	Unknown
MUC8	Unclassified	Unknown	Unknown
MUC9	Unclassified	Unknown	Unknown
MUC11	Transmembrane	Colon	7
MUC12	Transmembrane	Colon	7
MUC13	Unclassified	Whole GIT	Unknown
MUC15	Unclassified	Unknown	Unknown
MUC16	Unclassified	Unknown	Unknown
MUC17	Transmembrane	Duodenum, colon	7

(Williams *et al.* 2001). Mucus offers a number of ecologic advantages to intestinal bacteria. Bacteria are able to degrade mucus, which provides a direct source of carbohydrates and peptides, and exogenous nutrients (vitamins and minerals) (Deplancke and Gaskins 2001). The mucus also contains receptors recognising specific adhesion proteins (Gusils *et al.* 2004). Bacteria colonising the mucus can also avoid rapid expulsion through the intestine, imparting a growth advantage (Deplancke and Gaskins 2001). It is not known whether goblet cell numbers or mucus secretion is increased in response to bacterial mucolysis (Deplancke and Gaskins 2001).

1.8 Chemotherapy and Gastrointestinal Mucositis

Cytotoxic chemotherapy agents cause functional and structural changes to the GIT (Cunningham *et al.* 1985). Common gastrointestinal symptoms following chemotherapy include heartburn, abdominal pain, diarrhoea, bloating and nausea (Keefe *et al.* 2004). These symptoms arise as the result of the damage caused by chemotherapy agents (Duncan and Grant 2003). Abdominal pain is caused by the extensive damage occurring in the abdominal region. Diarrhoea and constipation are thought to be caused by the alteration in absorptive functions of cells, goblet cell and mucin distribution and composition, and bacterial interactions with these cells and metabolites of the drugs themselves (Ikuno *et al.* 1995; Takasuna *et al.* 1996). Cytotoxic drugs are known to act by inducing apoptosis in cancer, unfortunately apoptosis is also induced in the GIT (Keefe *et al.* 2000; Gibson *et al.* 2002). There are limited ways the mucosa and underlying layers of the GIT can respond to

damage. These are the same ways that chemotherapy causes damage to the GIT, including cell death, which leads to villous atrophy and crypt ablation in the small intestine, and crypt ablation in the large intestine (Keefe *et al.* 2004).

1.8.1 Stomach

Very little research has been conducted into chemotherapy-induced mucositis in the stomach. The reasons behind this are unclear, but are likely to be due to the difficulties in accessing the stomach. Despite this, it is known that this organ is affected by chemotherapy, with nausea and vomiting key side effects. Chemotherapy-induced gastric injury has been observed following treatment with Cyclophosphamide, Methotrexate and 5-Fluorouracil (Sartori *et al.* 2000). However, how cells respond following chemotherapy is unknown.

1.8.2 Small intestine

A significant amount of research has been conducted examining the response of the small intestine, in particular the jejunum, to cytotoxic chemotherapy. Cytotoxic agents act at different levels of the small intestinal crypt cell hierarchy (Ijiri and Potten 1983; Ijiri and Potten 1987). Despite the different sites of action, the end result is still the same: crypt hypoplasia, followed by crypt hyperplasia, then restoration of normal tissue again (Keefe *et al.* 2000; Gibson *et al.* 2003; Keefe *et al.* 2004). The epithelial stem cells in the small intestine become damaged and no longer divide or differentiate into specific cell lineages

following chemotherapy (Inomata *et al.* 2002; Gibson *et al.* 2003). Cell renewal is affected by this and the villous mucosa is not replaced, leading to a rapid loss of structure and function (Duncan and Grant 2003). Small intestinal weight is often decreased due to loss of structure after chemotherapy (Gibson *et al.* 2003), and villus and crypt hypoplasia is often evident (Keefe *et al.* 2000; Gibson *et al.* 2003). Pro- apoptotic members of the Bcl-2 family (Bak and Bax) have been shown to be increased in the small intestine after chemotherapy in rats (Bowen *et al.* 2005). Conversely, goblet cell numbers in the small intestine do not seem to alter after chemotherapy (Gibson *et al.* 2003).

1.8.3 Large intestine

Chemotherapy causes changes to the large intestine, although the research has been less extensive than the small intestine. Irinotecan is known to have a detrimental effect on the colon, where crypt cells undergo increased apoptosis, causing crypt hypoplasia (Keefe *et al.* 2004). The lamina propria becomes infiltrated with mononuclear cells and an increased number of eosinophils. The glandular and surface epithelium loses its columnar morphology and becomes cuboidal (Verburg *et al.* 2000). Goblet cells in the colon have been shown to exhibit a more diverse staining pattern after chemotherapy, with increased mucous production. Goblet cells decrease at the same time as severe architectural changes occur, and mucous secretion increases (Gibson *et al.* 2003).

1.9 Chemotherapy-Induced Diarrhoea

Chemotherapy-induced diarrhoea (CID) and constipation are well recognised side effects of cancer treatment (Saltz *et al.* 1996; Engelking *et al.* 1998; Wadler *et al.* 1998; Gwede 2003; Viele 2003; Benson *et al.* 2004), and can be accompanied with blood, mucous and pain (Gibson and Keefe 2006). Unfortunately, there has been very little research conducted in the underlying mechanisms. Of the total number of patients undergoing chemotherapy, approximately 20-40% experience severe diarrhoea (Takasuna *et al.* 2006). The numbers could be higher, but hidden, due to the awkwardness of discussing the subject. Most literature is based on clinical observation only, with very few scientific-based studies being conducted. The pathophysiology of CID has been recognised as being extensive, complex and regulated by a number of mechanisms (Gwede 2003; Viele 2003) which are yet to be understood. The pathophysiology of constipation is unclear, but is likely to be equally complex.

The chemotherapeutic agents known to cause diarrhoea are 5-Fluorouracil, Irinotecan, Methotrexate, taxanes, monoclonal antibodies and hormonal agents, with vinca alkaloids, platinums, Thalidomide and hormonal agents known to cause constipation (Gibson and Keefe 2006). CID is likely to be multifactorial and may be caused by combinations of varying factors (Table 1.3). The factors involved are likely to include altered gut motility, which leads to reduced transit time through the intestine and less water absorption; colonic crypt damage, the site of water absorption in the colon; changes to microflora, also

affecting absorption and other intestinal functions involving the microflora; and altered fluid transport in the colon (Gibson and Keefe 2006). There is increasing evidence to suggest diarrhoea may also be caused by other influences (Read 1993). These may be related to enteric nerve reflexes, and involve luminal stimuli (bile acids, bacterial products, mucosal inflammation) that acts via epithelial receptors to induce both secretion and propulsive motor activity in the intestine (Read 1993).

1.10 Electrolytes

Fluid transport in the colon is critical to the regulation of intestinal fluid and electrolyte balance, and is a final determinant to the development of diarrhoea. Altered gut motility after chemotherapy may lead to reduced transit time through the intestine, and less electrolyte and water absorption in the colon. Chemotherapy also damages the colonic crypts, the site of water absorption in the colon (Gibson and Keefe 2006). A study by Cunningham and colleagues showed variability in patient water and electrolyte absorption after treatment with cyclophosphamide, methotrexate and 5-FU (Cunningham et al. 1985). In general, sodium absorption levels decreased, chloride levels decreased and water absorption decreased, although no changes were deemed significant (Cunningham et al. 1985). If the colonic crypts are damaged, then water and electrolyte absorption can be reduced.

Table 1.3: Mechanisms of Diarrhoea

Diarrhoea Type	Mechanism for Diarrhoea	Potentially Caused by Chemotherapy
Decreased surface area of small bowel and colon (secretory)	↑ Secretion of electrolytes ↓ Absorption of electrolytes ↓ Absorption surface	Yes: early with reduced small bowel surface (around Day 2)
Increased motility (osmotic + secretory)	↓ Contact time	Unlikely
Decreased enzyme activity (osmotic)	Non-absorbable intraluminal molecules	Yes: if transient lactase deficiency
Increased mucous secretions (exudative)	Impaired colonic absorption Outpouring of cells + colloid	Yes: if infection Also with Irinotecan
Increased infective agents (infectious)	Can be any of these	Yes.

1.11 Mucin alteration

The number and appearance of goblet cells has been shown to not alter after irinotecan administration in the small intestine of the DA rat (Gibson *et al.* 2003). However, the same study showed that goblet cells in the large intestine exhibit a more diverse mucin composition after treatment with irinotecan. Mucus production is increased, despite a decrease in goblet cell numbers, which may play a role in the development of CID (Gibson *et al.* 2003). Treatment with irinotecan has also been shown to cause goblet cell hyperplasia with mucous hypersecretion in the caecum, considered to be a main cause of diarrhoea (Ikuno *et al.* 1995). Methotrexate has been shown to induce goblet cell depletion in the small intestine (Verburg *et al.* 2000). The same study also showed Muc-2 expression to be decreased during villous atrophy. A recent study of mucins and goblet cells in colitis suggests that the goblet cells and mucins may be regulated by interactions of specific bacterial peptides with the gastrointestinal mucosa. Bacterial peptides were shown to cause interleukin (IL)-8 and mucin secretion by colon epithelial cell lines (Leiper *et al.* 2001). The results from this study suggest a strong link between bacteria (intestinal flora) and mucin secretion, which have both been shown to be affected in chemotherapy-induced mucositis (Farrell *et al.* 1998; Meslin *et al.* 1999; Gibson *et al.* 2003; Stringer *et al.* 2006).

1.12 5-Fluorouracil

5-Fluorouracil (5-FU) is one of the most commonly used chemotherapy agents in clinical oncology practice (Fata *et al.* 1999). 5-FU is a fluorinated pyrimidine, and has several sites of action (Milles *et al.* 1962). The structure of 5-FU is an analogue of uracil, with a fluorine atom at the C-5 position instead of a hydrogen atom. The primary mechanism of action is to inhibit thymidylate synthetase (TS), an important enzyme in nucleotide metabolism as its inhibition cannot be avoided by an alternative pathway (Noordhuis *et al.* 2004). TS is the only way a methyl group can be added to the 5-position of the pyrimidine ring in the synthesis of thymidine (Noordhuis *et al.* 2004).

5-FU is converted to three main active metabolites (Figure 1.6): fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) (Longley *et al.* 2003; Noordhuis *et al.* 2004). The main activation of 5-FU is by conversion to fluorouridine monophosphate (FUMP), either directly by orotate phosphoribosyltransferase (OPRT) with phosphoribosyl pyrophosphate (PRPP) as the cofactor, or indirectly via fluorouridine (FUR) through the actions of uridine phosphorylase (UP) and uridine kinase (UK), respectively. FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which is either further phosphorylated to the active metabolite fluorouridine triphosphate (FUTP), or converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). Following this, FdUDP is either

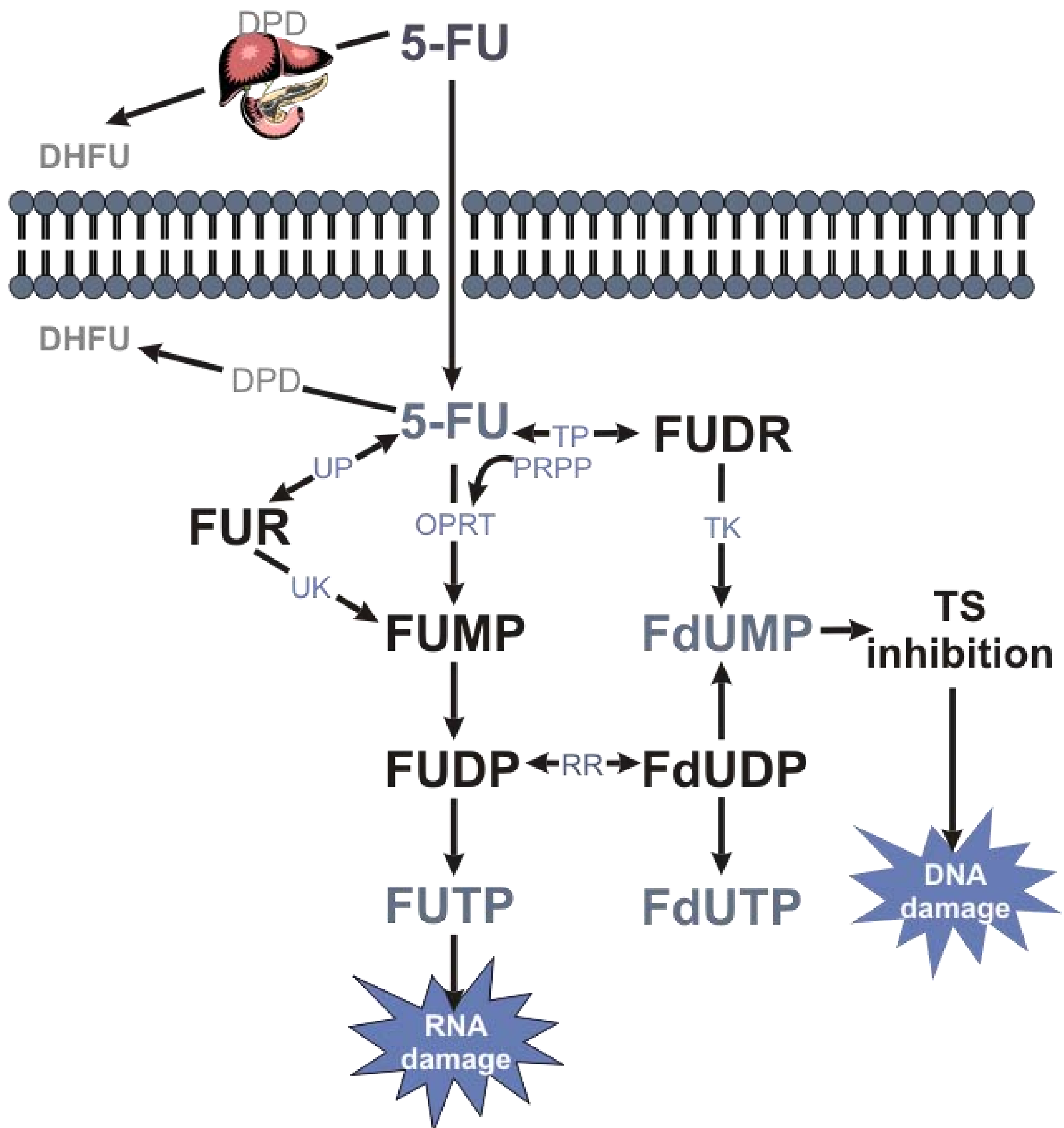


Figure 1.6 5-FU metabolism. 5-FU is converted to the 3 active metabolites FUTP, FdUTP and FdUMP. Abbreviations: FdUMP- fluorodeoxyuridine monophosphate; FdUTP - fluorodeoxyuridine triphosphate; FUTP - fluorouridine triphosphate; FUMP - fluorouridine monophosphate; OPRT - orotate phosphoribosyltransferase; PRPP - phosphoribosyl pyrophosphate; FUR – fluorouridine; UP - uridine phosphorylase; UK - uridine kinase; FUDP - fluorouridine diphosphate; FUTP - fluorouridine triphosphate; FdUDP - fluorodeoxyuridine diphosphate; RR - ribonucleotide reductase; FUDR - fluorodeoxyuridine; TK - thymidine kinase; DPD - Dihydropyrimidine dehydrogenase.

phosphorylated to the active metabolite FdUTP, or dephosphorylated to the active metabolite FdUMP (Longley *et al.* 2003; Noordhuis *et al.* 2004). An alternative activation pathway involves the catalytic action of thymidine phosphorylase to convert 5-FU to fluorodeoxyuridine (FUDR), which is then phosphorylated by thymidine kinase (TK) toFdUMP. Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil (DHFU) is the rate-limiting step of 5-FU catabolism in normal and tumour cells. Up to 80% of administered 5-FU is broken down by DPD in the liver (Longley *et al.* 2003; Noordhuis *et al.* 2004).

1.13 Irinotecan

Irinotecan hydrochloride (CPT-11) is a relatively new cytotoxic agent used to treat a variety of solid tumours. The primary mechanism of action is to inhibit DNA topoisomerase I (Araki *et al.* 1993; Ikuno *et al.* 1995; Takasuna *et al.* 1996). DNA damage is induced by trapping topoisomerase I during its normal action in regulating DNA structure (Alimonti *et al.* 2004). During DNA replication, topoisomerase I produces reversible single-strand (ss) breaks by cutting and reattaching double chain DNA. These breaks relieve the torsional strain generated by the advancing replication forks (Alimonti *et al.* 2004). Irinotecan causes severe diarrhoea (a dose-limiting side effect) in 60-80% of patients (Fittkau *et al.* 2004). Cholinergic, secretory diarrhoea occurs early, and can be prevented by the administration of atropine prior to irinotecan. A delayed diarrhoea also occurs, compounded by the action of β-glucuronidase making the irinotecan toxicity

worse (Gibson *et al.* 2003). Leukopaenia is another dose-limiting side effect of irinotecan, compromising the patient and resulting in opportunistic infection (Ikuno *et al.* 1995; Takasuna *et al.* 1996).

Irinotecan is converted by hepatic or gastrointestinal carboxylesterases to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), which is responsible for irreversible DNA damage (Takasuna *et al.* 1996; Alimonti *et al.* 2004). SN-38 has stronger antitumour activity (Takasuna *et al.* 1996), but is further processed by glucuronyltransferase to become SN-38 glucuronide (SN-38G), a less toxic form of SN-38 (Takasuna *et al.* 1996). However, SN-38G is transported to the intestine during bile excretion, and is able to be hydrolysed by β-glucuronidase to return to the toxic form, SN-38 (Takasuna *et al.* 1996) (Figure 1.5). CPT-11 and SN-38 bind to the topoisomerase I-DNA complex, preventing relegation of the DNA strand. This leads to double-strand (ds) DNA breakage and cell death. A number of the intestinal microflora have β-glucuronidase activity and may be responsible in part for the intestinal cytotoxicity of irinotecan (Takasuna *et al.* 1996). Bacterial β-glucuronidase is produced primarily by Enterobacteriaeceae (*E. coli*, *Salmonella spp.*, *Shigella spp.*, *Yersinia spp.*, *Citrobacter spp.*, *Hafnia spp.* and *Edwardia spp.*), and has been reported to be produced by *Flavobacterium spp.*, *Bacteroides spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Corynebacterium spp.* and *Clostridium spp.* (Tryland and Fiksdal 1998). This is supported by a recent study by Brandi and colleagues, where germ-free mice were shown to be more resistant to irinotecan than conventional mice (bearing their own microflora), with a reduction in intestinal damage and diarrhoea (Brandi *et al.* 2006).

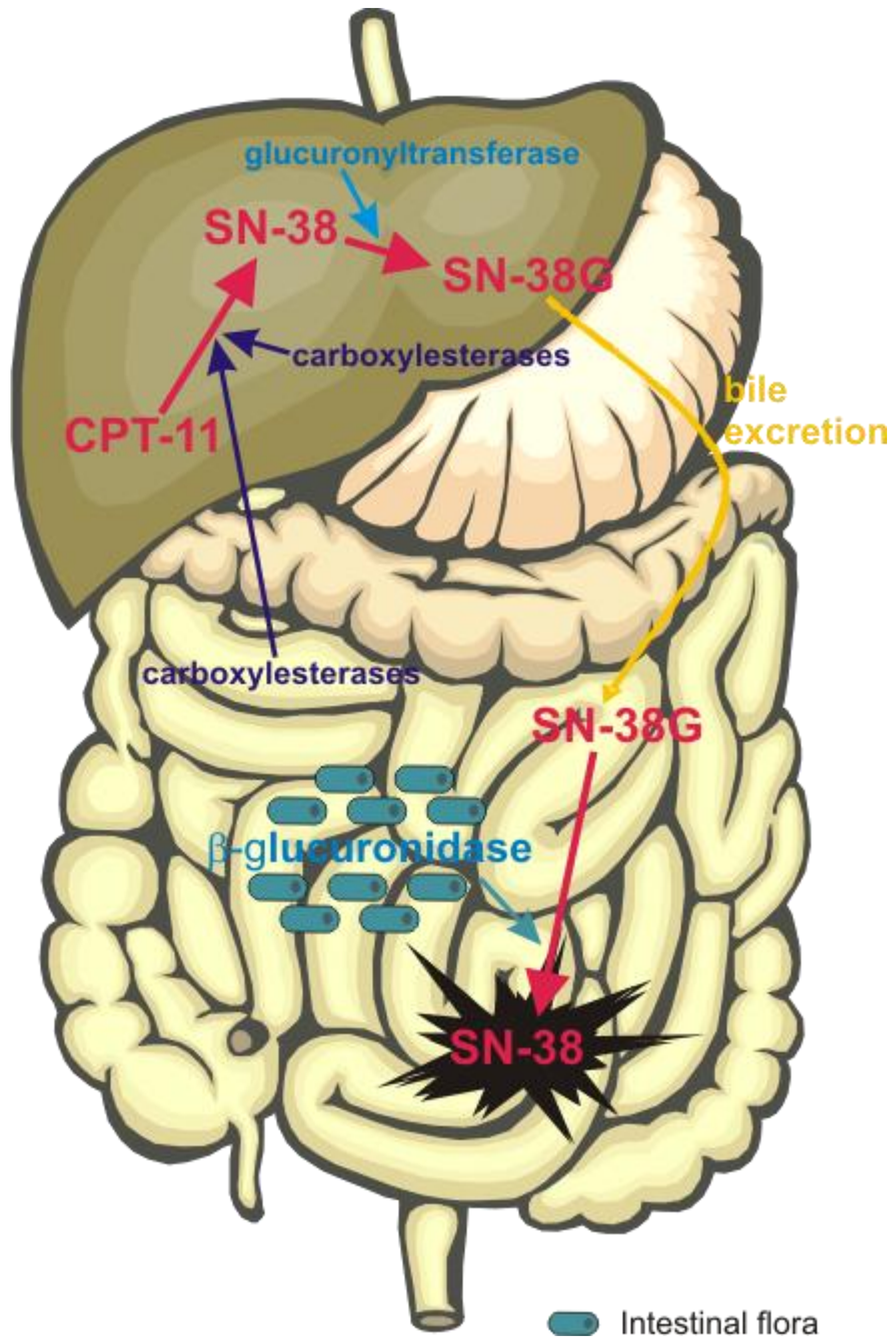


Figure 1.5 Irinotecan metabolism

There is little knowledge of the precise mechanism of induction of diarrhoea by CPT-11, although SN-38 has been implicated in the onset of diarrhoea (Takasuna *et al.* 1996). The small intestine has been shown to contain SN-38G as the constant primary metabolite of CPT-11 from 1-24h (Takasuna *et al.* 1996). However, the same study showed the large intestine to contain SN-38 over the same time period (except at 1 hour). SN-38 was shown to increase sharply in the large intestine after dosing with CPT-11, peaking at 5 hours. Irinotecan and SN-38 can be excreted in the faeces (60-70%), bile (25%) or urine (10-20%). The bile passes through the intestine, exposing this portion of SN-38G to bacterial β-glucuronidase, as well as the portion excreted in the faeces (Alimonti *et al.* 2004). A recent study has examined the effects of a β-glucuronidase inhibitor, D-saccharic acid 1.4-lactone (SAL), on mucosal damage caused by irinotecan. The results showed a reduction in mucosal damage, indicating this agent should be further examined (Fittkau *et al.* 2004). Another recent study has shown rifampicin influences the metabolism of irinotecan in a patient with small cell lung carcinoma (Yonemori *et al.* 2004), warranting further studies into the use of rifampicin with irinotecan.

1.14 Conclusions

Mucositis is a major oncological problem. Despite advances in the understanding of oral and small intestinal mucositis over recent years, large intestinal mucositis, including diarrhoea, has not been well defined and the underlying mechanisms of the condition are yet to be established. The majority of the literature available concerning diarrhoea is

based on clinical observations, with very little basic research existing. However, from the little research conducted, it is likely that the intestinal microflora play a role in the development of chemotherapy-induced diarrhoea. Treatment with irinotecan has also been shown to cause goblet cell hyperplasia with mucous hypersecretion in the caecum, considered to be a main cause of diarrhoea.

In conclusion, the relationship between chemotherapy-induced diarrhoea and the gastrointestinal microflora is extremely complex. There is a lack of knowledge which ties together the histological changes that the GIT undergoes following chemotherapy, changes in microflora, changes in mucin composition and secretion, and diarrhoea. Research is warranted into these areas in order to provide a detailed understanding of the inter-relationships which may exist. This may provide a platform for future research and targeted therapies.

1.15 Aims of study

The importance of the roles of gastrointestinal microflora and mucins in the development of chemotherapy-induced diarrhoea and mucositis has yet to be determined. The gastrointestinal microflora has been shown to be affected by chemotherapy, and have a possible role in the metabolism of certain chemotherapy agents (Araki *et al.* 1993; Takasuna *et al.* 1996). The aim of this thesis will therefore be to determine the role of the

gastrointestinal microflora in the development of mucositis following chemotherapy and correlate these changes to the incidence of diarrhoea and presence of overt pathological damage, and investigate temporal changes in mucin secretion and MUC gene expression following chemotherapy and determine if these changes may be a contributing factor in chemotherapy-induced mucositis.

2.0 Gastrointestinal microflora and mucins play a role in the development of 5-Fluorouracil-induced gastrointestinal mucositis in rats

2.1 Introduction

Previous studies have indicated that gastrointestinal microflora may be involved in the development of chemotherapy-induced mucositis and diarrhoea (Stringer *et al.* 2007). The microflora of the gastrointestinal tract (GIT) is a highly complex ecosystem consisting of both aerobic and anaerobic bacteria (Simon and Gorbach 1982; Simon and Gorbach 1984; Simon and Gorbach 1986), which have a number of key functions including epithelial protection, and metabolism of, bilirubin, intestinal mucins, pancreatic enzymes, fatty acids, bile acids, cholesterol and steroid hormones (Gustafsson 1982). Furthermore, gastrointestinal bacteria function to process nutrients, regulate intestinal angiogenesis, and work with the immune system (Umesaki and Setoyama 2000; Rhee *et al.* 2004). Previous research has shown that the microflora of the gastrointestinal tract also changes after treatment with irinotecan (another cytotoxic agent known to induce diarrhoea) (Stringer *et al.* 2007), therefore it is highly likely that treatment with 5-FU will also alter the gastrointestinal microflora.

The integrity of the 'normal' intestinal flora is maintained by the structure of mucins, by providing attachment sites for intestinal flora and pathogenic bacteria (Robbe *et al.* 2004), as well as simultaneously protecting the mucosa from bacterial overgrowth and/or penetration (Specian and Oliver 1991). The mucins also protect the epithelium

from digestion by acting as substrates for the enzymes produced by the intestinal flora, such as α-galactosidase, β-N-acetylgalactosaminidase, sialidase, β-glucuronidase, blood group degrading enzymes and proteases (Specian and Oliver 1991). The metabolism of mucin may serve to regulate the microecology of the intestinal luminal environment (Specian and Oliver 1991). Acidic mucins have been suggested to protect against the translocation of bacteria (Deplancke and Gaskins 2001). Mucus offers a number of ecologic advantages to intestinal bacteria. Bacteria are able to degrade mucus, which provides a direct source of carbohydrates and peptides, and exogenous nutrients (vitamins and minerals) (Deplancke and Gaskins 2001). The mucus also contains receptors recognising specific adhesion proteins (Gusils *et al.* 2004). Bacteria colonising the mucus can also avoid rapid expulsion through the intestine, imparting a growth advantage (Deplancke and Gaskins 2001). It is not known whether goblet cell numbers or mucus secretion is increased in response to bacterial mucolysis (Deplancke and Gaskins 2001).

Treatment with irinotecan has previously been shown to cause goblet cell hyperplasia with accompanying mucous hypersecretion in the caecum (Ikuno *et al.* 1995). Methotrexate has also been shown to induce goblet cell depletion in the small intestine (Verburg *et al.* 2000). A recent study of mucins and goblet cells in colitis suggests that the goblet cells and mucins may be regulated by interactions of specific bacterial peptides with the gastrointestinal mucosa (Leiper *et al.* 2001). Bacterial peptides have been shown to cause mucin secretion by colon epithelial cell lines (Leiper *et al.* 2001). These results suggest a strong link between bacteria (intestinal flora) and mucin

secretion, both of which have been shown to be affected in chemotherapy-induced mucositis (Farrell *et al.* 1998; Meslin *et al.* 1999; Gibson *et al.* 2003; Stringer *et al.* 2007). Therefore, the aim of this study was to determine if 5-FU-induced mucositis can be correlated with changes to the gastrointestinal microflora, the composition and secretion of mucins in the GIT, and goblet cell numbers and integrity.

2.2 Materials and Methods

2.2.1 Animals

The animals used in this study were female Dark Agouti (DA) rats, weighing between 150 and 170g. The rats were kept at 22±1˚C and subject to a 14:10 hr light-dark cycle. Approval for the use of animals was granted by the Animal Ethics Committees of the Institute of Medical and Veterinary Science (IMVS) 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). Animals were monitored twice daily for the duration of the study.

2.2.2 Experimental Design

Eighty-one rats were randomly assigned to groups. For each time point one group of rats (n = 6) received 5-FU and one group of control rats (n = 3) received no treatment. Administration of 5-FU involved intraperitoneal injection of 150mg/kg 5-Fluorouracil (Mayne Pharma Ltd, Melbourne, Australia). Groups of rats were killed via exsanguination and cervical dislocation while under deep 3% halothane in 100% O_2 anaesthesia, at times 30, 60 and 90 min, 2, 6, 12, 24, 48 and 72 h post treatment. Immediately prior to anaesthesia, faecal samples were aseptically collected by directly collecting the excreted faeces immediately as it left the rat into a sterile container in an area cleaned with 70% ethanol. Samples were frozen in liquid N_2 and stored at -70˚C until required. Prior to cervical dislocation, a blood sample was collected via cardiac puncture. The GIT (from the pyloric sphincter to the rectum) was dissected out and

separated into the small intestine (pyloric sphincter to ileocaecal sphincter) and colon (ascending colon to rectum). The small intestine was flushed with sterile, chilled distilled water. Two 1cm samples were collected at 25% of the length of the small intestine (jejunum) were collected for culture and histology. The colon was also flushed with sterile, chilled distilled water, and contents were collected for electrolyte analysis. Two 1cm samples were taken at 50% of the length of the colon for culture and histology. The stomach was dissected from the rat and contents emptied. Two small pieces (1cm x 0.5cm) of stomach were collected for culture and histology. All samples collected for culture were stored at -70˚C until required, and those for routine histological examination were fixed in 10% neutral buffered formalin.

2.2.3 Electrolyte analysis

Blood samples were centrifuged (Hereus, Finland) at 3000 rpm for 5 min. The serum was collected into a fresh tube and analysed by the Department of Clinical Pathology, IMVS, Adelaide, South Australia. Measurements for sodium, potassium, bicarbonate, chloride, anion gap, and measured osmolality were obtained from serum samples.

2.2.4 Histological examination

Fixed samples of stomach, jejunum, and colon were processed and embedded in paraffin. Sections of 4μm thickness were cut and mounted on glass slides. Routine haematoxylin and eosin (H&E) staining was performed. Sections were examined using light microscopy and reported on by specialist veterinary pathologist (Dr John Finnie, IMVS).

2.2.5 Alcian Blue-PAS stain

Sections were dewaxed in xylene and rehydrated through a graded series of alcohols. Sections were stained in Alcian Blue (1% Alcian Blue 8GX (CI 74240) in 3% glacial acetic acid) for 5 min, then rinsed in distilled water. Sections were oxidised in 1% periodic acid before washing in running water and rinsing in distilled water. Sections were treated for 15 min in Schiff's reagent and washed for 7 min in running water. Slides were dehydrated, cleared and mounted.

2.2.5.1 Quantitative Histology

To determine the effect of 5-FU on mucus secretion, goblet cells were counted. Decreased goblet cells indicated release of mucins from the mucosal surface, and cavitation of mucus cells is a sign of accelerated mucus secretion by compound exocytosis (Barcelo *et al.* 2000). Therefore, both the number of goblet cells and percentage of cavitated cells were analysed, according to a method previously described (Barcelo *et al.* 2000). Briefly, a cavitated cell is recognised by apical indentation into the intracellular store of mucus granules. Goblet cells and cavitated cells in crypts and villi that were deemed to be greater than 80% complete were counted, with a total of at least 15 villi/crypts per section analysed.

2.2.6 Culture of samples

To determine the flora of the DA rat treated with 5-FU, a variety of selective and non-selective media (Oxoid, Adelaide, Australia) was used in an attempt to identify as many

bacteria as possible from the GIT. Media used included horse blood agar (HBA) (Ellner *et al.* 1966); MacConkey (MAC); HBA + colymycin + nalidixic acid (CNA); MAC + CV; Chromogenic E. coli/coliform; CIN (Schiemann 1982); Raka Ray (Saha *et al.* 1974); TCBS (Davis *et al.* 1981); Xylose-Lysine-Desoxycholate (XLD) (Weissman *et al.* 1975); Campylobacter (Patton *et al.* 1981); Anaerobic (MacFaddin 1985); Anaerobic + nalidixic acid; and Anaerobic + nalidixic acid + vancomycin (Wren 1980) (details shown in Table 2.1). The mucosal surface of the tissue samples was scraped and faecal samples were homogenized with a sterile swab, and inoculated onto the top third of the plate. A flame-sterilised inoculating loop was used to streak in a zigzag fashion. All plates and broths were incubated at 35-37˚C, for 24-48 h. The level of growth of each bacterium was graded using a modified qualitative assessment technique (Bowen *et al.* 2005; Yeoh *et al.* 2005), using the following criteria: 0, no growth (NG); 1, very light growth (VLG) (less than 10 colonies); 2, light growth (LG) (growth in the original inoculation zone only); 3, moderate growth (MG) (growth in the first streak line) and; 4, heavy growth (HG) (growth in the second streak line or greater). All gradings were conducted in a blinded fashion by a single assessor (A.M.S.). This grading system has been validated in our laboratory using quality control organisms and sample organisms to ensure consistent and valid results.

Each different bacterium present was isolated and subcultured onto horse blood agar plates (Oxoid, Adelaide, Australia) for biochemical testing to assist in identification. The first identification step was a Gram stain, followed by a variety of catalase, oxidase (Barry and Bernsohn 1969), coagulase, indole, urease, growth in NaCl, motility

Table 2.1 Materials used for culture.

Media	Selectivity	References
Columbia HBA	Non-selective	Ellner et al. 1966
MAC	Gram negative	Murray et al. 1999
HBA + CNA	Gram positive	Murray et al. 1999
MAC + CV	Gram negative	Murray et al. 1999
Chromogenic E. coli/coliform	Coliforms	Kilian et al. 1979
CIN	Yersinia sp.	Schiemann 1982
Raka Ray	Lactobacillus sp.	Saha et al. 1974
TCBS	Vibrio sp.	Davis et al. 1981
XLD	Salmonella sp. and Shigella sp.	Weissman et al. 1975
Campylobacter	Campylobacter sp.	Patton et al. 1981
Anaerobic	Anaerobes	Wren 1980
Anaerobic + Nali	Anaerobes, enhanced Gram positive	Wren 1980
Anaerobic + Nali + Vanc	Anaerobes, enhanced Gram negative	Wren 1980

Abbreviations: HBA – Horse Blood Agar; MAC – MacConkey; CNA – Colymycin + Nalidixic Acid; CV – Crystal Violet; CIN – Yersinia; XLD – Xylose-Lysine-Desoxycholate; Nali – Nalidixic Acid; Vanc – Vancomycin.

(Cowen and Steele 1977), oxidation-fermentation (OF) test (Hugh and Leifson 1953), Lancefield grouping and PYRase (Chagla *et al.* 1993), using a standard microbiological dichotomous key, and depending on Gram stain result. If identification was not possible after biochemical tests were conducted, identification kits were used, in which a wider array of biochemical tests was used to identify the bacteria. For anaerobic bacteria the Remel RapID (Oxoid, Adelaide, Australia) kit was used and for Gram negative bacteria the Microbact 24E kit was used (Mugg and Hill 1981).

2.2.6.1 Bacterial Strains and culture conditions

The bacterial strains used as positive and negative controls in this study included *Bacteroides fragilis* ATCC 25285, *Bifidobacterium breve* ATCC 15700, *Clostridium botulinum* ATCC 13124, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 47077, *Lactobacillus acidophilus* ATCC 314 and *Staphylococcus epidermidis* ATCC 12228 (Cryosite, Lane Cove, NSW, Australia). Bacteria were cultured in nutrient broth at 37˚C overnight. Bacteria requiring anaerobiosis for growth were cultured at 37˚C in and anaerobic jar (Oxoid, Australia) in Cooked Meat broth (Oxoid), Thioglycollate broth (Oxoid) or MRS broth.

2.2.7 Extraction and purification of DNA from bacterial culture and faecal samples

2.2.7.1 DNA Extraction from Bacteria

DNA was extracted from known bacterial strains (Table 2.2) using a DNeasy Tissue Mini Kit (Qiagen). Briefly, bacteria were grown to log phase in nutrient broth. Cells ere harvested by centrifuging for 10 min at 5000 x *g*. Gram positive bacteria were lysed in enzymatic lysis buffer (20mM Tris-Cl, pH 8.0, 2mM sodium EDTA, 1.2% Triton® X-100, 20mg/mL lysozyme) at 37˚C, followed by proteinase K at 70˚C. Gram negative bacteria were lysed in provided buffer and proteinase K at 55˚C, followed by further lysis in buffer at 70˚C. For both gram positive and gram negative bacteria, DNA was bound to the membrane in the provided spin-column. The membrane was washed with 2 buffer solutions before DNA was eluted from the membrane into a centrifuge tube. DNA concentration was determined using a spectrophotometer (Eppendorf, Sydney, Australia) and diluted to a final working concentration of 10ng/μL.

2.2.7.2 DNA Extraction from samples

DNA was extracted from rat faecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Doncaster, Australia). Briefly, a pellet of rat faeces was homogenised in lysis buffer and heated at 70˚C. Samples were centrifuged and the supernatant collected in a centrifuge tube. Inhibitors were adsorbed to InhibitEX tablets before samples were centrifuged again and the supernatant collected. Proteins were digested with proteinase K and buffer. DNA was then bound to the membrane in the provided spin column. The membrane was washed with 2 wash buffers before DNA was eluted into a

Table 2.2 Cross reactivity and cycling details of primers used in this study.

Primer	Other reactive organisms	Anneal Temp	Extension	Cycles	Product Size (bp)
Bacteroides (Llayton *et al.* 2006)	***Bacteroides fragilis***, all Bacteroides genus	55°C 15s	20s	50	106
Bifidobacterium (Rinttila *et al.* 2004)	***Bifidobacterium breve***, *B. bifidum, B. adolescentis, B. longum, B. minimum, B. angulatum, B. catenulatum, B. pseudocatenulatum, B. dentium, B. ruminatum, B. thermophilum, B. subtile, B. bifidum, B. boum, B. animalis, B. choerinum, B. gallicum, B. pseudolongum, B. magnum, B. infantis, B. indicum, B. gullinarum, B. pullorum, B. saeculare, B. suis*	60°C 50s	30s	45	243
Clostridium (Rinttila *et al.* 2004)	***Clostridium botulinum***, *C. perfringens, C. homoproprionicam, C. cadaveris, C. intestinalis, C. putrificum, C. novyi, C. sporogenes, C. tyrobutyricum, C. kluyveri, C. ljungdahlii, C. scatologenes, C. acetireducens, C. subterminale, C. esterheticum, C. argentinense, C. chauvoei, C. sardiniensis, C. paraputrificum, C. longisporum, C. septicum, C. cellulovorans, C. barati, C. absonum, C. carnis, C. butyricum, Eubacterium cudayi, E. nitritogens, E. moniliforme, E. multiforme*	50°C 20s	30s	45	120
E. coli (Penders *et al.* 2005)	***E. coli***	57°C 15s	20s	45	95
Enterococcus (Rinttila *et al.* 2004)	***Enterococcus faecalis***, *E. faecium, E. asini, E. saccharolyticus, E. casseliflavus, E. gallinarum, E. dispar, E. flavescens, E. hirae, E. durans, E. pseudoavium, E. raffinosus, E. avium, E. malodoratus, E. mundtii, E. azikeevi, E. canis, E. gilvus, E. haemoperoxidus, E. hermanniensis, E. moraviensis, E. pallens, E. phoeniculicola, E. villorum, E. rottae*	61°C 15s	20s	60	144
Lactobacillus (Rinttila *et al.* 2004)	***Lactobacillus acidophilus***, *L. amylovorus, L. delbruecki, L. amylolyticus, L acetotolerans, L. crispatus, L. amylophilus, L. johnsonii, L. gasseri, L. fermentum, L. pontis, L. reuteri, L. mucosae, L. vaginalis, L. panis, L. oris, L. pentosus, L. plantarum, L. collinoides, L. alimentarius, L. farciminis, L. brevis, L. buchneri, L. kefiri, L. fructivorans, L. mali, L. animalis, L. murinus, L. ruminis, L. agilis, L. salivarius, L. aviaruis, L. sharpeae, L. manihotivorans, L. rhamnosus, L. casei, L. zeae, L. sakei, Pediococcus pentosaceus, P. parvulus, P. acidilactici, P. dextrinicus, Weissella halotolerans, W. confusus, W. paramesenteroides, W. hellenica, w. viridescens, W. kandleri, W. minor, Leuconostoc lactis*	50°C 20s	30s	45	341
Staphylococcus (Sakai *et al.* 2001)	***S. epidermidis***, *S. aureus, S. saprophyticus, S. cohnii, S. haemolyticus, S. hominis, S. lugdenensis, S. xylosus, S. wareni*	46°C 8s	20s	68	462

centrifuge tube. DNA concentration was determined using a spectrophotometer, and diluted to a final working concentration of 5ng/μL.

2.2.8 Real-time PCR

All primers used in this study have been used previously (Rinttila *et al.* 2004; Sakai *et al.* 2004; Penders *et al.* 2005; Layton *et al.* 2006) (Table 2.2). General optimisation of each set of primers was carried out to determine optimal working conditions in the Rotorgene rotary cycler (Corbett Research, Mortlake, NSW, Australia), using Quantitect SYBR Green Mastermix (Qiagen). PCR mixtures consisted of 1x Quantitect® SYBR Green Mastermix (Qiagen), 2.5ng/μL of each primer and 10ng of DNA in a volume of 10μL. The cycling parameters consisted of enzyme activation at 95˚C for 10 min, followed by cycles of 95˚C for 15 s melting, 15 s annealing (see Table 2.2 for annealing temperatures) and 72˚C for 20s extension. The SYBR green fluorescent signals were acquired at 72˚C. Standard curves were constructed from PCR reactions using 10-fold serial dilutions of known bacterial DNA. Data was analysed using Rotorgene 6.0 software (Corbett Research).

2.2.9 Bacterial susceptibility

Bacterial susceptibility to 5-FU was determined using a standard antibiotic susceptibility testing method (Bodet *et al.* 1985). The same standards from real time PCR experiments were used, and also *Streptococcus pneumonia* (ATCC 49619), *Psuedomonas aeruginosa* (ATCC 49189) . Briefly, serial dilutions of 5-FU were diluted in sterile water for injection, and 10μL was put onto blank discs (Oxoid). Sensitest

Agar plates were used for *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus pneumonia, Staphylococcus aureus* and *Staphylococcus epidermidis*. Anaerobic agar (Oxoid) was used for *Bacteroides fragilis*, *Bifidobacterium breve*, *Lactobacillus acidophilus* and *Clostridium botulinum*. Discs were placed onto a lawn culture of each bacterium, incubated overnight at 37°C, and zones of inhibition measured. All experiments were carried out in triplicate.

2.2.10 Statistical Analysis

Results were statistically analysed using the Mann-Whitney U test. For adjustments for multiplicity, the significance levels of the *P* values were determined according to the Bonferroni correction.

2.3 Results

2.3.1 Effects of 5-FU on electrolytes

Electrolyte levels were altered in rats treated with 5-FU (Figure 2.1). Serum sodium levels were lower in experimental rats after treatment compared with control rats. Bicarbonate levels were significantly higher 12 h after treatment ($p<0.005$). The anion gap was significantly lower in experimental rats 12 h ($p<0.002$). Serum potassium, chloride and measured osmolality levels did not change significantly after treatment (data not shown).

2.3.2 Histological changes caused by 5-FU

Pathological changes caused by 5-FU were seen in the stomach, jejunum and colon (Figure 2). A loose infiltration of eosinophils in the deep lamina propria was seen in the stomach, with occasional necrotic cells in the glandular epithelium 24 h after chemotherapy treatment. No significant changes were seen from 48 h. Mild villous clubbing and fusion, and enterocyte hyperplasia were seen in the jejunum 30 min after chemotherapy. Villous atrophy was apparent 90 min after chemotherapy, with apoptotic bodies evident in the crypts by 6 h. Villous atrophy and apoptotic bodies were present at 12 and 24 h, and by 72 h eosinophils and lymphocytes had infiltrated the lamina propria, with cytoplasmic vacuolation of the enterocytes. The colon exhibited numerous apoptotic bodies in the crypts 12-24 h after treatment.

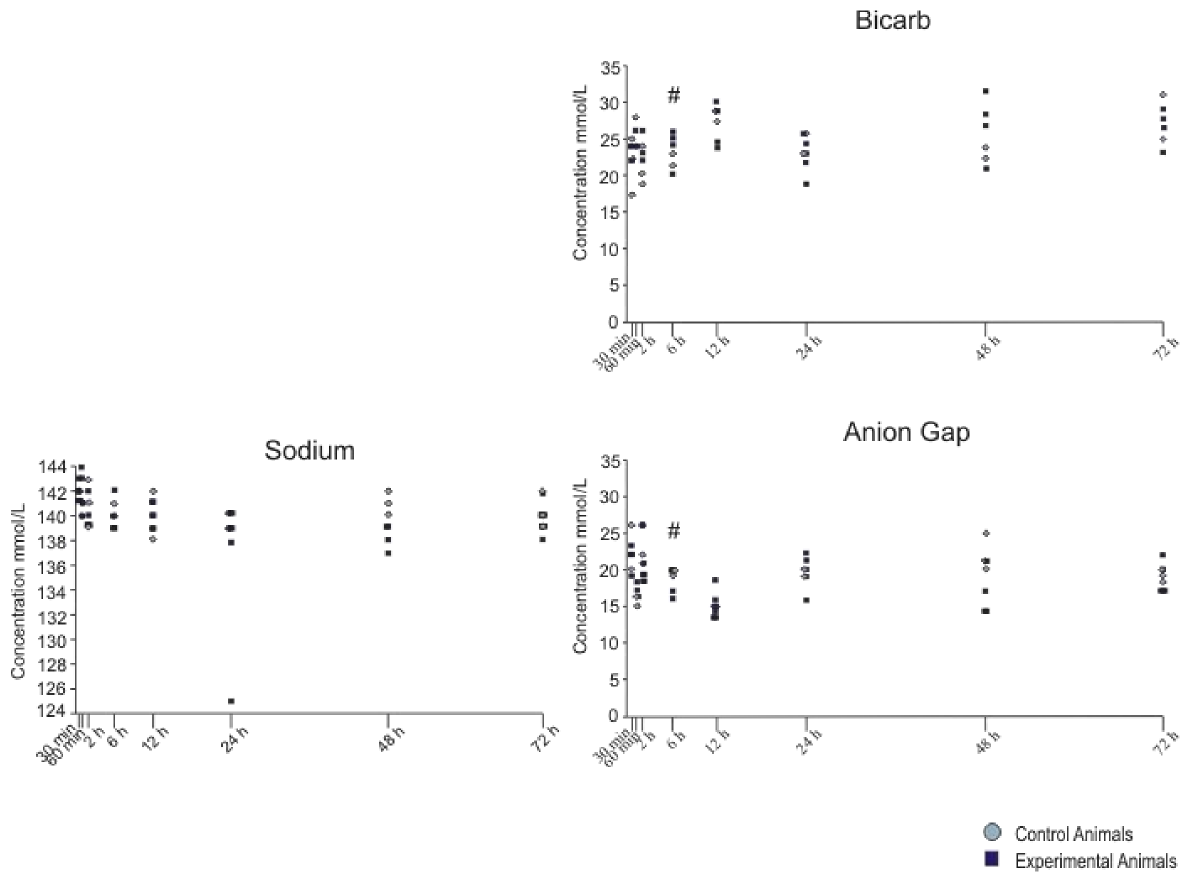


Figure 2.1 Electrolytes. A) Sodium. B) Bicarbonate. C) Anion Gap. Results displayed as Mean±SEM, # denotes significance with Bonferroni correction.

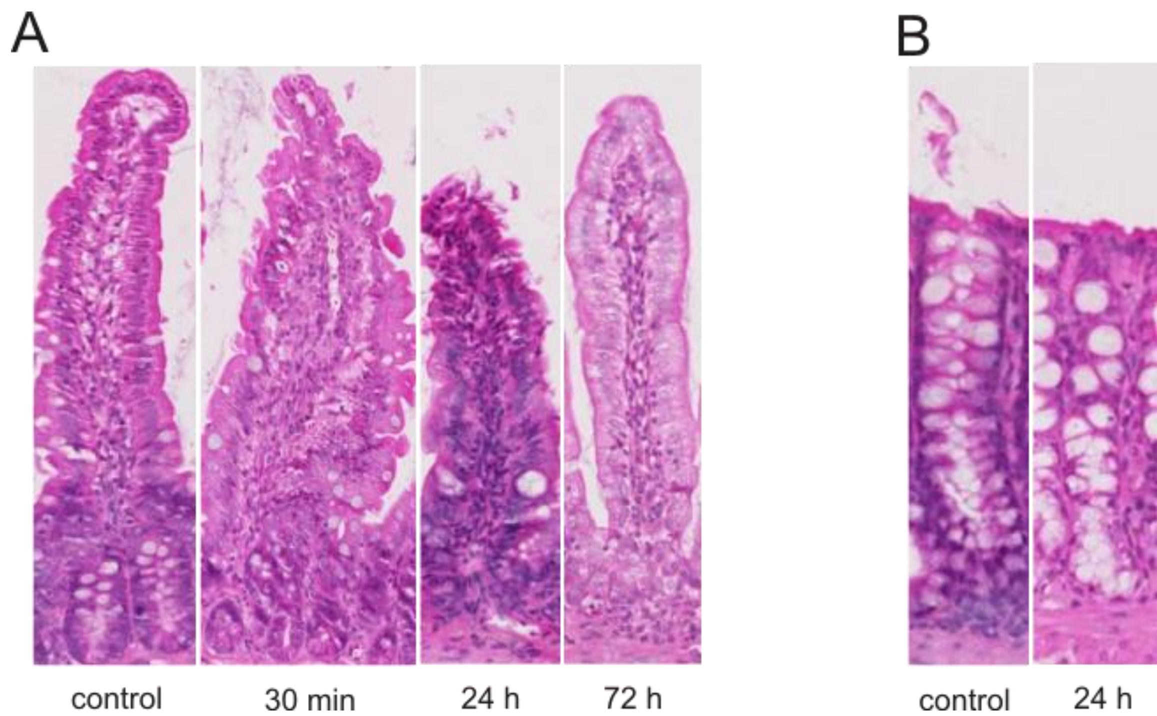


Figure 2.2 Pathology. A) Histological changes in the jejunum following treatment. B) Histological changes in the colon following treatment.

2.3.3 Goblet cell composition and distribution

2.3.3.1 Stomach

No noticeable changes to the mucin composition of the stomach were seen following treatment with 5-FU (data not shown).

2.3.3.2 Jejunum

In the jejunum, goblet cells became condensed at the base of the villi 30 min after treatment. These goblet cells began to 'move' up the villi until 2 h post treatment. At 24-48 h the goblet cells became enlarged and dilated (Figure 2.3). There were no changes in the composition of mucins following 5-FU treatment.

2.3.3.3 Colon

At 6 h following chemotherapy treatment, mixed mucins were only seen in the base of the crypts. By 12 h, the mucins at the base of the crypt were acidic, and the goblet cells were partially dilated. At 24 h following 5-FU administration the majority of mucins were mixed (with a few neutral) and the goblet cells were dilated.

2.3.4 Effect of 5-FU on mucin discharge

All goblet cells were counted per villus/crypt in the jejunum and colon. Mucus cells in the stomach could not be counted due to the histological structure. Goblet cell counts in untreated rats had 14.25 ± 0.73 (mean ± SEM) goblet cells/villus, with 37.37

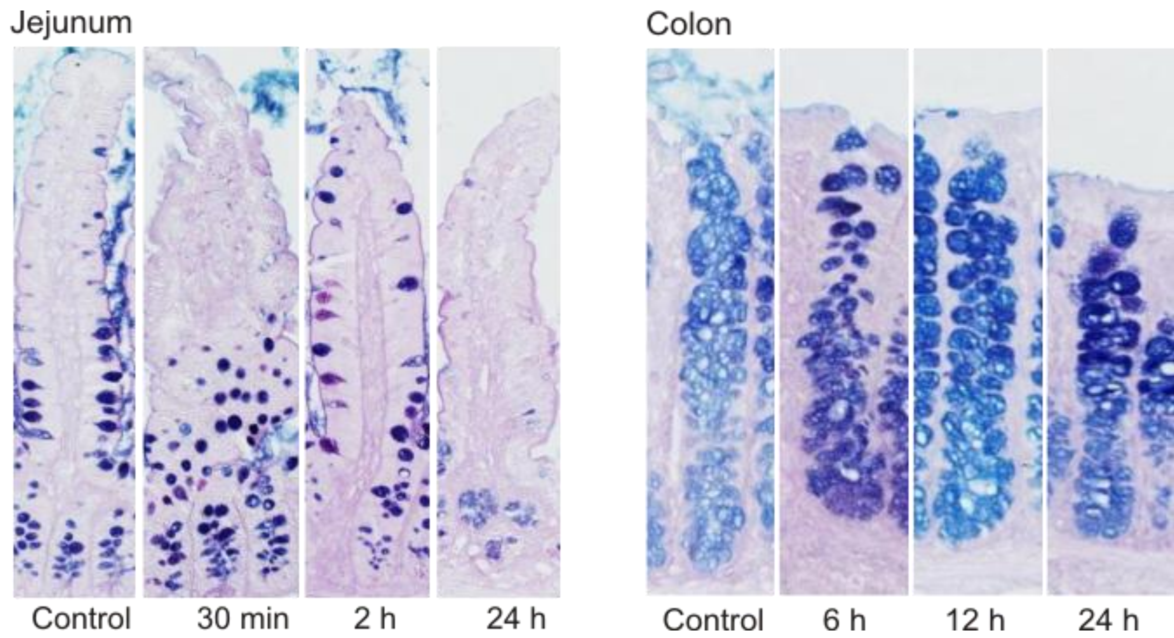


Figure 2.3 Alcian Blue-PAS Staining in the jejunum and colon, demonstrating changes in mucin composition and distribution following treatment.

± 2.40% (mean ± SEM) of those deemed to be cavitated. A mild increase in total goblet cells was apparent early after treatment (≤12 h), with a decrease from 24 h. Total goblet cells in the villi were decreased significantly at 72 h compared with control ($p<0.0006$) (Figure 2.4). The percentage of cavitated cells appeared to increase at 72 h after treatment, but this was not deemed significant after adjustment using Bonferroni correction. The jejunum crypts exhibited a significant decrease in total goblet cell numbers 48-72 h after treatment ($p<0.004$). The colon exhibited a general decrease in total goblet cell numbers after treatment with 5-FU, although not significant after performing Bonferroni correction. The percentage of cavitated cells did not change significantly after treatment (not shown).

2.3.5 Culture

Changes were seen in the flora of the stomach, jejunum, colon and faeces (Figure 2.5). The organisms identified were consistent with the expected gastrointestinal microflora of rats. The stomach mucosal surface of rats was found to harbour copious amounts of *Clostridium spp.* and *Lactobacillus spp.*, with a moderate amount of *Streptococcus spp.* Changes in levels of bacteria following 5-FU were seen. *Clostridium spp.* and *Escherichia spp.* levels decreased at 24 h. *Staphylococcus spp.* levels also decreased, but at 6 h post treatment.

The jejunal mucosal surface was found to accommodate relatively small amounts of organisms compared with the stomach, colon and faeces. The most prominent were

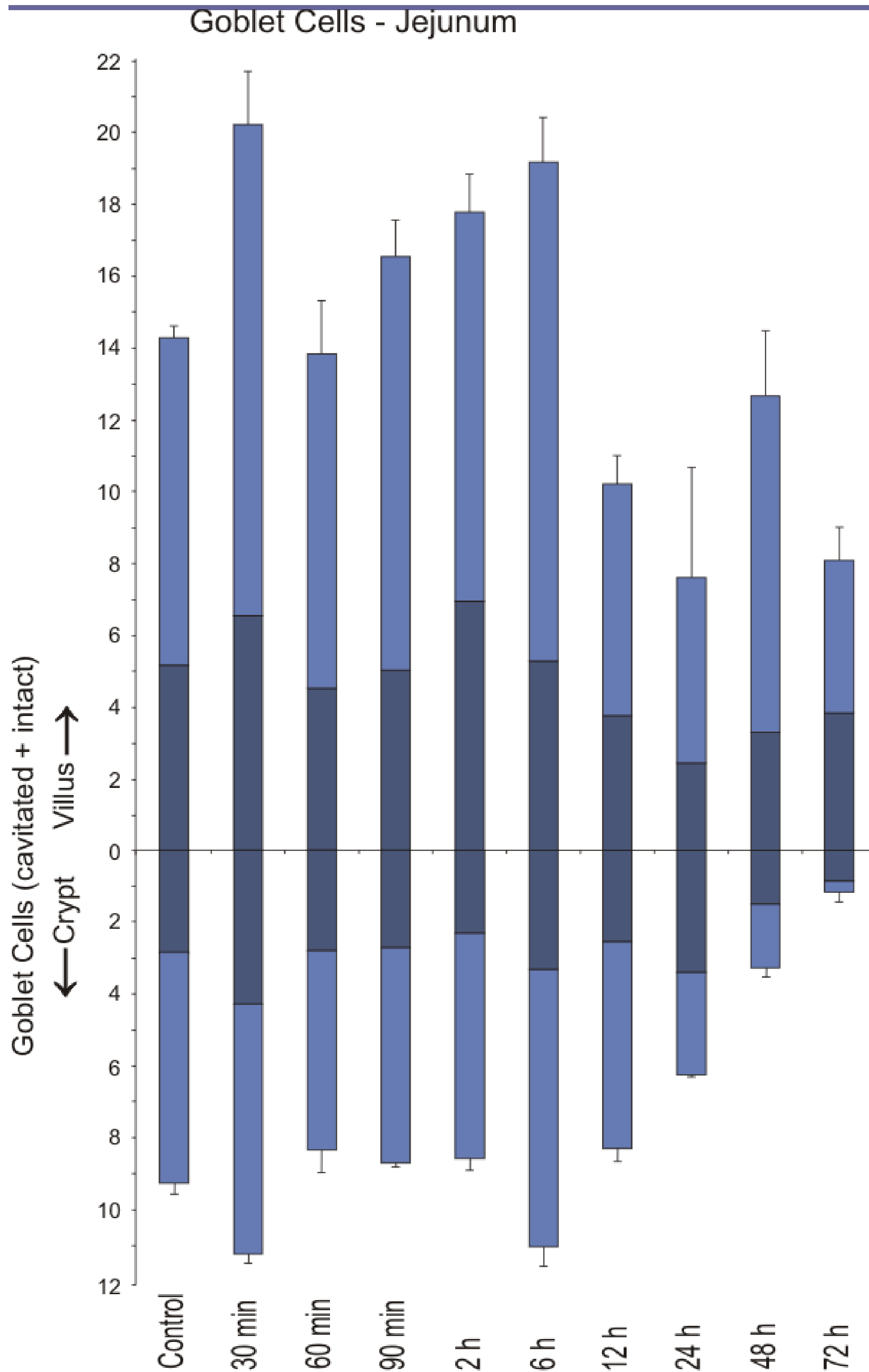


Figure 2.4 Goblet cells. Goblet cells counted in the jejunum, divided into villi and crypts, and into cavitating goblet cells and intact goblet cells, # denotes significance.

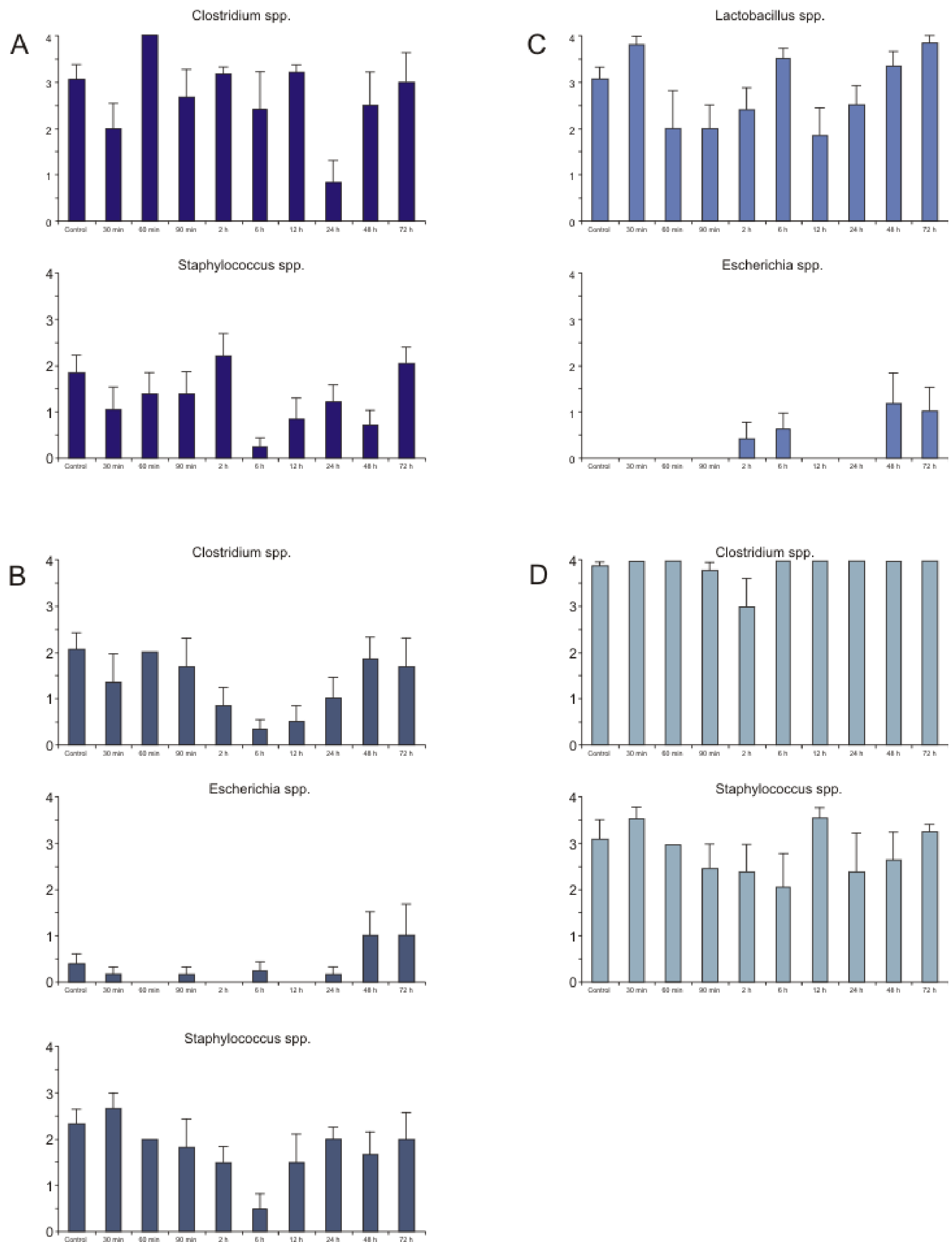


Figure 2.5 Qualitative microbiology. Changes to microflora in the A) stomach, B) jejunum, C) colon and D) faeces.

Clostridium spp., *Lactobacillus spp.* and *Streptococcus spp.* Changes were seen in levels of bacteria after chemotherapy. *Clostridium spp.* decreased from 6-12 h post treatment. *Lactobacillus spp.* decreased from 60 min post treatment, with a larger decrease at 6 h. *Escherichia spp.* peaked at 48-72 h post treatment. The mucosal surface of the colon in control rats was found to accommodate a large amount of organisms. The most prominent was *Lactobacillus spp.*, with smaller amounts of *Streptococcus spp.* and *Clostridium spp.* Changes were seen in the levels of bacteria following chemotherapy. There was an increase in *Clostridium spp.* after 24 h, and *Escherichia spp.* from 2-6 h and again from 48-72 h. *Lactobacillus spp.* decreased from 1-2 h and 12 -24 h. *Streptococcus spp.* fluctuated after chemotherapy.

In all rats, the bacterial content of the faeces was found to be higher than the colon, jejunum or stomach. The most prominent bacteria were *Clostridium spp.*, *Enterococcus spp.*, *Eubacterium spp.* and *Lactobacillus spp.* Changes were seen following treatment with 5-FU. *Clostridium spp.* decreased 2 h following treatment. *Escherichia spp.* decreased from 2-24 h post treatment. *Proteus spp.* decreased from 2-12 h post treatment. *Streptococcus spp.* decreased at 72 h.

2.3.6 Real Time PCR

Differences in *Bifidobacterium spp.*, *Bacteroides spp.*, *Clostridium spp.*, *E. coli*, and *Lactobacillus spp.* were seen in rats treated with 5-FU. However, following Bonferroni correction these were not deemed significant. The following trends were seen. *Bifidobacterium spp.* exhibited fluctuations between time points, *Bacteroides spp.*

decreased at 48 h, *Clostridium spp*. increased after treatment *E. coli* increased at 48 h, and *Staphylococcus spp*. increased at 24 h. *Lactobacillus spp*. decreased from 12-24 h, and *Enterococcus spp*. was decreased from 2-48 h after treatment (Figure 2.6).

2.3.7 Bacterial susceptibility to 5-FU

E. coli and *P. aeruginosa* were not susceptible to 5-FU at any concentration. *E. faecalis*, *S. pneumoniae*, *L. acidophilus*, *B. lactis*, *C. botulinum* and *S. epidermidis* showed susceptibility, as summarised in Table 2.3.

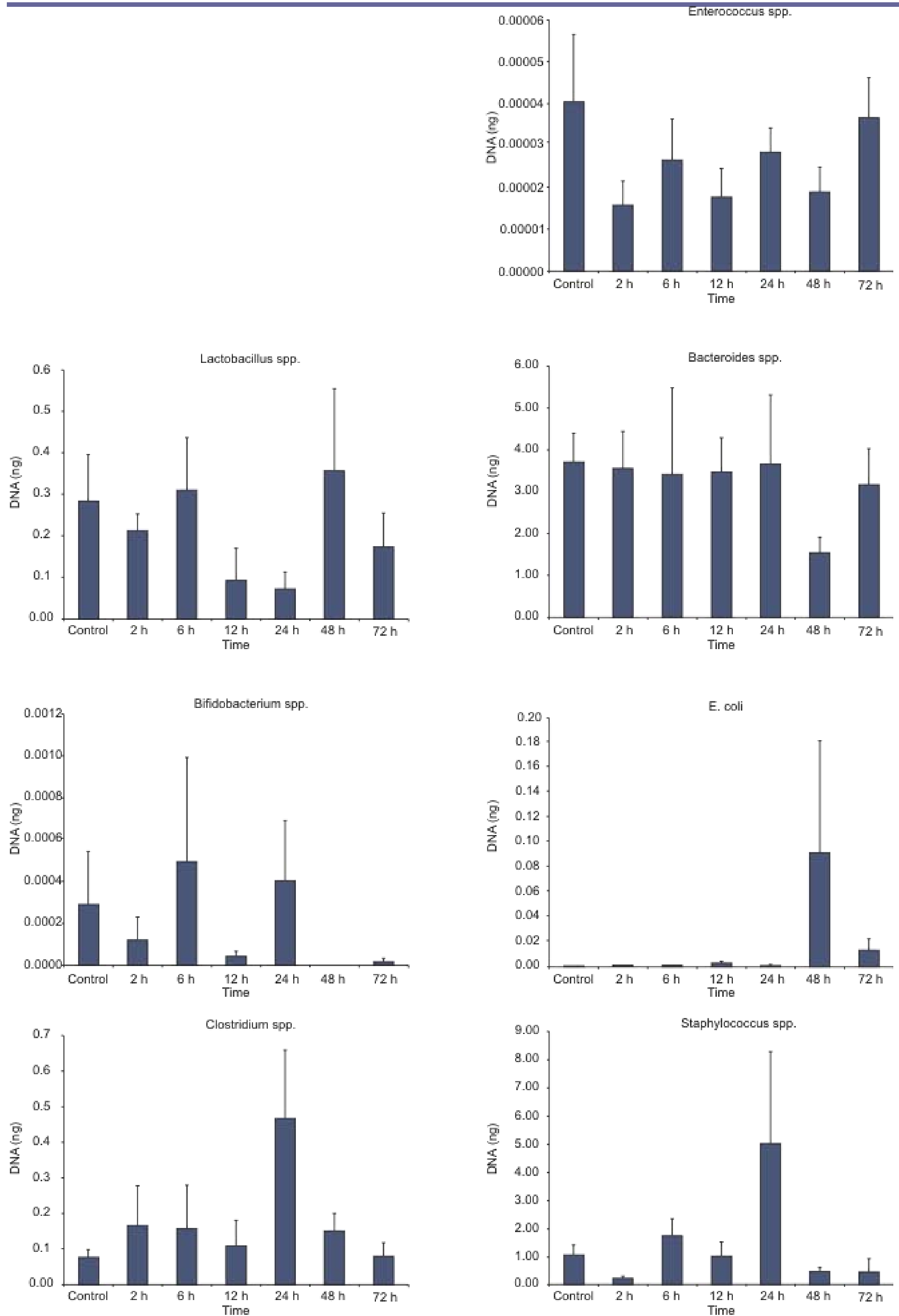


Figure 2.6 Quantitative PCR. Changes to faecal microflora.

Organism	5-FU dose	Zone of Inhibition (mm ± sem)	n
Streptococcus pneumoniae	250µg	Complete	
	100µg	27.8 ± 0.09	1
	10µg	21.8 ± 0.67	2
	1µg	1.51 ± 0.37	3
	0.1µg	Nil	
Staphylococcus aureus	250µg	17.97 ± 0.38	3
	100µg	15.70 ± 0.44	3
	10µg	8.09 ± 0.29	3
	1µg	Nil	
Pseudomonas aeruginosa	250µg	Nil	
Escherichia coli	250µg	Nil	
Staphylococcus epidermis	250µg	27.18 ± 0.34	3
	100µg	24.26 ± 0.90	3
	10µg	16.29 ± 0.40	3
	1µg	5.35 ± 0.08	3
	0.1µg	Nil	
Enterococcus faecalis	250µg	20.09 ± 0.36	3
	100µg	17.68 ± 0.51	3
	10µg	13.06 ± 0.76	3
	1µg	3.47 ± 0.13	3
Enterococcus faecalis (cont.)	0.1µg	Nil	
Serratia marcescens	250µg	7.40 ± 0.29	3
	100µg	3.60 ± 0.17	3
	10µg	Nil	
Bacteroides fragilis	250µg	Complete	
	100µg	Complete	
	10µg	23.6 ± 1.51	3
	1µg	Nil	
Clostridium botulinum	250µg	19.33 ± 0.46	3
	100µg	16.20 ± 1.29	3
	10µg	13.99 ± 1.30	3
	1µg	0.10 ± 1.10	3
	0.1µg	Nil	
Lactobacillus acidophilus	250µg	Complete	
	100µg	Nil	
Bifidobacterium lactis	250µg	Complete	
	100µg	Complete	
	10µg	5.11 ± 0.51	3
	1µg	Nil	

Table 2.2: Bacterial susceptibility to 5-FU.

2.4 Discussion:

5-FU is one of the most commonly used chemotherapy agents used in clinical oncological practice (Fata *et al.* 1999). However, side effects are prevalent, and result from the inhibition of rapidly dividing tissues, including bone marrow, haemopoietic cells and gastrointestinal mucosal cells (Fata *et al.* 1999). Clinical studies have shown 5-FU to cause mucositis, myelosuppression, nausea, emesis, and diarrhoea, associated with marked histopathological changes (Milles *et al.* 1962; Fata *et al.* 1999). However, the association between mucositis and the intestinal bacteria remains unclear. This study has demonstrated changes in intestinal bacteria and goblet cells which correlate with histological changes at early time points after 5-FU treatment. We have also shown a variety of other changes in electrolytes and correlated them with histological changes, suggesting that these components may play a crucial role in the development in the development of 5-FU-induced mucositis.

Histological evidence of damage was seen in the jejunum as early as 30 min after treatment in this study, with greatest damage seen 24 h after treatment in the stomach, jejunum and colon, and was consistent with previous studies (Milles *et al.* 1962; Fata *et al.* 1999). Mucin metabolism has previously been demonstrated to be influenced by multiple doses of 5-FU (Saegusa *et al.* 2007). This study has shown that a single dose of 5-FU also influences mucins, affecting composition, distribution and secretion. Goblet cells in the jejunum tended to decrease temporally after treatment with 5-FU, with crypt goblet cells being affected the most, decreasing significantly from 24-72 h. Cavitated cells can be counted as a measure of cells secreting mucin, and expressed as a

percentage of total goblet cells (Plaisancie *et al.* 1997). Cavitated cells in the jejunum crypts increased significantly from 24-72 h. However, cavitated cells in the villi did not change significantly, suggesting that mucin secretion in the jejunum as a result of treatment with 5-FU occurs primarily in the crypts. The implications of both decreased goblet cells and an increased percentage of cavitated cells could be detrimental to the small intestine. The protective capacity of the mucus barrier could be altered after the stored mucins have been depleted. The rapid secretion of mucus is thought to be caused by enteric neurotransmitters acting on epithelial cells (including goblet cells), such as acetyl choline (ACh), substance P (SP) and vasoactive intestinal peptide (VIP) (McConalogue and Furness 1994), which suggests that 5-FU absorption by the intestine may cause an up-regulation of neurotransmitter release from enteric neurons, resulting in increased mucin secretion. Goblet cells and cavitated cells did not change significantly in the colon, suggesting no significant change to the protective capacity of mucins in the colon.

This study also clearly demonstrated that changes in the microflora occur in the stomach, jejunum, colon and faeces. Culture methods showed decreases in *Clostridium spp.*, *Escherichia spp.* and *Staphylococcus spp.* in the stomach, and decreases in the jejunum in *Clostridium spp.* and *Lactobacillus spp.*, with an increase in *Escherichia spp.* 48-72 h after treatment. In the colon, *Clostridium spp.* and *Escherichia spp.* increased, but *Lactobacillus spp.* decreased. In the faeces, *Clostridium spp.* decreased, as did *Escherichia spp.* and *Proteus spp.* early after 5-FU treatment and *Streptococcus spp.* late after 5-FU treatment. Being a qualitative technique, the culture method makes it

difficult to gauge exactly how significant changes in bacteria are, and also to grow some fastidious organisms. Despite this, the technique gives a good indication of which organisms to target with other quantitative techniques. Real time PCR using primers directed specifically at groups of bacteria in the intestine was performed on DNA from snap frozen faecal samples. This technique gave an accurate quantification of the amount of DNA for each group of organisms tested in the faeces of treated and control rats. *Clostridium spp.* and *Staphylococcus spp.* increased after treatment. Other changes seen included decreases in *Bacteroides spp.* and *Lactobacillus spp.*, and a significant increase in *E. coli* (p=0.02). It is likely that these changes are seen due to a direct effect on the bacteria in the GIT from 5-FU. Susceptibility tests for 5-FU on bacteria showed that *E. coli* and *P. aeruginosa* were not susceptible, even when applied at the manufacturer's concentration. All other bacteria tested were susceptible to varying degrees. 5-FU is absorbed to a degree in the GIT (Fata *et al.* 1999; Inomata *et al.* 2002), making it likely to have its effect on bacteria at this time. However, pharmacokinetic studies have shown concentrations of 5-FU given intraperitoneally to be lower in the peritoneal fluid and colon tissue than the concentrations the bacteria were susceptible to in this study (Wei *et al.* 2008), suggesting the implication of other mechanisms. Patients undergoing chemotherapy with 5-FU are susceptible to infections as the result of myelosuppression. Clinical data suggests that infections are often caused by bacteria found in the intestinal tract, such as *E. coli* and *P. aeruginosa* (Tancrede and Andremont 1985; Wells *et al.* 1987; Nomoto *et al.* 1992; Nomoto *et al.* 1992). *E. coli* and *P. aeruginosa* were shown not to be susceptible to 5-FU. Other organisms involved in maintaining a balanced microecology in the intestine were

susceptible to 5-FU, which may allow the proliferation of non-susceptible bacteria and the opportunity for overgrowth and penetration of the damaged mucosa, causing host infections.

The loss of intestinal microflora also results in the loss of bacterial function in the gut, including processing of nutrients, regulation of intestinal angiogenesis, immune development in the GIT (Umesaki and Setoyama 2000; Rhee et al. 2004), protection, and metabolism of enzymes, fatty acids, bile acids, cholesterol, steroid hormones and intestinal mucins (Gustafsson 1982). These are important functions in the GIT, and their lacking could also result in decreased GIT functional and protective capabilities. Probiotics are known to exert beneficial effects to the host when ingested, and therefore could be useful in controlling the intestinal microflora during chemotherapy (Quigley 2007). We have shown that VSL#3 is able to reduce the severity and duration of CID in rats treated with irinotecan (Bowen *et al.* 2007). Further research into the role that probiotics could play in ameliorating mucositis should also be explored. The use of probiotics could be a viable option for replacing and/or preventing lost microflora and maintaining gut homeostasis. The careful use of targeted antibiotics against *E. coli* and *Clostridium spp*. may also be useful.

The changes in electrolytes seen in this study are also highly likely to be mucositis-related. The significant decrease in sodium at 48 h is likely to be due to gastrointestinal loss through decreased absorptive capacity as a result of damage caused by 5-FU. The significant increase in bicarbonate at 12 h is likely to be the result of a compensatory

mechanism. 5-FU is broken down to urea, CO_2 and α-fluoro-β-alanine, all of which are excreted in urine (1997). The temporary increase in blood CO_2 would be compensated by an excretion of H+ ions, and an increased reabsorption of $HCO3^-$, thus resulting in an increased serum bicarbonate level (Tortora and Grabowski 1993).

In conclusion, 5-FU causes intestinal mucositis, the effects of which are predominantly seen in the small intestine. This study has shown for the first time that 5-FU treatment results in changes to the intestinal microflora and mucin secretion, which are probably responsible in part for development of severe mucositis.

3.0 Chemotherapy-induced diarrhoea is associated with changes in the luminal environment in the DA rat

3.1 Introduction

Major progress has been made in recent years in understanding the mechanisms of oral (Sonis 1998; Sonis 2004; Sonis 2004; Gibson *et al.* 2005) and small intestinal mucositis (Gibson *et al.* 2002; Gibson *et al.* 2002; Gibson *et al.* 2003; Keefe *et al.* 2004; Bowen *et al.* 2005; Gibson *et al.* 2005). However, the mechanisms of large intestinal mucositis (including CID) remain insufficiently understood. The pathophysiology of diarrhoea is complex and likely to involve a number of mechanisms (Engelking *et al.* 1998; Viele 2003). The different types of diarrhoea related to cancer and cancer therapy include secretory, osmotic, malabsorptive, exudative and dysmotile (Engelking et al. 1998), although infectious diarrhoea, inflammatory diarrhoea and steattorhea may also be related.

Irinotecan hydrochloride (CPT-11) is a relatively new cytotoxic agent used to treat a variety of solid tumours. The primary mechanism of action is to inhibit DNA topoisomerase I (Araki *et al.* 1993; Ikuno *et al.* 1995; Takasuna *et al.* 1996; Cao *et al.* 1998). Irinotecan is converted by hepatic or gastrointestinal carboxylesterases to its active metabolite, SN-38, which is responsible for irreversible DNA damage (Alimonti *et al.*

2004) and has been implicated in the onset of diarrhoea. SN-38 is further processed to become SN-38 glucuronide, a less toxic form of SN-38 (Takasuna et al. 1996). However, SN-38 glucuronide is able to be enzymatically cleaved by β-glucuronidase to return it to the toxic form, SN-38 (Takasuna et al. 1996). A number of the intestinal microflora have β-glucuronidase activity and may be responsible in part for the intestinal cytotoxicity of irinotecan (Takasuna et al. 1996). Bacterial β-glucuronidase is produced primarily by Enterobacteriaceae (*E. coli*, *Salmonella spp.*, *Shigella spp.*, *Yersinia spp.*, *Citrobacter spp.*, *Hafnia spp.* and *Edwardia spp.*), and has been reported to be produced by *Flavobacterium spp.*, *Bacteroides spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Corynebacterium spp.* and *Clostridium spp.* (Tryland and Fiksdal 1998).

The microflora of the gastrointestinal tract (GIT) is a highly complex ecosystem consisting of both aerobic and anaerobic bacteria (Simon and Gorbach 1982; Simon and Gorbach 1984; Simon and Gorbach 1986). These microflora have a number of key functions including protection, as well as metabolism of bilirubin, intestinal mucins, pancreatic enzymes, fatty acids, bile acids, cholesterol and steroid hormones (Gustafsson 1982). Furthermore gastrointestinal bacteria function to process nutrients, regulate intestinal angiogenesis, and work with the immune system (Umesaki and Setoyama 2000; Rhee *et al.* 2004).

The aim of this study was to determine if changes in the luminal environment, specifically changes in gastrointestinal flora (especially those genera which are known to produce β-

glucuronidase and may interfere with the processing of irinotecan) coincided with subsequent diarrhoea in rats treated with irinotecan.

3.2 Materials and Methods

3.2.1 Animals

Animals used in this study were female Dark Agouti (DA) rats, weighing between 150 and 170 g. Rats were housed in Perspex cages at a temperature of 22±1˚C and subject to a 14 hour light/10 hour dark cycle. Approval for the use of animals was granted by the Animal Ethics Committees of the Institute of Medical and Veterinary Science (IMVS), 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). Due to the nature of the diarrhoea induced by irinotecan, animals were monitored 4 x daily and if any animal showed certain criteria (as defined by the Animal Ethics Committees) they were euthanised. These criteria included a dull ruffled coat with accompanying dull and sunken eyes, cold to touch with no spontaneous movement and a hunched appearance.

3.2.2 Experimental plan

Eighty-one rats were randomly assigned to groups. For each time point there was one group of six (6) rats receiving irinotecan and one group of three (3) control rats receiving no treatment. Rats receiving irinotecan received 0.01mg/kg subcutaneous atropine (to reduce cholinergic reaction to irinotecan) prior to (within 2 min) administration of 200 mg/kg intraperitoneal irinotecan. Irinotecan (kindly supplied by Pfizer, Kalamazoo, USA)

was administered in a sorbitol/lactic acid buffer (45mg/mL sorbitol/0.9mg/mL lactic acid, pH 3.4), required for activation of the drug, at time designated 0 h. Groups of rats were killed using 3% halothane in 100% O_2 anaesthesia and cervical dislocation at times 30 and 60 min, 2, 6, 12, 24, 48 and 72 h post-treatment. Immediately prior to anaesthesia, faecal samples were aseptically collected by directly collecting the excreted faeces immediately as it left the rat in a sterile container in an area cleaned with 70% ethanol. Samples were stored at -70˚C. Prior to cervical dislocation, a blood sample was collected via cardiac puncture. The GIT (from the pyloric sphincter to the rectum) was dissected out and separated into the small intestine (pyloric sphincter to ileocaecal sphincter) and colon (ascending colon to rectum). The small intestine was flushed with chilled, sterile distilled water, and 1 cm samples taken at approximately 50% of the length, were collected for culture and routine histology. The colon was also flushed with chilled sterile, distilled water and contents were collected for electrolyte analysis. Two 1 cm samples of colon, taken at approximately 50% of the length were collected for culture and histology. The stomach was dissected from the rat and contents emptied and discarded. Two small pieces (1cm x 0.5cm) of stomach were collected for culture and histology. All samples collected for culture were stored at -70°C until required, and those for routine histological examination were fixed in 10% neutral buffered formalin

3.2.3 Diarrhoea assessment

All animals were checked four times daily and diarrhoea recorded according to previous gradings (Gibson et al. 2003; Gibson et al. 2005). This was graded as 0, no diarrhoea; 1, mild diarrhoea (staining of anus); 2, moderate diarrhoea (staining over top of legs and lower abdomen) and; 3, severe diarrhoea (staining over legs and higher abdomen, often with continual anal leakage). All diarrhoea assessments were conducted in a blinded fashion by two investigators (A.M.S. and R.M.L.)

3.2.4 Electrolyte analysis

Blood and colonic flush samples were centrifuged (Haereus, Finland) at 3000 rpm for 5 min. The serum from the blood samples and supernatant from the flush samples were collected into fresh tubes and analysed by the Department of Clinical Pathology at the Institute of Medical and Veterinary Science (IMVS), Adelaide, South Australia. Measurements for sodium, potassium, bicarbonate, chloride and osmolarity were measured from serum samples, and sodium and potassium only were measured from the colonic flush samples.

3.2.5 Histological examination

Samples of stomach, jejunum and colon were collected and fixed in 10% neutral buffered formalin for routine histological examination. Fixed samples were processed and embedded in paraffin. Sections of 4μm thickness were cut and mounted on glass slides.

Routine haematoxylin and eosin (H&E) staining was performed. Briefly, the wax was dissolved with xylene and sections rehydrated before staining in Lillie-Mayer's haematoxylin for 10 min. After differentiating in 1% acid alcohol and bluing in Scott's tap water, sections were counterstained in eosin, dehydrated, cleared and mounted, and then examined using light microscopy and reported on by a professional veterinary pathologist.

3.2.6 Culture of samples

To determine the flora of the Dark Agouti rat, a variety of selective and non-selective media (Oxoid, Adelaide, Australia) were used in an attempt to identify as many bacteria as possible from the GIT. For full details of this technique, please refer to ***2.2.6 Culture of samples*** (page 42) and **Table 2.1** (page 44).

3.3 Results

3.3.1 Diarrhoea

Diarrhoea was observed in 23% of treated rats from 2 h after treatment (Figure 3.1). Mild diarrhoea was seen in 23% of experimental rats at 2-6 h and at 12 h 30% of rats had mild and 5% of rats had moderate diarrhoea. This increased to 39% of rats having mild and 12% having moderate diarrhoea at 24 h. At 48 h 20% of rats had mild diarrhoea. Late onset diarrhoea was apparent at 72 h after treatment with 33% of treated rats having mild diarrhoea. No control rats had diarrhoea at any time point investigated.

3.3.2 Electrolyte Analysis

Electrolyte levels were altered in rats treated with irinotecan. Serum sodium, chloride and bicarbonate levels were lower in the experimental rats compared with control rats. The anion gap was higher in experimental rats than control rats, and serum potassium levels were also higher in experimental rats (Figure 3.2).

Faecal sodium levels were similar for both experimental and control rats for the early time points. However, between 12-48 h there was a peak in faecal sodium levels in experimental rats. Faecal potassium levels between experimental and control rats were similar until 72 h, where experimental levels began to increase (Figure 3.3). The serum osmolarity levels (Figure 3.4) were lower in experimental rats that control rats, with the largest difference at 6-24 h.

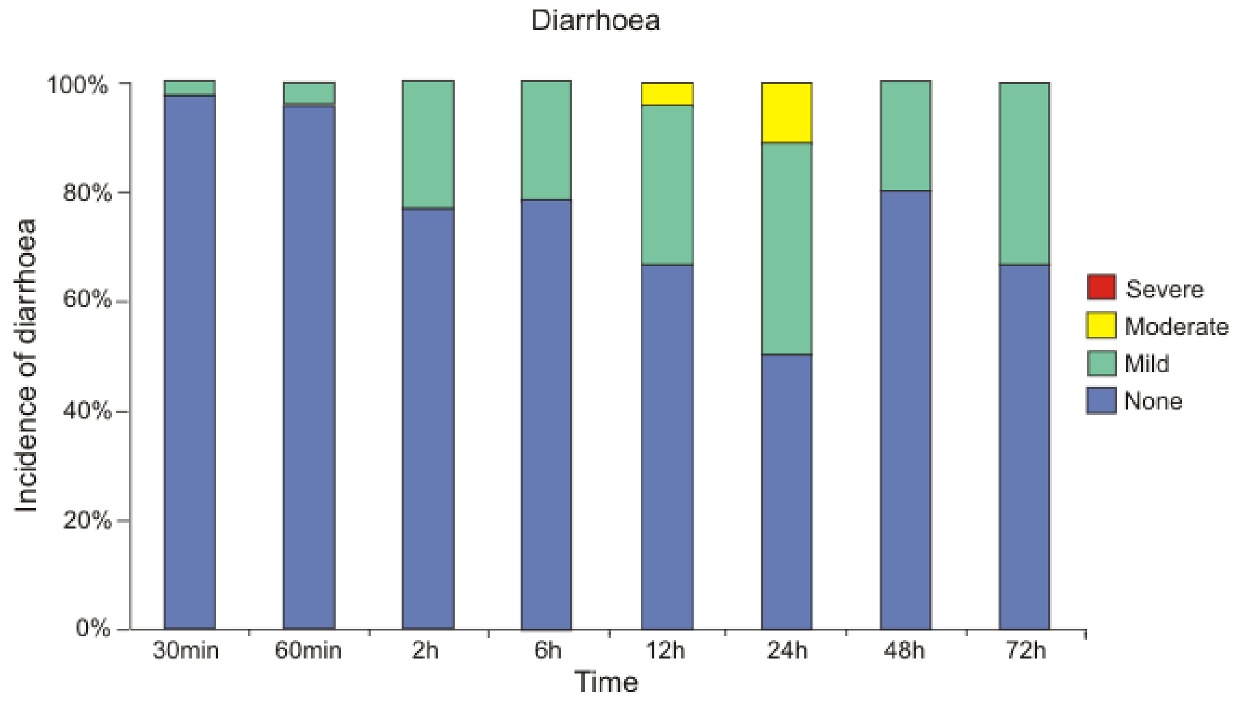


Figure 3.1 Incidence of diarrhoea

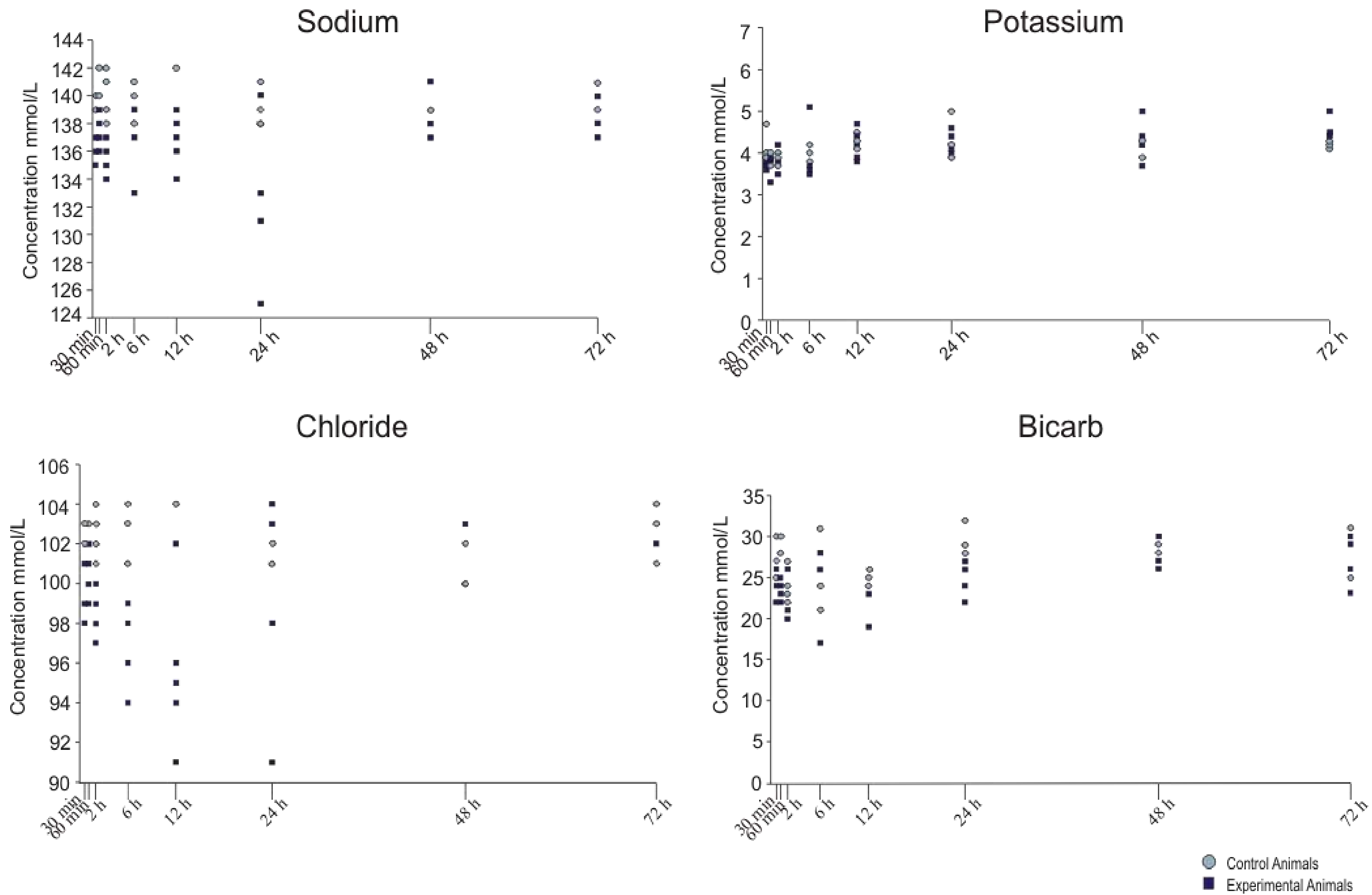


Figure 3.2 Serum electrolytes. Sodium and chloride levels were reduced early in rats treated with Irinotecan. Bicarbonate levels were reduced at later time points after treatment. Potassium levels increased after treatment with Irinotecan.

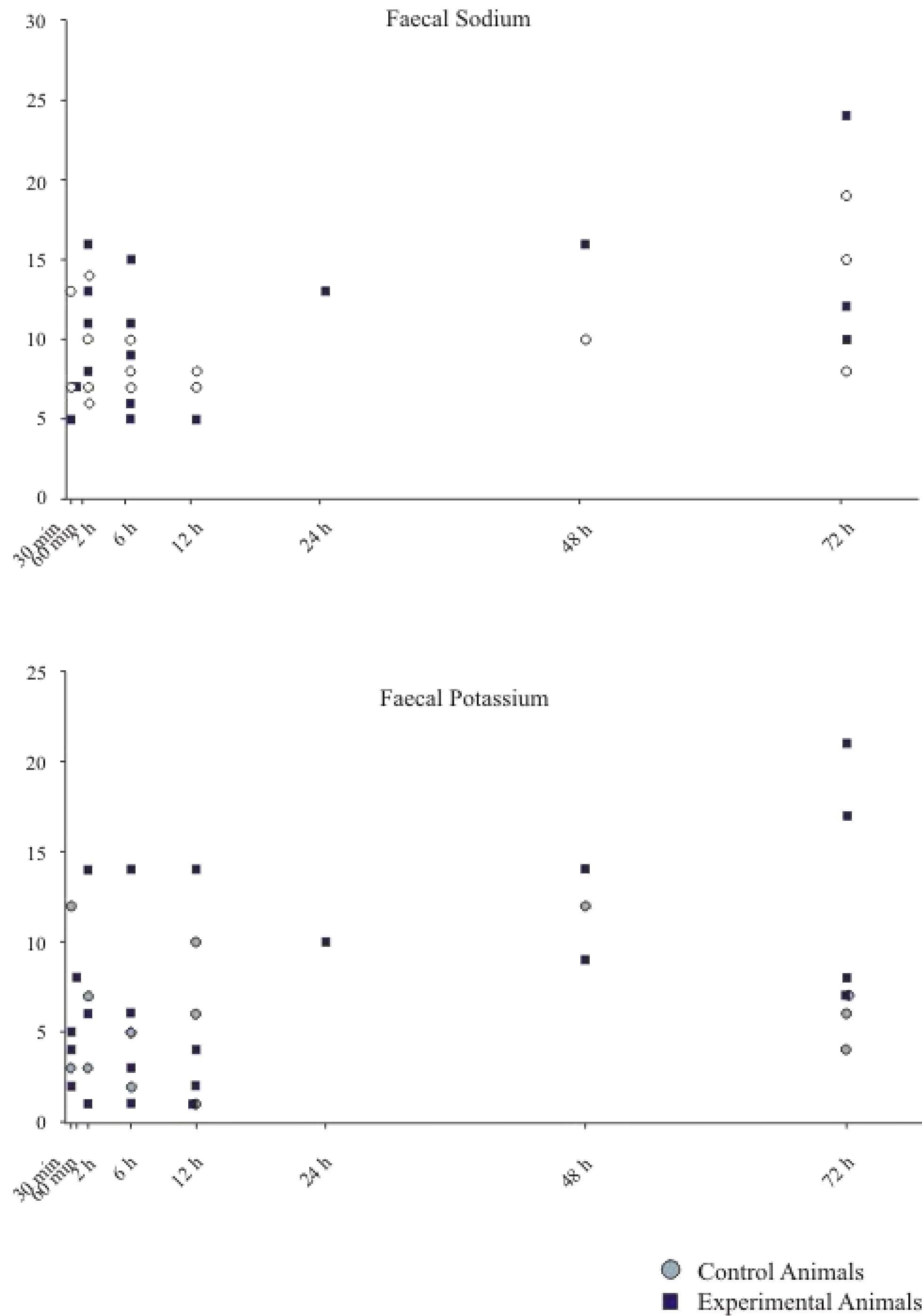


Figure 3.3 Faecal electrolytes. Sodium levels increased at 24h and 48h in rats.

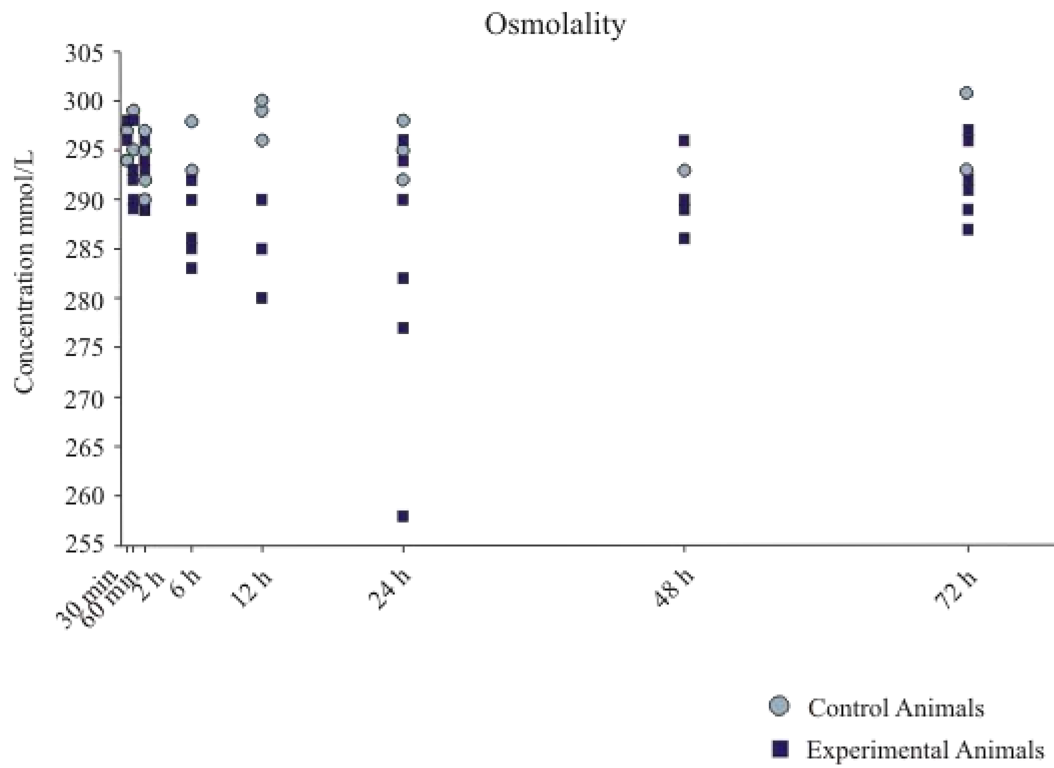


Figure 3.4 Serum osmolality levels were lower in rats treated with irinotecan. The largest difference is seen between 6-24 h.

3.3.3 Histology

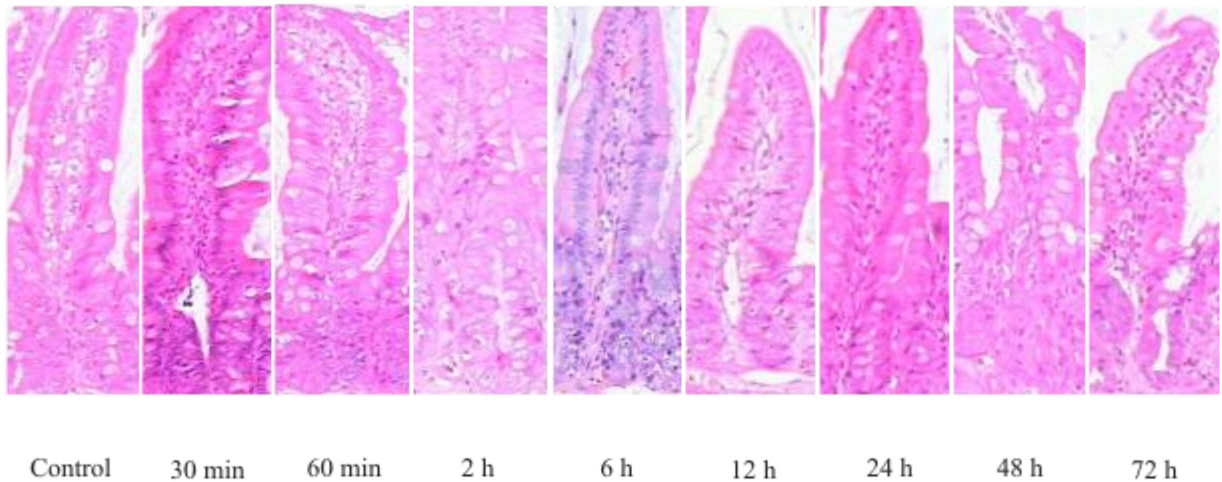
Pathological changes caused by irinotecan were seen in the colon, jejunum (Figures 3.5) and stomach (not shown). There were no histological changes in control rats or in experimental rats between 30 min and 2 h post-chemotherapy. At 6 h, changes in the colon included patchy, but widely distributed apoptosis of enterocytes, especially in the mid and basal regions of the crypts. Dilated crypt lumina were lined with attenuated epithelium and occasional debris in the lumen was observed at 48 h and 72 h (more severe at 72 h) with condensation of the stroma also observed at 72h.

The jejunum showed no histological changes in control rats, or in experimental rats between 30 min and 60 min post-chemotherapy. Between 2 h and 72h, patchy widespread apoptosis of basal crypt enterocytes was observed. Extensive damage was observed from 2 h. However, the most severe histological changes were seen at 6 h following chemotherapy. The severity of changes decreased from 12 h to 72 h.

The stomach showed no histological changes in control rats, before 2 h, and after 24 h post-chemotherapy. At 2 h, patchy but widely distributed apoptosis of individual basal glandular epithelial cells was seen, with slight inflammation. The affected cells were more numerous and superficially disposed at 6 h, and at 12 h glandular epithelial degeneration was patchy and very limited.

3.3.4 Culture

Changes were seen in the flora of the stomach, jejunum, colon, and faeces of rats treated with chemotherapy. The majority of these changes were observed at 6, 12 and 24 h after treatment. The organisms identified were consistent with the expected gastrointestinal microflora population of rats.



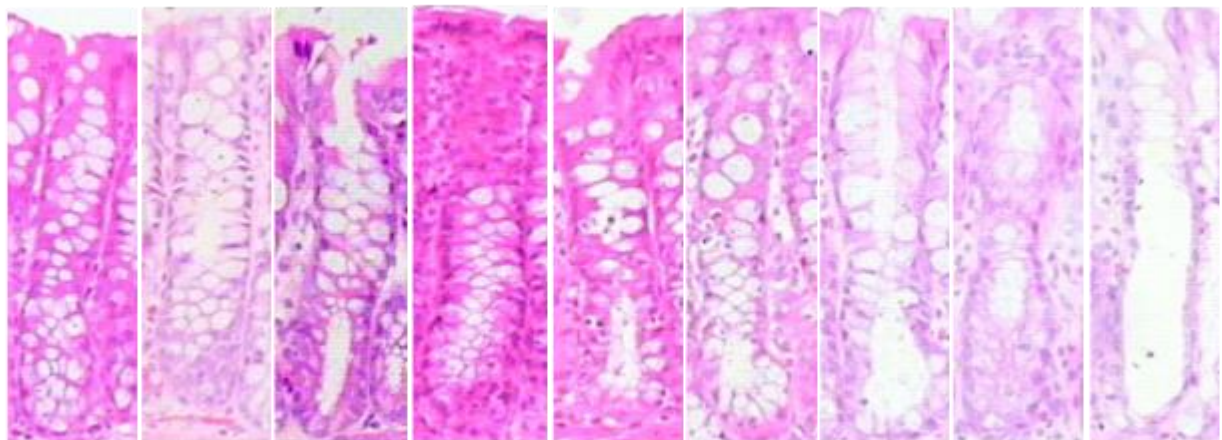


Figure 3.5 Histopathology. Histopathological changes are seen in the jejunum and colon following treatment with Irinotecan. Changes begin to be evident at 6 h after treatment in the jejunum, with apoptosis in the crypt cells. At 48 h the villi become blunted and there is a loss of normal architecture. In the colon there are also apoptotic cells at 6 h and by 72 h there is complete crypt ablation.

3.3.4.1 Stomach

The stomach mucosal surface of all rats was found to harbour copious numbers of *Lactobacillus spp.*, *Enterococcus spp.* and *Staphylococcus spp.* Changes in levels of bacteria following chemotherapy were seen (Figure 3.6). *Enterococcus spp.* levels peaked at 2-6 h. *Peptostreptococcus spp.* levels peaked 30-60 min after the administration of Irinotecan. *Serratia spp.* decreased early, but peaked at 2 h. *Staphylococcus spp.* fluctuated considerably, with a peak observed at 12 h.

3.3.4.2 Jejunum

The jejunal mucosal surface of all rats was found to accommodate relatively small numbers of organisms compared with the stomach, colon and faeces. The most prominent were *Enterococcus spp.*, *Lactobacillus spp.*, *Staphylococcus spp.* and *Serratia spp.* Changes were seen in bacterial levels following chemotherapy (Figure 3.7). There was a peak at 2 h in Clostridium spp., Enterococcus spp. and Lactobacillus spp. There was also a peak in *Serratia spp.* at 2 h. *Peptostreptococcus spp.* levels decreased at 2 h. *Bifidobacterium spp.* and *Prevotella spp.* were detected only between 2-12 h, whereas *Pseudomonas spp.* was not detected after 2 h, and Proteus spp. was undetected from 2-48 h.

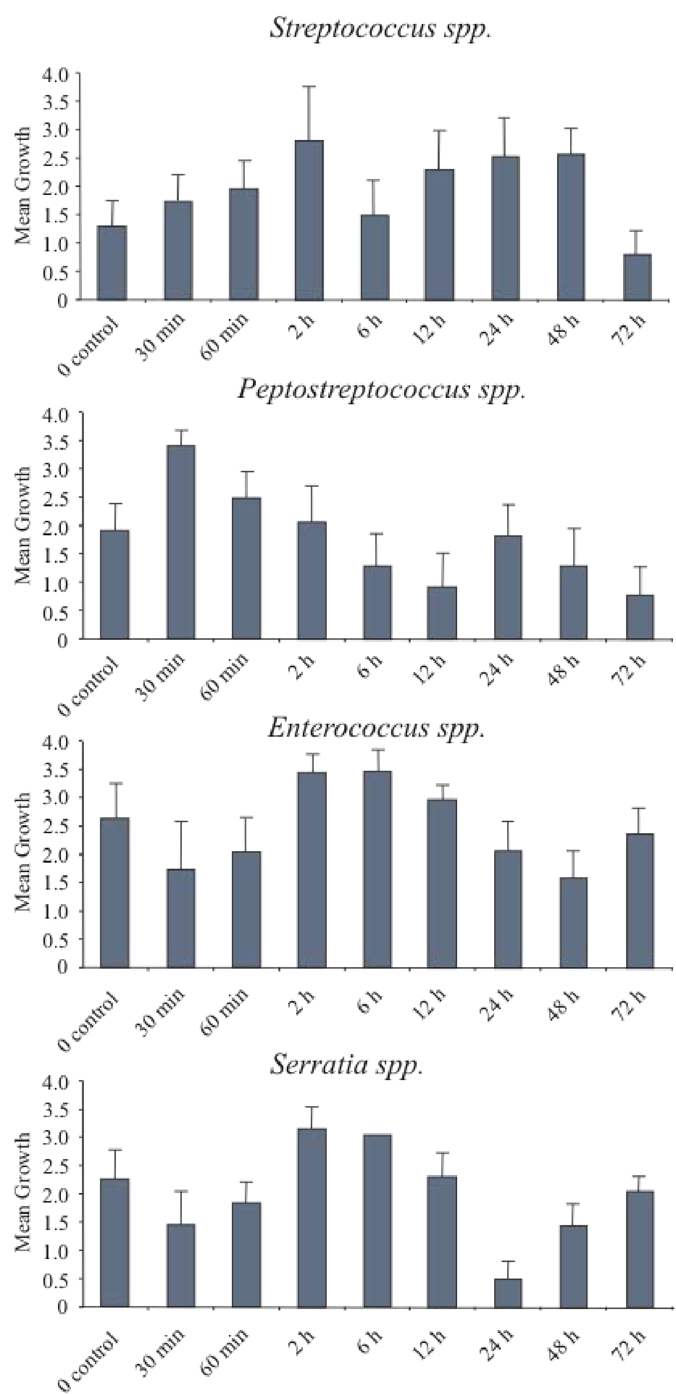


Figure 3.6 Bacterial changes in the stomach after treatment with irinotecan.

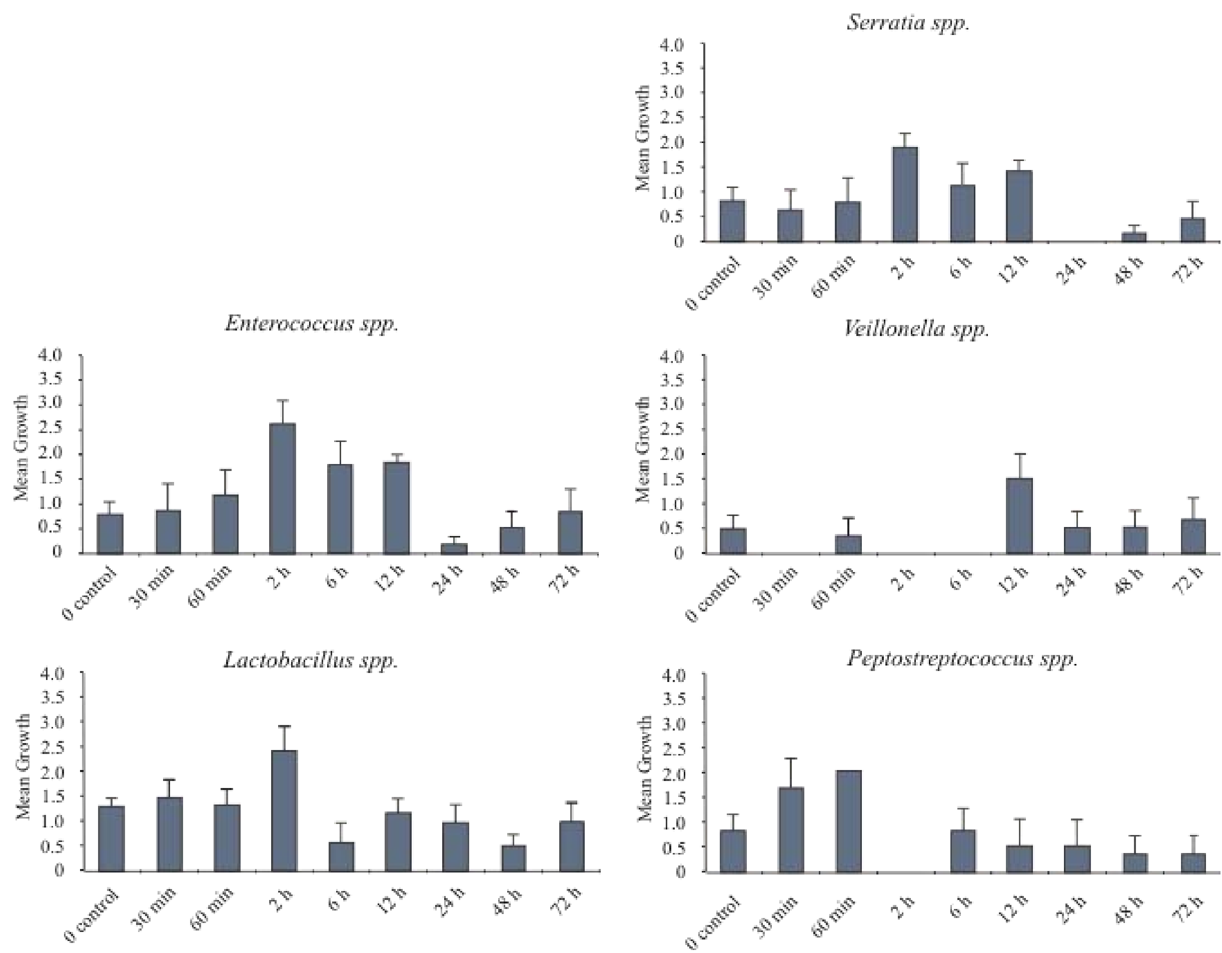


Figure 3.7 Bacterial changes in the jejunum following treatment with irinotecan.

3.3.4.3 Colon

The mucosal surface of the colon in all rats was found to accommodate a large number of organisms. The most prominent in the control rats were *Enterococcus spp*. and *Lactobacillus spp*. Changes were seen in the bacterial levels following chemotherapy (Figure 3.8). There was an increase in the levels of *Escherichia spp*. between 6-24 h. *Clostridium spp*. showed an increase at 2 h, *Enterococcus spp*. increased at 6 h, and Serratia *spp*. increased between 60 min-24 h. *Staphylococcus spp*. increased at 60 min, and again at 48 h. *Peptostreptococcus spp*. levels increased early, at 30-60 min. *Veillonella spp*. were undetected at 30 min, and 2-6 h. *Lactobacillus spp*. increased slightly over time. *Bacillus spp*. increased at 6 h. *Proteus spp*. and *Streptococcus spp*. were both unable to be detected at 2 h.

3.3.4.4 Faeces

In all rats, the bacterial content of the faeces was found to be higher than the colon, jejunum or stomach. The most prominent bacteria were *Lactobacillus spp.*, *Enterococcus* spp*.,* Escherichia spp*.,* Proteus spp*.,* Serratia spp*., and* Staphylococcus spp*.* Changes were detected in the faecal flora following chemotherapy (Figure 3.9). Proteus spp. levels were highest at 24-72 h. Bacillus spp. were undetected at 12 h and 72 h. Bifidobacterium spp. were also undetected at 60 min, and 48-72 h. Clostridium spp. were undetected at 60 min, but reached their highest level at 2 h. Peptostreptococcus spp. levels were highest at 30-60

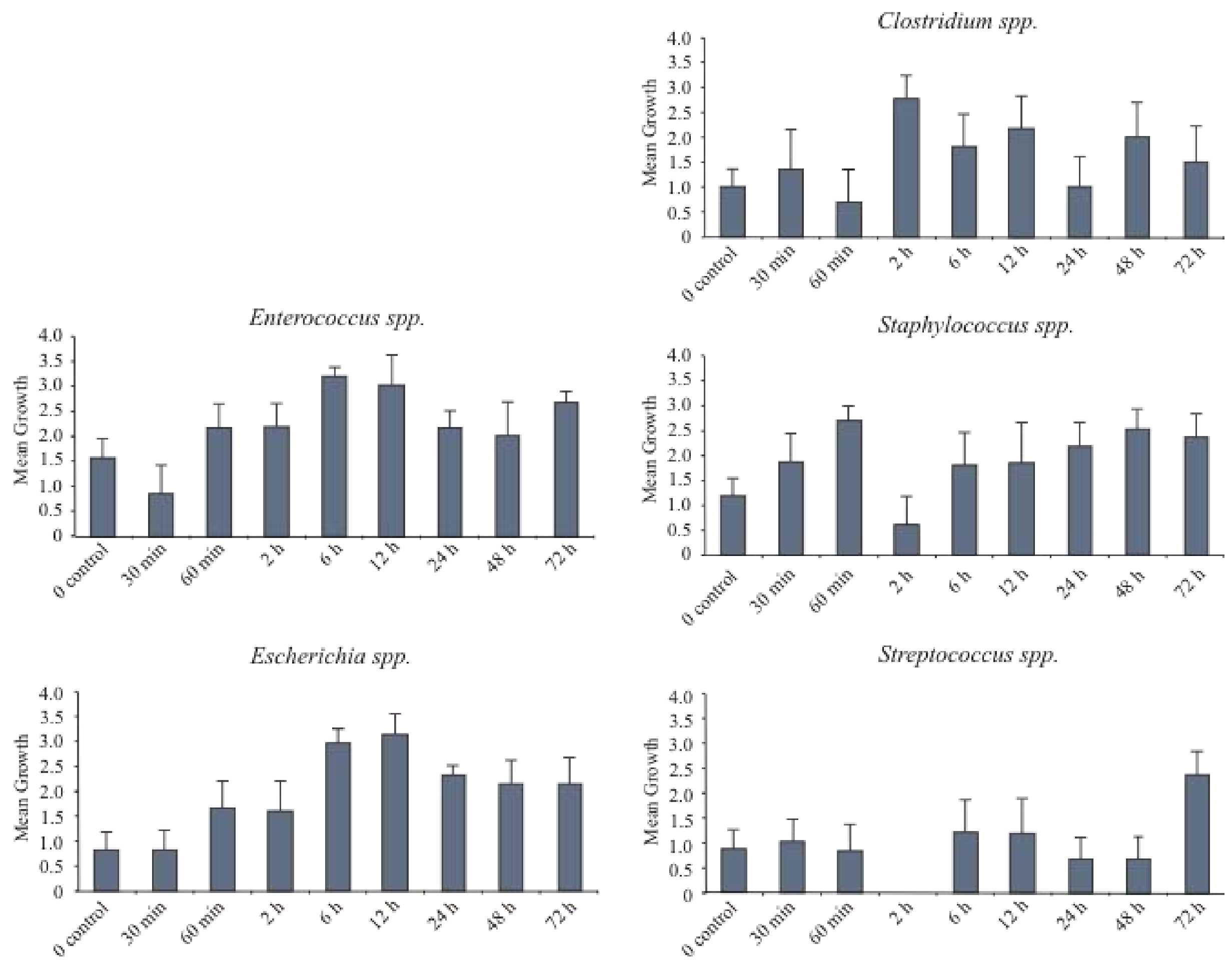


Figure 3.8 Bacterial changes in the colon after treatment with irinotecan.

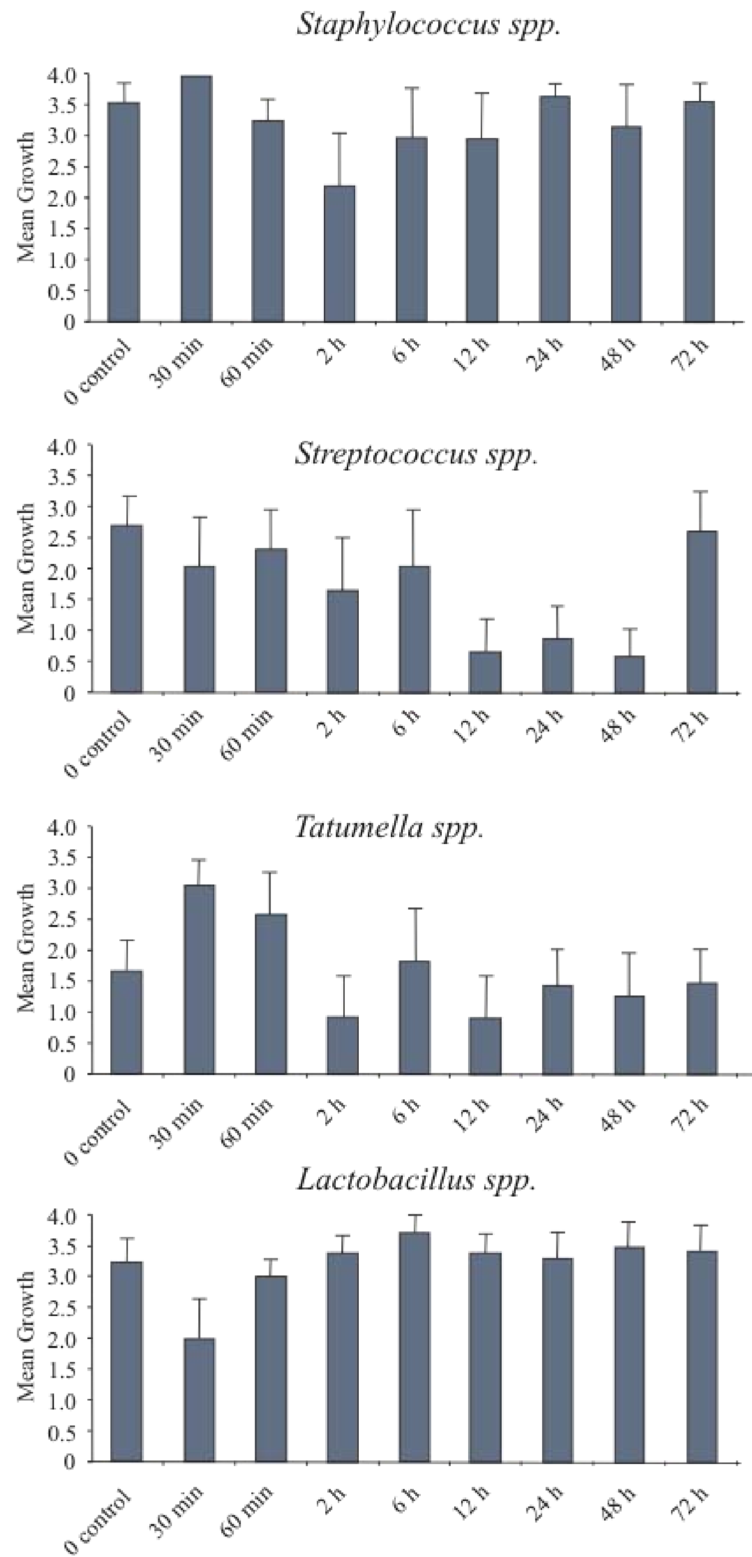


Figure 3.9 Bacterial changes to faeces after treatment with irinotecan.

min. *Escherichia spp*. levels fluctuated substantially, and *Lactobacillus spp*. levels reached a low point at 30 min, prior to returning to constant levels for the remaining timepoints. *Enterobacter spp.* reached a small peak at 2 h. *Veillonella spp.* were unable to be detected following chemotherapy until 12 h. *Actinobacillus spp*. were detected in control rats, but were not able to be detected in any of the treated rats.

3.4 Discussion

Several possible mechanisms have been proposed for the development of irinotecan-induced diarrhoea. These range from changes in the architecture and absorption rates (Araki et al. 1993; Ikuno et al. 1995) of the large intestine, to increases in intestinal β-glucuronidase levels due to changes in intestinal bacteria (Takasuna et al. 1996). The present study was unique in that it investigated changes in gastrointestinal architecture and correlated these changes with changes in gastrointestinal flora and faecal and serum electrolyte levels. Previous studies have also shown epithelial proliferation to be altered and apoptosis to be increased (Gibson et al. 2003; Gibson et al. 2005). A key finding of this study was that changes to the gastrointestinal microflora following irinotecan treatment can occur as early as 30 min after treatment. Previous research has shown that cytotoxic radiotherapy induces p53 (Martin *et al.* 2000) within 2 h and cytotoxic chemotherapy induces p53 within 40 mins (Ishizuka *et al.* 2003). It is therefore not surprising that chemotherapy also alters gastrointestinal microflora rapidly, and may be having a direct effect on the bacteria.

The present study showed that changes occur in the composition of the gastrointestinal microflora after treatment with irinotecan. Changes differed between regions of the GIT, with some of the larger changes occurring in bacteria of the colon. This is suggestive of some relationship between these changes and the incidence of diarrhoea. It is likely that the changes in bacteria may be a result of the altered ecology of the luminal environment

throughout the entire GIT, and severe damage to the gastrointestinal architecture. Irinotecan may alter pH levels in the stomach, allowing certain types of bacteria to proliferate or inhibit the growth of others, depending on the bacterial tolerance of acidic conditions (Simon and Gorbach 1982). Oxygen levels in the lumen of the GIT may also be altered, which may promote or inhibit different bacteria (Simon and Gorbach 1986). However, there is no evidence at this point in time to indicate which of these events actually occurs first, or evidence to confirm which events are direct and/or indirect results of irinotecan.

Bacteria lining the stomach mucosal surface were shown to change over time after chemotherapy, the key changes being an increase in *Enterococcus spp*. at 2-6 h, an increase in *Peptostreptococcus spp*. at 30-60 min, and an increase in *Serratia spp*. at 2 h. The microflora bound to the mucosa of the stomach (and protected from the acidic environment) are likely to be increased due to the architecture (and protective functions) of the mucosa being compromised by irinotecan at these time points. Mucosal-bound microflora of the jejunum are likely to be increased due to decreased motility, architectural damage and greater overflow of bacteria from the stomach. This is demonstrated by an increase in *Enterococcus spp*., *Lactobacillus spp*., *Clostridium spp*. and *Serratia spp*. at 2 h (similar to the stomach). These findings correlate with the severe architectural changes observed in the jejunum, especially at 2-12 h. Previous studies (also using 200mg/kg irinotecan in DA rats) have shown a peak in apoptosis in the jejunum at 6

h, as well as villus and crypt hypoplasia in the jejunum at 24 h (Gibson et al. 2003; Gibson et al. 2005).

This study has also demonstrated that a number of the microflora of the colon increase following treatment with irinotecan. Extensive histological changes were observed in the colon at 6 h (also time of onset of cholinergic diarrhoea), and from 48-72 h (time of onset for diarrhoea). This coincides with changes seen in previous studies (Gibson et al. 2003; Gibson et al. 2005) where 200mg/kg irinotecan in DA rats causes a peak in apoptosis in the colon at 6 h. The luminal environment in the colon is usually of a reduced nature, allowing the domination of anaerobes (Evaldson *et al.* 1982). An increase in oxygen levels allows the proliferation of aerobic bacteria, in particular those producing β-glucuronidase, including *Escherichia spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, and *Clostridium spp.* (Takasuna et al. 1996) Lactic acid bacteria such as *Lactobacillus spp.* have been suggested to inhibit β-glucuronidase activity (Sreekumar and Hosono 2000). *Lactobacillus spp.* also increased, but only slightly. The changed luminal environment of the GIT results in altered absorption and other functions (Geibel 2005). Changes to absorption are likely to alter the composition of the faecal excrement, including the composition of microflora present. Not all of the faecal microflora producing β-glucuronidase increase after chemotherapy. The information from this study adds to other studies, such as one by Takasuna and colleagues. They suggested the importance of the intestinal microflora in chemotherapy-induced mucositis and characteristic late-onset diarrhoea of irinotecan,

looking at β-glucuronidase activity during the time of diarrhoea (Takasuna et al. 1996). Our study looks at earlier time points', adding more information to what is known to date.

Changes to the luminal environment, and subsequent changes to absorption are the likely cause of altered electrolyte levels after chemotherapy treatment. Faecal sodium levels change significantly after irinotecan treatment, as do faecal potassium levels, with both peaking at 24 h, when diarrhoea incidence is also at its highest level. Water follows the electrolytes into the lumen in an attempt to restore balance, resulting in diarrhoea. Serum sodium, chloride and osmolarity levels are significantly altered. Treated rats have lower sodium levels than control rats for the duration of the experiment; osmolarity levels are also lower, reaching the lowest level at 24 h, coinciding with the diarrhoea incidence, and chloride levels are significantly lower, reaching the lowest level at 12 h, when diarrhoea incidence is also high.

In conclusion, irinotecan treatment causes changes in the flora of the stomach, jejunum, colon and faeces of rats, which coincides with the development of diarrhoea. These changes in flora may have systemic effects, and in particular may contribute to the development of chemotherapy-induced mucositis. The luminal environment is also altered by irinotecan, and as a result may favour different genera of bacteria, allowing them to proliferate. The bacteria producing β-glucuronidase are among those that increase, resulting in SN-38G being converted back to SN-38 at an increased rate, causing significant damage and causing diarrhoea. Absorptive function in the intestines is

decreased, increasing faecal sodium and electrolyte levels, which may contribute to the irinotecan induced diarrhoea. Further studies are being undertaken to quantify the changes in gastrointestinal microflora, and investigate other consequences of a changed luminal environment.

4.0 Faecal microflora and β-glucuronidase expression are altered in an irinotecan-induced diarrhoea model in rats

4.1 Introduction

Chemotherapy-induced diarrhoea (CID) is a well recognised side effect of cancer treatment (Saltz *et al.* 1996; Engelking *et al.* 1998; Wadler *et al.* 1998; Gwede 2003; Viele 2003; Benson *et al.* 2004), and can be accompanied by blood, mucus and abdominal pain (Gibson and Keefe 2006). Very little research has been conducted into the causes of CID with the majority of information available based on clinical observations with very little basic science existing. However, the pathophysiology behind CID is extensive, complex and likely to be the result of a number of mechanisms (Engelking *et al.* 1998; Gwede 2003; Viele 2003).

Irinotecan is a relatively new cytotoxic agent utilised in the treatment of solid tumours, the primary action being to inhibit DNA topoisomerase I (Araki *et al.* 1993; Ikuno *et al.* 1995; Takasuna *et al.* 1996; Takasuna *et al.* 2006). Severe diarrhoea is a dose-limiting side effect in 60-80% of patients receiving irinotecan (Fittkau *et al.* 2004). Cholinergic, secretory diarrhoea occurs early, although this can be managed by blocking neurons containing acetyl choline (ACh) in the enteric nervous system with atropine. Delayed severe diarrhoea also occurs, which is one of the main dose-limiting side effects of irinotecan treatment (Gibson *et al.* 2003). To date the mechanism underlying this delayed

diarrhoea remains unclear, although it has been reported that intestinal bacteria may play a role in its development. Leukopaenia is another dose-limiting side effect, often resulting in opportunistic infection (Ikuno *et al.* 1995; Takasuna *et al.* 1996).

Irinotecan is converted by hepatic or gastrointestinal carboxylesterases to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), responsible for irreversible DNA damage, and stronger antitumour activity (Takasuna *et al.* 1996; Alimonti *et al.* 2004). SN-38 is subsequently conjugated in the liver by glucuronyltransferase to SN-38 glucuronide (SN-38G), a less toxic metabolite, and is excreted into the gastrointestinal tract via bile. SN-38G is susceptible to hydrolysis by β-glucuronidase to return to SN-38, increasing the presence of SN-38 in the gastrointestinal tract, further contributing to toxicity (Takasuna *et al.* 1996; Takasuna *et al.* 2006). Irinotecan and SN-38 bind to the topoisomerase I-DNA complex, leading to double strand (ds) breakage and cell death.

Intestinal microflora have β-glucuronidase activity and may be responsible in part for the intestinal toxicity of irinotecan (Takasuna *et al.* 1996; Brandi *et al.* 2006; Takasuna *et al.* 2006). Enterobacteriaeceae (*Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Yersinia spp.*, *Citrobacter spp.*, *Hafnia spp.* and *Edwardsiella spp.*) are primary producers of β-glucuronidase, and *Flavobacterium spp.*, *Bacteroides spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Corynebacterium spp.* and *Clostridium spp.* have also been reported to produce β-glucuronidase (Tryland and Fiksdal 1998). Previous research has shown some of these bacteria are altered in the gastrointestinal tract (GIT) after treatment with

Irinotecan (Stringer *et al.* 2007). Of particular interest in this study are E. coli, Bacteroides spp., Staphylococcus spp., and Clostridium spp., all reported to produce β-glucuronidase. Also of interest are Lactobacillus spp., suggested to inhibit β-glucuronidase (Sreekumar and Hosono 2000), and Bifidobacterium spp., reported to be beneficial and have protective properties towards the gut mucosal barrier (Kleessen and Blaut 2005).

The purpose of this study was to quantify bacteria known to produce or inhibit β-glucuronidase at various intervals after treatment with Irinotecan; to investigate the expression of β-glucuronidase in the GIT; and to determine if bacteria known to colonise the GIT are susceptible to irinotecan.

4.2 Materials and Methods

4.2.1 Animals and Experimental plan

The animals used in this study were the same animals from **Chapter 3.0**. For details of animal conditions and experimental plan refer to ***3.2.1 Animals*** and **3.2.2 Experimental plan** (page 70).

4.2.2 Diarrhoea assessment

All animals were checked four times daily and diarrhoea recorded according to previous gradings (Gibson et al. 2003; Gibson *et al.* 2005). This was graded as 0, no diarrhoea; 1, mild diarrhoea (staining of anus); 2, moderate diarrhoea (staining over top of legs and lower abdomen) and; 3, severe diarrhoea (staining over legs and higher abdomen, often with continual anal leakage). All diarrhoea assessments were conducted in a blinded fashion by two investigators (A.M.S. and R.M.L.).

4.2.3 Immunohistochemistry

Sections were cut from paraffin blocks at 4μm and mounted onto silane-coated glass slides. Sections were dewaxed in xylene and brought to water via a graded series of alcohols. Antigen retrieval was carried out using 10 mmol/L citrate buffer (pH 6.0) and heat, with sections subjected to microwave (mw) irradiation in a domestic microwave with a carousel, microwaved on HIGH (930W) until boiling, followed by LOW (650W) for 10 min. Endogenous peroxidases were blocked with 3% H_2O_2 in methanol. Non-

specific antibody binding was blocked with 20% normal goat serum (NGS) (Sigma) in PBS applied at room temperature (RT) for 30 mins.

Sections were incubated with primary antibody, goat anti-*E. coli* β-Glucuronidase (Molecular Probes, USA) diluted in 5% NGS at 4°C overnight. Sections were incubated sequentially with biotinylated goat anti-rabbit immunoglobulin (IgG) antibody (Vector Laboratories, USA) and 5μL/mL Vectastain ABC kit solution (Vector Laboratories., USA). For visualisation of bound antibodies, sections were incubated with 3'3-diaminobenzidine (DAB) (Zymed) for up to 5 mins, or until a brown precipitate appeared on positive control sections. Nuclei were counterstained with haematoxylin. Sections were examined using light microscopy. Negative controls were carried out by incubating with 5% NGS instead of the primary antibody solution.

Qualitative assessment of sections was carried out by scoring the intensity of staining as follows: 0, negative; 1, weak; 2, moderate; 3, strong; 4, very intense. This qualitative assessment of staining has previously been validated by published grading system (Krajewski *et al.* 1996; Krajewska *et al.* 1997; Krajewska *et al.* 2002; Bowen *et al.* 2005; Yeoh *et al.* 2005).

4.2.4 Real Time PCR

4.2.4.1 DNA extraction from bacteria

DNA was extracted from standard bacterial samples using a DNeasy Tissue Mini Kit (Qiagen). For details refer to *2.2.7.1 DNA extraction from bacteria* (page 46).

4.2.4.2 DNA extraction from samples

DNA was extracted from rat faecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Doncaster, Australia). For details refer to *2.2.8.2 DNA Extraction from samples* (page 46).

4.2.4.3 Real-time PCR

Real-time PCR was carried out on a Corbett Rotorgene 3000 rotary cycler (Corbett Research, USA). Primers used in this study have been used previously (Edwards *et al.* 2001; Matsuki *et al.* 2002; Matsuki *et al.* 2004; Rinttila *et al.* 2004; Sakai *et al.* 2004; Penders *et al.* 2005; Layton *et al.* 2006) (Table 2.2, page 47). For details refer to ***2.2.8 Real time PCR*** (page 48).

4.2.5 Bacterial susceptibility

Bacterial susceptibility to irinotecan was determined using a standard antibiotic susceptibility testing method (Bodet *et al.* 1985). Briefly, serial dilutions of irinotecan were diluted in sorbitol/lactic acid buffer (45mg/mL sorbitol/0.9mg/mL lactic acid, pH 3.4), required for activation of the drug, and 10µL was put onto blank discs (Oxoid). Sensitest Agar plates were used for *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus pneumoniae* and *Staphylococcus epidermidis*. Anaerobic agar (Oxoid) was used for *Bacteroides fragilis*, *Bifidobacterium lactis* and *Lactobacillus acidophilus*. Discs were placed onto a lawn culture of each bacterium, incubated overnight at 37°C, and zones of inhibition were measured. All experiments were carried out in triplicate.

4.2.6 Statistical analysis

Results were statistically analysed using the Mann-Whitney U test. For adjustments for multiplicity, the significance levels of the P values were determined according to the Bonferroni correction.

4.3 Results

4.3.1 Diarrhoea

Early onset diarrhoea was observed in 23% of treated rats 2 h after treatment (Figure 4.1). Mild diarrhoea was seen in 23% of experimental rats between 2-6 h and by 12 h 30% of rats had mild diarrhoea and 5% of rats had moderate diarrhoea. At 24 h this had increased to 39% of rats having mild diarrhoea, and 12% having moderate diarrhoea. At 48 h 20% of rats had mild diarrhoea. Late onset diarrhoea was apparent 72 h after treatment with 33% of treated rats having mild diarrhoea. No control rats had diarrhoea at any time point investigated.

4.3.2 Immunohistochemistry

β-glucuronidase was expressed at a consistently low level in the gastrointestinal tract of control rats, with staining seen in the cytoplasm of enterocytes (Figure 4.2). In the stomach, β-glucuronidase expression did not alter after irinotecan treatment (data not shown). However, in the jejunum, β-glucuronidase expression increased from 2 h in the villi, with the largest increase seen at 72 h. Furthermore increased expression was seen in the jejunal crypts at both 24 h and 72 h after irinotecan treatment (Figure 4.3). In the colon, β-glucuronidase expression increased moderately 12-24 h after irinotecan treatment. β-glucuronidase expression had also increased considerably by 72 h after irinotecan treatment (Figure 4.3).

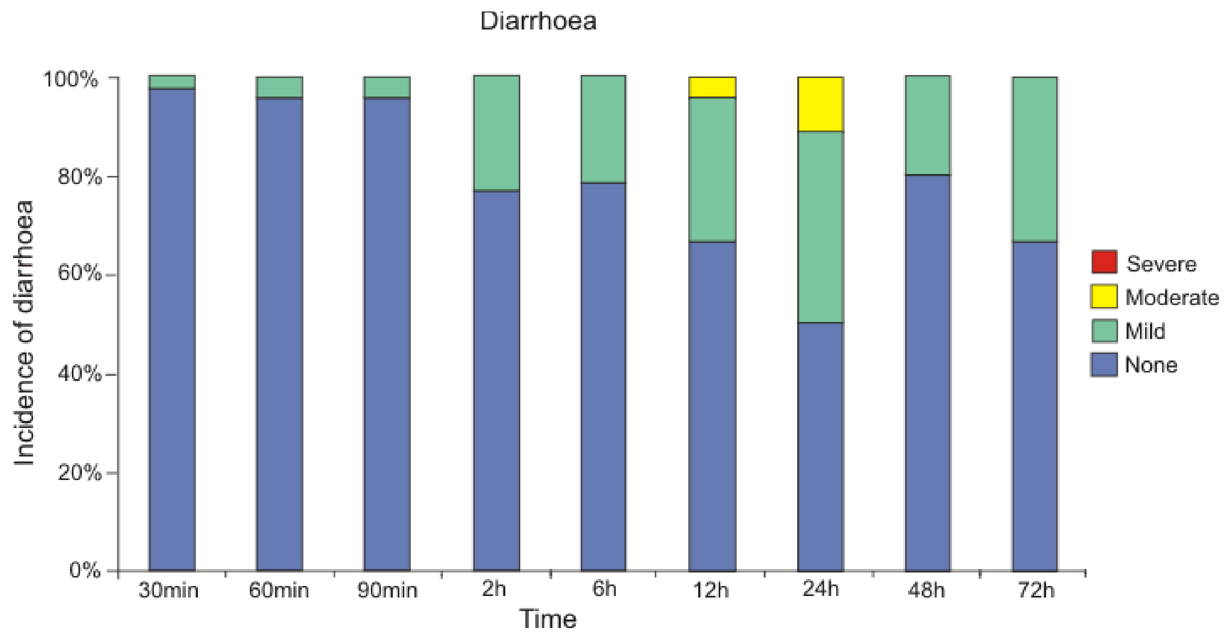


Figure 4.1 Incidence of diarrhoea.

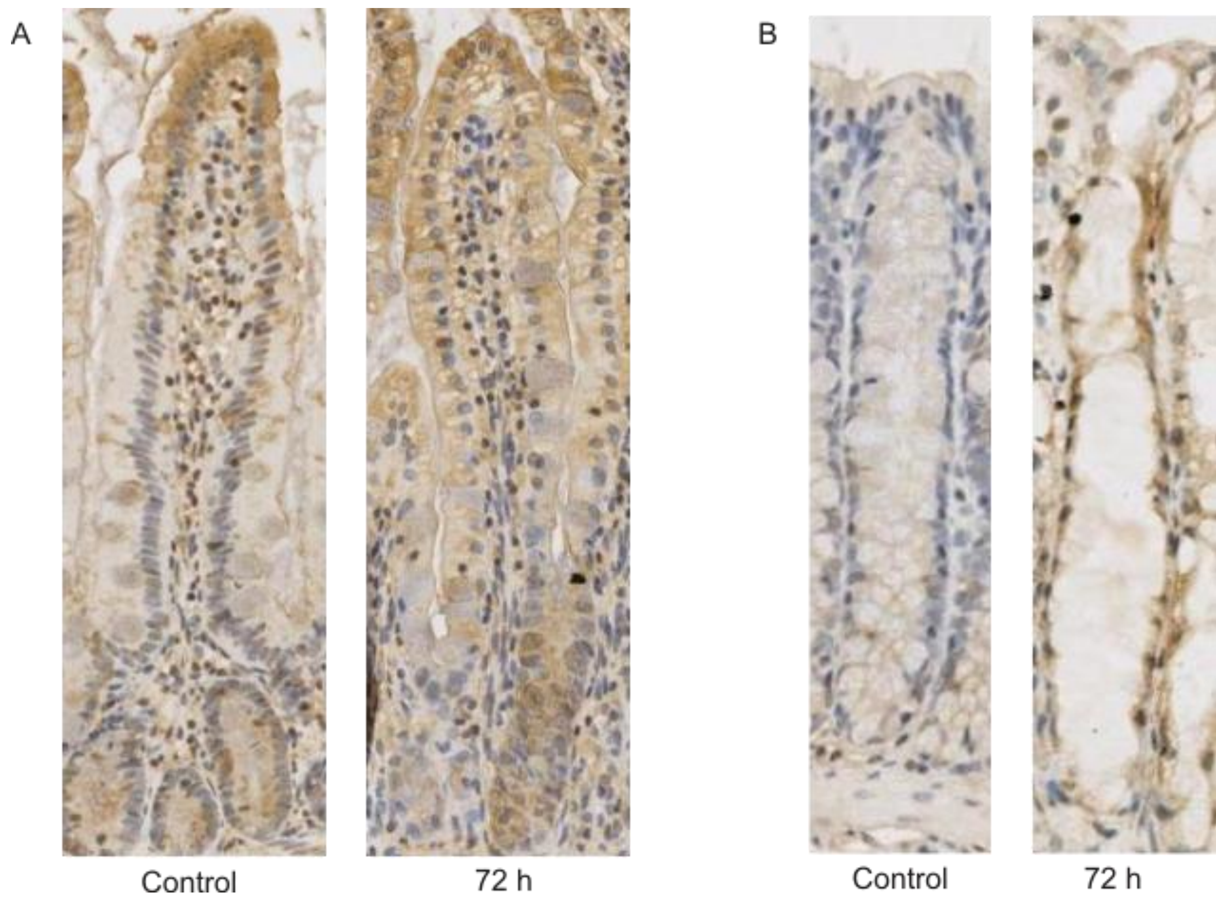


Figure 4.2 β-glucuronidase immunohistochemistry. Staining of A) Jejunum and B) Colon in both control and treated rats 72 h after treatment.

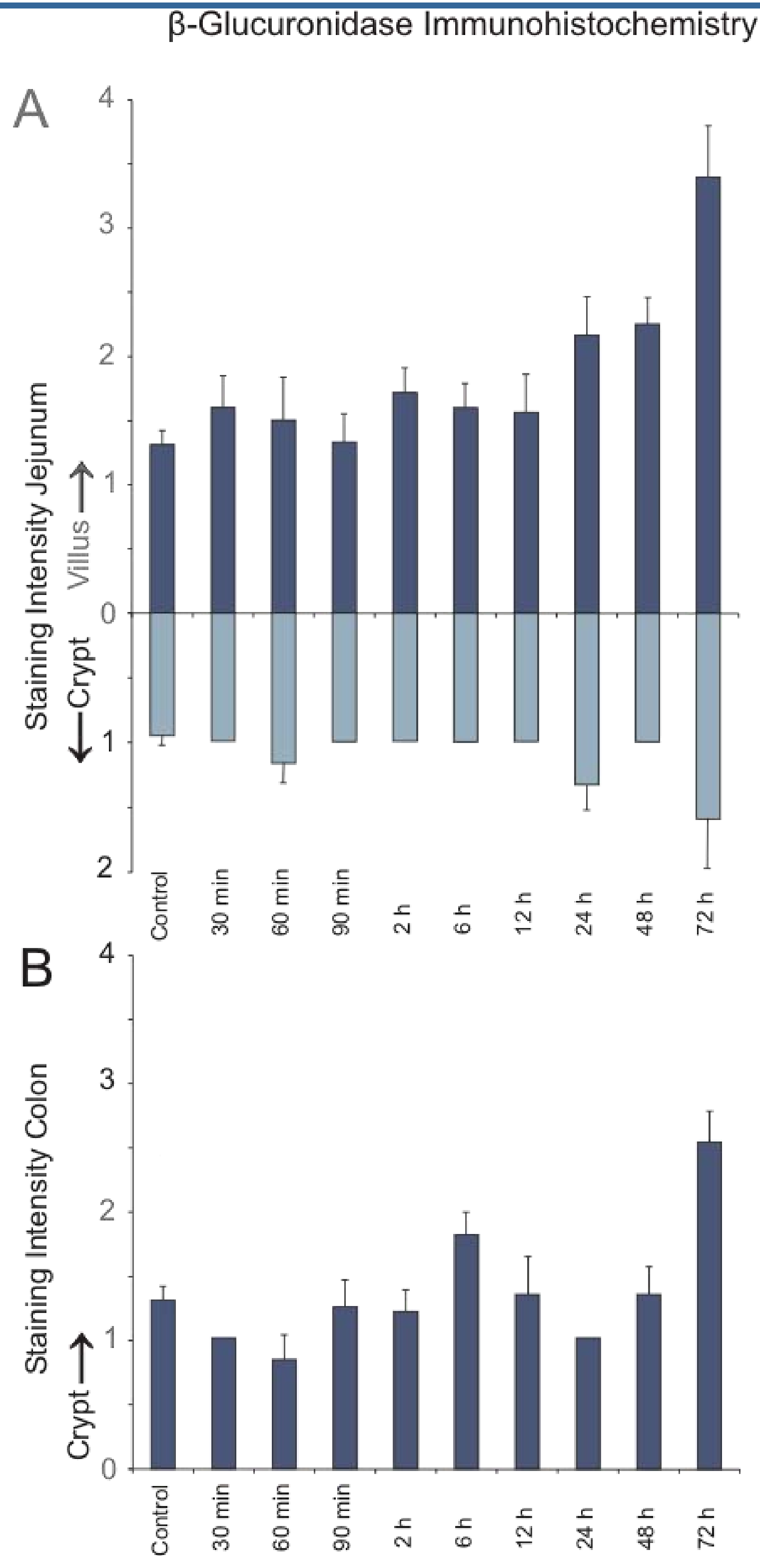


Figure 4.3 β-glucuronidase immunohistochemistry grading of intensity of staining. A: Jejunum villi and crypts. B: colon crypts. Results are expressed as mean (± standard deviation [SD]).

4.3.3 Real-time PCR

Real-time PCR assays for 6 bacterial genera known to either be 'beneficial' to the GIT, or produce β-glucuronidase were used to quantify bacteria in rat faecal samples using a standard curve technique. Of the β-glucuronidase-producing bacteria, *Bacteroides spp.* decreased from 6-24 h, and at 72 h, *Staphylococcus spp.* increased from 2-12 h, *Clostridium spp.* increased at 48 h, and *E. coli* increased from 24-48 h. Of the 'beneficial' bacteria, *Lactobacillus spp.* decreased from 12-48 h, and *Bifidobacterium spp.* was decreased at all times points after irinotecan treatment (figure 4). Statistical analysis showed significance ($p<0.05$) between groups mean for *E. coli*, *Staphylcoccus spp.* and *Lactobacillus spp.*

4.3.4 Bacterial susceptibility to Irinotecan

Irinotecan was found to have no inhibitory effects on the tested bacteria. No zones of inhibition were observed at any concentration of the drug (data not shown).

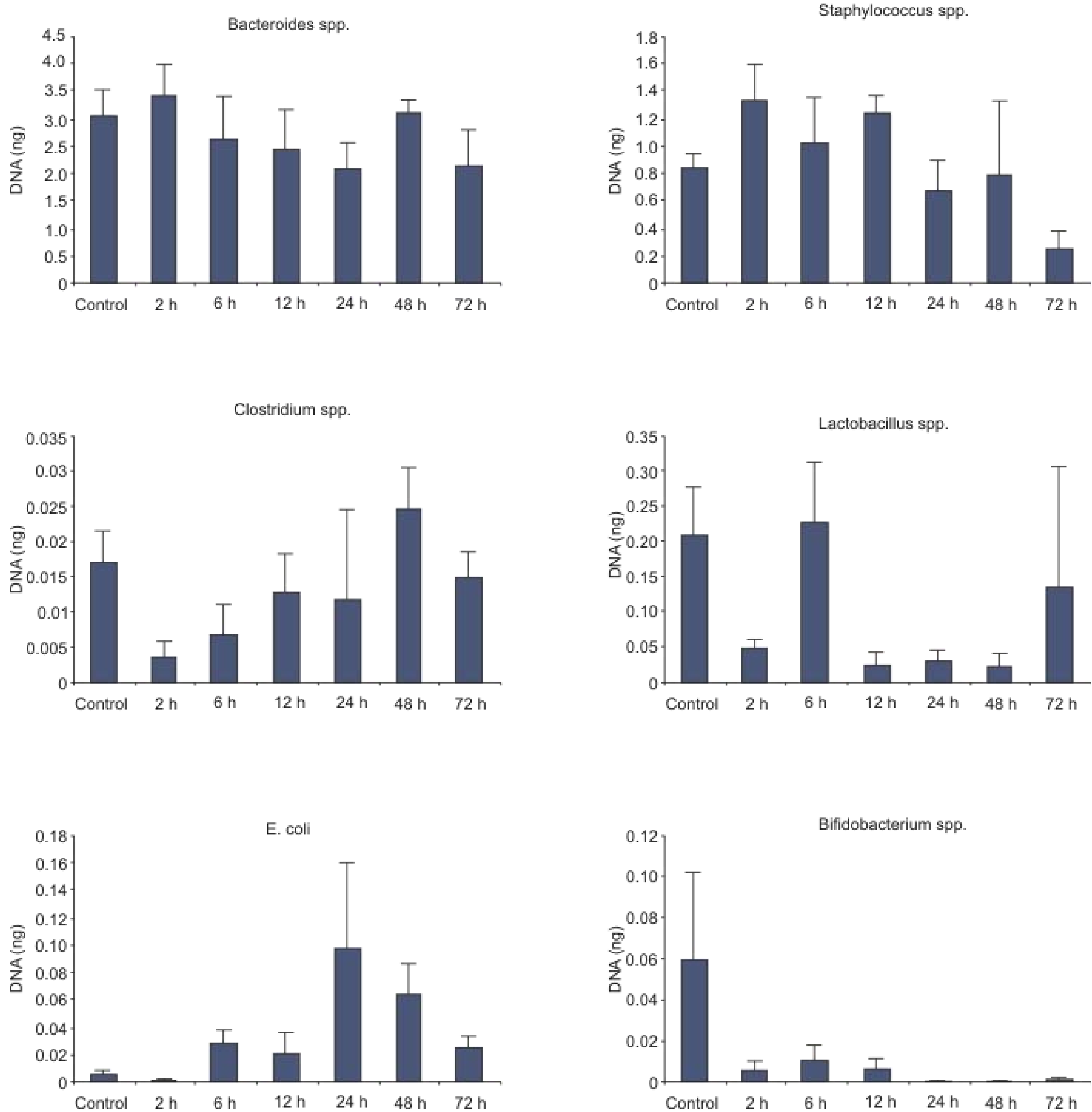


Figure 4.4 Quantification of bacterial DNA in faecal samples using real time PCR. Results are expressed as mean (± SD).

4.4 Discussion

Irinotecan-induced mucositis manifesting as diarrhoea is a severe dose-limiting side effect, with large cost to health services, and is often life-threatening (Elting *et al.* 2007) . Several mechanisms have been proposed for the development of irinotecan-induced diarrhoea, ranging from changes in the architecture and absorption rates of the large intestine (Araki et al. 1993; Ikuno et al. 1995), to increases in intestinal β-glucuronidase levels caused by changes in intestinal bacteria (Takasuna et al. 1996; Takasuna et al. 2006). The present study has, for the first time, quantified bacterial changes in rats following irinotecan treatment. It also shows there are significant differences in the levels of bacteria in rats receiving chemotherapy vs. chemotherapy-naïve rats in faecal samples. Furthermore, this study has been able to compare the incidence of diarrhoea and β-glucuronidase expression in enterocytes, and changes in the faecal flora of rats, in particular those with β-glucuronidase activity (*E. coli*, *Staphylococcus spp.*, *Bacteroides spp.*, *Clostridium spp.* (Takasuna et al. 1996)), those that have been suggested to inhibit β-glucuronidase activity (*Lactobacillus spp.* (Sreekumar and Hosono 2000)), and those that have general beneficial effects to the intestine (*Bifidobacterium spp.* (Kleessen and Blaut 2005)).

Previously, intestinal microflora have been shown to be altered after treatment with irinotecan in rats using qualitative culture methods (Stringer et al. 2007). This study extends this research by confirming these changes with quantitative real time PCR. A significant increase in *E. coli* (a β-glucuronidase producing bacterium) correlates directly

with the onset of diarrhoea. Only one group of β-glucuronidase producing bacteria (*Bacteroides spp.*) decreased after treatment, although this group of bacteria encompasses the largest proportion of the intestinal microflora under normal conditions (Gorbach 1971; Evaldson *et al.* 1982; Simon and Gorbach 1982; Simon and Gorbach 1984). The other β-glucuronidase producing bacteria comprise a smaller proportion of the intestinal microflora under normal conditions (Gorbach 1971; Evaldson et al. 1982; Simon and Gorbach 1982; Simon and Gorbach 1984). Interestingly, the beneficial bacteria tested were decreased at this time, reducing the protective effects of the microflora, and decreasing colonisation resistance. The previously suggested inhibitory effects of *Lactobacillus spp.* against β-glucuronidase (Sreekumar and Hosono 2000) are also reduced, suggesting the increase in β-glucuronidase activity may be multifactorial.

The expression of β-glucuronidase was increased in the jejunum, with expression in the villi seen from 2 h after treatment, with the largest increase seen at 72 h, when late onset diarrhoea became evident. Increased expression was also seen in the crypts, with an increase at 24 h and 72 h, occurring when both the early (cholinergic) and late onset diarrhoea was evident. Increased β-glucuronidase expression was also seen in the colon, again with a considerable increase observed 72 h after treatment, again when late onset diarrhoea was evident. Increases in β-glucuronidase producing bacteria were predominantly seen 48 h following treatment, 24 h prior to considerable increases in β-glucuronidase expression in the intestine (72 h), suggesting that the increases in β-

glucuronidase producing bacteria leads to the increased β-glucuronidase expression in the intestine.

These findings are highly significant as they confirm for the first time that some intestinal bacteria and β-glucuronidase expression are increased following irinotecan treatment, resulting in augmented toxicity from β-glucuronidase activity. These results now provide a target for further research into therapeutic developments for treatment and/or prevention of CID. Takasuna and colleagues (Takasuna et al. 2006) have already explored the relationship between antibiotics and the β-glucuronidase inhibitor, baicalin, with mixed results, Further research into tailored antibiotic treatment targeting specific β-glucuronidase producing bacteria such as *E. coli*, *Staphylococcus spp.* and *Clostridium spp.*, should now be conducted. Furthermore probiotics are known to exert beneficial effects to the host when ingested, and therefore could be useful in controlling the intestinal microflora during chemotherapy (Quigley 2007). We have shown that VSL#3 is able to reduce the severity and duration of CID in rats (Bowen *et al.* 2007). Further research into the role that probiotics could play in ameliorating CID should also be explored. The aim now is to determine if the same effects on the microflora are seen in patients in a clinical setting, allowing translation from animal studies to the oncology clinic.

In conclusion, irinotecan-induced diarrhoea may be caused by an increase in some β-glucuronidase producing bacteria. However, this increase may also be caused by irinotecan, further exaggerating the toxicity of the drug, and emphasising the need for

these specific bacteria to be therapeutically targeted for successful treatment regimens to be accomplished.

5.0 Irinotecan-induced mucositis is associated with changes in intestinal mucins.

5.1 Introduction

Irinotecan is used to treat a variety of solid tumours, through the inhibition of DNA topoisomerase I. However, unfortunately it is linked with severe mucositis and diarrhoea (Armand et al. 1995; Ikuno et al. 1995; Takasuna et al. 1996; Gibson et al. 2003; Brandi et al. 2006; Stringer et al. 2007). The metabolism of irinotecan has been described previously (Smith et al. 2006). Irinotecan and SN-38 bind to the topoisomerase I-DNA complex, leading to double strand (ds) breakage and cell death. We have previously shown that irinotecan treatment decreases goblet cells in the large intestine (Gibson et al. 2003). However, mucus production appears to be increased, which may contribute to the development of CID (Gibson et al. 2003). A more recent study has examined the effects of 5-Fluorouracil (5-FU), a chemotherapeutic agent causing mucositis (Saegusa et al. 2007), and demonstrated that the mucin content of the jejunum is altered by 5-FU.

Goblet cells are highly polarised exocrine cells that synthesise and secrete mucins (Specian and Oliver 1991; Robbe et al. 2004). The baseline secretion of mucins consists of the periodic exocytosis of mucin granules (Kurosumi et al. 1981), effecting the slow continual release of mucins, maintaining a mucus blanket (Specian and Oliver 1991), protecting the epithelium from mechanical and chemical stress (Robbe et al. 2004; Smirnov et al. 2004). The mucus layer also allows transport between the luminal

contents and epithelium (Smirnov et al. 2004). The structure of mucins allows the maintenance of the normal intestinal flora, by providing attachment sites for intestinal flora and pathogenic bacteria (Robbe et al. 2004), and simultaneously protecting the mucosa from bacterial overgrowth and/or penetration (Specian and Oliver 1991). Goblet cell differentiation is thought to be regulated by kruppel-like factor (KLF) 4 (previously known as GKLF), an epithelial zinc-finger protein, also implicated in growth arrest and down regulation of cell proliferation (Katz et al. 2002).

Muc genes (Muc1-17) are regulated by cytokines, bacterial products and growth factors (Smirnov et al. 2004). The biosynthesis of mucins is affected by conditions or agents affecting the differentiation of precursor cells into goblet cells, and those that uncouple the process of protein synthesis (such as fasting or malnutrition) (Smirnov et al. 2004). The Muc gene family contains members which are dissimilar, with two structurally and functionally distinct subfamilies: secreted gel-forming mucins (Muc2 and Muc5AC) and transmembrane mucins (Muc1, Muc3, and Muc4). Of particular interest for this study are Muc1, Muc2, Muc3, Muc4 and Muc5AC; this represents at least one transmembrane and one secreted mucin for each region of the GIT.

The aims of this study are to determine if the expression patterns of mucins (Muc1, Muc2, Muc3, Muc4 and Muc5AC), and/or Klf4 alter in the GIT of rats following treatment with irinotecan, as well as to establish if these changes are comparable with changes seen in goblet cells and the incidence of diarrhoea.

5.2 Materials and Methods:

5.2.1 Animals

The animals used in this study were the same animals from **Chapter 3.0**. For details of animal conditions and experimental plan refer to ***3.2.1 Animals*** and ***3.2.2 experimental plan*** (page 70).

5.2.2 Diarrhoea assessment

Diarrhoea was assessed as described previously. For full details refer to ***3.2.3 Diarrhoea assessment*** (page 72).

5.2.3 Mucin Staining

5.2.3.1 Alcian Blue-PAS stain

Alcian Blue-PAS staining was carried out as described previously. For full details refer to ***2.2.5 Alcian Blue-PAS stain*** (page 42).

5.2.3.2 Quantitative Histology

To determine the effect of irinotecan on mucus secretion, goblet cells were counted as described previously. Decreased goblet cells indicated release of mucins from the mucosal surface, and cavitation of mucus cells is indicative of accelerated mucus secretion by compound exocytosis (Barcelo et al. 2000). Therefore, both the number of intact goblet cells and cavitated cells were analysed, according to a method previously described (Barcelo et al. 2000). Briefly, a cavitated cell is recognised by apical

indentation into the intracellular store of mucus granules. Goblet cells and cavitated cells in crypts and villi that were deemed to be greater than 80% complete were counted under high power magnification, with a total of at least 15 villi/crypts per section analysed. Analysis was conducted in a blinded fashion.

5.2.3.3 High Iron Diamine stain

To investigate the distribution of sulphated mucins high iron diamine staining was performed. Sections were dewaxed in xylene and rehydrated through a graded series of alcohols. Sections were stained for 18-24 h in high iron diamine solution (2.4% N,N-dimethyl-meta-phenylenediamine dihydrochloride, 0.4% N,N-dimethyl-para-phenylenediamine dihydrochloride, 2.8% v/v 10% ferric chloride). Following staining, slides were washed in running water for 2 min. Sections were then counterstained with 1% Alcian Blue in 3% glacial acetic acid for 5 min, then washed before being dehydrated, cleared and mounted. Analysis was conducted in a blinded fashion.

5.2.4 Muc3 antisera production

Antisera production for rat Muc3 has been previously described (Khatri et al. 2001). Briefly, a peptide sequence from within the COOH-terminal region of rat Muc3, namely residues 135-148 (LKAQYTPGFDNTLD) (Khatri et al. 2001), was synthesised (Auspep Pty Ltd, Parkville, Australia) and used to develop a polyclonal antibody in a New Zealand rabbit, carried out by the Veterinary Services Division, IMVS, Adelaide, Australia. Antibody activity was detected in a test serum with an ELISA assay, with

pre-bleed serum used as a negative control. Protein G purification was carried out to increase specificity, and aliquots were stored at -20˚C.

5.2.5 Immunohistochemistry

Sections were cut from paraffin blocks at 4μm and mounted onto silane-coated glass slides. Sections were dewaxed in xylene and brought to water via a graded series of alcohols. Antigen retrieval was carried out using 10 mmol/L citrate buffer (pH 6.0) and heat, with sections subjected to microwave (mw) irradiation in a domestic microwave with a carousel, microwaved on HIGH (930W) until boiling, followed by LOW (650W) for 10 min. Endogenous peroxidases were blocked with 3% H_2O_2 in methanol. Non-specific antibody binding was blocked with either 20% normal goat serum (NGS) or normal horse serum (NHS) (Sigma) in PBS applied at room temperature (RT) for 30 min.

Sections were incubated with primary antibody diluted in the corresponding serum (5%) (Table 5.1). Sections were incubated sequentially with secondary antibody (Vector Laboratories, USA) and 5μL/mL Vectastain ABC kit solution (Vector Laboratories, USA). For visualisation of bound antibodies, sections were incubated with 3'3-diaminobenzidine (DAB) (Zymed) for up to 5 min, or until a brown precipitate appeared on positive control sections. Nuclei were counterstained with haematoxylin. Sections were examined using light microscopy. Negative controls were carried out by incubating with 5% serum instead of the primary antibody solution.

Table 5.1: Details of primary antibodies used

Primary Antibody	Blocking Serum	Secondary Antibody	Incubation Conditions	Antibody Concentration	Manufacturer
Anti-Mucin 1	NHS	Horse anti-goat	4°C overnight	2.0 µg/mL	Santa Cruz Biotechnology
Anti-Mucin 2	NGS	Goat anti-rabbit	22°C 1 h	0.3 µg/mL	Santa Cruz Biotechnology
Anti-Mucin 3	NGS	Goat anti-rabbit	4°C overnight	0.01 µg/mL	This study
Anti-Mucin 4	NGS	Goat anti-mouse	4°C overnight	5.0 µg/mL	Zymed Laboratories
Anti-Mucin 5AC	NGS	Goat anti-mouse	4°C overnight	1:100 dilution	Abcam
Anti-KLF 4	NHS	Horse anti-goat	4°C overnight	2.0 µg/mL	R&D Systems

Qualitative assessment of sections was carried out by scoring the intensity of staining as follows: 0, negative; 1, weak; 2, moderate; 3, strong; 4, very intense. This qualitative assessment of staining has previously been validated by published grading system (Krajewski et al. 1996; Krajewska et al. 1997; Krajewska et al. 2002; Bowen et al. 2005; Yeoh et al. 2005). Quantitative assessment of sections was carried out by counting the stained cells in villi/crypts that were deemed to be at least 80% under high power magnification, with at least 15 complete villi/crypts counted per section. Analysis was conducted in a blinded fashion.

5.2.6 Statistical Analysis

Results were statistically analysed using the Mann-Whitney U test. For adjustments for multiplicity, the significance levels of the *P* values were determined according to the Bonferroni correction.

5.3 Results

5.3.1 Diarrhoea

Cholinergic diarrhoea was observed in 23% of treated rats 2 h after treatment, despite treatment with atropine (Figure 5.1). Mild diarrhoea was seen in 23% of experimental rats between 2-6 h and by 12 h 30% of rats had mild diarrhoea and 5% of rats had moderate diarrhoea. At 24 h this had increased to 39% of rats having mild diarrhoea, and 12% having moderate diarrhoea. At 48 h 20% of rats had mild diarrhoea. Late onset diarrhoea was apparent 72 h after treatment with 33% of treated rats having mild diarrhoea. No control rats had diarrhoea at any time point investigated.

5.3.2 Goblet cell composition and distribution

Stained mucins did not significantly change in the stomach after irinotecan treatment. Stained mucins in the jejunum decreased in both the crypts and villi from 90 min after chemotherapy, with few stained mucins present by 48 h (Figure 5.2). Mucins became more sulphated in treated rats. Mucin composition did not alter significantly in the colon after irinotecan. However, high iron diamine staining showed mucins in the colon changed from being predominantly carboxylated (blue/green) to sulphated (brown) (Figure 5.2).

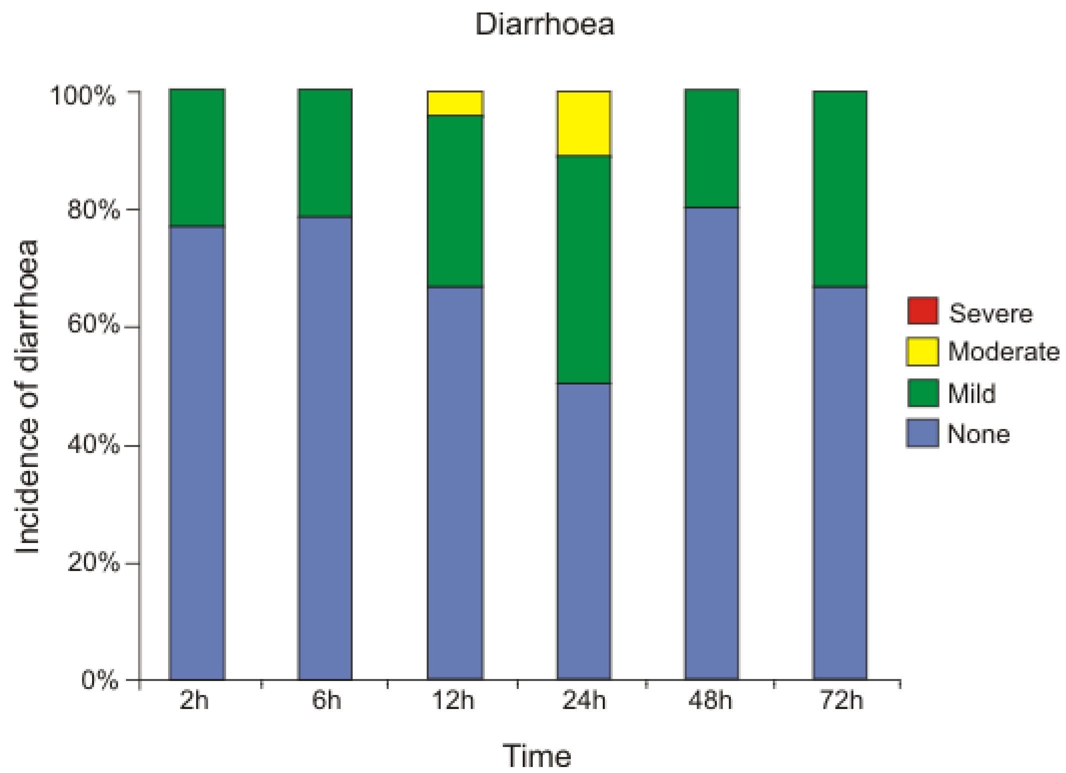


Figure 5.1 Incidence of diarrhoea.

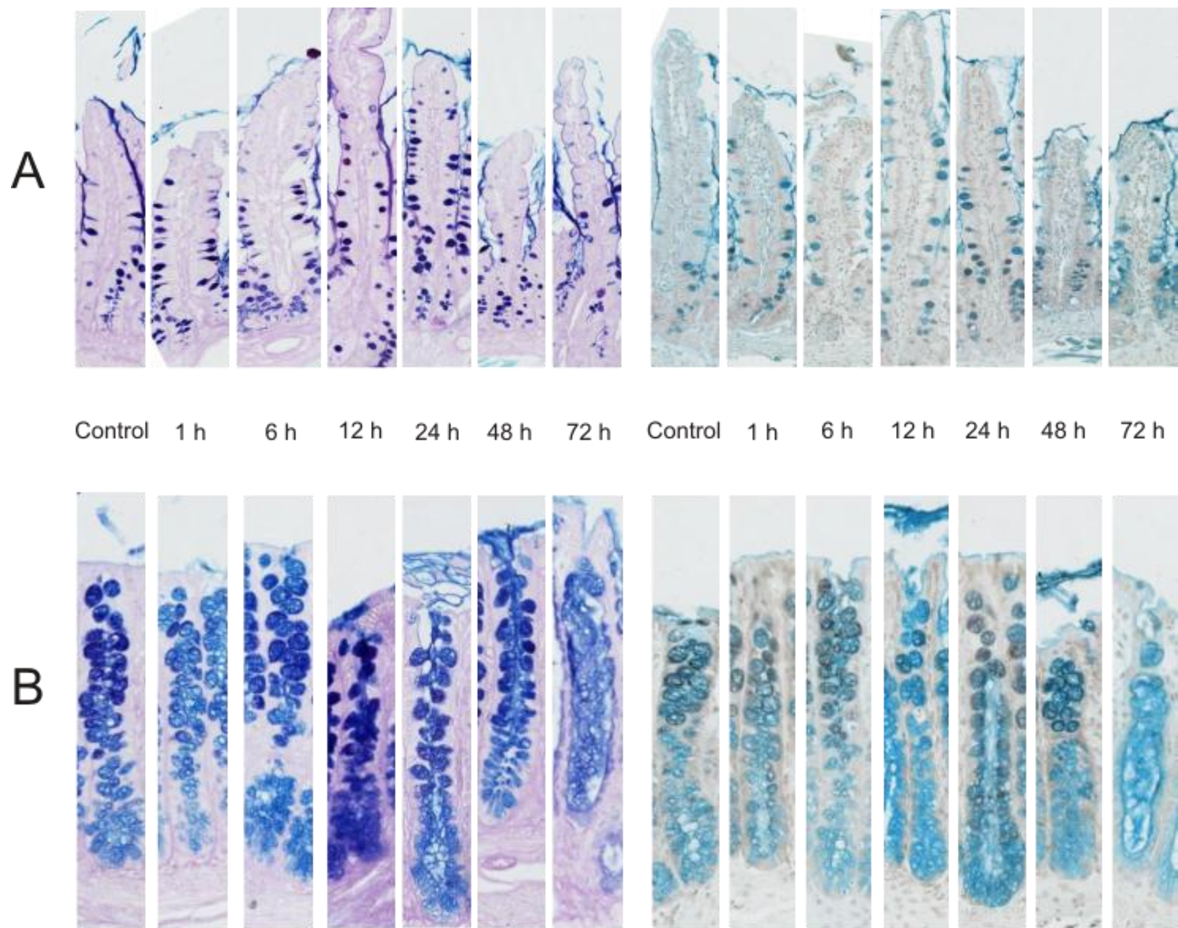


Figure 5.2 Alcian blue-PAS staining and High iron diamine staining of sections of A) jejunum and B) colon.

5.3.3 Effect of irinotecan on mucin discharge

Goblet cell counts in untreated rats showed 9.9 ± 0.3 (mean ± SD) cells/crypt in the jejunum, with 16.7 ± 2.9% deemed to be cavitated, and 13.2 ± 0.8 cells/villus, with 34.3 ± 2.7% deemed to be cavitated. The number of intact goblet cells in the crypt decreased significantly at 90 min ($p<0.004$), 6-12 h ($p<0.003$), and 48 h ($p<0.002$). Cavitated goblet cells increased significantly at 72 h ($p<0.002$). Intact goblet cells in the villus decreased significantly 12 h ($p<0.005$) and 72 h ($p<0.002$) after treatment. Cavitated goblet cell numbers fluctuated after treatment (Figure 5.3).

Total goblet cells in the colon of untreated rats numbered 36.9 ± 1.0 cells per crypt, with 7.9 ± 0.7% cavitated. The total number of intact goblet cells decreased significantly at 72 h after treatment ($p<0.0008$). Cavitated goblet cells increased significantly from 24-72 h ($p<0.005$) (Figure 5.3).

5.3.4 Expression of Mucins

5.3.4.1 Muc1

Muc1 was expressed by mucous cells in the stomach, selected goblet cells in the jejunum and around goblet cells in the colon. There were no differences in expression observed at any time point after chemotherapy in the stomach (data not shown). Differences between group means of counted Muc1 positive cells in the jejunum villi and crypts were not deemed to be significant at any time point following chemotherapy. The colon did not show any significant change in the number of

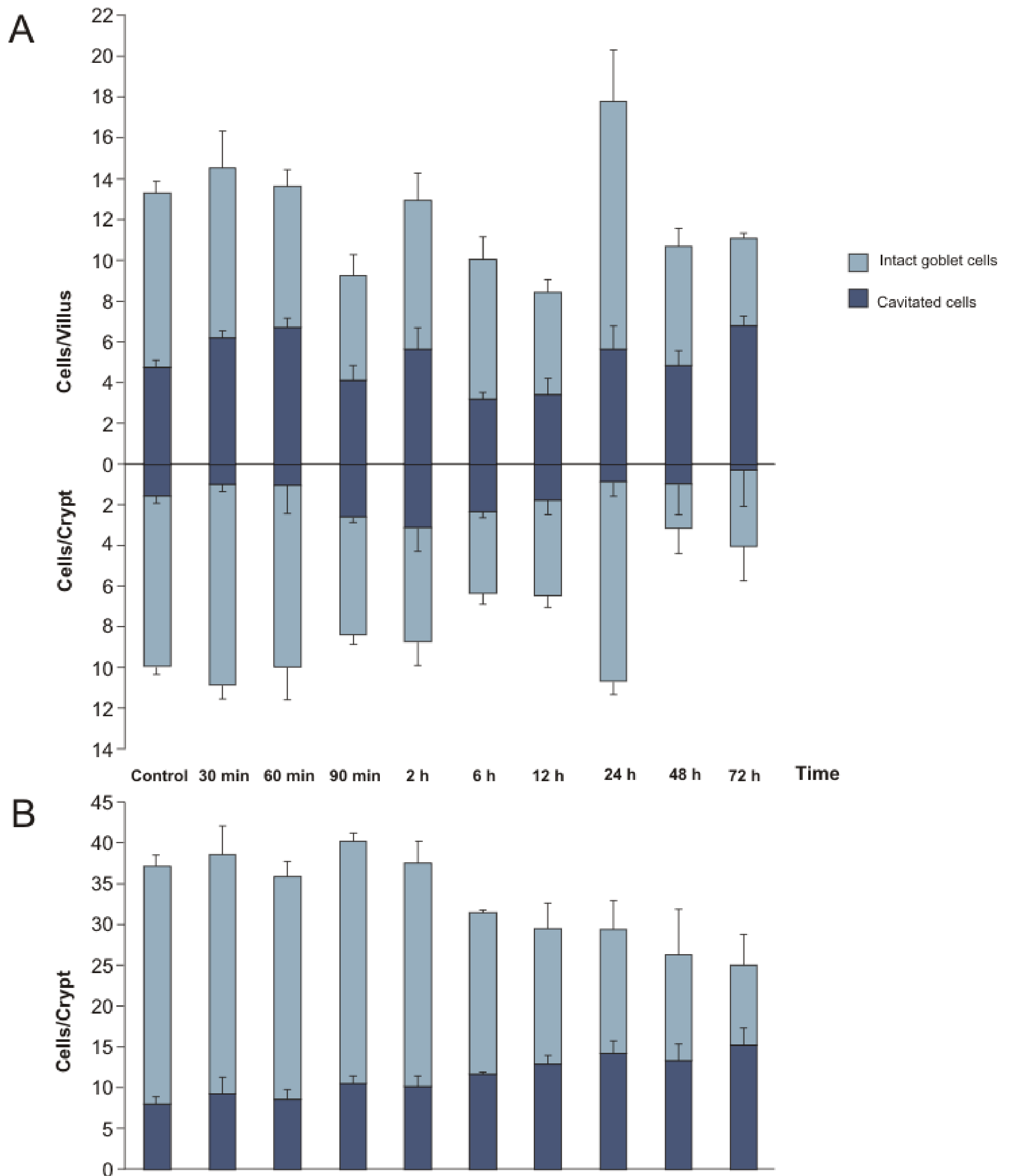


Figure 5.3 Goblet cell counts of intact and cavitated cells. A) Crypts and villi of jejunum (mean ± SD). B) Crypts of colon (mean ± SD).

Muc1 positive cells (Figure 5.4). The intensity of staining for Muc1 was low for all control and treated rats (data not shown).

5.3.4.2 Muc2

Muc2 was expressed by most goblet cells in the jejunum and colon (Figure 5.5). The number of Muc2 positive cells significantly decreased at 12 h ($p<0.003$) after treatment in the jejunum villi, with a significant decrease seen in the crypts at 24-48 h ($p<0.001$). A significant decrease was seen in the colon from 24-72 h ($p<0.0007$). The staining was moderate at all time points investigated.

5.3.4.3 Muc3

A specific antibody for Muc3 was produced and purified. Specificity was tested using ELISA (Figure 5.6). Muc3 was expressed by epithelial cells in the apical region of villi in the jejunum (Figure 5.6). There was a significant decrease in Muc3 positive cells from 48-72 h ($p < 0.003$). The staining was intense.

5.3.4.4 Muc4

Muc4 was expressed by most goblet cells in the jejunum and colon (Figure 5.7). In the jejunum, a decrease (not significant) in Muc4 positive cells was observed in the villi from 48-72 h. Significant decreases were seen in the crypts at 1 h ($p<0.003$), 6 h ($p<0.003$), and 48-72 h ($p<0.004$). Differences between group means in the colon were

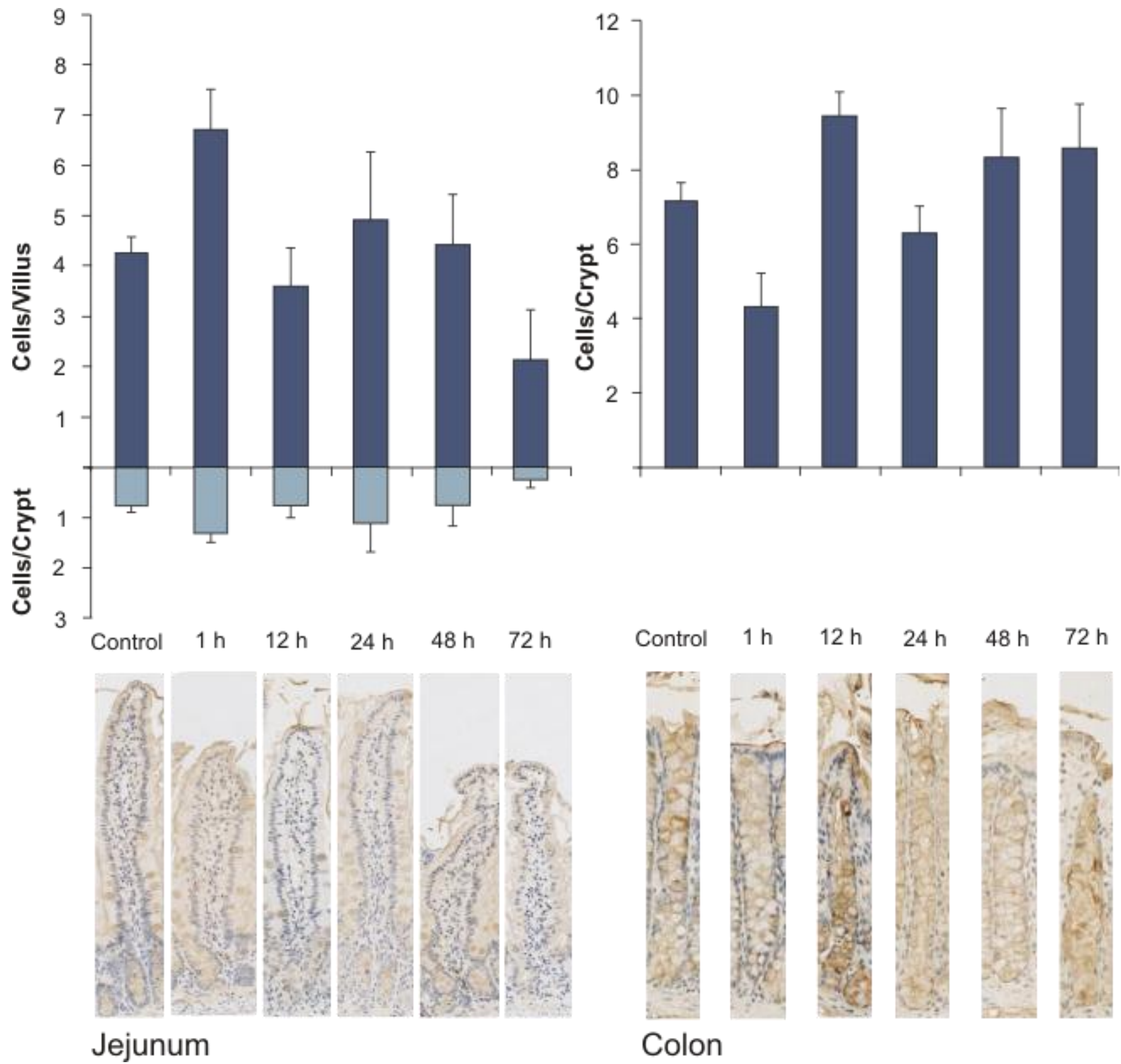


Figure 5.4 Muc 1 Immunostaining. Counts of stained cells per crypt/villus in the jejunum and crypts in the colon (mean ± SD). Representative images of staining in crypts/villi in the jejunum and crypts in the colon.

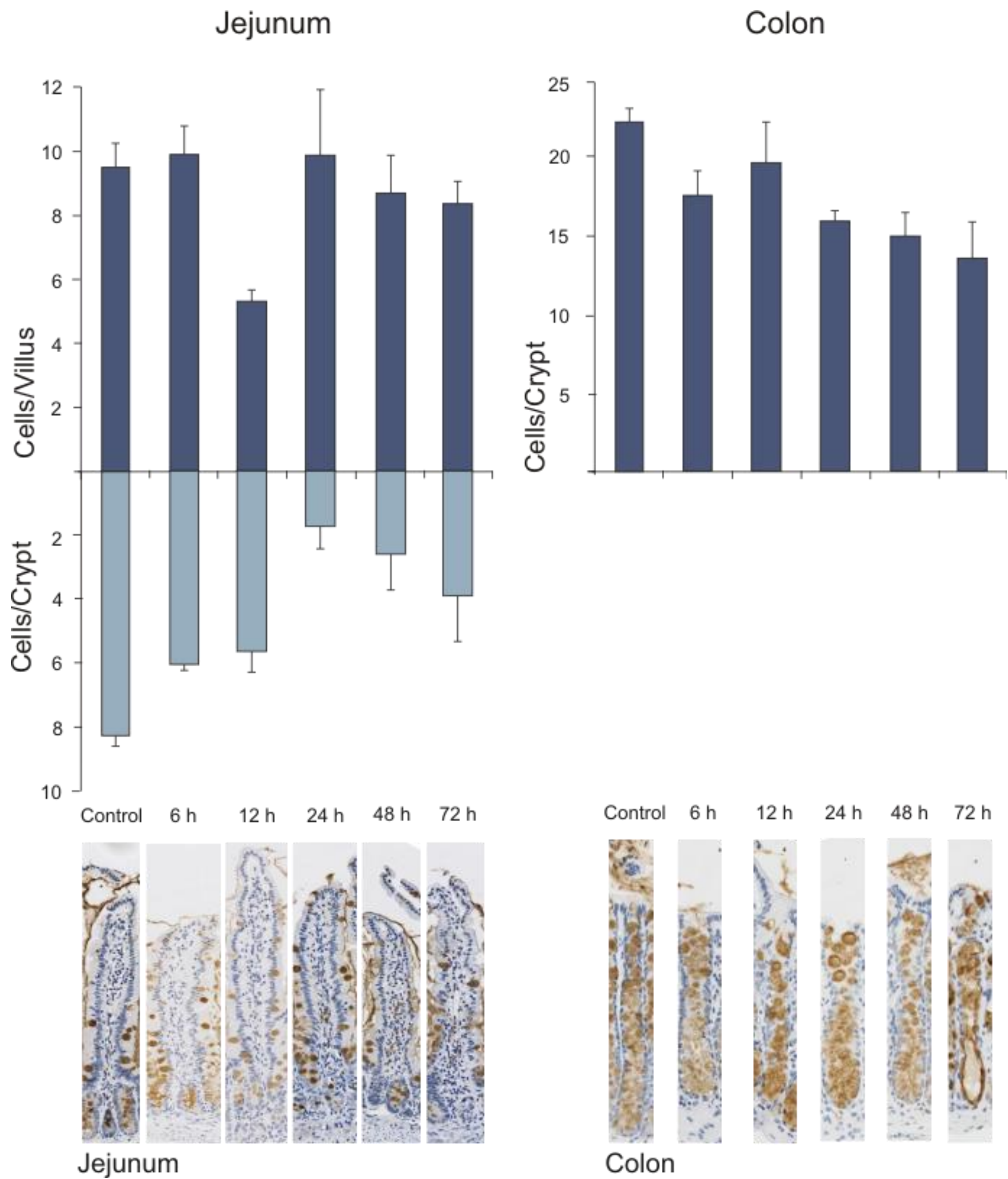


Figure 5.5 Muc 2 Immunostaining. Counts of stained cells per crypt/villus in the jejunum and crypts in the colon (mean ± SD). Representative images of stained goblet cells in crypts/villi in the jejunum and crypts in the colon.

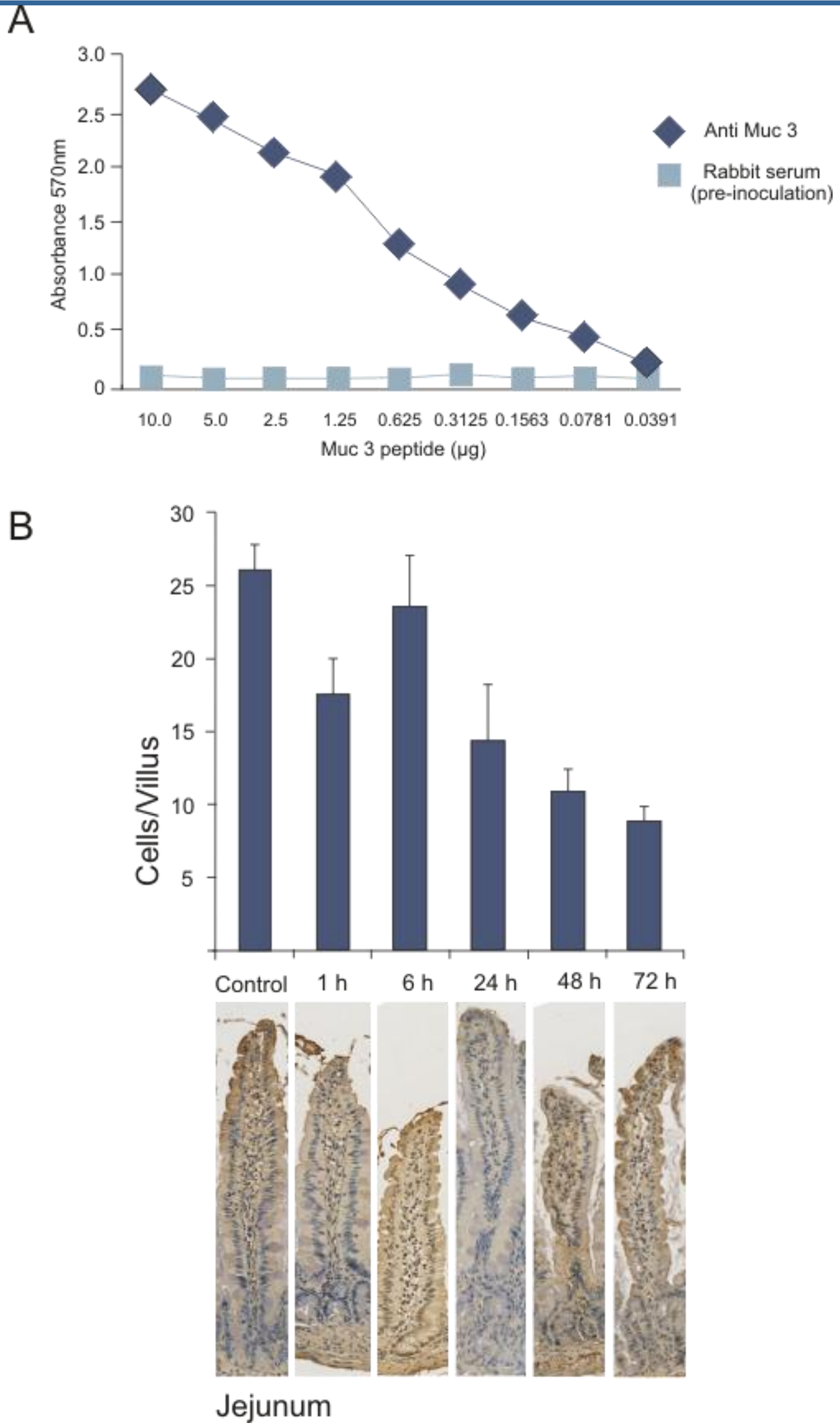


Figure 5.6 A) Antibody activity and specificity ELISA. B) Muc 3 Immunostaining. Counts of stained cells per villus in the jejunum (mean ± SD). Representative images of epithelial staining in villi in the jejunum.

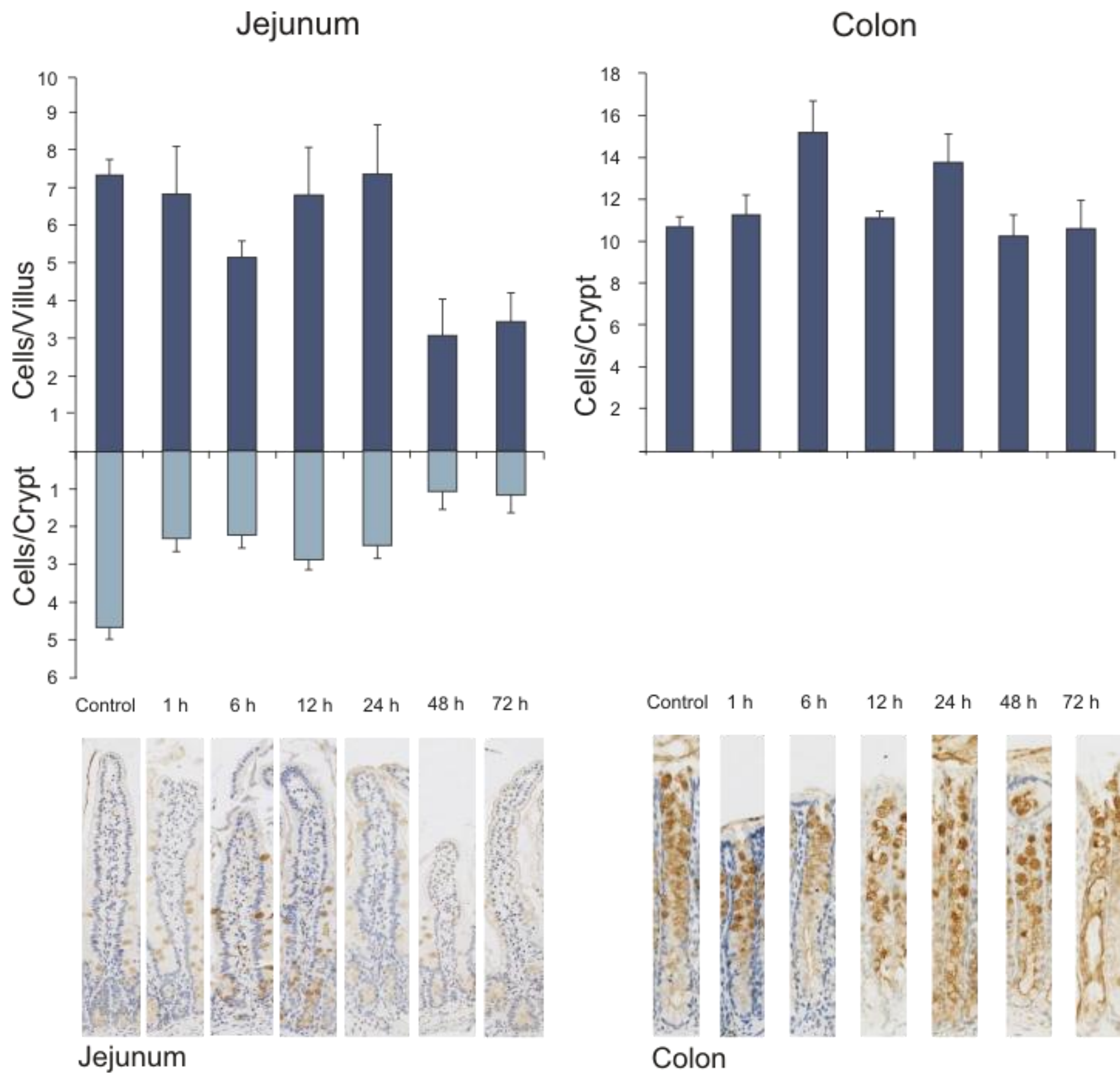


Figure 5.7 Muc 4 Immunostaining. Counts of stained cells per crypt/villus in the jejunum and crypts in the colon (mean ± SD). Representative images of stained goblet cells in crypts/villi in the jejunum and crypts in the colon.

not deemed significant. The staining was mostly weak-moderate, with a decrease in intensity in the villi from 30-60 min, and from 24-48 h after treatment,and in the crypts a decrease from 30-60 min, and from 12-72 h after treatment. Staining intensity in the colon increased at later time points after chemotherapy.

5.3.4.5 Muc5AC

Muc5AC was expressed in the apical region of the gastric pits in the stomach (Figure 5.8). Due to the nature of the architecture of the stomach, stained cells could not be counted. Staining intensity was graded as an alternative. The intensity of staining decreased at 72 h after irinotecan treatment.

5.3.5 Expression of Klf4

Klf4 was expressed in the stomach, jejunum and colon. In the stomach Klf4 was expressed in the depths of gastric pits. Staining intensity was graded, as for Muc5AC, demonstrating a decrease in the intensity of staining from 48 h after irinotecan treatment, with staining not detectable at 72 h. In the jejunum and colon, Klf4 cells were counted in the villi and crypts respectively. Klf4 was expressed in enterocytes in the apical region of villi, and in the apical region of colonic crypts. In the villi, there was a significant decrease in Klf4-expressing cells from 60-90 min ($p<0.005$). In the colon there were no significant changes. However, an increase (not significant) was detected at 24 h after irinotecan treatment (Figure 5.9).

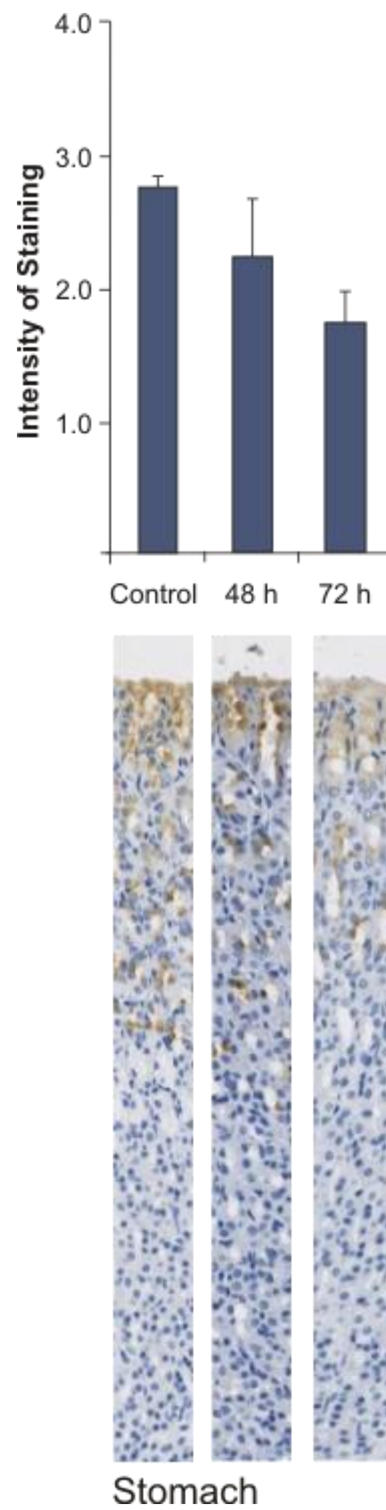


Figure 5.8 Muc 5AC Immunostaining in the stomach. Gradings of staining intensity (mean ± SD) and representative staining in apical region of gastric pits.

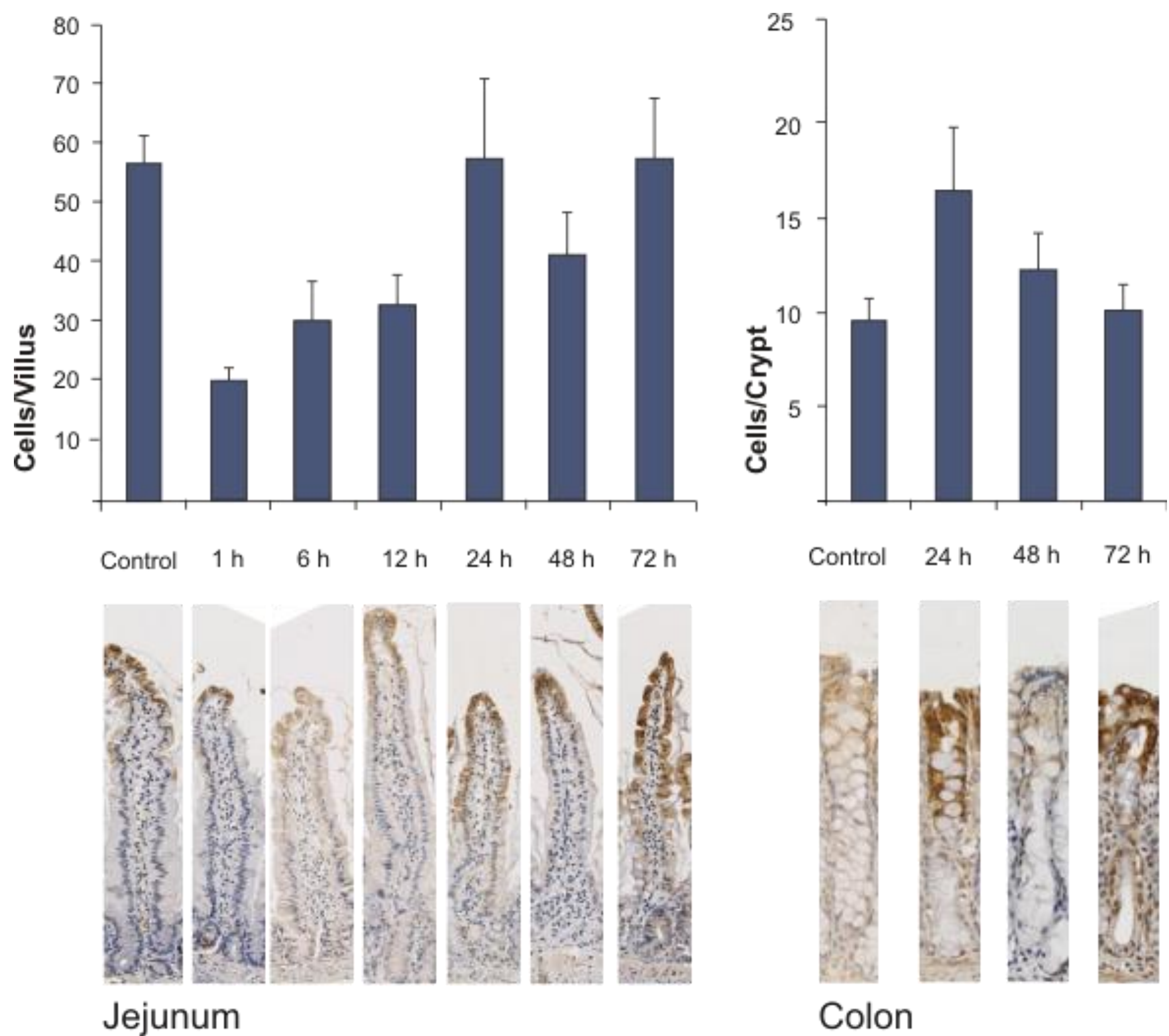


Figure 5.9 Klf4 Immunostaining of goblet cells in the jejunum, and epithelial cells other than goblet cells in the apical region of the colon, and counts of cells per villus in the jejunum and crypt in the colon.

5.4 Discussion

Irinotecan-induced mucositis manifesting as diarrhoea is a severe dose-limiting side effect, with large cost to health services, and is often life-threatening (Elting et al. 2007). Several mechanisms have been proposed for the development of irinotecan-induced diarrhoea, ranging from changes in the architecture and absorption rates of the large intestine (Araki et al. 1993; Ikuno et al. 1995), to increases in intestinal β-glucuronidase levels caused by changes in intestinal bacteria (Takasuna et al. 1996; Takasuna et al. 2006). This study demonstrates for the first time that mucin secretion may also be involved in the development of mucositis and subsequent diarrhoea, with a significant increase in cavitated cells after treatment, as well as altered mucin expression.

The involvement of mucins has not previously been examined in the mucositis setting. This study has shown for the first time that mucin expression alters throughout the gastrointestinal tract following treatment with irinotecan, suggesting a link between mucin expression and mucositis. This may represent a reduction in mucin production, which in turn results in less mucin storage in goblet cells. Less mucin storage may cause the decreased staining of goblet cells seen in this study. An alternative theory is that the goblet cells could be damaged by the cytotoxic agent, resulting in decreased mucin production. Both scenarios could contribute to a compromised protective mucous layer, exposing the epithelium to damage from noxious agents present in the lumen, and bacterial degradation.

This study has demonstrated an increase in sulphated mucins in the colon. Altered sulphate content of mucins can be detrimental (Strous and Dekker 1992; Roediger et al. 1997; Nieuw Amerongen et al. 1998). As sulphate is thought to confer resistance to enzyme degradation, decreased sulphation is unfavourable (Nieuw Amerongen et al. 1998). Despite this, increased sulphation may also be detrimental, increasing the sulphate residues available for sulphate reducing bacteria to utilise allowing these bacteria to proliferate and increasing the presence of sulphides (toxic to the intestinal mucosa) (Roediger et al. 1997). We have previously shown an increase in some bacterial genus' following treatment with irinotecan (Stringer et al. 2007; Stringer et al. 2008), and although sulphur-reducing bacteria were not examined, it is possible that they may be increased also.

Other studies using irinotecan in rats have looked briefly at goblet cells and noted increases in mucin secretion (Gibson et al. 2003; Bowen et al. 2007). This study extends these studies by showing decreased intact cells (indicating release of mucin) (Barcelo et al. 2000), and increased cavitated cells (a hallmark of accelerated mucus secretion) (Barcelo et al. 2000). The decrease in goblet cells (release of mucin) occurs just prior to the occurrence of extensive pathological damage seen in this study, a peak in nuclear factor kappa B (NFκB) expression (Bowen et al. 2007; Logan et al. 2008), and peaks in expression of pro-inflammatory cytokines tumour necrosis factor (TNF), interleukin (IL)-1β and IL-6 (Logan et al. 2008). The peak in cavitated goblet cells in the jejunum crypts represents accelerated mucus secretion at 48 h, preceding the delayed onset mucositis and diarrhoea seen with irinotecan in rats (Gibson et al. 2003;

Gibson et al. 2005; Gibson et al. 2007). The decrease in intact goblet cells and increase in cavitated cells in the colon also precedes the delayed onset mucositis and diarrhoea associated with irinotecan (Gibson et al. 2003; Gibson et al. 2005; Gibson et al. 2007). This increase in cavitated cells (accelerated mucus secretion) (Barcelo et al. 2000) correlates with the incidence of diarrhoea, indicating that the accelerated mucin secretion in the colon may be a contributing factor in the development of diarrhoea.

The incomplete effectiveness of atropine in this study indicates a neurotransmitter other than acetylcholine (ACh) may be mediating the increased mucin secretion. Apical mucin and chloride secretion is achieved by the mechanical stimulus of villous cells, by non-neuronal mechanisms to provide non-specific protection of the mucosa, protecting against the passage of food and protecting the epithelium from damage. Neuronal modulation is yet to be conclusively demonstrated (Laboisse et al. 1996). However, goblet cells are specialised secretory cells endowed with a variety of receptors coupled to intracellular signaling pathways that regulate exocytotic machinery, and located in the vicinity of enteric nerves, enteroendocrine cells and immune cells, suggesting neurotransmitters, hormones and inflammatory mediators are potentially involved (Plaisancie et al. 1998). Efforts towards understanding the regulation of mucus secretion from goblet cells have been made, but the knowledge about chemical transmitters regulating mucus secretion remains limited (Laboisse et al. 1996). Recent studies have shown vasoactive intestinal peptide (VIP, a neurotransmitter) increases mucus secretion in the intestine, as well as serotonin (a neurotransmitter, also known as 5-HT), prostaglandin E2 (PGE_2), IL-1β (a pro-

inflammatory cytokine), and sodium nitroprusside (SNP, a nitric oxide generator) (McConalogue and Furness 1994; Plaisancie et al. 1998) .

Nitric oxide may be implicated in the control of gastrointestinal mucin secretion (Plaisancie et al. 1998). Research has shown an increase in inducible nitric oxide synthase (iNOS), and subsequently nitric oxide (NO) during 5-fluorouracil-induced mucositis (Leitao et al. 2007). Chemotherapy agents generate reactive oxygen species (ROS) which directly damage cells and stimulate transcription factors, including nuclear factor kappa-B (NF-κB), in turn up-regulating genes resulting in the production of pro-inflammatory cytokines (including TNF, IL-1β and IL-6) (Leitao et al. 2007). More recently the same cytokines have been shown to be involved in chemotherapy-induced mucositis using the same dose of irinotecan as this study (Logan et al. 2008).

Another possibility that may contribute to the increase in mucin secretion is the disruption of actin in damaged cells in the intestine, including goblet cells. Actin is present along the apical surface of goblet cells, and is distributed differently from adjacent absorptive cells (Oliver and Specian 1990). Studies have shown that actin filament disruption does not alter the morphology of goblet cells, but does cause the acceleration of mucin granules through goblet cells to the apical surface, where they are then secreted (Oliver and Specian 1990). This is indicative that a loss of actin results in the acceleration of baseline (slow, sustained release of mucins to counteract loss in mucus layer) mucus secretion (Oliver and Specian 1990).

Klf4 (an epithelial zinc-finger protein) is thought to be involved in the differentiation of goblet cells (Katz et al. 2002). Klf4 has also been implicated in down regulation of cell proliferation and growth arrest (Oliver and Specian 1990). Studies have shown that Klf4-null mice have mature goblet cells in the colon reduced 90% relative to total epithelial cells (Katz et al. 2002). The present study indicates that Klf4 expression is altered in the stomach, jejunum and colon following treatment with irinotecan, suggesting that changes in the differentiation of goblet cells from multipotent stem cells could be in part responsible for changes in mucin expression and secretion. However, little research has been conducted on Klf4, and the exact extent of its function is yet to be elucidated, let alone its involvement in mucositis. Further studies to explore the potential significance between Klf4 and mucositis are now warranted.

In conclusion, this study has shown for the first time that irinotecan causes an increase in mucin secretion and a net decrease in mucin-producing goblet cells, and the expression of Muc1, Muc2, Muc3, Muc4 and Muc5AC in the GIT is altered following treatment. Increased mucin secretion is likely to be related to altered mucin gene expression, and may contribute to chemotherapy-induced diarrhoea.

6.0 Irinotecan-induced mucositis manifesting as diarrhoea corresponds with an amended intestinal flora and mucin profile

6.1 Introduction

Irinotecan is effective in treating a wide variety of solid tumours, through the inhibition of DNA topoisomerase I. However, it is linked with severe mucositis and diarrhoea (Armand et al. 1995; Ikuno et al. 1995; Takasuna et al. 1996; Gibson et al. 2003; Brandi et al. 2006; Stringer et al. 2007). Cholinergic, secretory diarrhoea occurs early, although this can be managed by blocking neurons containing acetyl choline (ACh) in the enteric nervous system with atropine. Delayed severe diarrhoea also occurs; this is one of the main dose-limiting side effects of irinotecan treatment (Gibson et al. 2003). The metabolism of irinotecan has been described previously (Smith et al. 2006), however briefly, irinotecan and SN-38 bind to the topoisomerase I-DNA complex, leading to double strand (ds) breakage and cell death. Previous research has shown that bacterial β-glucuronidase plays a crucial role in the intestinal toxicity of irinotecan (Takasuna et al. 1996; Takasuna et al. 2006). Bacterial β-glucuronidase is produced primarily by Enterobacteriaceae (*E. coli*, *Salmonella spp.*, *Shigella spp.*, *Yersinia spp.*, *Citrobacter spp.*, *Hafnia spp.* and *Edwardia spp.*), and has been reported to be produced by *Flavobacterium spp.*, *Bacteroides spp.*, *Staphylococcus spp.*, *Streptococcus spp.*, *Corynebacterium spp.* and *Clostridium spp.* (Tryland and Fiksdal 1998).

Irinotecan has previously been shown to alter the intestinal microflora up to 72 h after treatment, both qualitatively (Stringer et al. 2007) and quantitatively (Stringer et al. 2008), in particular, bacteria known to produce β-glucuronidase (including *E. coli*). We have also shown that irinotecan treatment decreases goblet cells in the large intestine at time points up to 72 h (Chapter 5.0). However, mucus secretion is increased at this time, which may contribute to the development of late onset irinotecan-induced diarrhoea (Chapter 5.0). The exact mechanisms of late onset diarrhoea are still unclear.

The aim of this study was to determine if the early changes seen in the luminal environment, specifically changes in gastrointestinal flora (especially those genera which are known to produce β-glucuronidase and may interfere with the processing of irinotecan) and intestinal mucin, were also observed during irinotecan-induced late onset diarrhoea in rats treated with irinotecan.

6.2 Materials and Methods

6.2.1 Animals

Animals used in this study were female Dark Agouti (DA) rats, weighing between 150 and 170 g. Rats were housed in Perspex cages at a temperature of 22±1˚C and subject to a 14 hour light/10 hour dark cycle. Approval for the use of animals was granted by the Animal Ethics Committees of the Institute of Medical and Veterinary Science (IMVS), 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). Due to the nature of the diarrhoea induced by irinotecan, animals were monitored 4 times daily and if any animal showed certain criteria (as defined by the Animal Ethics Committees) they were euthanized. These criteria included a dull ruffled coat with accompanying dull and sunken eyes, cold to touch with no spontaneous movement and a hunched appearance.

6.2.2 Experimental plan

Twenty seven rats were randomly assigned to groups. For each time point there was one group of six (6) rats receiving irinotecan and one group of three (3) control rats receiving no treatment. Rats receiving irinotecan received 0.01mg/kg subcutaneous atropine (to reduce the cholinergic reaction) immediately prior to administration of 200 mg/kg intraperitoneal irinotecan (previously shown to induce diarrhoea (Logan et al. 2007; Stringer et al. 2007)). Irinotecan (kindly supplied by Pfizer, Kalamazoo, USA) was administered in a sorbitol/lactic acid buffer (45mg/mL sorbitol/0.9mg/mL lactic

acid, pH 3.4), required for activation of the drug, at time designated 0 h. Groups of rats were killed using 3% halothane in 100% O_2 anaesthesia and cervical dislocation at times 96, 120 and 144 h post irinotecan treatment. Immediately prior to anaesthesia, faecal samples were aseptically collected by directly collecting the excreted faeces immediately as it left the rat in a sterile containers in an area cleaned with 70% ethanol. Samples were frozen in $N_{2(l)}$ and stored at -70˚C. The GIT (from the pyloric sphincter to the rectum) was dissected out and separated into the small intestine (pyloric sphincter to ileocaecal sphincter) and colon (ascending colon to rectum). The small intestine was flushed with chilled, sterile distilled water, and 1 cm samples taken at approximately 50% of the length, was collected for histological and microbiological procedures. The colon was also flushed with chilled sterile, distilled water. Samples (1 cm) of colon, taken at approximately 50% of the length were also collected for histology and microbiology. The stomach was dissected and contents emptied and discarded. Small pieces (1cm x 0.5cm) of stomach were collected for histology and microbiology. All samples for histological examination were fixed in 10% neutral buffered formalin, processed and embedded in paraffin. All samples for microbiology were stored at -70˚C until required.

6.2.3 Diarrhoea assessment and Bodyweight

Diarrhoea was assessed as described previously. For full details refer to ***3.2.3 Diarrhoea assessment*** (page 72).

6.2.4 Electrolyte analysis

Blood samples were centrifuged (Hereus, Finland) at 3000 rpm for 5 min. The serum was collected into a fresh tube and analysed by the Department of Clinical Pathology, IMVS, Adelaide, South Australia. Measurements for sodium, potassium, bicarbonate, chloride, anion gap, and measured osmolality were measured from serum samples.

6.2.5 Histological examination

Samples were paraffin-embedded and stained as previously described. For full details refer to ***3.2.5 Histological examination*** (page 72).

6.2.6 Alcian Blue-PAS stain

Sections were stained with Alcian Blue-PAS as described previously. For full details refer to ***2.2.5 Alcian Blue-PAS stain*** (page 42).

6.2.6.1 Quantitative Histology

To determine the effect of irinotecan on mucus secretion, goblet cells were counted as previously described. For full details refer to *2.2.5.1 Quantitative Histology* (page 42).

6.2.7 Immunohistochemistry

Immunohistochemistry was used to detect β-glucuronidase, Muc1, Muc2, Muc3, Muc4 and Klf4 expression, as previously described. For full details please refer to ***4.2.3***

Immunohistochemistry (page 98), 5.2.5 Immunohistochemistry (page 114) and Table 5.1 (page 115).

6.2.8 Culture of samples

To determine the flora of the DA rat treated with irinotecan, a variety of selective and non-selective media (Oxoid, Adelaide, Australia) was used in an attempt to identify as many bacteria as possible from the GIT. For detailed methodology please refer to ***2.2.6 Culture of samples*** (page 42) and ***Table 2.1*** (page 44).

6.2.9 Extraction and purification of DNA from bacterial culture and faecal samples

6.2.9.1 DNA extraction from bacteria

DNA was extracted from standard bacterial samples using a DNeasy Tissue Mini Kit (Qiagen). For full details refer to *2.2.7.1 DNA extraction from bacteria* (page 46).

6.2.9.2 DNA Extraction from samples

DNA was extracted from rat faecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Doncaster, Australia). For full details refer to *2.2.7.2 DNA Extraction from samples* (page 46).

6.2.10 Real-time PCR

Real-time PCR was carried out on a Corbett Rotorgene 3000 rotary cycler (Corbett Research, USA). Primers used in this study have been used previously (Edwards et al. 2001; Matsuki et al. 2002; Matsuki et al. 2004; Rinttila et al. 2004; Sakai et al. 2004;

Penders et al. 2005; Layton et al. 2006) (**Table 2.2**, page 47). For details refer to ***2.2.8 Real-Time PCR*** (page 48).

6.2.11 Statistical analysis

Results were statistically analysed using a Mann-Whitney U test. For adjustments for multiplicity, the significance levels of the *P* values were determined according to the Bonferroni correction.

6.3 Results

6.3.1 Clinical signs

Diarrhoea occurred after irinotecan treatment as expected (Figure 6.1). Mild diarrhoea was seen in 23% of rats from 0-24 h (early, cholinergic). This reduced to 6% at 48 h, before increasing at 72 h, with 28% of rats experiencing mild diarrhoea, 5% with moderate diarrhoea and 6% with severe diarrhoea. There was a further increase at 96 h with 33% of rats experiencing mild diarrhoea, 11% with moderate diarrhoea and 6% with severe diarrhoea. All diarrhoea was resolved by 120 h after irinotecan administration.

Untreated control rats gained weight gradually and consistently over the experimental period. Rats treated with irinotecan decreased in weight until 96 h, with significant differences between control and treated rats at 48 h ($p<0.007$), 72 h ($p<0.0007$), 96 h ($p<0.002$), and 120 h ($p<0.007$). From 120 h, chemotherapy-treated rats gained weight (Figure 6.1).

6.3.2 Electrolytes

Rats treated with irinotecan exhibited altered electrolytes profiles compared with control rats (Figure 6.2). Sodium and bicarbonate levels were decreased in treated rats 96 h after treatment. Potassium levels were higher in treated rats at all time points, and anion gap was raised 96 h after treatment. Osmolality levels were lower in treated rats at all time points investigated.

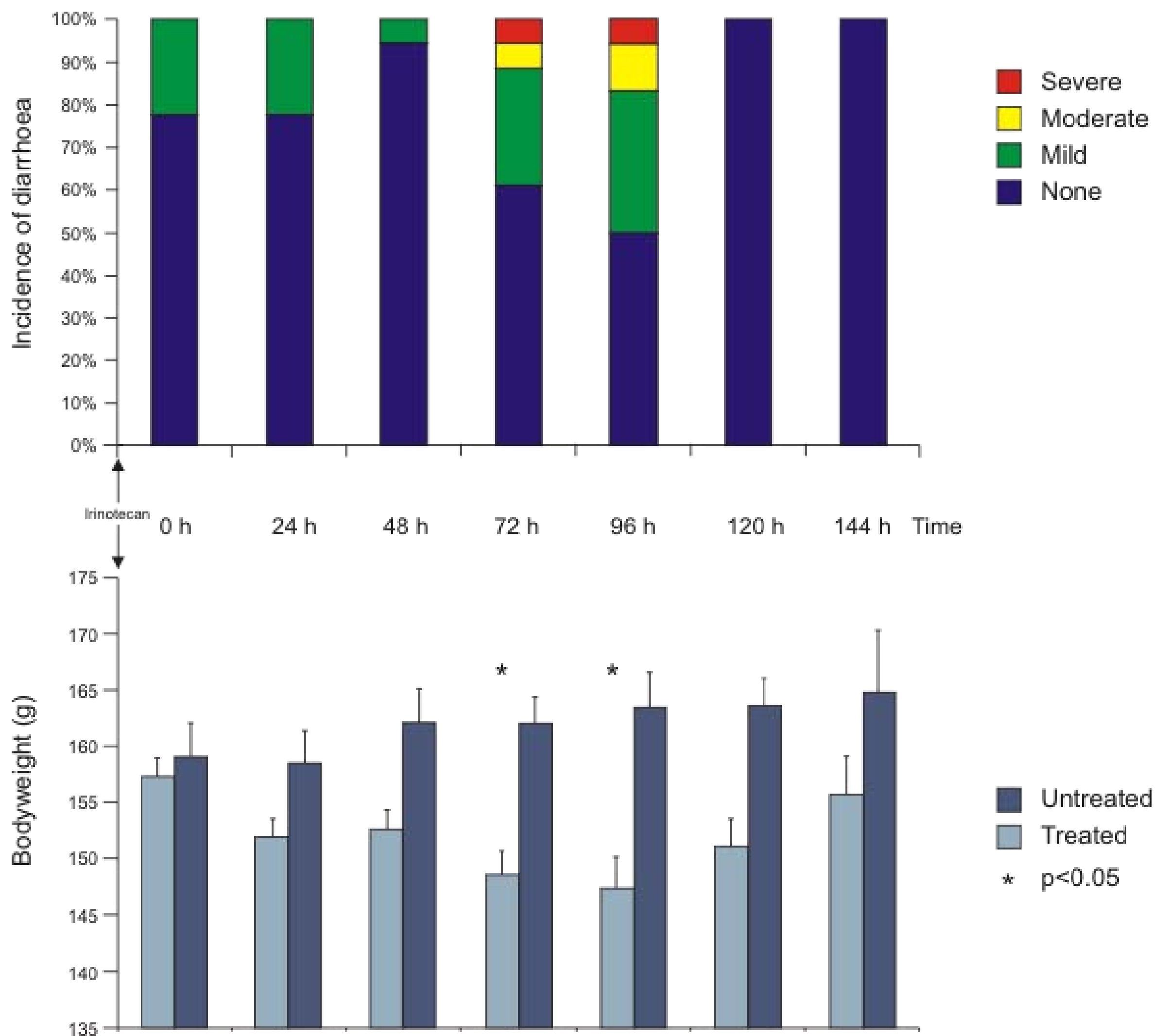


Figure 6.1 Clinical signs. Incidence of diarrhoea (top) and bodyweight (g) (bottom).

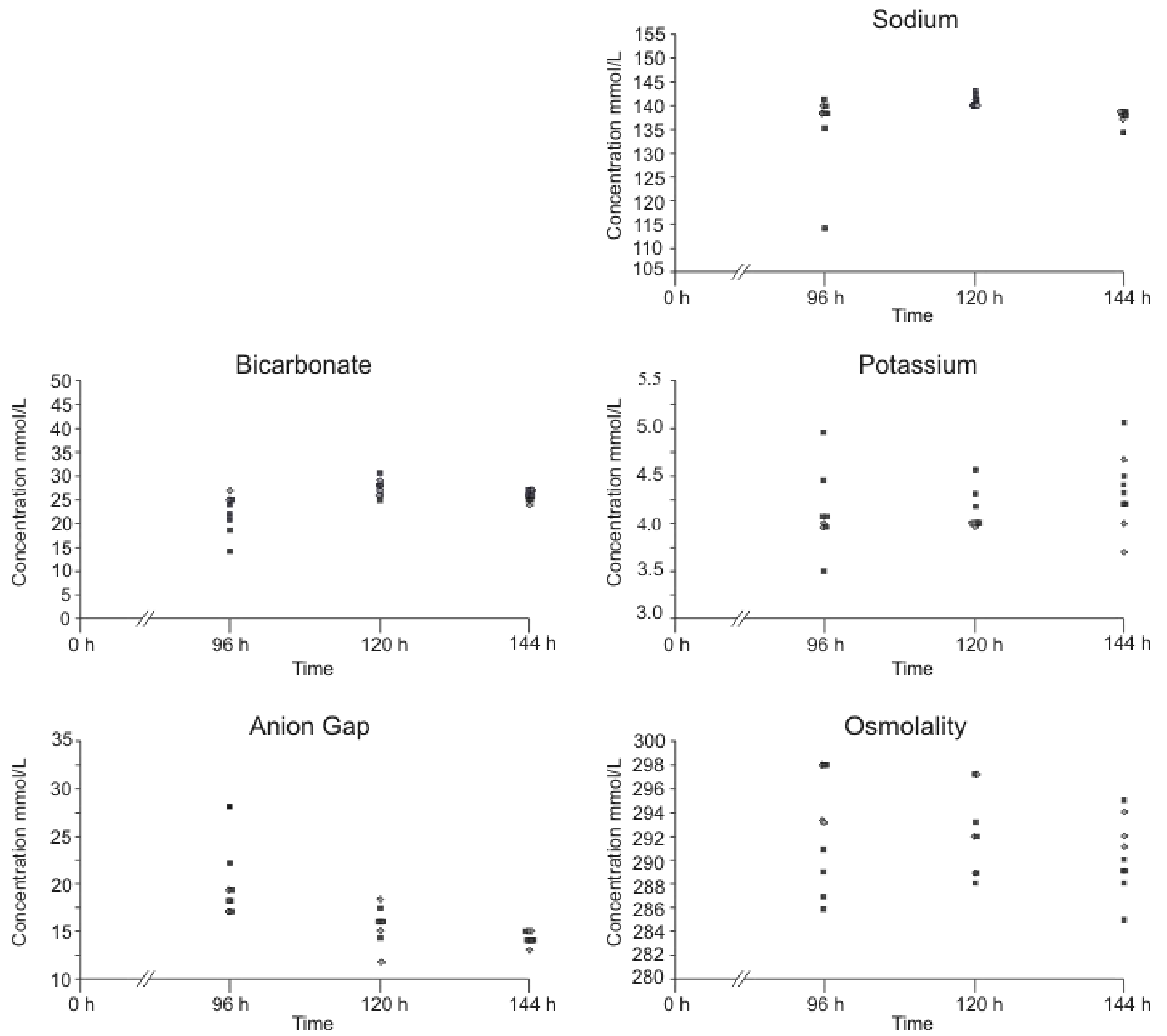


Figure 6.2 Serum electrolyte analyses.

6.3.3 Histology

Pathological changes were seen following irinotecan treatment in the jejunum and colon (Figure 6.3). The jejunum showed increased mitotic activity at 96 h, and occasional apoptotic bodies from 96-144 h. The colon showed considerable damage from 96-120 h, with patchy crypt degeneration, dilated crypts with attenuated epithelium, and a few desquamated epithelial cells polymorphonuclear cells in the lumina. There was also evidence of increased hyperplasia and mitotic figures.

6.3.4 Goblet cells

6.3.4.1 Jejunum

The total number of goblet cells increased over time after irinotecan treatment, with a significant difference between at 120 h ($p<0.004$) and 144 h ($p<0.0125$). The number of intact goblet cells did not alter significantly after treatment. However, the number of cavitated goblet cells increased over time, with significance reached at 96 h ($p<0.007$) and 144 h ($p<0.0125$) (Figure 6.4).

6.3.4.2 Colon

The total number of goblet cells decreased significantly at 96 h ($p<0.0125$). However, numbers returned to control levels by 144 h. The number of intact goblet cells was significantly decreased at 96 h also ($p<0.007$). The number of cavitated cells did not alter significantly, although the percentage of cavitated cells increased significantly at 96 h ($p<0.011$).

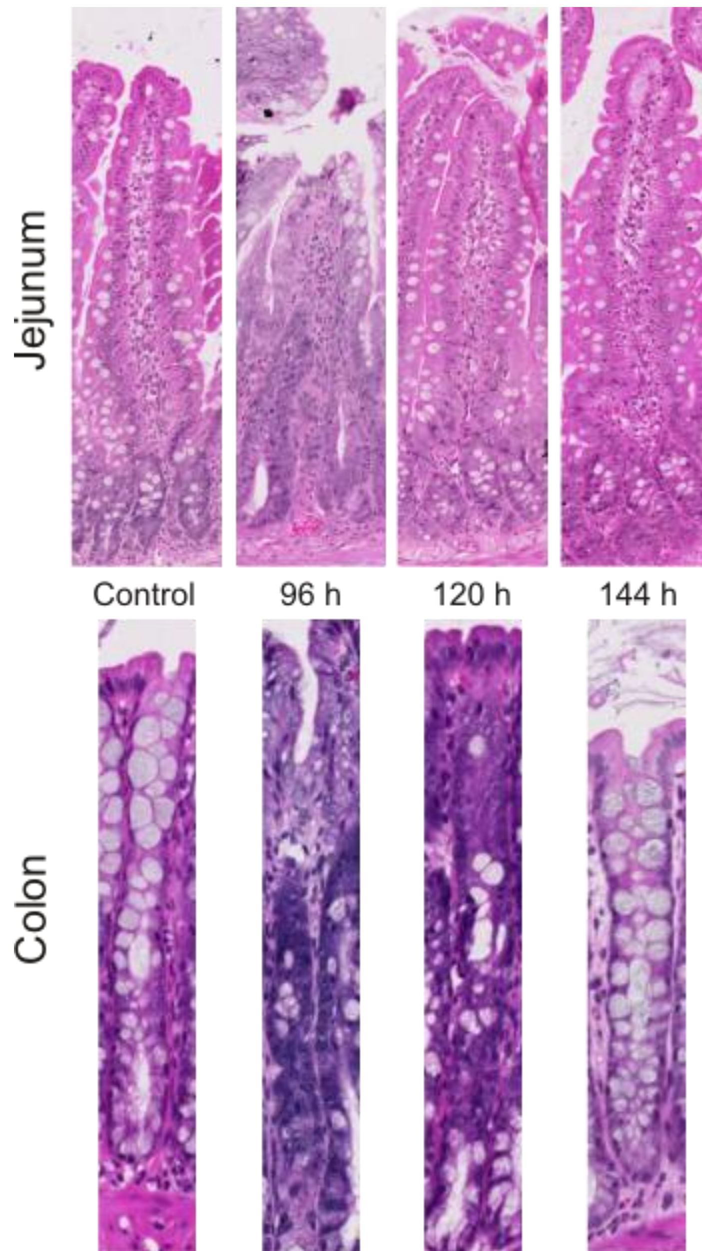


Figure 6.3 Histopathological changes seen in the jejunum and colon following treatment with irinotecan.

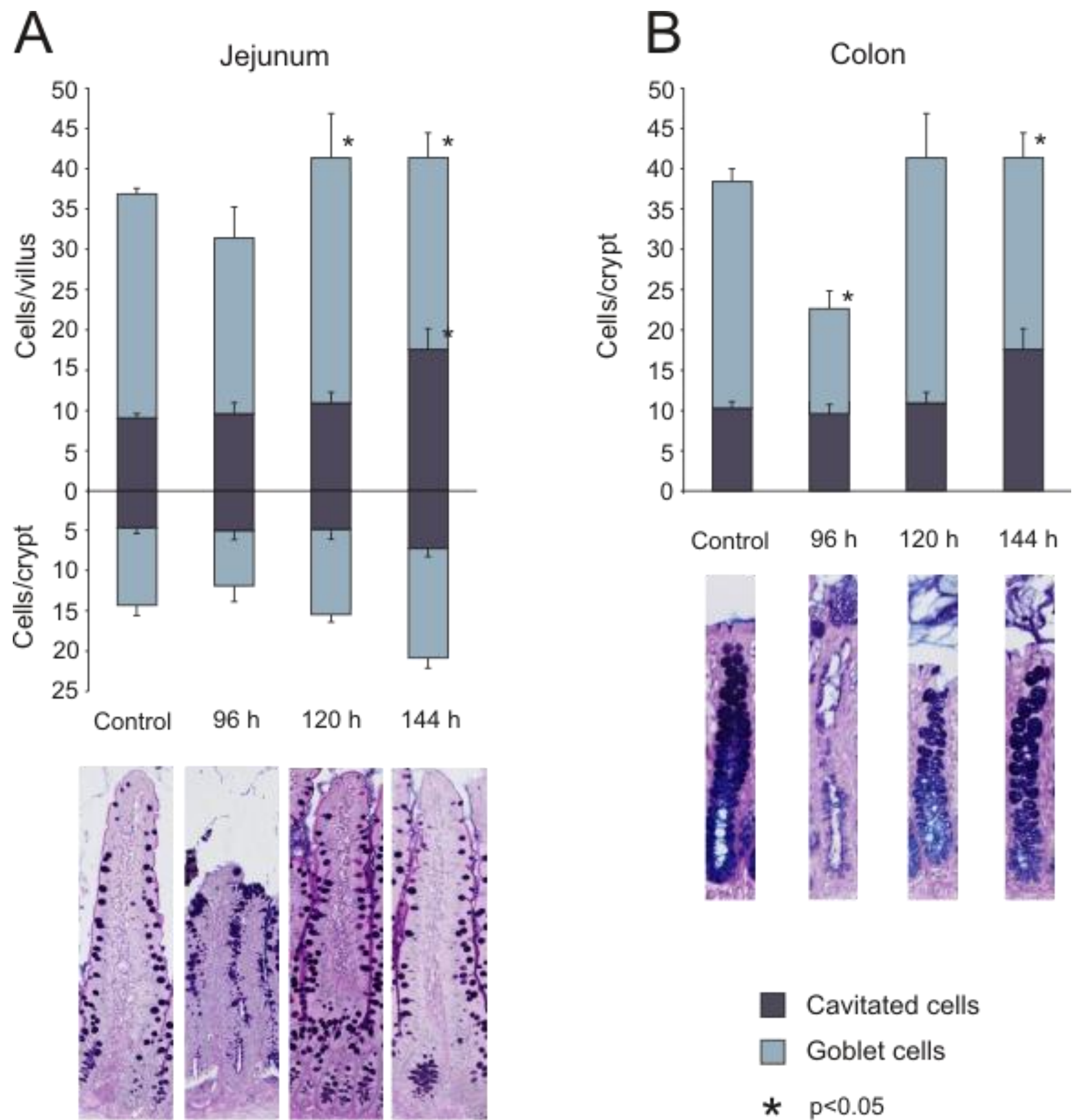


Figure 6.4 Alcian Blue-PAS staining and goblet cell counts in the A) jejunum and B) colon. Counts expressed as mean ± SD.

6.3.5 Mucin expression

Muc1 expression did not alter significantly in the jejunum. There was a decrease in the number of Muc1 positive cells in the crypts at 96 h, however this number did not achieve significance. Like the jejunum Muc1 expression did not alter significantly in the colon.

The expression of Muc2 did not alter significantly in the jejunum or colon at any time point investigated. No significant changes to Muc3 expression were detected in the jejunum at any time point investigated. Muc3 was not expressed in the colon. Muc4 expression was significantly decreased at 96 h in the jejunum villi ($p<0.004$) (Figure 6.5). However, expression in the jejunum crypts and colon was not significantly altered at any time point investigated. Klf4 expressing cells were significantly reduced in the jejunum villi at 96 h ($p<0.0004$) and 144 h ($p<0.0002$). There were no changes in the jejunum crypts or colon.

6.3.5 Culture

Changes were seen in the bacteria of jejunum and colon of rats that received irinotecan. The majority of these changes were seen from 96-120 h after treatment. In the jejunum, there were increases in *Escherichia spp.* with a peak at 96 h, *Clostridium spp.* from 96-144 h and *Staphylococcus spp.* with a peak at 144 h. Changes were also seen in the colon with increases in *Escherichia spp.* from 96-144 h, *Clostridium spp.* with a small peak at 96 h, *Enterococcus spp.* from 120-144 h, *Serratia spp.* with a peak at 120 h, *Staphylococcus spp.* from 120-144 h (Figure 6.6).

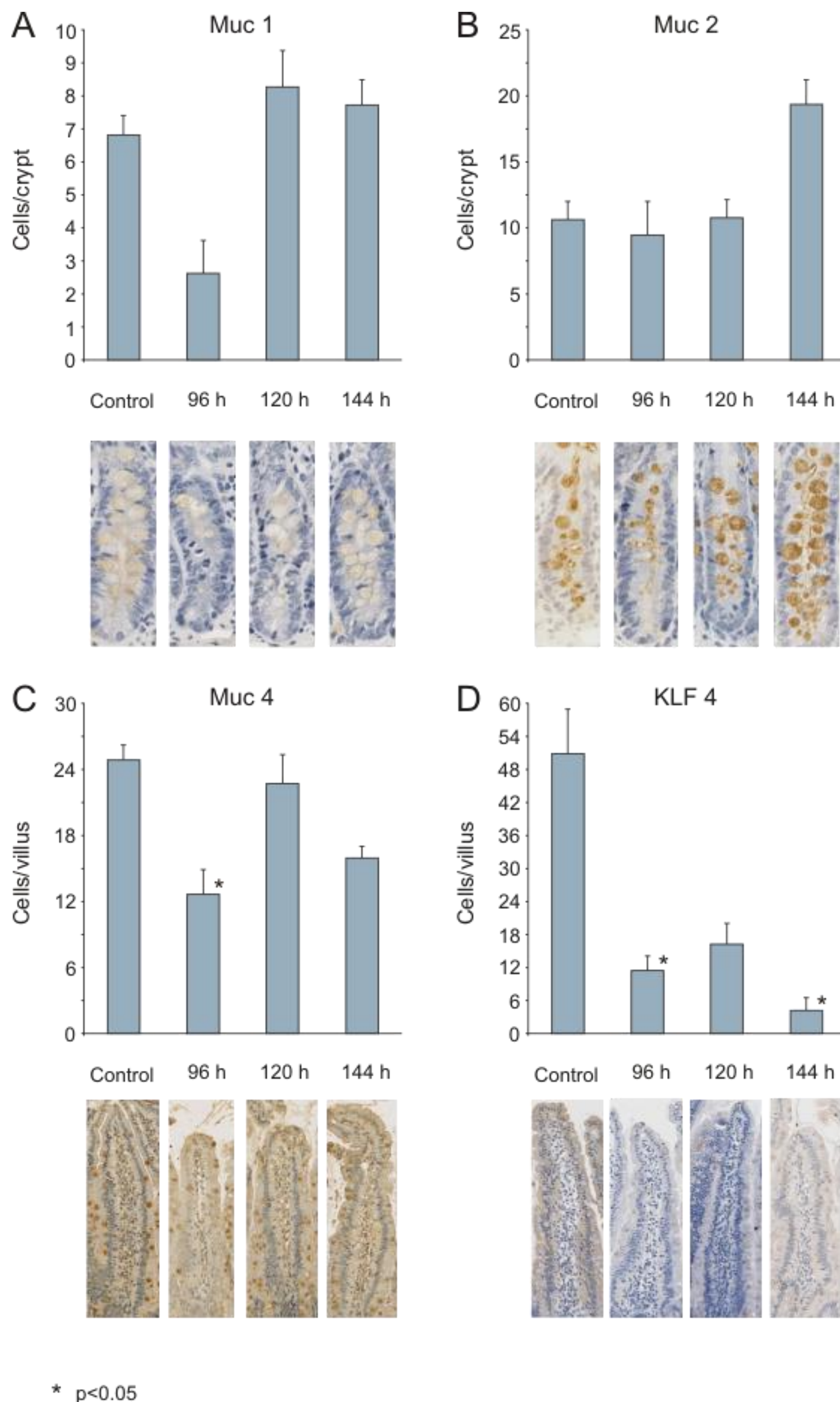


Figure 6.5 Mucin immunostaining in the jejunum. A) Muc1 expression in the crypts. B) Muc2 expression in the crypts. C) Muc4 expression in the villi. D) Klf4 expression in the villi.

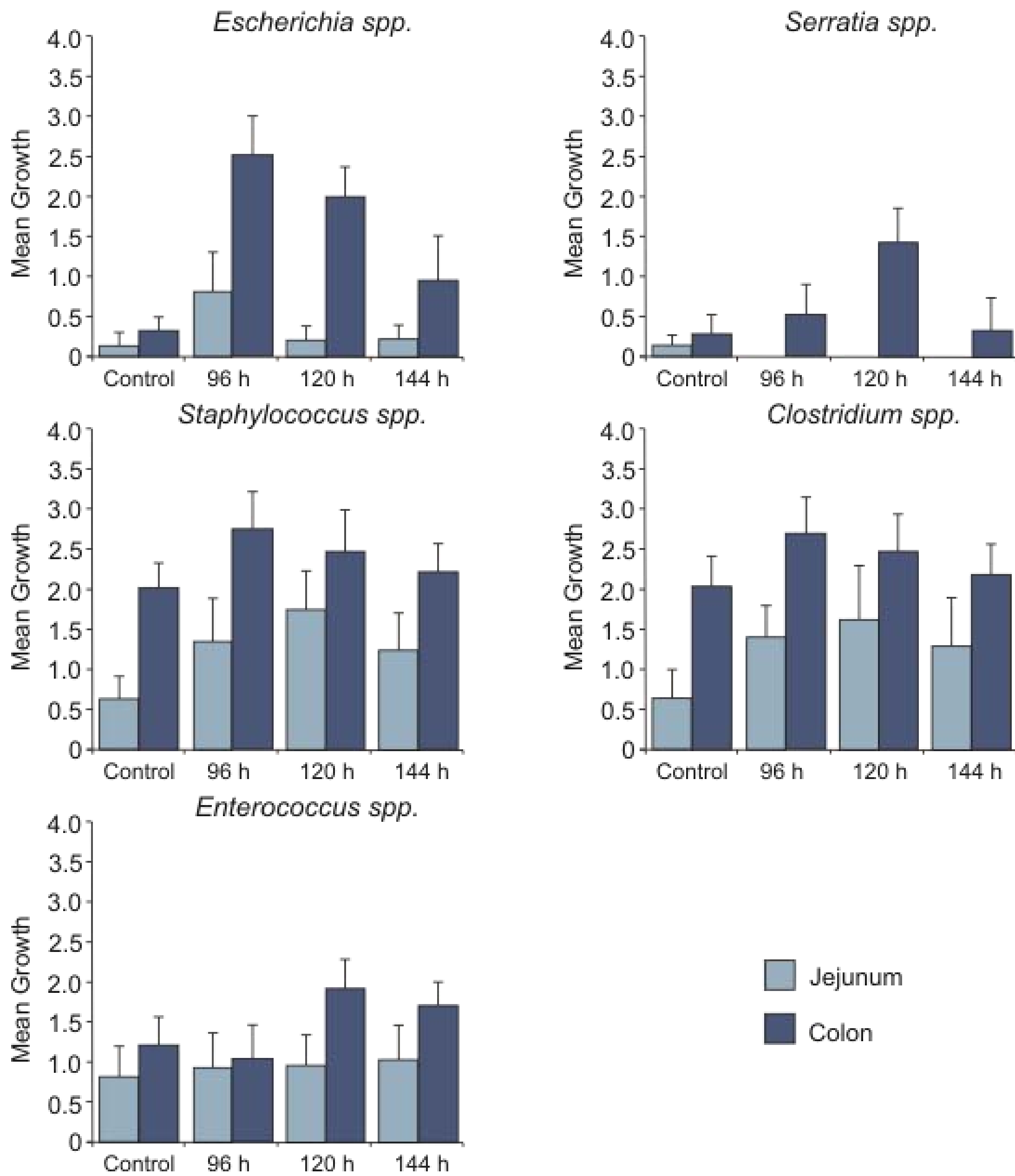


Figure 6.6 Qualitative microbiology results for the jejunum and colon.

6.3.6 Quantification of faecal flora

No significant changes were seen in the faecal flora of rats. However, *E. coli* (produces β-glucuronidase) exhibited a tendency to peak at 96 h, and *Bifidobacterium spp.* (beneficial intestinal function) exhibited a tendency to decrease after treatment at all time points (Figure 6.7).

6.3.7 β-glucuronidase expression

Expression of β-glucuronidase was increased in the colon from 96-120 h after treatment, with sections demonstrating a clear increase in staining intensity (Figure 6.7). This increase was correlated with the incidence of severe diarrhoea, and a peak in *E. coli*.

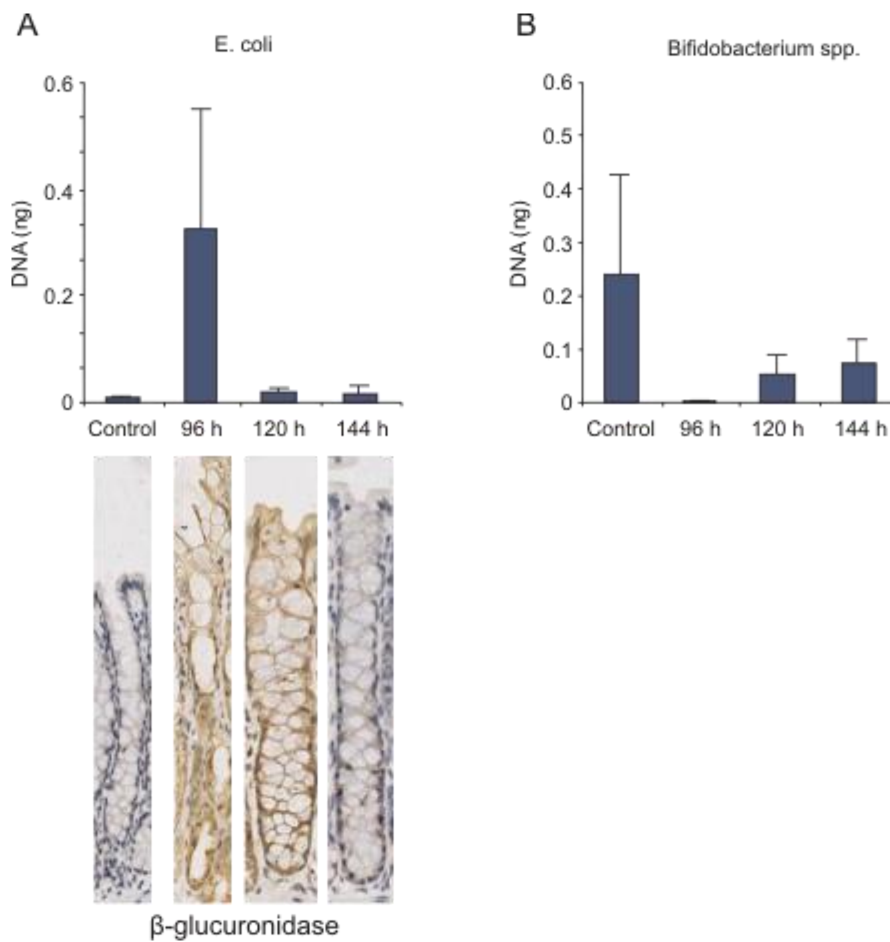


Figure 6.7 Quantitative real time PCR results showing A) an increase in E. coli and β-glucuronidase expression in the colon after treatment; and B) a decrease in Bifidobacterium spp. after treatment.

6.4 Discussion

Irinotecan-induced mucositis manifesting as diarrhoea is a severe dose-limiting side effect, with large cost to health services, and is often life-threatening (Elting et al. 2007). Several mechanisms have been proposed for the development of irinotecan-induced diarrhoea, ranging from changes in the architecture and absorption rates of the large intestine (Araki et al. 1993; Ikuno et al. 1995), to increases in intestinal β-glucuronidase levels caused by changes in intestinal bacteria (Takasuna et al. 1996; Takasuna et al. 2006). The present study was unique in that it investigated a variety of changes, including gastrointestinal architecture, gastrointestinal microflora, intestinal mucins, and serum electrolytes for the duration of delayed onset mucositis and diarrhoea.

We have previously shown that there are significant differences in the levels of bacteria in rats receiving chemotherapy vs. chemotherapy-naïve rats in faecal samples at early time points (up to 72 hours) (Stringer et al. 2007) (unpublished data). This study extends this work and demonstrates that E. coli increased in the gastrointestinal tract at late time points (up to 144 hours) after treatment with irinotecan. Extensive histological changes were observed in the colon from 96-120 h which correlates with the time of onset for delayed severe diarrhoea. The luminal environment in the colon is usually of a reduced nature, allowing the domination of anaerobes (Evaldson et al. 1982). An increase in oxygen levels allows the proliferation of aerobic bacteria, especially *E. coli,* which is a known producer of β-glucuronidase (Takasuna et al. 1996).

Increased β-glucuronidase expression was seen in the colon, with a considerable increase observed 96-120 h after treatment, again, correlating with moderate-severe

late onset diarrhoea. An increase in E. coli (which is known to produce β-glucuronidase) was observed 96 h following treatment. These findings are highly significant as they confirm for the first time that intestinal bacteria and β-glucuronidase expression are increased following irinotecan treatment, resulting in augmented toxicity from β-glucuronidase activity.

It is known that changes to the luminal environment of the GIT result in altered absorption and other functions (Geibel 2005). Changes to absorption are likely to alter the composition of the faecal excrement, including the composition of microflora present. The information from this study supports current theories (Takasuna et al. 1996), which suggests that the intestinal microflora play a fundamental role in the devlopment of chemotherapy-induced mucositis. In particular the intestinal microflora that express β-glucuronidase activity play a key role in the characteristic late-onset diarrhoea observed following irinotecan (Takasuna et al. 1996). Qualitative changes to the microflora after irinotecan treatment have been demonstrated previously, indicating changes to a variety of bacteria composing the intestinal microflora (Stringer et al. 2007). Changes to the luminal environment, and subsequent changes to absorption are likely to cause altered electrolyte levels after chemotherapy treatment (Stringer et al. 2007).

Previous studies using irinotecan in rats have looked at goblet cells and noted an increased presence of mucus at the apical surface of the intestine (Gibson et al. 2003; Bowen et al. 2007). This study has characterised goblet cell changes after irinotecan treatment, and also extends previous studies by showing increased goblet cells in the

jejunum after treatment (with increased cavitated cells, indicating an accelerated increase in mucin secretion). There is a decrease in the number of goblet cells after treatment in the colon. Decreased goblet cells indicate release of mucins from the mucosal surface, and cavitation of mucus cells is a sign of accelerated mucus secretion by compound exocytosis (Barcelo et al. 2000). Both the increase in cavitated cells in the jejunum and decrease in normal goblet cells in the colon correlate with the incidence of diarrhoea, indicating accelerated mucus secretion in the jejunum, and mucin release in the colon may be contributing to the diarrhoea. Cells expressing Muc4 were decreased significantly in the jejunum at 96 h, corresponding with the increased cavitated cells, suggesting that mucus secreted may consist of Muc4 mucins.

These results now provide a target for further research into therapeutic developments for treatment and/or prevention of CID. Takasuna and colleagues (Takasuna et al. 2006) have already explored the relationship between antibiotics and the β-glucuronidase inhibitor, baicalin, with mixed results. Probiotics are known to exert beneficial effects to the host when ingested, and therefore could be useful in controlling the intestinal microflora during chemotherapy (Quigley 2007). We have previously shown that VSL#3 is able to reduce the severity and duration of CID in rats (Bowen et al. 2007). Further research into the role that probiotics could play in ameliorating CID should also be explored. The role of anti-secretory agents may also prove to be of use in averting excessive mucus secretion. The aim now is to determine if the same effects on the microflora and mucins are seen in the clinical setting, in

patients with CID following irinotecan treatment, allowing translation from animal studies to the oncology clinic.

In conclusion, irinotecan-induced diarrhoea may be caused by an increase in some β-glucuronidase producing bacteria, especially *E. coli*, exacerbating the toxicity of active metabolites. Accelerated mucus secretion and mucin release may also be contributing to the delayed onset diarrhoea.

7.0 Chemotherapy-induced diarrhoea and changes in the faecal flora of cancer patients

7.1 Introduction

Diarrhoea is a major clinical manifestation of alimentary mucositis. The underlying pathology of oral (Sonis 2004; Sonis 2004; Sonis et al. 2004) and small intestinal mucositis has been well studied (Gibson et al. 2002; Gibson et al. 2003; Bowen et al. 2005; Gibson et al. 2005), although the mechanisms contributing to diarrhoea are poorly understood (Engelking et al. 1998; Gwede 2003; Viele 2003; Gibson and Keefe 2006; Stringer et al. 2007). Regimens, for colorectal cancer in particular, illustrate that the diarrhoea component of mucositis is one of the most common and clinically important aspects of toxicity. The absolute percentage of patients that have CID as a result of their treatment has yet to be fully defined, although general estimates place 10% of patients with advanced cancer as being afflicted (Wadler et al. 1998; Gibson and Keefe 2006). This represents a significant clinical, and importantly, economic burden in oncology. The presence of any mucositis during a cycle of chemotherapy significantly increases the risk of dose reduction, the frequency of infections and bleeding, and increases the length and cost of hospitalisation. Reductions in treatment doses lead to reduced survival (Savarese et al. 1997).

Chemotherapy regimens cause diarrhoea to varying degrees. In particular those that contain fluouropyrimidines and irinotecan appear to cause extensive diarrhoea, with

incidences being reported to be as high as 50-80% when these drugs are administered (Saltz et al. 1996; Wadler, Benson et al. 1998; Viele 2003). It has been reported that the mechanisms differ between chemotherapeutic agents (Wadler, Benson et al. 1998), although limited supportive evidence has been cited. The majority of basic research conducted to elucidate the mechanisms behind CID has focused on irinotecan, with few studies existing using other drugs.

Much of the information regarding CID in the published literature is based on clinical observations with very little basic science. Recent research conducted in our laboratory has attempted to correct this anomaly and has found a correlation between CID and changes to the intestinal microflora in our animal model (Stringer, Gibson et al. 2007; Stringer et al. 2007). The purpose of this study was to translate the previous data obtained from animal studies into a pilot clinical study. The primary aim was obtain preliminary data to determine if changes in microflora observed in animal models of chemotherapy-induced mucositis and diarrhoea compared with clinical data, and to determine if the faecal flora of patients with CID was displaced from that of healthy controls.

7.2 Patients and Methods

7.2.1 Patients

All patients recruited for this study were inpatients due to mucositis manifesting as diarrhoea. Sixteen patients (6 males and 10 females) with a median age of 71 years (range 36-82) receiving chemotherapy provided informed consent. Patients receiving concurrent radiotherapy were excluded from the study to eliminate radiotherapy-induced diarrhoea. All other medications were recorded for each patient. The malignancies (number of patients) included: breast cancer (two), colorectal cancer (eleven), laryngeal cancer (one), oesophageal cancer (one), and melanoma (one). There were not enough subjects to perform a subset analysis based on the individual chemotherapy agents, as many different chemotherapy regimens were used for these patients. In addition to these subjects, stool samples from two healthy volunteers (1 male and 1 female) were obtained for the study (Table 7.1). This study was approved by the Ethics of Human Research Committee of the Royal Adelaide Hospital and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrolment in the study.

This was a non-invasive study, with patients requested to provide stool samples and blood samples, taken after the onset of CID. The analysis of blood samples was carried out by the Institute of Medical and Veterinary Science, where complete blood examination (CBE) and biochemical analyses were performed and compared with internal normal reference ranges. Stool samples were analysed in the laboratory using conventional culture techniques and quantitative real time PCR.

7.2.2 Culture of samples

To determine the which bacteria were present in the faeces of patients experiencing CID, a variety of selective and non-selective media (Oxoid, Adelaide, Australia) was used in an attempt to identify as many bacteria as possible from the GIT. For full details refer to 2.2.6 Culture of samples (page 42) and Table 2.1 (page 44).

7.2.3 Extraction and purification of DNA from Bacterial culture and faecal samples

7.2.3.1 DNA Extraction from bacteria

DNA was extracted from standard bacterial samples (Table 1) using a DNeasy Tissue Mini Kit (Qiagen). For full details refer to 2.2.7.1 DNA extraction from bacteria (page 46).

7.2.3.2 DNA Extraction from samples

DNA was extracted from faecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Doncaster, Australia). For full details refer to *2.2.7.2 DNA extraction from samples* (page 46).

7.2.3.3 Real-time PCR

Real-time PCR was carried out on a Corbett Rotorgene 3000 rotary cycler (Corbett Research, USA). Primers used in this study have been used previously (Edwards et al. 2001; Matsuki et al. 2002; Matsuki et al. 2004; Rinttila et al. 2004; Sakai et al. 2004;

Penders et al. 2005; Layton et al. 2006) (**Table 2.2**, page 47). For details refer to **2.2.8 Real-Time PCR** (page 48).

7.3 Results

7.3.1 Complete blood examination

The CBE revealed that 50% of patients with CID were anaemic. Erythrocytopaenia was also evident, observed in 43% of patients. Thrombocytopaenia was evident in 37% of patients. Leucocytopaenia was present in 56% of patients, with 55% of these patients displaying neutropaenia and 88% displaying lymphocytopaenia. However, lymphocytopaenia was present in 75% of all patients.

7.3.2 Electrolyte analysis

Biochemical analysis showed 62% of patients with mild hyponatraemia, 50% with hypokalaemia, 18% with hypochloraemia and 18% with hyperchloraemia. Low bicarbonate levels were exhibited in 50% of patients, and 6% of patients had low and 18% had high anion gaps.

7.3.3 Faecal microflora analysis

The overall culture analysis revealed that 75% of patients had a decreased anaerobic component of their microflora, with respect to both the level of growth and diversity of species present. No marked differences between gender were observed. One patient presented with no detectable bacteria in culture, and another patient exhibited a presence of Staphylococcus spp. only in their faecal samples. Interestingly, both of these patients received a form of antiobiotic prior to sample collection. There was a large difference in the diversity of bacteria present in the microflora between patients experiencing CID and healthy controls, and also between individuals (Figure 7.1).

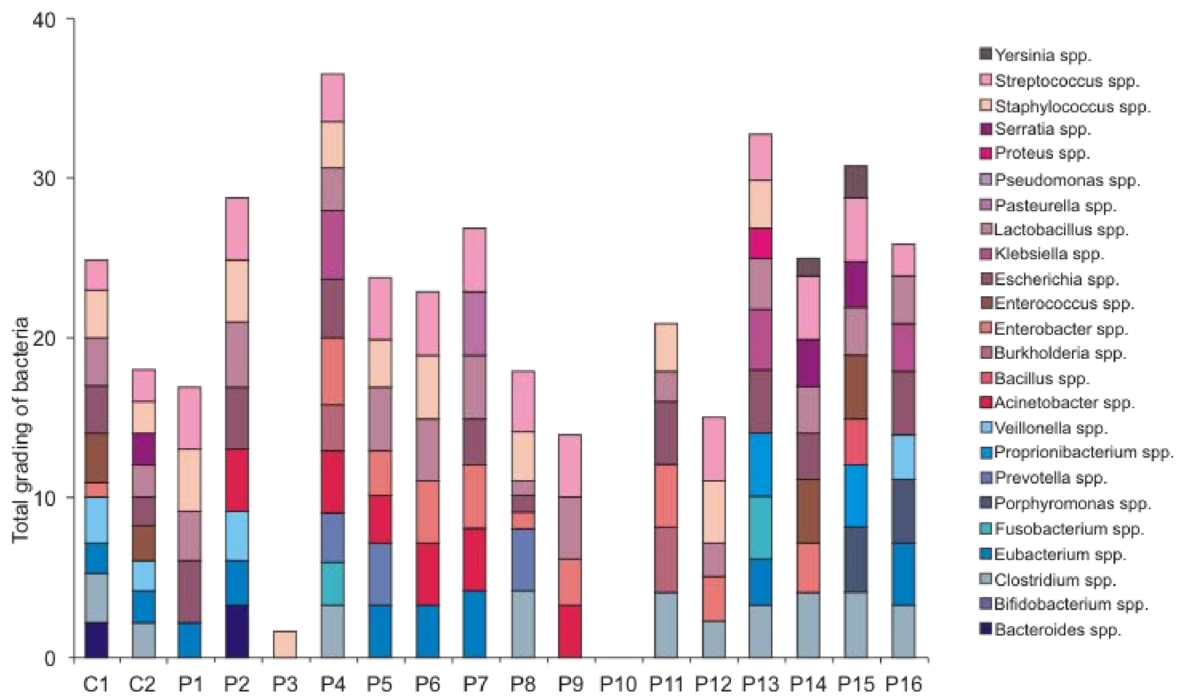


Figure 7.1 Microflora composition of patients experiencing chemotherapy-induced diarrhoea (P1-16) and healthy controls (C1-2).

Quantitative real time PCR demonstrated differences in the amount of bacteria present in the faecal samples of patients with CID and healthy controls. The majority of patients experiencing CID showed a decrease in *Clostridium spp*. (with some exceptions), *Lactobacillus spp.* (with some exceptions), *Bifidobacterium spp.* (one exception) and *Bacteroides spp*. (all patients) and *Enterococcus spp*. (all patients) (Figure 7.2). Increases were also observed in *E. coli* and *Staphylococcus spp.* (with some exceptions) (Figure 7.3).

7.3.4 Antibiotics

A number of patients (Patients 1, 3, 7, 10, 12 and 15) received antibiotics prior to collection of a faecal sample. Collectively, these patients did not exhibit any marked differences or trends with respect to the changes in microflora, demonstrated with either culture techniques or quantitative real time PCR, compared with patients who did not receive antibiotics.

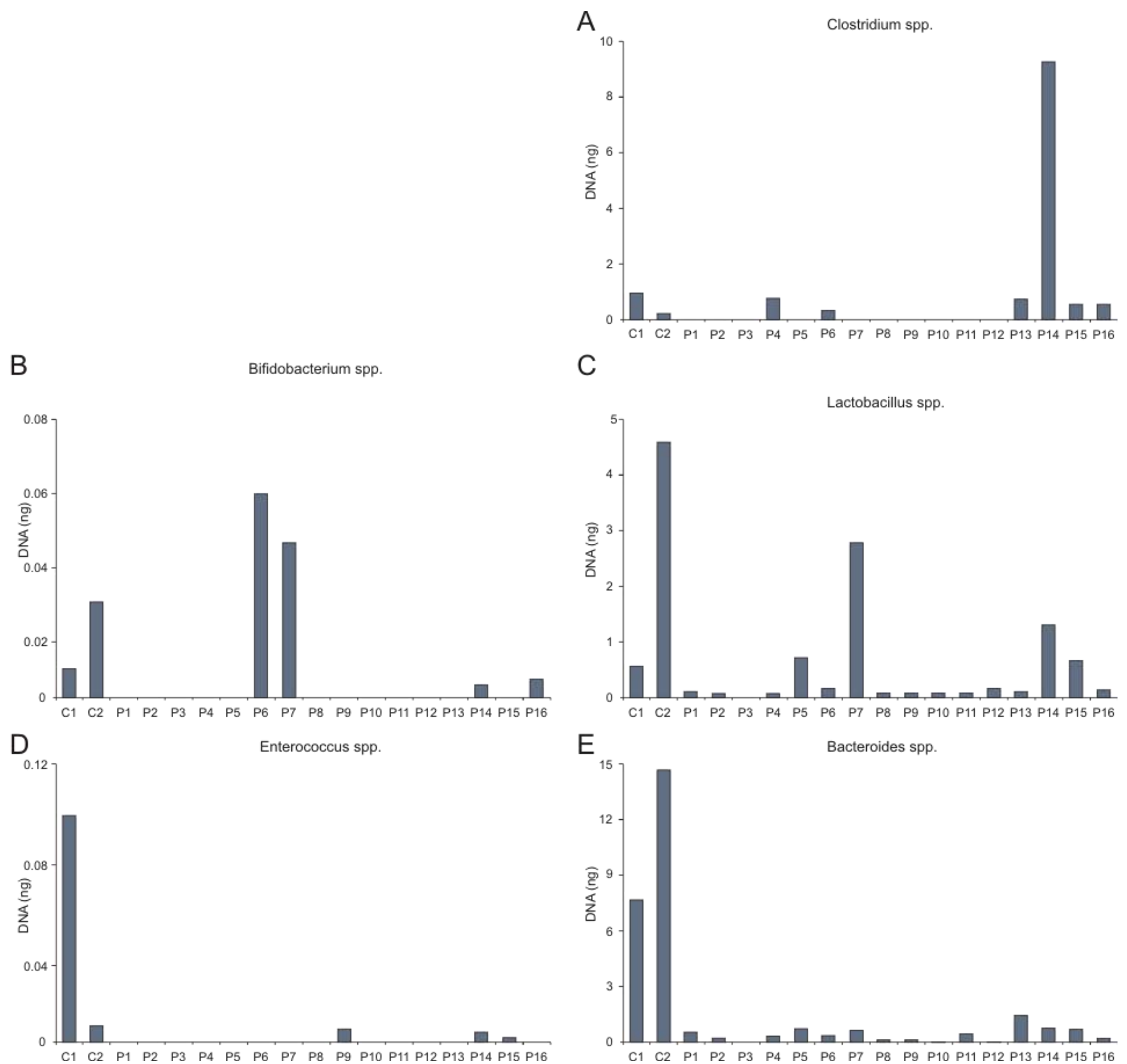


Figure 7.2 Decreases in A) *Clostridium spp.*, B) *Bifidobaterium spp.*, C) *Lactobacillus spp.*, D) *Enterococcus spp.* and E) *Bacteroides spp.* were observed in the majority of patient experiencing CID.

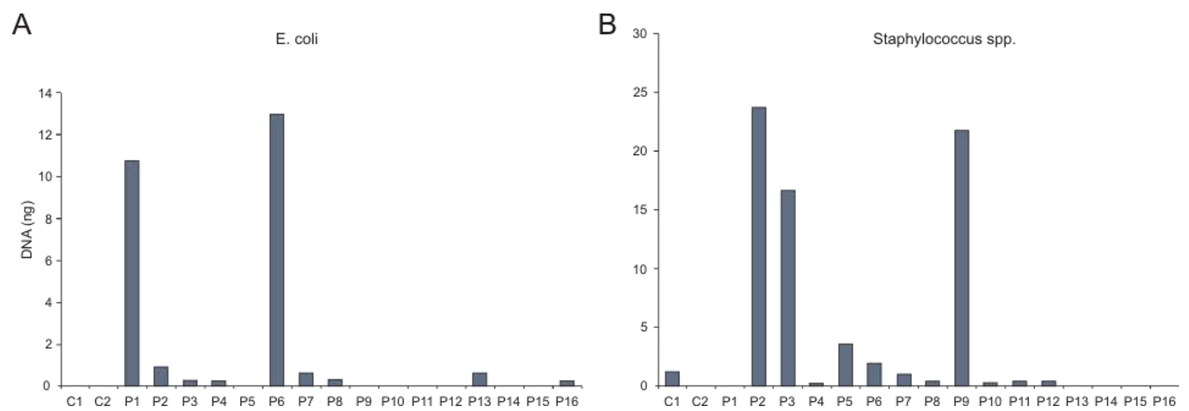


Figure 7.3 Increases in A) *E. coli* and B) *Staphylococcus spp.* were observed in the majority of patients experiencing CID.

7.4 Discussion

Chemotherapy-induced diarrhoea (CID) is a well-recognised side effect of cancer treatment (Saltz, Shimada et al. 1996; Engelking, Rutledge et al. 1998; Wadler, Benson et al. 1998; Gwede 2003; Viele 2003; Benson et al. 2004). Unfortunately, there has been very little research conducted in the underlying mechanisms. Of the total number of patients undergoing chemotherapy, approximately 20-40% experience severe diarrhoea (Takasuna et al. 2006). The numbers could be higher, but hidden, due to the awkwardness of discussing the subject. Most of the current literature is based on clinical observation only, with very little scientific-based studies being conducted. The pathophysiology of CID has been recognised as being extensive, complex and regulated by a number of mechanisms (Gwede 2003; Viele 2003) which are yet to be completely understood. The chemotherapeutic agents known to cause diarrhoea are 5-Fluorouracil, Irinotecan, Methotrexate, taxanes, monoclonal antibodies and hormonal agents, among others (Gibson and Keefe 2006). In the present clinical study, many of these agents are the causative agent for CID. Previous research has shown intestinal microflora to be involved in chemotherapy-induced mucositis in animal models (Berg 1983; Bodet et al. 1985; Von Bultzingslowen et al. 2003; Brandi et al. 2006; Bowen et al. 2007; Stringer, Gibson et al. 2007). This is the first pilot clinical study to examine the faecal microflora in conjunction with CID based on the results from previous animal studies.

Marked changes were observed in the microflora of patients experiencing CID. It is likely that these changes are playing an important role in the development of CID in

these patients, as numerous studies have indicated that intestinal microflora may be involved in chemotherapy-induced diarrhoea and mucositis (Berg 1983; Bodet, Jorgensen et al. 1985; Takasuna et al. 1996; Von Bultzingslowen, Adlerberth et al. 2003; Brandi, Dabard et al. 2006; Bowen, Stringer et al. 2007; Stringer, Gibson et al. 2007). The changes observed varied between patients, and this may be due to a variety of reasons, including but not limited to natural variation between subjects, due to age, gender, race and dietary influence (Heller and Duchmann 2003). However, when genders were differentiated there was no difference. There were not enough enrolled patients in this pilot clinical study to differentiate into age groups. Race and dietary influence were unknown.

The individual effects of each chemotherapy agent may be another reason for the variability observed. Previous animal studies have demonstrated changes to intestinal microflora in animal models utilising chemotherapy agents (Achord 1969; Berg 1983; Von Bultzingslowen, Adlerberth et al. 2003; Brandi, Dabard et al. 2006; Stringer, Gibson et al. 2007), with different changes observed with each chemotherapy agent used. It is likely that the differences seen may be due to the different classes and subsequent different mechanisms of action of chemotherapy agents. Irinotecan is an example of one agent where the intestinal flora are thought to play a role in the actual metabolism of the drug (Takasuna, Hagiwara et al. 1996; Sandmeier et al. 2005; Brandi, Dabard et al. 2006; Hidaka et al. 2007; Stringer, Gibson et al. 2007). The metabolism of irinotecan has been published previously (Smith et al. 2006). Briefly, the intestinal microflora are thought to produce β-glucuronidase, which is able to hydrolyse SN-38G

(the less toxic metabolite from irinotecan), once transported to the intestine, to SN-38 (the toxic metabolite) resulting in the characteristic damage and diarrhoea (Araki et al. 1993; Ikuno et al. 1995; Takasuna, Hagiwara et al. 1996; Smith, Figg et al. 2006).

Whilst alteration to microflora is unlikely to be the sole foundation of CID, it is likely to substantially contribute. The intestinal bacteria provide a variety of functions that mammalian species have not yet evolved in exchange for residence and access to nutrients in the luminal contents (Wexler 2007). Protection is one such function, where the intestinal microbiome is able prevent colonisation by pathogens (Gustafsson 1982; Neish 2002). Metabolism of bilirubin, intestinal mucins, pancreatic enzymes, fatty acids, bile acids, cholesterol, carbohydrates and steroid hormones are also important features required for normal gut function (Umesaki et al. 1997; Cassel et al. 2008). Other functions of gastrointestinal bacteria include nutrient processing, regulation of intestinal angiogenesis, and immune functions (Umesaki and Setoyama 2000; Cassel, Sutterwala et al. 2008). *Bifidobacterium spp.* is known to colonise the gastrointestinal tract soon after birth (McCartney et al. 1996), and is also known to play a pivotal role in maintaining the microbial balance in the healthy gastrointestinal tract (Masco et al. 2006). Alteration of the normal ecosystem can lead to either an overgrowth of some species of bacteria increasing the risk of infection, or a depletion of some species, with a subsequent loss of bacterial functions in the gastrointestinal tract (Neish 2002). Chemotherapy-induced changes to the microflora are likely to result in diminished bacterial function in the gut, which may contribute to intestinal damage, in turn contributing to CID.

CID is likely to be multifactorial and may be caused by combinations of varying factors (Gibson and Keefe 2006). The other factors involved are likely to include altered gut motility, which leads to reduced transit time through the intestine and less water absorption; colonic crypt damage, the site of water absorption in the colon; changes to microflora, also affecting absorption and other intestinal functions involving the microflora; and altered fluid transport in the colon (Gibson and Keefe 2006). There is increasing evidence to suggest diarrhoea may also be caused by other influences (Read 1993). These may be related to enteric nerve reflexes, and involve luminal stimuli (bile acids, bacterial products, mucosal inflammation) that acts via epithelial receptors to induce both secretion and propulsive motor activity in the intestine (Read 1993).

A recent study has proposed that abrupt changes in the microbiome may result in excessive generation of reactive oxygen species (ROS) in the epithelium (Kumar et al. 2007). The previously proposed, and now widely accepted, 5-phase model of mucositis involves an up-regulation and message generation phase (Sonis 2004). In this model, ROS are implicated in the up-regulation of nuclear factor kappa B (NFκB), and subsequent up-regulation of pro-inflammatory cytokines, inducing inflammation, apoptosis and tissue injury (Sonis 2004). The changes to intestinal microflora observed in this study may be partly responsible for initiating the NFκB pathway, resulting in extensive intestinal damage and diarrhoea.

In conclusion, CID is associated with marked changes in the intestinal microflora. These changes may result in diminished bacterial functions within the gut, altering gut function and initiating intestinal damage, resulting in the onset of diarrhoea.

8.0 General Discussion

8.1 Introduction

Mucositis is a major oncological problem, a result of the treatment of malignant disease with chemotherapeutic agents (Mitchell and Schein 1984; Cunningham *et al.* 1985; Keefe *et al.* 1997; Keefe *et al.* 2000; Keefe *et al.* 2004) and radiotherapy (Duncan and Grant 2003; Keefe *et al.* 2004). The entire gastrointestinal tract (GIT) can be affected by mucositis, with recipients of radiotherapy, and/or chemotherapy exhibiting symptoms such as pain, nausea, heartburn, ulceration, abdominal pain, bloating, vomiting, diarrhoea and constipation (Keefe *et al*. 1997; Keefe *et al*. 2000; Keefe *et al*. 2004). Major progress has been made in recent years in understanding the mechanisms of oral (Sonis 1998; Sonis 2004; Sonis 2004; Gibson *et al.* 2005) and small intestinal mucositis (Gibson *et al.* 2002; Gibson *et al.* 2003; Keefe *et al.* 2004; Bowen *et al.* 2005). However, the large intestine becomes severely damaged following chemotherapy (Keefe 2004; Keefe *et al*. 2004), and further research into the mechanisms of large intestinal mucositis is still required.

8.2 Chemotherapy-induced mucositis and diarrhoea

Mucositis was thought originally to develop entirely as the consequence of epithelial injury (Sonis 2004). However, it is now known that the pathobiology is more intricate,

involving a series of steps, each of which entails complex signalling pathways (Sonis 2004). Briefly, damage (both DNA and non-DNA) is initiated by chemotherapy or radiotherapy, releasing reactive oxygen species (ROS) and inducing the up-regulation of various transcription factors. The resultant pathways are amplified, including apoptosis and inflammatory pathways, leading to ulceration and resulting in additional pro-inflammatory cytokines and damaging enzymes, and increasing vulnerability to bacterial translocation. Healing occurs once radiotherapy and chemotherapy treatment cycles cease (Sonis 2004; Sonis 2004).

This thesis has examined in mucositis, in particular chemotherapy-induced diarrhoea following 5-FU and irinotecan treatment. Furthermore, it has investigated the changes that these cytotoxic agents cause in the jejunum and colon, specifically the changes induced in the goblet cells. Both 5-FU and irinotecan were investigated as they are known to be causative agents for chemotherapy-induced diarrhoea (Gibson and Keefe 2006; Stringer et al. 2007).

The studies investigating the effects of 5-FU entailed in this thesis have demonstrated changes in intestinal bacteria and goblet cells which correlate with histological changes at early time points (up to 6 hours) after 5-FU treatment. Changes in electrolytes were also demonstrated, suggesting that 5-FU-induced mucositis may be the result of several factors. Furthermore, the studies investigating irinotecan entailed within this thesis have utilised an animal model to elucidate some of the mechanisms underlying chemotherapy-induced mucositis and diarrhoea, including increased β-glucuronidase

expression, possibly brought about by increases in β-glucuronidase producing intestinal bacteria, and mucin secretion. In addition, similar changes in intestinal bacteria were demonstrated in a pilot clinical study, supporting the hypothesis that similar mechanisms may translate to the clinic.

8.3 Gastrointestinal microflora

The studies performed here have clearly demonstrated that changes in the gastrointestinal microflora occur following chemotherapy in the stomach, jejunum, colon and faeces, with different profile changes observed between drugs. 5-FU produced decreases in most bacteria in most regions of the GIT, with the exception of *E. coli,* which increased after treatment. It is likely that these changes are seen due to a direct effect on the bacteria in the GIT from 5-FU. Bacterial susceptibility tests for 5-FU were performed and demonstrated that *E. coli* and *P. aeruginosa* were not susceptible, even when applied at the manufacturer's concentration. However, all other bacteria tested were susceptible to varying degrees. It is known that 5-FU is absorbed to a degree in the GIT (Fata et al. 1999; Inomata et al. 2002). Therefore it is likely to exert an effect on bacteria in the GIT. However, pharmacokinetic studies have shown concentrations of 5-FU given intraperitoneally are lower in the peritoneal fluid and colon tissue than the concentrations the bacteria were susceptible to in this study (Wei et al. 2008), suggesting the implication of other mechanisms.

Patients undergoing chemotherapy with 5-FU are susceptible to infections as the result of myelosuppression, and clinical data suggests that infections are often caused by bacteria found in the intestinal tract, such as *E. coli* and *P. aeruginosa* (Tancrede and Andremont 1985; Wells et al. 1987; Nomoto et al. 1992; Nomoto et al. 1992). Organisms involved in maintaining a balanced microecology in the intestine were susceptible to 5-FU, which may allow the proliferation of non-susceptible bacteria and the opportunity for overgrowth and penetration of the damaged mucosa, causing host infections. The loss of intestinal microflora also results in the loss of bacterial function in the gut, including processing of nutrients, regulation of intestinal angiogenesis, immune development in the GIT (Umesaki and Setoyama 2000; Rhee et al. 2004), protection, and metabolism of enzymes, fatty acids, bile acids, cholesterol, steroid hormones and intestinal mucins (Gustafsson 1982). These are important functions in the GIT, and their lacking could also result in decreased GIT functional and protective capabilities.

The composition of the gastrointestinal microflora changed after treatment with irinotecan also, although the changes differed from those observed with 5-FU. Changes differed between regions of the GIT, with some of the larger changes occurring in bacteria of the colon. This study has shown there are significant differences in the levels of bacteria in rats receiving chemotherapy vs. chemotherapy-naïve rats in faecal samples. Furthermore, this study has been able to correlate the incidence of diarrhoea and β-glucuronidase expression in enterocytes, and changes in

the faecal flora of rats, in particular those with β-glucuronidase activity (*E. coli*, *Staphylococcus spp.*, *Bacteroides spp.*, *Clostridium spp.* (Takasuna et al. 1996)), those that have been suggested to inhibit β-glucuronidase activity (*Lactobacillus spp.* (Sreekumar and Hosono 2000)), and those that have general beneficial effects to the intestine (*Bifidobacterium spp.* (Kleessen and Blaut 2005)). This is suggestive of some relationship between these changes and the incidence of diarrhoea. It is likely that the changes in bacteria may be a result of the altered ecology of the luminal environment throughout the entire GIT, and severe damage to the gastrointestinal architecture caused by the chemotherapy agents.

The expression of β-glucuronidase is thought to be important in the irinotecan setting, due to its action on the active metabolites of irinotecan. Expression was increased in the small and large intestine at times when the incidence of diarrhoea was highest, and architectural damage in the small and large intestine was extensive. These findings are highly significant as they confirm for the first time that some intestinal bacteria and β-glucuronidase expression are increased following irinotecan treatment, resulting in augmented toxicity from β-glucuronidase activity.

The changes observed in the microflora were also able to be translated into a clinical study, where faecal samples of patients with CID were also shown to be altered from control subjects. This study was not drug-specific, and although there were patients receiving 5-FU and/or irinotecan, there were also patients receiving a variety of other

chemotherapeutic agents, indicating that specific bacterial changes may be drug-specific, but that changes in the intestinal flora as a whole entity may be involved with all forms of chemotherapy-induced diarrhoea. The intestinal microbiome has been implicated in the pathogenesis of other gastrointestinal disorders, including ulcerative colitis and Crohn's disease (Takaishi et al. 2007), suggesting it may also be implicated in chemotherapy-induced mucositis and diarrhoea.

8.4 Gastrointestinal mucins

The number and appearance of goblet cells has been shown to not alter after irinotecan administration in the small intestine of the DA rat (Gibson et al. 2003). However, the same study showed that goblet cells in the large intestine exhibit a more diverse mucin composition after treatment with irinotecan. Mucus production is increased, despite a decrease in goblet cell numbers, which may play a role in the development of CID (Gibson et al. 2003). Treatment with irinotecan has also been shown to cause goblet cell hyperplasia with mucous hypersecretion in the caecum, considered to be a main cause of diarrhoea (Ikuno et al. 1995). Bacteria (intestinal flora) and mucin secretion have both been shown to be affected in chemotherapy-induced mucositis (Farrell et al. 1998; Meslin et al. 1999; Gibson et al. 2003; Stringer et al. 2007). The results from the present study suggest there may be a strong link between intestinal flora and mucins in the pathogenesis of chemotherapy-induced mucositis and diarrhoea.

Mucin metabolism has previously been demonstrated to be influenced by multiple doses of 5-FU (Saegusa et al. 2007). The present study has shown that a single dose of 5-FU also influences mucins, affecting composition, distribution and secretion. Goblet cells in the jejunum tend to decrease temporally after treatment with 5-FU, with crypt goblet cells being affected the most, decreasing significantly from 24-72 h. A decrease in goblet cells is indicative of the apical release of mucin. Cavitated cells represent accelerated mucus secretion (Plaisancie et al. 1997). Cavitated cells in the jejunum crypts increased significantly from 24-72 h. Cavitated cells in the villi did not change significantly, suggesting that mucin secretion in the jejunum as a result of treatment with 5-FU occurs primarily in the crypts. The implications of both decreased goblet cells and an increased percentage of cavitated cells could be detrimental to the small intestine. The protective capacity of the mucus barrier could be altered after the stored mucins have been depleted. The rapid secretion of mucus is thought to be caused by enteric neurotransmitters acting on epithelial cells (including goblet cells), such as acetyl choline (ACh), substance P (SP) and vasoactive intestinal peptide (VIP) (McConalogue and Furness 1994), which suggests that 5-FU absorption by the intestine may cause an up-regulation of neurotransmitter release from enteric neurons, resulting in increased mucin secretion. Goblet cells and cavitated cells did not change significantly in the colon, suggesting no significant change to the protective capacity of mucins in the colon.

Previous studies using irinotecan in rats have looked briefly at goblet cells and noted increases in mucin secretion (Gibson et al. 2003; Bowen et al. 2007). This study extends these studies by showing decreased intact goblet cells (indicating release of mucin) (Barcelo et al. 2000), and increased cavitated cells (a hallmark of accelerated mucus secretion) (Barcelo et al. 2000) in the jejunum crypts. The decrease in goblet cells (release of mucin) occurs just prior to the occurrence of extensive pathological damage, a peak in nuclear factor kappa B (NFκB) expression (Bowen et al. 2007; Logan et al. 2008), and peaks in expression of pro-inflammatory cytokines tumour necrosis factor (TNF), interleukin (IL)-1β and IL-6 (Logan et al. 2008). The peak in cavitated goblet cells in the jejunum crypts represents accelerated mucus secretion at 48 h, preceding the delayed onset mucositis and diarrhoea seen with irinotecan in rats (Gibson et al. 2003; Gibson et al. 2005; Gibson et al. 2007). The decrease in intact goblet cells and increase in cavitated cells in the colon also precedes the delayed onset mucositis and diarrhoea associated with irinotecan (Gibson et al. 2003; Gibson et al. 2005; Gibson et al. 2007). The increase in cavitated cells (accelerated mucus secretion) (Barcelo et al. 2000) correlates with the incidence of diarrhoea, suggesting that accelerated mucin secretion in the colon may be contributing to the diarrhoea.

8.5 Future Directions

The present study has demonstrated the association of intestinal microflora and mucins with the development of chemotherapy-induced mucositis and diarrhoea. Bacterial susceptibility testing demonstrated that bacteria were not significantly affected when

exposed to 5-FU and irinotecan. The primary area for future research arising from this thesis is to identify the pathways which may be involved in altering various factors in the GIT which may be responsible for changes to the microbiome.

This study has also revealed an array of targets which can now be pursued for therapeutic intervention against mucositis. Probiotics are known to exert beneficial effects to the host when ingested, and therefore could be useful in controlling the intestinal microflora during chemotherapy (Quigley 2007). A probiotic cocktail, VSL#3, has demonstrated the potential to reduce the severity and duration of CID in rats treated with irinotecan (Bowen et al. 2007). Further research into the role that probiotics could play in ameliorating mucositis should now be explored. The use of specific probiotic bacteria could be a viable option for replacing and/or preventing lost microflora and maintaining gut homeostasis. The careful use of targeted antibiotics against *E. coli* may also be useful.

Research into the potential of anti-secretory agents or agents blocking specific enteric neurotransmitters to avert excess mucus secretion may also have potential in the chemotherapy-induced mucositis and diarrhoea setting. However, the specific mechanisms involved in intestinal mucus secretion would need to be identified.

8.6 Conclusions

In conclusion, there is clear evidence that 5-FU treatment results in changes to the intestinal microflora and mucin secretion, which may be responsible in part for the development of severe mucositis. There is also clear evidence that irinotecan treatment causes changes to intestinal microflora, which coincides with the development of diarrhoea. The increase in β-glucuronidase producing bacteria may compound the toxicity of irinotecan. The intestinal flora of cancer patients experiencing CID is also noticeably different to that of healthy subjects. Irinotecan causes changes to mucin secretion, and the specific expression of Muc2, Muc4 and Klf4, suggesting that secretory control by the enteric nervous system may also be affected by chemotherapy. This research has extended the understanding of chemotherapy-induced mucositis and diarrhoea, complex side effects of chemotherapy. Furthermore, this study has identified new areas for future research into chemotherapy-induced diarrhoea.

9.0 References

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