The Effect of Dietary Lipids on the Heightened Risk for Developing Colon Cancer in the Animal Model of Inflammatory Bowel Disease

BY

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Sabia Jihad Dahleh

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

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LIST OF ABBREVIATIONS

ACF	aberrant crypt foci
AIN-76A	American Institute of Nutrition-76 adult diet
ALA	alpha-linolenic acid
AOM	azoxymethane
ANOVA	Analysis of Variance
CD	Crohn's Disease
CGN	carrageenan
COX	cycloxygenase
DHA	docosahexaenoic acid
DMH	1,2 dimethylhydrazine
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EPA	eicosahexaenoic acid
ERK	extracellular signal regulated kinase
GALT	gut associated lymphoid tissue
HFC	high fat corn oil diet
HFCC	high fat corn oil diet with carrageenan treatment
HFCN	high fat corn oil diet with no carrageenan
HFF	high fat flaxseed oil diet
HFFC	high fat flaxseed oil diet with carrageenan treatment
HFFN	high fat flaxseed oil diet with no carrageenan
IBD	inflammatory bowel disease
LFC	low-fat corn oil diet
LFCC	low fat corn oil diet with carrageen treatment
LFCN	low-fat corn oil diet with no carrageenan
LT	leukotriene
MAP-K	mitogen-activating protein kinases
mRNA	messenger RNA
NSAIDS	non steroidal anti-inflammatory drugs
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PG	prostaglandin
PMN	polymorphonuclear leukocytes
PMSF	phenylmethylsulfonylflouride
RNA	ribonucleic acid
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SMP	skim milk powder
TBS-T	tris buffered saline plus tween
TGF	transforming growth factor

TNF	tumour necrosis factor
TNSB	trinitrobenzene sulphonic acid
TX	thromboxane
UC	ulcerative colitis

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ABSTRACT

The main objectives were to determine if a diet rich in n-6 or n-3 fatty acids would alleviate the risk of developing colon cancer in the carrageenan induced animal model of inflammatory bowel disease and affect the generation of molecules associated with inflammation. Two studies were conducted to meet these objectives. In study-1 male Sprague Dawley rats were fed one of three diet: low fat corn oil (LFCN), high fat corn oil (HFCN), high fat flaxseed oil (HFFN) without carrageenan or with carrageenan at 2% by weight (LFCC, HFCC, HFFC) for two weeks prior to injection with Azoxymethane (15mg/kg) and four weeks after injection. Their colons were evaluated for aberrant crypt foci (ACF). In the second study rats were fed specific diets as described in study-1 for six weeks and colonic mucosae were processed to assess the levels of cyclooxygenases (COX-1/2), transforming growth factor beta (TGF-beta 1/2), and mitogen activated protein kinases (MAPK 42/44). The number of ACF was highest in the HFCN group compared to the LFCN or HFFN groups. In contrast among the carrageenan fed groups, the LFCC group had the highest number of ACF compared to the HFCC or HFFC groups. The HFCN or HFFN diet groups exhibited significantly higher COX-2 compared to LFCN whereas HFFN had the highest level of COX-1 compared to LFCN or HFCN. Among the carrageenan fed groups COX-2 was significantly higher in the LFCN only and had no effect on COX-1. TGF-beta-1 was higher in the LFCN or LFCC groups than in the other groups. TGF-beta 2 significantly increased in the HFCC and HFFC groups compared to the rest of the groups. The MAP-K level was consistently lower in the HFFN group compared to the other groups. These findings demonstrated that n-6 or n-3 rich diets affected the growth of ACF differently in the inflamed verus non-inflamed colons. Biochemical parameters measured in the normal and inflamed mucosa demonstrated also that the HF n-3 and n-6 diets mediate these responses by different mechanisms. These findings support the potential of dietary lipids to serve as therapeutic agents in the inflammatory bowel disease.

CHAPTER 1

LITERATURE REVIEW

"The role of Science is not just to define a repertoire of facts, but to determine their extension, their dependence, their harmony and their role."

Claude Bernard, 1876.

1.1 Ulcerative Colitis: An Overview

One in five Canadians suffers from chronic gastrointestinal problems. They experience a wide range of symptoms such as abdominal pain, bloating, gas, diarrhoea and constipation. Although the symptoms are similar, gastrointestinal disorders fall into broad categories. Inflammatory bowel disease (IBD) is the most life threatening of those disorders. Unlike other forms of gastrointestinal disorders, inflammation and ulceration are clearly visible in IBD. IBD is an umbrella term referring to two chronic diseases that cause inflammation of the intestine: Crohn's disease (CD) and ulcerative colitis (UC). CD is a disease that causes inflammation in any part of the intestine. UC is the inflammation of the large intestine, mostly the colon. In this thesis, UC will be used as the model inflammatory bowel disease and will form the main thrust of this review.

1.1.1 Epidemiology

A recent epidemiological study reported that the incidence, or number of new cases reported each year, of ulcerative colitis in Manitoba is 14.3 for every

100,000 people (Bernstein et al., 1999). The prevalence, or overall number of cases, of ulcerative colitis in Manitoba is 169.7 per 100,000 people in 1998, a significant increase since 1989 from 127 cases per 100,000 (Bernstein et al., 1998). Manitoba has a population of 1.14 million people; therefore, approximately 163 new cases of ulcerative colitis are annually expected to be diagnosed. In comparison, the annual incidence of ulcerative colitis in the United States and northern European countries is 5-10 per 100,000 people, and the prevalence is 100 per 100,000 of the population (Kelly, 1992).

It is difficult to accurately compare epidemiological studies on ulcerative colitis in different geographical regions because of the variability in disease definitions used. However, most resources agree that the rate of incidence and prevalence for ulcerative colitis are highest in Scandinavian countries, Great Britain and North America, and lowest in Asia, Africa and South America (Targan and Shanahan, 1994).

Men are slightly more at risk for ulcerative colitis than women (Bernstein et al., 1998). Ulcerative colitis is most commonly diagnosed in people between 20-29 years (Bernstein et al., 1999). Smoking, oral contraceptives and non-steroidal anti-inflammatory drugs have been associated with decreased incidence of UC (Targan and Shanahan, 1994). The etiology of ulcerative colitis has been linked to genetic disposition, infections, environmental agents and immunological factors (Fenoglio-Preiser et al., 1999).

Manitoba has a high incidence of ulcerative colitis in comparison to other geographical regions. UC is a debilitating disease that affects young people

predominantly, and is presently increasing in prevalence. The etiology of this disease remains a controversy, and a non-surgical treatment has still not been established. For these reasons, research on ulcerative colitis and its possible management by dietary means remains an unexplored area.

1.1.2 Pathophysiology

1.1.2.1 Mucosal immune system

The gut is considered to be the largest immune organ in the body. It is an important subdivision of the immune system, and 25% of the intestinal mucosa is lymphoid tissue, also called gut-associated lymphoid tissue (GALT) (Kagnoff, 1987). The GALT contains three major lymphoid compartments that can be distinguished on the basis of anatomic and morphologic differences. These compartments include: the Peyers' patches, the intestinal lamina propria and the intraepithelial lymphocytes (Kagnoff, 1987).

The Peyer's patches are located on the mucosa and are in close proximity to the intestinal lumen where they initiate the mucosal immune reaction (Targan and Shanahan, 1994). Epithelial cells called M cells surround the patches and have fenestrations allowing bacteria and viruses into the patches (Figure 1). The antigens allowed into the interior of the Peyer's patches in turn activate immune cells, including T cells, macrophages, and B cells (Kagnoff, 1987). The activated cells quickly travel to mesenteric lymph nodes, and then consequently to the systemic circulation.

The lamina propria contains B-lymphocytes, plasma cells, T cells, macrophages, eosinophils, and mast cells dispersed in the lymphatic rich

connective tissue (Targan and Shanahan, 1994). The intraepithelial lymphocytes are available in the villi at a ratio of 1:6 with epithelial cells (Figure 2). They are located near the basement membrane and increase in population and mitotic activity with the presence of antigens (Kagnoff, 1987).

The gastrointestinal mucosa under normal conditions is already in a state of controlled inflammation. During ulcerative colitis, this controlled inflammation goes array, and increases in inflammatory cells and mediators magnify the inflammatory process and injure the intestinal tissue.

1.1.2.2 The inflammatory process

A food or bacterial antigen existing in the intestinal lumen causes the gut's mucosal immune system to be triggered. This immune system reaction is initiated in the Peyer's patch as the antigen flows through the M cells' fenestrations and activates the B-lymphocytes. The presence of the antigen automatically causes increased intestinal blood flow and capillary permeability (Roediger, 1988). This assists in the proceeding immune reaction, which is the release and infiltration of multiple inflammatory cells from the lamina propria of the intestinal lumen (Roediger ,1988). These inflammatory cells in turn release inflammatory mediators that amplify the inflammatory reaction via chemotaxis, increased permeability and blood flow.

Figure 1. Peyer's patches in a human colon. Three M cells are shown in this picture overlying human Peyer's patches (Johnson, 1987)

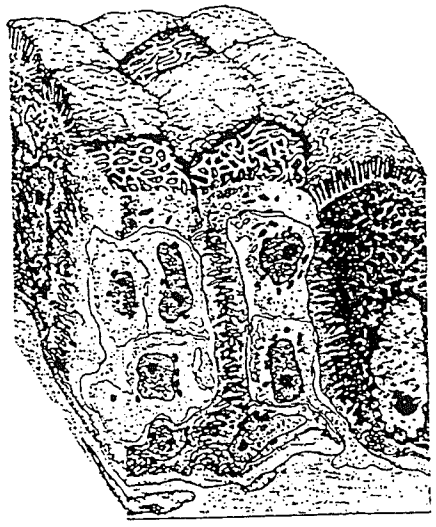
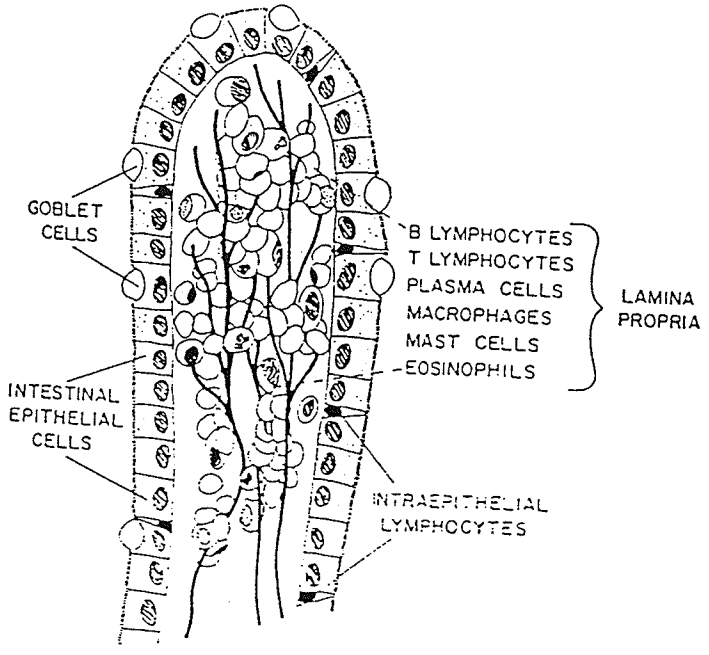


Figure 2. Schematic representation of immune cells in an intestinal villus. Intraepithelial cells are situated between intestinal epithelial cells. B and T lymphocytes, plasma cells, macrophages, mast cells, esonophils are interspersed in the vascular tissue (Johnson, 1987)

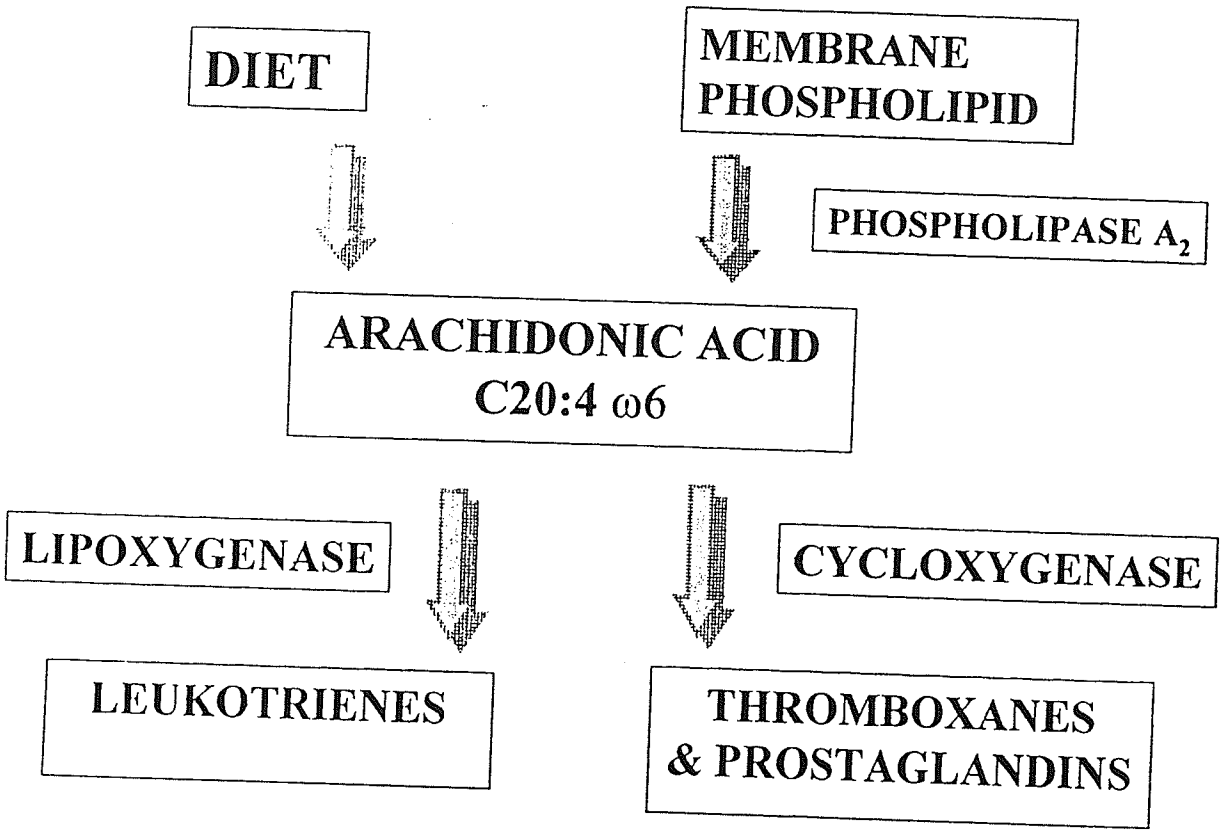


1.1.2.3 Eicosanoids: inflammatory mediators

The eicosanoids are a family of hormone-like compounds that have become important due to their multiple pharmacological and physiological functions. The body synthesizes eicosanoids from the precursor polyunsaturated fatty acid arachidonic acid. Arachidonic acid is either obtained from phospholipids of the plasma membrane or from the diet through the essential fatty acids linoleic or linolenic acid. The eicosanoids consist of the prostaglandins and thromboxanes (synthesized through the cycloxygenase enzyme pathway) or the leukotrienes (synthesized through the lipoxygenase pathway) (Figure 3). Cycloxygenase products are found in all cells, while lipoxygenase products are predominantly found in immune cells, monocytes, macrophages and polymorphonuclear leukocytes (PMNs) (Donowitz, 1985).

The prostaglandins (PG) have various physiological roles in the intestine ranging from modulating colonic function to maintaining mucosal integrity. Prostaglandins can be either pro-inflammatory or anti-inflammatory mediators. PGE_2 is a potent vasodilator that regulates the blood flow in the intestine (Neilson and Rask-Masden, 1996). Some prostaglandins have been shown to stimulate intestinal motility and secretion; for example, studies administering PGE_1 orally found that it accelerated transit rate through the small intestine and colon (Nylander and Mattsson, 1975). PGE_2 has been shown to increase epithelial cell release of chloride, which may contribute to the diarrhoea witnessed in UC (Singer et al., 1998). Higher levels of prostaglandin were found to accumulate in

Figure 3. The cycloxygenase and lipoxygenase enzyme pathway.



ulcerative colitis patients' inflamed mucosa and rectal dialysates (Stenson et al., 1992), and it is generally accepted that there is increased formation of prostaglandins in active colitis state (Yamashita, 1993). Increased prostaglandin production could be the result of high metabolism of arachidonic acid by infiltered activated inflammatory cells and by the colonic tissue itself. Several prostaglandins, especially PGE_2 and TXB_4, may contribute to the inflammatory process in UC by enhancing local vasodilation and edema formation (Neilson and Rask-Masden, 1996). Whether PGE_2 is pro-inflammatory or anti-inflammatory remains to be established.

The lipoxygenase products have a prominent role as inflammatory mediators in the intestine. They stimulate locomotion, lysosomal release and superoxide production in human leukocytes, with LTB_4 being the most potent stimulator (Neilson and Rask-Masden, 1996). Studies have shown a substantial increase in the production of LTB_4 in patients with UC (Henderson, 1994). LTB_4 is found in inflammatory cells, especially neutrophils, macrophages and mast cells. It appears that this leukotriene is integral to the amplification of the inflammatory process as it acts as a potent chemotactic agent attracting numerous PMNs (Ross, 1993). Furthermore, PMNs are a main source of LTB_4 (Shumert et al., 1988). PGs from the E and I series are anti-inflammatory mediators that inhibit LTB_4 synthesis by PMNs, and oppose biological effects of LTB_4 by inhibiting PMN migration and secretion (Neilson and Rask-Masden, 1996).

There remains a lot to be discovered about the varying biological roles of eicosanoids. Future pharmacological interventions that may inhibit pro-inflammatory eicosanoids to control inflammatory responses in diseased states are still being sought.

1.1.2.4 Clinical manifestations of UC

Ulcerative colitis is a chronic, non-infectious inflammatory disease affecting only the colonic mucosa, and in some rare cases the rectal mucosa. The entire colon is inflamed in 90% of UC patients (Ghosh et al., 2000). UC is characterized by cycles of exacerbation and remissions; remissions have been reported to vary from months to years. Upon clinical examination, an UC patient will present an extended and tender abdomen. Patients with active UC almost always have rectal bleeding or bloody diarrhea. Most UC patients complain of mild to no symptoms; in fact, research has stated that 50% of patients are asymptomatic, 30% display mild symptoms and 20% have moderate to severe symptoms (Ghosh et al., 2000). A patient with UC will suffer urgency, incontinence, lower abdominal cramps and pain with defecation. Other common extra intestinal symptoms experienced include tiredness, weight loss, malaise and fever. Approximately 2-7% of patients develop a chronic liver disease associated with ulcerative colitis called sclerosing cholangitis (Ghosh et al., 2000).

There is no single curative drug for ulcerative colitis. Mild to moderate UC attacks are usually managed with sulfasalazine. This drug may affect the immune

response by inhibiting cycloxygenase pathways of arachidonic acid metabolism and hence weakening lymphocytes' function (Sands, 2000). If this drug proves to be ineffective for a patient, then corticosteroids are recommended to help control painful symptoms. This drug greatly decreases the production of pro-inflammatory cytokines, impedes arachidonic acid metabolism and inhibits leukocyte function (Sands, 2000). Furthermore, antibiotics such as metronidazole are sometimes recommended for mild to moderate UC patients. These antibiotics are believed to have an immunomodulatory effect (Sands, 2000). However, patients who fail to respond clinically to drugs are referred to surgical management. Presence of colonic carcinoma is also an indication for surgery. The colectomy procedure is curative, and no further inflammatory episodes can occur (Ghosh et al., 2000).

1.1.2 Increased Risk for Colon Cancer

Many studies have been performed to estimate the risk of colon cancer in ulcerative colitis patients. A meta-analysis summarizing colon cancer incidence rates in ulcerative colitis patients from all studies performed since 1925 reported that the risk for colitis patients is 2% at 10 years, 8% at 20 -years and 18% after 30 years of disease (Eaden et al., 1999). The overall incidence of colorectal cancer in UC is falling (Eaden et al., 1999); this could be due to the increased use of the colectomy procedure as a curative therapy.

There are several factors that increase the risk of cancer in UC patients. Patients with extensive or total colon disease are at increased risk (Eaden and

Mayberry, 2000). It is rare to find colorectal malignancy when duration of disease is less than 10 years. Geographical location has been associated with risk, and incidence has been reported to be highest in North America and United Kingdom (Eaden et al., 1999). Furthermore, the small group of UC patients with sclerosing cholangitis is at increased risk for malignancy (Ghosh et al., 2000).

1.2 Carrageenan-Colitis Model

Carrageenan (CGN) is a sulphated polysaccharide obtained by aqueous extraction of red seaweeds (*Eucheuma spinosum*). CGN is commercially available either in a native undegraded form or in partially degraded forms of lower molecular weight and higher water solubility. Undegraded CGN has obtained popularity in the food industry as a result of its milk reactivity, the ability to bind casein (Ishioka et al, 1987). It is found as a food additive in milk, pudding, yoghurt and even infant formula. Interest in CGN increased after Europeans began using its degraded form to treat peptic ulcers due to its ability to inhibit proteolytic action of pepsin (Ishioka et al., 1987).

Watt and Marcus were among the first researchers to report that the administration of degraded CGN induces ulcerative colon lesions in guinea pigs (1969), rats (1971) and rabbits (1970) that were similar in characteristics to human ulcerative colitis. These colitis-like lesions morphologically had numerous crypt abscesses, necrosis of the crypt epithelium, increased mucous in adjacent

glands, lymphocytic infiltration in the lamina propria and atypical epithelial hyperplasia (Mottet, 1972).

Researchers have reported using various differing treatments of CGN to induce colitis in animals (Table 1). Marcus and Watts (1969) added 5% degraded CGN to drinking water and found that it caused ulceration in caecum, colon and rectum in a 4-6 week treatment. Fabian and colleagues (1973) reported that clinical symptoms of colitis, such as blood in stool, appeared in 5% fed rats in 3-7 days. Kitano and colleagues (1986) reported observing loose stool in rabbits 2 weeks after the start of 1% CGN in water treatment. They also reported that colitis-like lesions were induced within 7-8 weeks using the 1% CGN in drinking water treatment. Research has shown that the administration of degraded CGN in the diet, in contrary to drinking water, presented earlier changes in proliferation (Ishioka et al., 1987).

Long-term treatments of degraded CGN have shown its effects as a carcinogenic factor; hence, CGN can be used to illustrate the relationship between inflammation and carcinogenesis. Kitano and colleagues (1986) reported finding carcinomas in situ, or lesions of high grade dysplasia originating in the flat mucosa. Kawuara and colleagues (1982) studied the effects of

Table 1. Summary of a number of studies performed using different treatments of carrageenan to induce colitis in animals.

Treatment	Animal	Dosage (w/v)	Duration	Researcher
Degraded carrageenan	Rabbits	1% drinking fluid	9 wks	Al- Suhail et al, 1984
Degraded carrageenan	Guinea pigs	5% drinking fluid	5 days	Jensen et al, 1985
Degraded carrageenan	Rabbits	1% drinking fluid	7-8 wks + 28 months	Kitano et al, 1986
Degraded carrageenan	Young adult guinea pigs	1.2% - 3% drinking fluid	2 wks	Marcus & Watt, 1989
Degraded carrageenan	Newly weaned guinea pigs	3% drinking fluid	3-7 days	Marcus & Watt, 1989
Degraded carrageenan	Mice	2% drinking fluid	4 wks	Ziljlstra et al, 1992
Degraded carrageenan	Sprague-Dawley rats	2% drinking fluid	2,4,6,&8 wks	Pricolo et al, 1996

degraded CGN on intestinal carcinogenesis in rats treated with 1,2 dimethylhydrazine (DMH). It seemed that degraded CGN in the diet enhanced intestinal carcinogenesis induced by DMH in rats.

CGN causes an inflammatory response because the intestinal macrophages' lysosomal enzymes cannot digest endocytosed CGN. This results in the congestive swelling of lysosomes, leakage of enzymes into mucosal tissue, inflammation and the ultimate death of the cells (Abraham et al., 1974). Intestinal microorganisms also may have a role in the development of CGN-induced colitis (Onderdonk, 1985).

There are various other chemicals used to induce experimental colitis in animals. The oral administration of other sulphated polysaccharides such as amylopectin sulphate and dextran sulphate has shown similar effects to carrageenan (Tessner et al., 1998). Chemical irritations by rectal application of diluted acetic acid or administration of trinitrobenzene sulphonic acid (TNBS) are other reported experimental colitis models (Kim and Berstad, 1992).

1.3 Colon Cancer

The incidence of colon cancer has been increasing while the mortality rate has remained constant (Semelka and MacEwan, 1987). Colon cancers arise in the epithelial cells outlining the colon's lumen. Carcinomas are the common type of cancer found in the colon and rectum. Other types of cancers such as squamous cell carcinoma, sarcoma, lymphoma and carcinoid tumours are very

rare, and altogether constitute less than 2% of all colorectal cancer. Colorectal cancers begin as polyps, also known as adenomas.

It is generally believed that the transformation of the normal epithelial cell into a cancer cell is a multistage process that is initiated with specific genetic cell alterations affecting the control of epithelial growth, differentiation and programmed cell death (Smalley and DuBois, 1997). The colon's epithelial cells are known to be fast growing cells that are renewed rapidly; in fact, humans completely replace their colonic epithelium every 4-8 days (Lipkin et al., 1999). However, the progression of normal mucosa to adenoma and then to a carcinoma occurs over large period of time, sometimes 10 years or more (Smalley and DuBois, 1997). Under normal conditions, the labile epithelial cells undergo a cell cycle that is regulated by checkpoint mechanisms that allow time for cell DNA repair (Ford and Pardee, 1999). Defective checkpoint mechanism and cell cycle control result in further genetic mutations and progression of carcinogenesis (Ford and Pardee, 1999).

Lifestyle and environmental factors, such as diet, play an integral part in influencing each stage of the slow progression of colorectal cancer from normal colonic mucosa (Figure 4) (Shike, 1999). Epidemiological studies have demonstrated that individuals from countries with low incidence of colorectal cancer gain increased risk of the disease when they move to countries with a high incidence (Shike, 1999). This fact has lead to the strong hypothesis that environmental factors, diet and nutrition are key modulators in the development of colon cancer. This is not surprising since the colon's epithelial cells come into

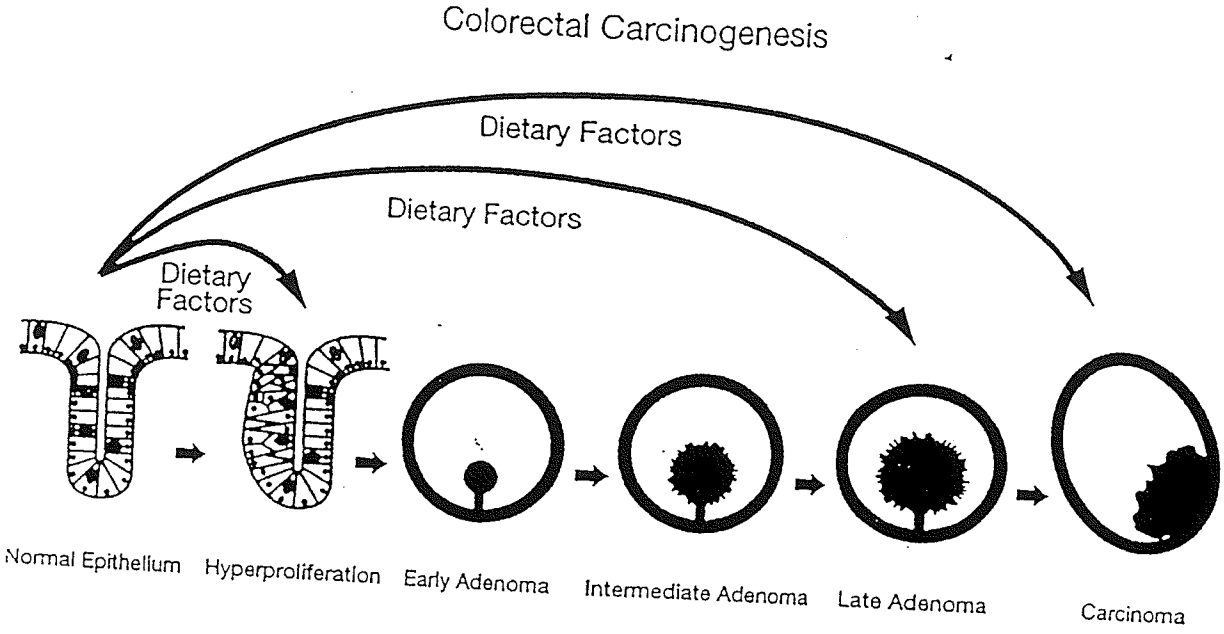
close and ongoing contact with all nutrients from the lumen. Research has associated several dietary factors with colon cancer, these include: excess calories, high fat content, low intake of fibre and calcium (Shike, 1999). Based on many studies, high total fat in diet increases risk of colon cancer by increasing excretion of bile acids, which are believed to act as carcinogens or promoters (Willet, 2000). Interest in dietary factors that may potentially inhibit or promote the progression of colorectal cancer continues to increase.

1.4 Colon Cancer Model: Azoxymethane

In 1963, Laquer and colleagues discovered that feeding rats large quantities of cycasin derived from the cycad flower produced adenocarcinomas in the animals' colons. Cycasin, a naturally occurring form of methylazoxymethanol, was later identified as a colon specific carcinogen. Today, dimethyhydrazine or its metabolite azoxymethane (AOM), which is used to induce tumours in rodents, is the most commonly used model for colon cancer. This model has been valuable in the study of factors that affect the initiation and development of colon cancer.

DMH is oxidized to azomethane that is then converted to AOM. AOM requires metabolic activation before it is able to act as a true carcinogen (Greene et al., 1987). This activation process is summarized in Figure 5. AOM is converted to methylazoxymethanol by N-hydroxylation, and the final product of the activation process is methyldazodium, which is capable of methylating

Figure 4. The influence of dietary factors on the development of the different stages of colon cancer (Shike, 1999).



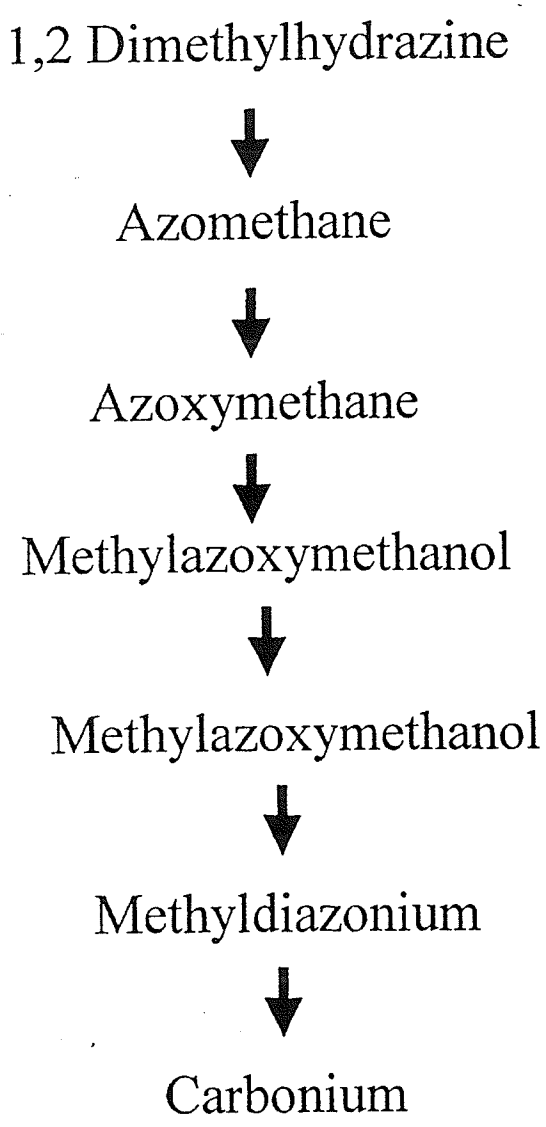
DNA, RNA or protein and hence initiating the carcinogenic process (Greene et al., 1987). It is hypothesized that the methyl groups of DMH are important in its organ specificity and tumour yield (Druckrey, 1970). The liver takes up DMH roughly one hour after injection, and the epithelial cells take up DMH three hours after injection. DMH and its metabolites are transported to intestinal cells by the blood.

1.5 Aberrant Crypt Foci: a Biomarker of Colon Cancer

Aberrant Crypt Foci (ACF) are putative preneoplastic lesions observed on the colonic mucosal surface of carcinogen treated rodents (Bird, 1987). Colon carcinogens, such as azoxymethane, specifically induce ACF in rats and mice (McLellan and Bird, 1988). ACF with identical structural characteristics as in the experimental animal were later identified and quantified in the human colon (Pretlow et al., 1991; Roncucci et al., 1991). The ACF animal model has been recognized as a potential biomarker of colon cancer by many studies, and has been utilized to identify numerous chemopreventative agents in colon cancer. Furthermore, the ACF model has been valuable in providing insight to the pathogenesis of carcinogenesis (Bird, 1995).

Carcinogenesis is a multi-step process summarized into three phases: initiation, promotion and progression (Bird, 1995). During the initiation phase of carcinogenesis, an aberrant crypt forms when a normal crypt exposed to a

Figure 5. This diagram illustrates the activation process of 1,2 dimethylhydrazine to a true carcinogen (Greene at al, 1987).



carcinogen gains growth advantage. The aberrant crypt differs from surrounding normal crypts because of its enlarged size, thicker epithelial lining, asymmetrical and expanded luminal opening, and increased pericryptal zone (Bird, 1987; Bird, 1998) (Figure 6). During the promotion phase, the aberrant crypt expands into clusters of crypts, or foci, with abnormal morphological characteristics. As the carcinogenesis process progresses, the aberrant crypt foci develop into adenomas and adenocarcinomas.

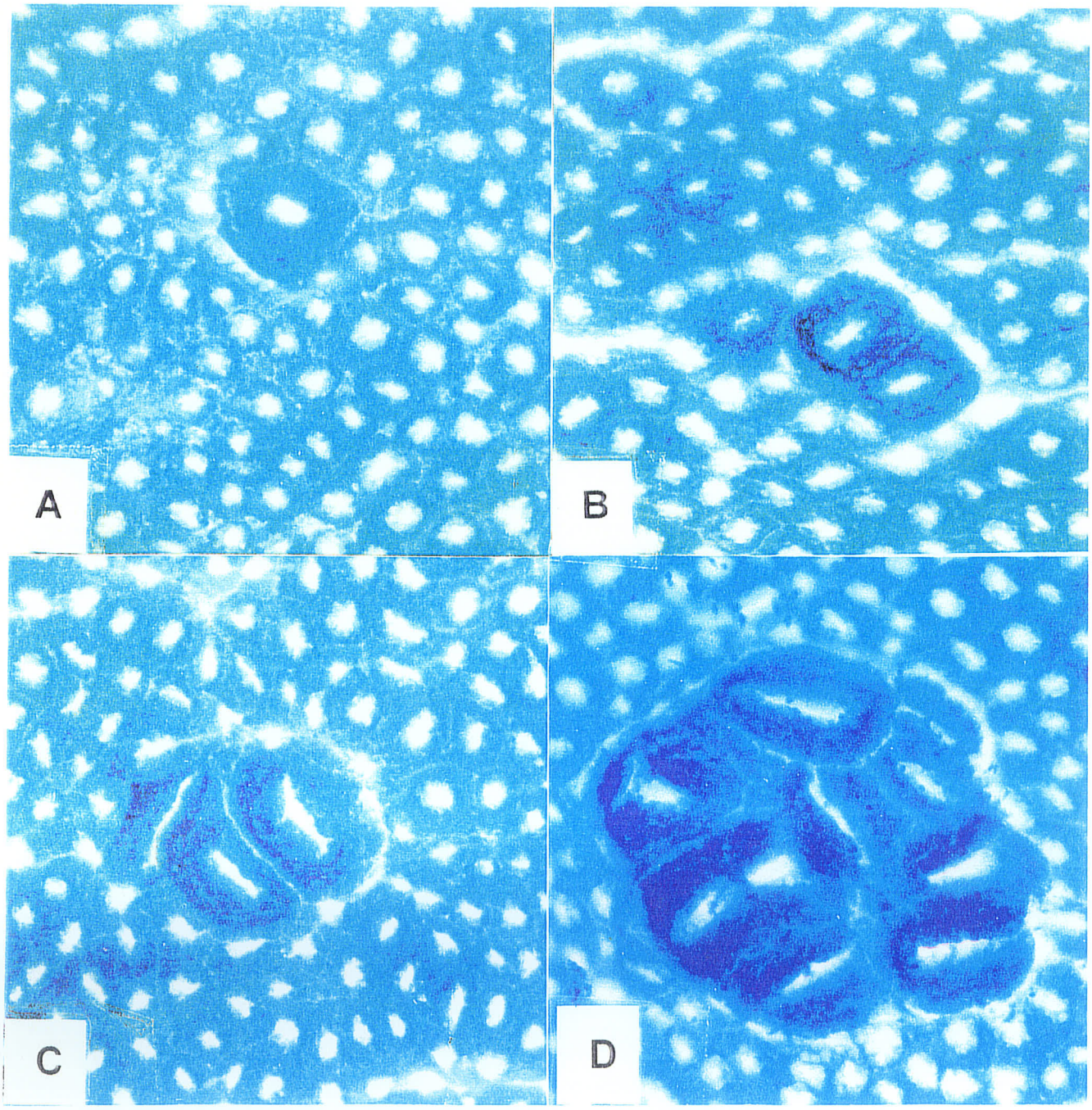
A simple method was developed to observe ACF on carcinogen-treated rodent colon. Fixed, unsectioned colonic mucosa stained with methylene blue and viewed under a microscope at a magnification of 40x reveals morphologically abnormal crypts (McLellan and Bird, 1988). These abnormal crypts begin to appear in rodents two weeks after a single injection of azoxymethane (McLellan and Bird, 1988). The number, size and crypt multiplicity of ACF is dependent on various variables including age, sex and strain of animals, dose and frequency of induced carcinogen, colonic region observed, and duration of study (Bird and Good, 2000).

Adult rats at about 50 weeks of age demonstrate increased susceptibility to azoxymethane-induced ACF compared to young rats at around 4 weeks of age (Magnuson et al., 2000). Male rats exhibit more ACF than female rats (Ochiai et al., 1996); and therefore, estrogen may play a protective role in the female rat. The genetic background and species of laboratory animals determines susceptibility to colon carcinogens (Rosenberg and Liu, 1995) and hence to colon cancer development and ACF induction. In fact, it has been reported that

DMH-treated F-344 rats were more susceptible to long-term administration of carcinogen compared to Sprague-Dawley rats (Nauss et al., 1987). The increased frequency of carcinogen injections was observed to enhance ACF growth and number significantly only after longer experimental durations (Bird and Good, 2000). Furthermore, total number of ACF observed increased with time (Bird, 1995). ACF were noted to be generally more prominent in the rectal end than the ceacal end of the treated rat (Bird, 1995). This observation was related to the varying susceptibility of colonic regions to the colon carcinogen.

The ACF model has been frequently utilized to identify colon cancer preventative or causative agents by their capability of enhancing or inhibiting ACF growth and number (Bird, 1995). The effect of varying dietary fat compositions has been studied lately. It has been found that a high fat diet (high fat beef tallow: 1% corn oil + 22% beef tallow) compared to a low fat diet (1% corn oil + 4% beef tallow) is conducive for the development of high multiplicity ACF (Bird and Good, 2000). A study investigating the effect of dietary lipids with different fatty acid composition on the growth and development of ACF found that a high fat fish oil diet, compared to low fat corn oil and high fat corn oil diets, retarded the growth of ACF from microscopic to macroscopic lesions (Good et al., 1998). It has also been observed that ACF in carcinogen-treated colons of rats fed DHA compared to control have a significantly decreased total number and crypt multiplicity (Takahashi et al., 1994). The literature agrees that essential fatty acids may have a preventative role in colon cancer, but no research to my

Figure 6. This picture shows a topographic view of methylene blue stained ACF. "A" shows an ACF with 1 crypt, "B" shows an ACF with 2 crypts, "C" shows an ACF with 3 crypts, "D" shows an advanced crypt with more than 3 crypts. ACF with more than 3 crypts were almost not observed in the studies presented in this thesis. Pictures were taken from slides made in this study.



knowledge has investigated the effect of a diet high in alpha-linolenic acid, such as flaxseed oil, on the carcinogenesis process using the ACF model.

1.6 Flaxseed Oil: Speculated Benefits

Flaxseed is the richest available source of alpha-linolenic acid (ALA), the precursor to the long chain ω–3 essential fatty acids eicosapentanoic acid (EPA) and decosapentanoic acid (DHA). Only 15% of ALA is converted to EPA and approximately 5% is converted to DHA in humans (Cunane, 1995). The composition of flaxseed oil, as provided by the Flax Council of Canada, is approximately 57% ω-3 polyunsaturated fatty acids (linolenic acid), 16% ω-6 polyunsaturated fatty acids (linoleic acid), 18% monounsaturated fatty acids and 9% saturated fatty acids. Therefore, flaxseed oil is low in saturated fats, provides only a moderate amount of monounsaturated fats, and has a high ratio of ω–3 to ω –6 fatty acids.

ALA has been shown to modulate eicosanoids synthesis (Ferretti and Flanagen, 1996); and hence, it has been speculated that ALA exhibits anti-inflammatory actions. Eicosanoids, such as PGE_2, LTB_4, TXA_2, metabolized from arachidonic acid, are pro-inflammatory mediators. Ingestion of ALA suppresses the biosynthesis and level of arachidonate in the tissues (Caughey, 1996). Alternatively, increased membrane phospholipid levels of ALA, EPA and DHA in inflammatory cells results in the biosynthesis of anti-inflammatory eicosanoids, such as prostaglandins of the 3- and 5- series. Furthermore, dietary ALA influences the immune response through cytokines. Some studies have shown

that the pro-inflammatory cytokines TNF and IL-1 are significantly reduced in people eating omega-3 fatty acids (James et al., 2000). Essential fatty acids may also have a wound healing effect on intestinal epithelium in ulcerative colitis (Ling and Griffiths, 2000).

Drugs capable of modulating the immune system such as azathioprine, 6-mercaptopurine are commonly employed in the management of IBD. However, some patients do not respond to these therapies, or develop side effects, which precludes their continued use. The modulation of dietary fatty acid intake is alternative therapeutic modality. This therapy, referred to as "immunonutrition" by some authors, aims to modify leukotriene and prostaglandin production and down regulate the intestinal immune system (James et al., 2000).

To our knowledge no studies have been conducted to investigate the effect of flaxseed oil or 18:3 n-3 rich diet on ulcerative colitis; however, a fish oil supplement high in EPA and DHA has been researched extensively. Studies examining the anti-inflammatory effect of fish oil supplementation have demonstrated a mild beneficial role in ulcerative colitis. Omega-3 fatty acid dosages ranging from 3.2 to 6.0-grams/ day, for diet durations of 8 weeks to 4 months, showed decreased clinical symptoms, improved histological parameters and decreased medication dosage in ulcerative colitis patients (Lorenz et al., 1989; McCall et al., 1989; Salmon et al., 1990; Stenson et al., 1992). An animal study using TNBS-treated rats supplemented with omega-3 fatty acids showed decreased colonic inflammatory mediators but no effect on mucosal lesions (Ling and Griffiths, 2000).

Researchers report that fish oil has only a modest treatment effect on active ulcerative colitis and does not seem to be beneficial in maintaining ulcerative colitis in remission (Young-in, 1996). This modest effect however can be used therapeutically as corticosteroid sparing (Hawthorne et al., 1992). It is speculated that flaxseed oil will have similar effects as fish oil.

1.7 Role of Cycloxygenase in Inflammation

Cycloxygenase (COX) is a family of enzymes that exists in two isoforms: COX-1 and COX-2. There are considerable distinctions between the two isoforms in terms of their distribution in the body and their functions in disease states. COX-1 is the constitutive isoform expressed in many tissues, such as the intestines and the colon (Singer et al., 1998). This isoform is thought to produce prostaglandins (PG) that partake in acute inflammatory responses, that maintain the GI integrity and that aid in blood clotting (Mitchel et al., 1995; Simon, 1999; Singer et al., 1998). COX-1 is expressed in epithelial cells in the lower half of the crypts in normal colons and UC, showing that COX-1 is expressed as cells become more differentiated (Singer et al., 1998).

COX-2 is the inducible isoform expressed by inflammatory cells such as macrophages during chronic inflammation (Singer et al., 1998). This isoform may also be induced in some tissues during non-inflammatory situations, for example, in the cerebral cortex, endometrium or fetal tissue (Reuter et al., 1996). COX-2 is induced by pro-inflammatory cytokines and intestinal bacterial infections (Singer

et al., 1998). COX-2 is believed to produce PGs that mediate pain and sustain the inflammatory progression (Simon, 1999). It is reported that COX-2 is expressed in surface epithelial cells in inflamed areas in UC (Singer et al., 1998).

Non-steroidal inflammatory drugs (NSIADs) have been known to cause GI ulceration and bleeding in pre-existing ulcers. After the discovery of the cycloxygenase isoforms, the method by which NSAIDs cause mucosal injury was investigated by several studies. A large body of evidence supports the fact that COX-2 inhibition has a gastrointestinal sparing effect. Most of the studies performed to support this hypothesis were not performed on a pre-existing intestinal inflammation, for example, ulcerative colitis. However, when selective COX-2 inhibitors were fed to TNBS colitis-induced rats, exacerbation of inflammation and consequent colon perfusion and death occurred (Reuter et al., 1996). This result was explained by the possibility that COX-2 is responsible for producing anti-inflammatory PGs that preserve mucosal integrity and promote wound healing (Reuter et al., 1996). These prostaglandins are assumed to also decrease pro-inflammatory cytokines, such as TNF and leukotriene B_4, that have been connected to the pathogenesis of ulcerative colitis (Reuter et al., 1996).

Gilroy and colleagues (1999), using carrageenan-induced pleurisy in rats as an inflammation model, describe two distinct inflammatory stages. The early inflammatory stage is dominated by the polymorphonuclear leucocytes (PMNs) for up to 12 hours. These cells are then replaced by roaming mononuclear cells (macrophages) during a later inflammatory stage; the MNs dominate this stage for up to 48 hours. It is hypothesized that COX-2 is pro-inflammatory during the

early inflammatory phase when PMNs are pre-dominant, and anti-inflammatory during the later inflammatory phase when MNs are pre-dominant through its production of a different group of prostaglandins (Gilroy et al., 1999). This is an interesting finding suggesting that NSAIDs, or selective COX-2 inhibitors, use should be adapted according to the phase of inflammation the patient is at.

The precise role of the cycloxygenase isoforms in inflammation remains controversial in many ways. COX-2 inhibitors have been proven to be beneficial for inflammation in rheumatoid arthritis and osteoarthritis. However, several studies have confirmed that COX-2 inhibition in intestinal inflammatory conditions has detrimental effects on inflammation, such as the reported UC exacerbation and relapse exacerbation (Kauffmann and Taubin, 1987; Rampton et al., 1983; Reuter et al., 1996). Whether Gilroy and colleagues' (1999) analogy of the different inflammatory phases and COX-2 functions applies to intestinal inflammation requires further study, but remains an interesting hypothesis and possibility. However, Ajeubor and colleagues (2000) elucidated to a similar analogy when they found an increase in the anti-inflammatory PGD_2 along with increased COX-2 expression early in the inflammatory reaction of TNBS-induced colitis. COX-2 expression remained high as PGD_2's levels decreased after 24 hours of induction. On the other hand, the pro-inflammatory PGE_2 was witnessed to have increased after 72 hours of induction. It is believed that perhaps other cytokines responsible for inducing COX-2 may be facilitating its production of anti-inflammatory or pro-inflammatory prostaglandins (Ajeubor et al., 2000).

PGs produced through COX-2 may play a role in the increased risk of colon cancer in ulcerative colitis. This raises the possibility that inhibition of COX-2 may reduce the risk of malignancy.

Biological Actions of Transforming Growth Factor β

Transforming growth factor β (TGFβ) is a member of a superfamily of growth regulatory factors (Shao et al., 1999) that demonstrate diverse inducive or inhibitory actions (Haung et al., 1995). TGFβ is a 25-kDa homodimer with two disulfide-linked subunits of 12.5 kDa (Adam et al., 2000). This cytokine and its receptors have been found in nearly all the cells in the body (Gerard et al., 2000). However, it is reported that platelets and bone are the major sources of TGFβ in humans (Taipale et al., 1998).

Three isoforms of TGFβ have been found in mammalian tissue, these include: TGFβ1, TGFβ2, and TGFβ3. Each of these isoforms is encoded by a specific gene, and is expressed primarily in different cells. For example, TGFβ1 mRNA is mostly expressed in endothelial and hemotopoietic cells while TGFβ2 mRNA is expressed mainly in epithelial and neuronal cells (Gerard et al., 2000). Few differences have been found in the biological actions of the TGFβ isoforms (Taipale et al., 1998). However, the three isoforms have different binding affinities for the TGFβ receptors (Gerard et al, 2000). TGFβ receptors are located in the upper crypts of the epithelial cells (Ohtani et al, 1995).

TGFβ has various biological actions that have only recently been uncovered. TGFβ inhibits the proliferation or growth of some cells, especially epithelial cells (Koli et al., 1996). It does this by blocking the cell cycle during the G1 (gap 1) phase when the cell is growing and its proteins are being synthesized (Massague, 1990). Furthermore, this cytokine has been noted to also be immunosuppressive because of its inhibitory effects on the proliferation of T, B, NK cells and macrophages (Taipale et al., 1998). However, not all of TGFβ's effects are negative towards the inflammatory process. TGFβ is a potent chemotaxic agent for T cells; and therefore, it increases cell migration during inflammation (Adam, 2000). TGFβ has also been reported to stimulate extracellular matrix formation (Taipake et al,, 1998). TGFβ is produced and released in a latent form that cannot react with its receptors to produce its biological effects (Koli et al, 1996). This latent form is an inactive complex associated with a binding protein (Taipale et al, 1998).

TGFβ has been associated with several diseases, including cancer, heart disease, diabetes, inflammatory bowel disease and fibrotic disease of the kidney, liver and lung (Adam et al., 2000; Lawrance et al., 1999, Gerard et al., 2000;). TGFβ's dual action as stimulator and/or inhibitor of the inflammatory response makes it an implicated factor in the uncontrolled mucosal inflammation witnessed in UC. The pathology of UC is linked to mucosal infiltration of inflammatory cells, and the chemotactic TGFβ has the ability to regulate and modulate these cells.

Babyatsky and colleagues (1996) showed that the TGFβ protein expression was higher in active UC patients than control and inactive UC, and

Babyatsky and colleagues (1996) showed that the TGFβ□ protein expression □was higher in active UC patients than control and inactive UC, and that TGFβ1 mRNA expression in the UC patients was found to be highest in epithelial cells with most inflammatory cell infiltration. On the contrary, Chowdhury and colleagues (1996) reported low expression of TGFβ1□ mRNA in chronic UC patients compared to normal mucosa, and they hypothesized that this low expression could be the reason behind the stubborn unrelenting inflammation in UC since TGFβ1 has been said to play a role in tissue repair.

There are several propositions that could explain the differences in findings between these two studies. Perhaps during persistent and chronic disease states, as investigated by Chowdhury and colleagues (1996), the need for tissue repair and inflammatory cells has peaked and TGFβ □is being synthesized, secreted and used immediately, leaving little time for it to be stored and expressed in the tissue. Another speculation is that TGFβ1 has various roles and hence expression levels in the progressive stages of UC inflammation severity. It may be that TGFβ's□ role is integral to the initiation of inflammation (during the migration of inflammatory cells) and to the remission of inflammation (during mucosal repair and restoration). Furthermore, it is necessary to consider that the main source of TGFβ1 is the inflammatory cell itself, and Lawrance and colleagues (1999) suggest that the increased expression of TGFβ1 depends on the presence and location of infiltrates. Nevertheless, further research investigating the role of this cytokine in inflammation is still needed. However, inflammation is not the only pathological process of UC that TGFβ□ has been

Since UC patients are at increased risk for colon cancer, it is also important to point out that this immunosuppressive and proinflammatory cytokine has a repeatedly verified role in carcinogenesis. In a normal cell, TGFβ acts to inhibit growth and proliferation by interfering with the G1 phase of the growth cycle. During carcinogenesis, mutations may cause cells to resist growth arrest with decreased sensitivity to TGFβ; hence, cells demonstrate uncontrolled proliferation. During carcinogenesis, there is increased secretion of TGFβ by the tumor itself. Eventually, as the cancer metastasizes, TGFβ is stated to cause increased immunosuppression, angiogenesis and ECM production and accumulation (Gerard et al., 2000) (Figure 7).

Research has shown that dietary lipids may play a role in the modulation of growth factors (Fernandes et al.,1994, Harbige et al., 2000). For example, it was noted that fish oil lowers the levels of TGFβ mRNA (Chanrasekar et al., 1995). Adam and colleagues (2000) have also shown that dietary lipids with different fatty acid compositions modified the protein expression of TGFβ1, illustrating a significant difference between increased TGFβ1 expression in high fat beef tallow and decreased TGFβ1 expression in high fat corn oil groups.

The exact mechanism by which the dietary lipids are able to alter TGFβ1 expression is still unknown. However, it is reported that TGFβ may play a role in magnifying or inducing COX-2 expression of intestinal epithelial cells (Shao et al., 1999). Whether COX enzymes regulate TGFβ remains to be seen. How the arachidonic acid metabolites may influence TGFβ's expression is unclear.

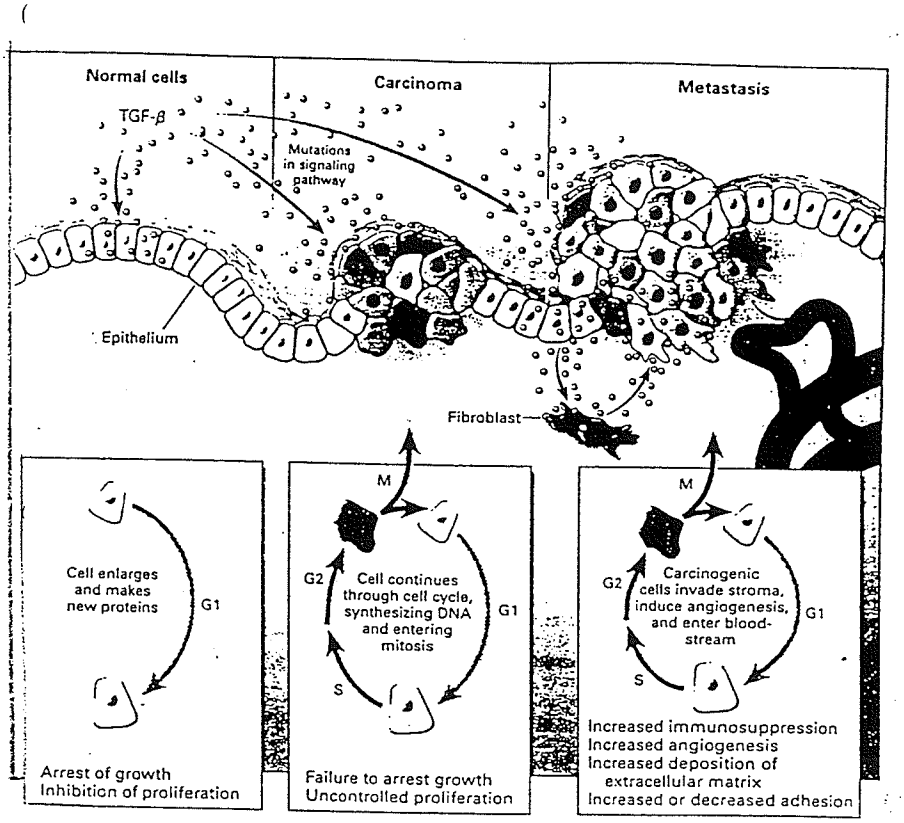
5-lipoxygenase's inhibition, and the resulting decrease in leukotriene production, seems to increase TGFβ1 (Kozubik et al., 1997).

1.9 Mitogen-activating protein kinase (MAPK) signaling pathway

The first members of the MAPK family to be discovered were the p42/p44 extracellular signal-regulated kinases (ERK) (Nebreda, 2000). A number of other MAPKs have been identified since, and it is believed that the different MAPKs respond to distinct stimuli and induce specific biological responses (Nebreda, 2000). For example, the p42/p44 MAPKs are thought to preferentially regulate cell growth and differentiation (Schaeffer and Weber, 1999). However, MAPK pathways produce responses that are cell specific. Johnson and colleagues (1999) demonstrated that p42/p44 ERKs are activated in polymononuclear neutrophils to decrease their cytotoxicity during inflammation.

The MAPK signalling cascades allow cells to respond to growth factors, cytokines and environmental stress (Martin-Blanco, 2000). TGF-β has been shown in different cell types to be capable of activating MAPK. The ERK pathway is rapidly stimulated by TGF-β1, a growth inhibitor (Choi, 2000). Therefore, it is suggested that p42/p44 MAPKs not only transport cell growth responses but also growth inhibitory responses (Choi, 2000).

Figure 7. Role of transforming growth factor β in cancer (Gerard et al., 2000). During carcinogenesis, there is a decreased sensitivity to TGFβ and cells proliferate uncontrollably. The tumour itself secretes increased amounts of TGFβ which causes in turn increased immunosuppression, angiogenesis and ECM production and accumulation.



1.10 Summary

The gut is considered the body's largest immune system. It is also the mostly likely organ to demonstrate exaggerated inflammatory responses. Chronic, consistent inflammation has been associated with increased risk of cancer development. Carrageenan is a sulphated polysaccharide that when ingested in a certain dosage by rats induces colitis, a chronic inflammatory response in the colon. The interrelationship between inflammation and the increased risk of cancer can be investigated by inducing colon cancer in the rats using azoxymethane.

Colitis is a difficult disorder to clinically manage; therefore, interest in alternative therapies has been increasing. Flaxseed oil has been implicated as a having a beneficial role in ameliorating inflammatory bowel disease symptoms. This beneficial role is related to flaxseed oil's high composition of alpha-linolenic acid, an essential fatty acid that may modulate eicosanoids synthesis and provide anti-inflammatory mediators.

The effects of flaxseed oil's lipid composition on inflammation may be witnessed at the molecular level. Research has repeatedly demonstrated that the cycloxygenase enzymes are influenced by lipid composition. These enzymes are responsible for producing inflammatory mediators, such as prostaglandins and leukotrienes. Inflammatory mediators in intestinal inflammation cause severe mucosal damage. Transforming growth factors are implicated in uncontrolled mucosal damage. They are known to be immunosuppressive and to inhibit epithelial cell growth and proliferation. Whether lipid composition may modulate

TGF-β expression in epithelial cells remains to be seen. The ERK signalling pathway transduces the biological responses of the cycloxygenase produced inflammatory mediators and the transforming growth factors.

CHAPTER 2

THE EFFECT OF DIETARY LIPIDS AND CARRAGEENAN ON MUCOSAL INFLAMMATION AND GROWTH OF ACF IN RAT COLON

3.1 Introduction

Dietary lipids have been implicated in the modulation of immune responses. The effects of both the amount and type of dietary fat on inflammation have been investigated. For example, rats fed a fat-rich diet (15% fat) compared to control diet (4% fat) showed a decreased inflammatory response (Lopes et al., 2000). Furthermore, omega 3 polyunsaturated fats are proposed to reduce inflammation. The potential anti-inflammatory effects of fish oil, a marine source of omega 3 fatty acids, on intestinal inflammation have been studied extensively. Most researchers conclude that supplementation with fish oil offers a modest reduction in intestinal inflammation (Lornez et al., 1989; McCall et al., 1989; Salmon et al., 1990; Stenson et al., 1992).

Epidemiological and animal studies have both supported the concept that the type and amount of dietary fat plays a role in the development of colon cancer (Reddy, 1994; Willet, 2000). Diets high in fat exhibit more colon cancer promoting effects than diets low in fat (Reddy, 1994). Animal studies have shown that while diets high in corn oil, safflower oil and beef tallow promote colon carcinogenesis, diets high in fish oil do not have this tumor promoting effect (Bird and LaFave, 1995).

Inflammation has been reported to enhance colon carcinogenesis. A long-term treatment with carrageenan (CGN), an agent that causes colitis-like

symptoms in animals, has been found to be a carcinogenic factor (Kawuara et al., 1982; Kitano et al., 1986). Patients suffering from long-standing ulcerative colitis are reported to have an increased risk for colon cancer (Eaden and Mayberrry, 2000). The role of dietary factors in modulating the effect of inflammation and its associated risk of developing colon cancer has not been investigated.

As described previously (see Chapter 1.5), ACF induced by a colon specific carcinogen are purported preneoplastic lesions. The ACF system has been used to identify chemopreventative agents in colon cancer (Bird, 1995). Degraded CGN when administered in the diet of animals cause colitis (Ishioka et al., 987; Watt and Marcus, 1971). Therefore, the ACF system and diets containing CGN can be utilized to assess the effect of inflammation on the risk of developing colon cancer and the potential role of dietary lipids on these processes.

The main objective of this study was to assess whether dietary lipids - varying in the levels of n-6 and n-3 will affect the induction and growth of ACF in the presence or absence of inflammatory responses. The experimental diets were a low fat corn oil, a high fat corn oil (n-6 rich) and a high fat flax oil (n-3 rich) with or without CGN. It was hypothesized that a high flax oil diet, due to its high content of n-3 alpha linolenic acid (18:3 n-3), will reduce inflammation, which in turn will lower the heightened risk of developing colon cancer whereas a high corn oil diet will not. This hypothesis is based on the proposal that 18:3 n-3 will reduce production of pro-inflammatory eicosanoids from 20:4 n-6.

3.2 Material and Methods

Animals

Weanling male Sprague-Dawley rats purchased from Charles River (Montreal, Canada) were used in this experiment. All procedures involving the animals were approved by the University of Manitoba Animal Care Committee and were conducted according to the guidelines of the Canadian Council of Animal Care (1993). Animals were housed in wire meshed stainless steel cages with sawdust bedding (two or three rats per cage) in a room under controlled environmental conditions (21° C and 50% humidity) and photoperiod (12-h light/dark cycle). Rats had free access to food and water *ad libitum*. Rats were allowed to acclimatize to conditions for several days before experimental diets and agents were introduced. Body weight of animals was measured using an electric, portable top loader balance at the start of the experiment, weekly, and at termination of animals.

Diets

The experimental diets were prepared based on the AIN-76A diet (American Institute of Nutrition, 1977) with some alterations as described in Appendix A. Three diets were made with different fat compositions. The difference in fat content between the low fat and high fat diets was 18% fat by weight; this was compensated for using cornstarch (Appendix A). Furthermore,

the amount of cellulose, vitamin mix, mineral mix and casein in the diets was adjusted to ensure that animals were fed isocaloric diets.

The low fat corn oil (LFC) diet was 5% corn oil by weight. The LFC diet was used as the control diet in the experiments. The high fat corn oil (HFC) diet was 23% corn oil by weight. The high fat flaxseed oil (HFF) diet was 14% corn oil and 9% flaxseed oil by weight. Flaxseed oil is roughly 73% unsaturated fatty acids (Appendix G: Flax Council of Canada); therefore, this oil needs to be stored carefully as it is at increased risk of rancidity. For this reason, the HFF diet's 23% fat composition was split into a 2:3 (flaxseed oil: corn oil) ratio. All diets were stored at 4 °C to prevent oxidation. Fresh diets were made weekly.

Cornstarch and dextrose were purchased from the University of Manitoba Food Services and Moonshiners (Winnipeg, MB), respectively. Flaxseed oil was purchased from Omega Nutrition (Vancouver, BC) and corn oil from Safeway (Mazola, Etioboke, ONT). All other dietary ingredients were purchased form Harlan Teklad (Madison, WI).

Carrageenan

The sulphated polysaccharide degraded λ- carrageenan (Sigma Chemical, Co., St. Louis, MO, USA) was used to induce colitis in the rats. Carrageenan was added to 2% by weight to the experimental diet.

Colon Carcinogen

The colon specific carcinogen azoxymethane (AOM) was dissolved in 0.9% saline and used in experiment. Animals were injected with AOM (Sigma Chemical Co., St. Lious, MO) s.c. once 2 weeks into the study at a concentration of 15 mg/kg BW.

Tissue Preparation

Animals were killed by CO_2 asphyxiation. Colons of AOM-treated animals (n=50) were excised, clamped from one side, and phosphate-buffered saline (BPS) was infused into colons using a syringe. This procedure allowed the colon walls to stretch. The BPS was then flushed through. Next, the colon was cut along the longitudinal axis from the rectal to the cecal end. Colons were fixed flat, mucosal side up, on filter paper immersed in 70% ethanol. Colons were stored at 4 °C for at least 24 hours before being analyzed.

Aberrant Crypt Foci

To score colons for existing ACF, the colon was first sectioned into 4 cm segments using a scalpel to mark the tissue. The colon was then dipped into a Petri dish flooded with 0.2% methylene blue stain for 2-3 minutes. The stained colon strip was next placed on a slide, mucosal side up, and viewed under a light microscope at x4-10 objective.

Various scoring methods were used to make a comparison between the different diet groups:

A. Quantification of ACF:

Starting from the colon's rectal end and proceed to the cecal end, ACF were identified and scored per 4 cm section. This allowed us to assess ACF distribution throughout different colon regions.

B. ACF crypt multiplicity.

The number of crypts existing in each ACF was also scored. An ACF with 2 crypts was identified as having a crypt multiplicity of 2. The multiplicity value of an entire colon or group was determined by calculating the average number of the mean number of crypts in a focus per colon or group. For example, if the following ACF existed in a colon: 3 ACF1 (with one crypt), 3 ACF2, and 1 ACF4, then the average crypt multiplicity for that colon would be: $[(3 \times 1) + (3 \times 2) + (1 \times 4)]/ 7 = 1.86$.

Peyer's Patches

The Peyers' patches are the lymphoid organs of the intestine, and were used as an index of extent of inflammation in the gut. Peyer's patches on colon sections were recognized as small oval lumps. The size of and PCNA labelling pattern of Peyer's patches were assessed. The length and width of the Peyer's patches were measured using a translucent ruler. The areas of these oval-protruding nodules were calculated as an ellipse area ($\pi \times W \times L$: W, width, L, length).

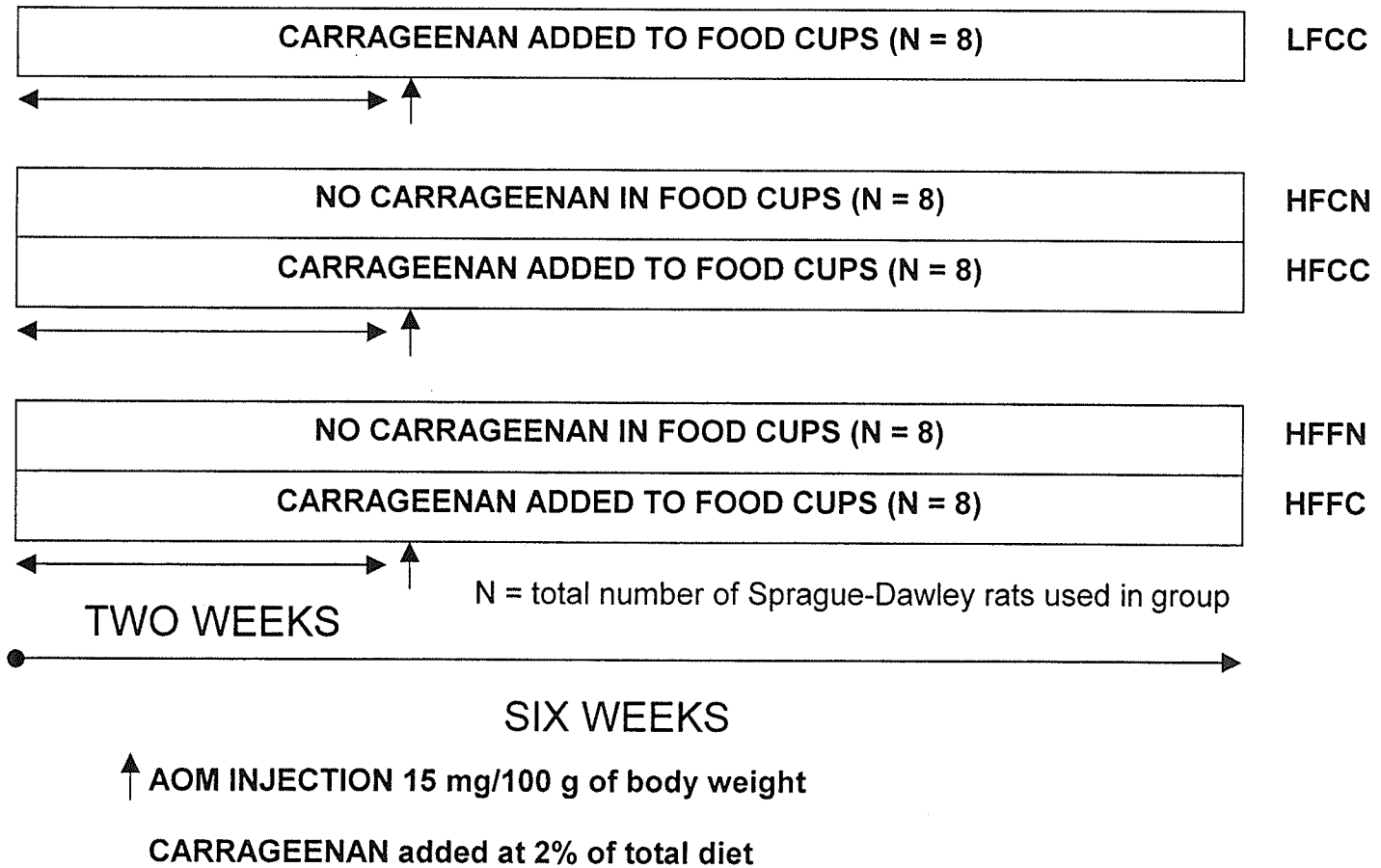
Colonic Inflammation

Stained colonic tissue slides were observed for histological indicators of inflammation. Histological indicators of colonic inflammation induced by CGN reported by researchers include epithelial degeneration, crypt abscesses, increased mucous, infiltrated lymphocytes, and increase in macrophages (Fabian et al., 1973; Ishioka et al., 1987; Mottet, 1972). These parameters were looked at in this study.

Experimental Design

The study's protocol is provided in Figure 8. Male weanling Sprague-Dawley rats were randomly allocated to 6 different groups. Three groups (LFCC, HFCC, HFFC) received CGN in diets that contained different dietary lipid compositions. CGN was added at 2% of total weight of diet. Researchers have reported that CGN induced colitis symptoms appear 2-4 weeks after CGN administration (Kitano et al., 1986; Marcus and Watts, 1969). The remaining three groups (LFCN, HFCN, HFFN) were control groups and were not given carrageenan in their different diets. The animals were injected once with AOM in fresh saline (15 mg/kg) two weeks after diets were initiated. It has been reported that one injection of AOM induces ACF within two weeks of treatment (McLean et al., 1991). After 6 weeks of feeding the experimental diets, animals were terminated with CO_2 asphyxiation and their colon were excised.

Figure 8. Schematic presentation of experimental protocol. Animals were injected with a colon carcinogen after receiving the carrageenan treated diet or normal diet for two weeks. The experiment lasted for 6 weeks. LFCN, low fat corn oil diet with no carrageenan treatments, LFCC, low fat corn oil diet with carrageenan treatment; HFC, high fat corn oil diet, HFF, high fat flaxseed oil diet.



Statistical Analysis

Statistical analysis of the data was performed using the statistics program primer of biostatistics, 4/E version 4.0 software in Windows. Analysis of variance and Duncan's Multiple Range Test at $p \leq 0.05$ were used to determined differences between the groups.

3.3 Results

Body weights of the injected animals

Animals fed the high fat diets weighed on average more than the animals fed the low fat diet (Table 2). Animals receiving CGN treatment experienced some diarrhoea after 3 weeks CGN administration. However, there were no significant differences between the weights of the normal and CGN-treated animals receiving the same diet compositions.

Total Aberrant Crypt Foci (TACF) enumerated

The TACF were enumerated and analyzed starting from the rectal end up to the cecal end. The results describe the total number of ACF and the distribution of ACF throughout the whole colon and in its different colonic regions (Table 3A-D). Three 4 cm segments were sectioned from colons of all animals.

Total colon ACF (1-12 cm):

TACF throughout the whole colon was enumerated to identify total lipid effect throughout colon, regardless of colon region (Table 3A). The trend in TACF

Table 2. Mean weights of AOM-injected animals.

Diet Group [1]	Animal Weights [2]
LFCN	415 ± 49
HFCN	429 ± 41
HFFN	435 ± 41
LFCC	404 ± 16
HFCC	476 ± 21
HFFC	437 ± 47

1. LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil. LFCN, low fat corn oil with no carrageenan treatment; LFCC, low fat corn oil with carrageenan treatment.
2. There were no significant differences in the weights of animals from different diet groups and treatments.

Table 3A. The number and growth features of ACF in Sprague Dawley rats after 6 Weeks Intervention with LFC, HFC and HFF Diets with or without Carrageenan in the 1-12 cm colon

Diet Group	TACF	ACF1	ACF2	ACF3
LFCN	147.9± 21.5	112.2 ± 13.4	31.1 ± 6.9	4.4 ± 2.6
LFCC	158.7 ±22.7	98.2 ± 14.2	44.5 ± 9.2	10.0 ± 4.1
HFCN	177.0 ± 23.7	119.7 ± 13.9	48.7 ± 11.8	7.1 ± 2.3
HFCC	103.9 ± 17.0	74.4 ± 12.9	25.5 ± 5.6	3.2 ±1.2
HFFN	147.1 ± 24.4	102.6 ± 17.1	39.1 ± 8.3	5.4 ±1.8
HFFC	100.0 ± 16.9	73.8 ± 11.7	21.6 ± 5.6	2.8 ± 0.8

1. Values are expressed as means ± SEM
2. There was no significant differences between the values in the same column
3. Abbreviations are as follows: TACF, total aberrant crypt foci; ACF1-3, aberrant crypt foci with one, two or three apparent crypts; LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil. LFCN, low fat corn oil with no carrageenan treatment; LFCC, low fat corn oil with carrageenan treatment.

was: HFFC < HFCC < HFFN < LFCN < LFCC < HFCN. No significant differences were apparent between the groups. Among the non CGN groups TACF in the LFCN = HFFN < HFCN. However, among the CGN fed groups, thehighest number of TACF was in the LFCC > HFCC = HFFC. The distribution of ACF with different crypt number clearly demonstrated that HFFC and HFCC has the lowest number of ACF with 3 or more crypts.

First 4 cm colon section:

The TACF and their distribution according to their crypt multiplicity in the first 4 cm of the colon are shown in Table 3B. The CGN treated colon had lower TACF than non-CGN treated tissue in all diet groups, but the only HFFC had significantly less TACF than HFFC. TACF was highest in the HFCN group, and was significantly higher than the LFCC or HFFC groups. The HFCC group has the lowest number of TACF. The trend in TACF showed LFCN < HFFN < HFCN.

Second 4 cm colon section:

The TACF and their distribution according to their crypt multiplicity in the second 4 cm of the colon are shown in Table 3C. The CGN treated tissue in the high fat diet groups had lower TACF than the normal tissue of the same diet groups, but only HFFC was statistically lower than LFCC. However, the CGN treated tissue of the low fat diet had higher TACF than all groups. It was important to note that the highest TACF was recorded for the LFCC group

followed by the HFCN group. LFCC had a significantly higher ACF3 number than all other diet groups.

Third 4 cm colon section:

The TACF and their distribution according to their crypt multiplicity in the third 4 cm of the colon are shown in Table 3D. When comparing the CGN-treated and normal tissue, only HFCC has significantly lower TACF than HFCN. The HFCN diet group consistently had the highest TACF among the normal mucosa diet groups across the colon. The HFCC group had the lowest number of TACF. The LFCN group, which is known to have low overall risk, also had a higher number of TACF. HFCC value was significantly lower than LFCN, LFCC and HFCN only.

Trends across the colon section:

Total number of ACF in all diet groups was combined for each section and analyzed for significant differences between first, second and third sections of colon (TACF: 240.4, 243.9, 350.3 respectively). The first and second sections showed similar mean TACF. The third section contained significantly more TACF than the first 2 sections of the colon.

Total ACF within each diet group was enumerated and percentage of ACF existing in each section of the diet group was calculated (Table 4). Regional differences in ACF percent among the non-CGN treated diet groups showed a

Table 3B. The number and growth features of ACF in Sprague Dawley rats after 6 Weeks Intervention with LFC, HFC and HFF Diets with or without Carrageenan in the 1-4 cm colon section

Diet Group	TACF	ACF1	ACF2	ACF3
LFCN	41.6 ± 6.2^{abc}	32.4 ± 3.1^{ab}	8.1 ± 2.7^{ab}	1.0 ± 0.9
LFCC	32.9 ± 5.1^{bc}	22.1 ± 3.2^{bc}	7.9 ± 1.6^{ab}	1.0 ± 0.5
HFCN	53.9 ± 4.0^{a}	37.3 ± 2.0^{a}	14.8 ± 3.3^{a}	1.6 ± 0.9
HFCC	39.4 ± 7.5^{abc}	27.8 ± 4.9^{abc}	10.6 ± 3.0^{ab}	0.7 ± 0.3
HFFN	47.3 ± 5.9^{ab}	32.5 ± 4.6^{ab}	11.9 ± 1.8^{ab}	2.6 ± 0.8
HFFC	25.3 ± 4.9^{c}	18.8 ± 3.1^{c}	4.6 ± 1.8^{b}	0.8 ± 0.3

1. Values are expressed as means ± SEM
2. Values in the same column sharing a common superscript are not significantly different ($p \leq 0.05$)
3. Abbreviations are as follows: TACF, total aberrant crypt foci; ACF1-3, aberrant crypt foci with one, two or three apparent crypts; LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil. LFCN, low fat corn oil with no carrageenan treatment; LFCC, low fat corn oil with carrageenan treatment.

Table 3C. The number and growth features of ACF in Sprague Dawley rats after 6 Weeks Intervention with LFC, HFC and HFF Diets with or without Carrageenan in the 4-8 cm colon section

Diet Group	TACF	ACF1	ACF2	ACF3
LFCN	37.0 ± 4.5^{ab}	28.3 ± 3.27	7.9 ± 1.1^{bc}	0.9 ± 0.4^{b}
LFCC	61.1 ± 9.8^{a}	35.13 ± 4.79	18.6 ± 3.7^{a}	4.4 ± 1.8^{a}
HFCN	52.1 ± 11.6^{ab}	34.8 ± 6.23	15.4 ± 5.4^{ab}	1.9 ± 0.6^{b}
HFCC	28.7 ± 4.9^{ab}	22.7 ± 3.57	5.0 ± 1.7^{c}	0.7 ± 0.5^{b}
HFFN	37.4 ± 6.4^{ab}	27.0 ± 4.73	9.3 ± 1.9^{bc}	0.9 ± 0.3^{b}
HFFC	27.6 ± 4.2^{b}	20.9 ± 3.21	6.1 ± 1.2^{c}	0.5 ± 0.2^{b}

1. Values are expressed as means ± SEM
2. Values in the same column sharing a common superscript are not significantly different ($p \leq 0.05$)
3. Abbreviations are as follows: TACF, total aberrant crypt foci; ACF1-3, aberrant crypt foci with one, two or three apparent crypts; LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil. LFCN, low fat corn oil with no carrageenan treatment; LFCC, low fat corn oil with carrageenan treatment.

Table 3D. The number and growth features of ACF in Sprague Dawley rats after 6 Weeks Intervention with LFC, HFC and HFF Diets with or without Carrageenan in the 8-12 cm colon section

Diet Group	First Colon Section (8-12 cm)			
	TACF	ACF1	ACF2	ACF3
LFCN	69.3 ± 10.8[a]	51.5 ± 7.0[a]	15.1 ± 3.1	2.5 ± 1.3[ab]
LFCC	64.7 ± 7.8[a]	41.0 ± 6.2[ab]	18.0 ± 3.9	4.6 ± 1.8[a]
HFCN	71.0 ± 8.1[a]	47.6 ± 5.7[a]	18.5 ± 3.1	3.6 ± 0.8[ab]
HFCC	35.8 ± 4.6[b]	23.9 ± 4.4[b]	9.9 ± 0.9	1.8 ± 0.4[ab]
HFFN	62.4 ± 12.1[ab]	43.1 ± 7.8[a]	17.9 ± 4.6	1.9 ± 0.7[ab]
HFFC	47.1 ± 7.8[ab]	34.1 ± 5.4[ab]	10.9 ± 2.6	1.5 ± 0.3[b]

1. Values are expressed as means ± SEM
2. Values in the same column sharing a common superscript are not significantly different ($p \leq 0.05$)
3. Abbreviations are as follows: TACF, total aberrant crypt foci; ACF1-3, aberrant crypt foci with one, two or three apparent crypts; LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil. LFCN, low fat corn oil with no carrageenan treatment; LFCC, low fat corn oil with carrageenan treatment.

Table 4. Percentage of TACF within each diet group found in each of 1-4cm, 4-8 cm and 8-12 cm colon sections in Sprague Dawley rats after 6 Weeks Intervention with LFC, HFC and HFF Diets Treated with Carrageenan

Diet Group	%ACF[a] in Colon Section		
	1-4cm section	4-8 cm section	8-12 cm section
LFCN	28.1	25	46.9
LFCC	20.7	38.5	40.8
HFCN	30.5	29.4	40.1
HFCC	37.9	27.6	34.5
HFFN	32.2	25.4	42.4
HFFC	25.3	27.6	47.1

a. %ACF is calculated as #TACF in section of diet group/ #TACF in all colon of diet group

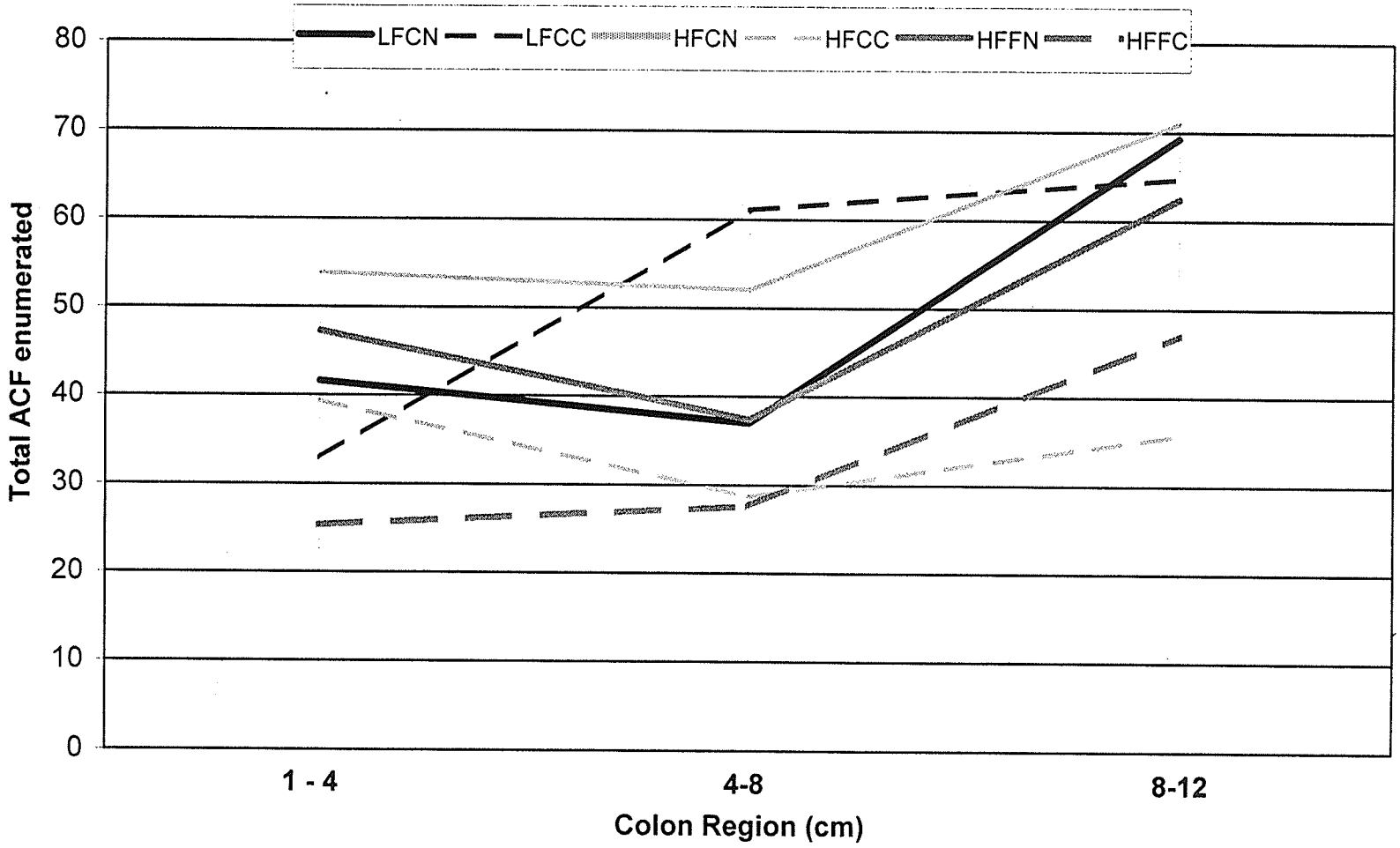
marked difference along the colon. The 8-12 cm region harboured the highest percentage of TACF (LFCN, HFCN, HFFN: 46.9%, 40.1%, 42.4% respectively) compared to the other 2 colon regions. Regional changes that occurred in the CGN-treated tissue were influenced by the type of lipids. This is due to the LFCC diet group that increased in ACF in the 4-8 cm section. The 4-8 cm section contained the same number of TACF as the 8-12 cm section, which is the opposite to the results from the LFCN diet group that had almost increased two-fold between the 2^{nd} and 3^{rd} colon sections.

A general trend in the distribution of ACF along the length of the colon is shown in Figure 9. It shows that distal colonic regions act differently than the proximal regions. With the exception of LFCC, the 8-12 cm section of the colon had the highest number of TACF. It also shows that generally the 4-8 cm section of the colonic regions the reference CGN treatments was most dependent on diet. This is the region that showed reduction (HFCC and HFFC) or increase (LFCC) in the TACF compared to 1-4cm. The highest fat feeding generally exerted a lowering of ACF throughout the colonic length in CGN fed animals.

Crypt Multiplicity (CM):

The first colon section and the third colon section demonstrated no significant differences between any of the groups with regards to their crypt multiplicity. There was a significant difference between the LFCC (1.44 ± 0.03) group and the HFCC group (1.25 ± 0.02) in the second colon section.

Figure 9. A line chart illustrating total ACF trends between diet groups and colon regions



ACF2 and ACF3 numbers were relatively low throughout the study. These numbers are speculated to show more significance if allowed to grow in volume over time (McLellan and Bird, 1988). However, HFCN demonstrated high numbers of ACF>1 throughout the colon. On the contrary, HFFC demonstrated low numbers of ACF>1 throughout the colon. There was a large difference in multiplicity in the second colon section between the HFC normal and carrageenan groups as noted by the dramatically lower in ACF2 for the HFCC group. A dramatic difference was also witnessed in the low fat groups in the second section as the number of ACF2 and ACF3 in the LFCC group increased by more than two-fold and four-fold respectively in the LFCN group.

Peyer's patches

The average length and width of Peyer's patches measured in colons of different diet groups was calculated (Table 5). The HFCN diet group had Peyer's patches with the smallest average length and width compared to the other diet groups.

The carrageenan treated groups showed longer and larger area patches on average than the control groups, although there was a significant differences between the two groups at $p < 0.05$ (mean areas ± SEM (cm^2): average area of patches from tissue of combined non-treated diet groups (LFCN, HFCN, HFFN): 5.0 ± 1.0; average area of patches from tissue of combined CGN-treated diet groups (LFCC, HFCC, HFFC): 7.2 ± 1.3).

Proliferative status and histological indicators of inflammation

Colon tissue treated with CGN showed an apparently larger number of PCNA stained proliferating cells positioned higher up in the crypts compared to normal tissue when viewed under a microscope, regardless of diet (Figure 9). Tissues treated with CGN compared to control tissues showed various morphological inflammation indicators such as crypt abscesses and villi blunting, epithelial degeneration, and muscle thickening (Figure 9).

3.4 Discussion

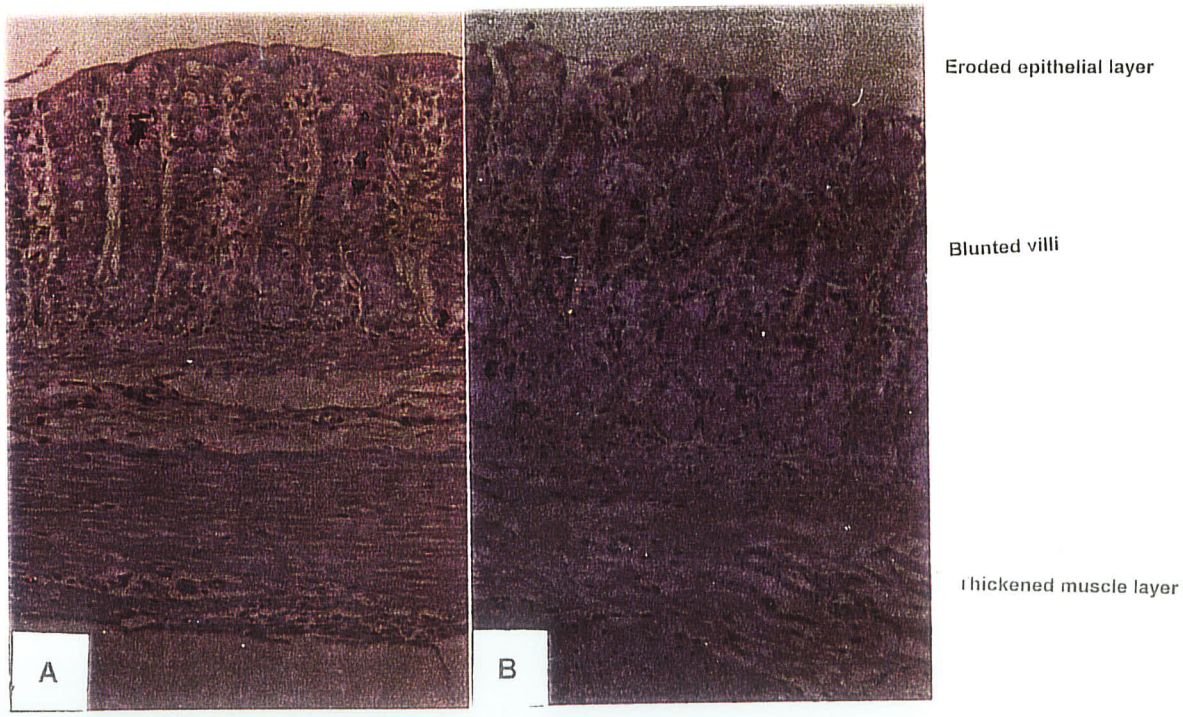
The CGN-induced colitis model has been known for decades, and the present study proved that it remains a simple, non-invasive and effective animal model for colonic chronic inflammation. The Sprague-Dawley rats ingesting a diet treated with CGN (2% by weight) exhibited some clinical symptoms, reported previously by researchers (Fabian et al., 1973; Kitano et al., 1986), such as moderate diarrhoea. We did not see melena - black, tarry stool resulting from intestinal bleeding - because we purposely used chronic feeding protocol rather than acute exposure to necrotic agents including CGN. In this study, these symptoms were observed after 3 weeks of initiating diets that contained CGN. The animals receiving CGN in their diets did not experience significant weight loss, and their mean weights were not significantly lower than the mean weights of the control animals.

Table 5. Average measured length and width of Peyers' Patches found on colonic mucosa. Peyers' patches were measured using a translucent ruler to assess inflammatory response.

Diet Group [1]	Length (cm) [2]	Width (cm) [2]
LFCN	1.0	0.53
LFCC	1.2	0.72
HFCN	0.85	0.41
HFCC	1.27	0.50
HFFN	1.01	0.59
HFFC	1.45	0.49

1. Abbreviations are as follows: LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil. LFCN, low fat corn oil with no carrageenan treatment; LFCC, low fat corn oil with carrageenan treatment.
2. There was no significant differences between the values in the same column. There was also no significant differences between the areas of the samples. When areas of all samples from carrageenan treated groups was combined and statistically compared to areas of all samples from non-CGN treated groups, CGN treated groups had statistically larger Peyers' patches ($p < 0.05$).

Figure 10. Immunohistochemical staining of PCNA in CGN-treated tissue and control tissue. Pictures were taken at higher magnification (x 125). Both tissues shown were taken from the low fat corn oil diet group. Picture A is of PCNA stained control tissue, and picture B is of PCNA stained CGN-treated tissue. In picture A, well formed long villi, a thin muscular layer and normal epithelial layer identify the standard healthy colon. In picture B, villi blunting and abscesses, thick muscular layer when compared to normal at same magnification, and eroded epithelial layer identify the inflamed colon. The differences are very apparent.



When the rats' colons were observed under a microscope, the CGN response was apparent morphologically. The CGN treated colons demonstrated lymphocytic infiltration, atrophy or denudation of the epithelial surface, increased goblet cells and mucous surface, and intense crypt abscesses. These features were not apparent in the colon tissue of the control groups. Furthermore, when assessing the tissues' proliferative status, one can observe the high number of proliferating cells near the epithelium in the CGN treated tissue.

ACF is considered a sensitive tumour marker, and previous research has demonstrated that ACF trends may predict incidence of colonic tumour in animals (McLellan and Bird, 1988). In our study, the LFCN group has lower numbers of TACF and ACF3 throughout the colon compared to HFCN. This is a trend that predicts what numerous studies have already documented: low fat diet groups yield fewer tumours than the high fat corn oil groups. Therefore, one may interpret an ACF trend as an indicator of tumour incidence regardless of statistical significance. The high fat corn oil diet (23% w/w) exhibited a higher number of TACF than the low fat corn oil diet (5% w/w) in non-CGN AOM treated colons. This finding and the findings of a number of studies assert the notion that ACF is a sensitive marker of tumour outcome.

The findings of the present study supported the previously stated hypothesis in part only. The HFF diet did lower the number of ACF in the CGN fed group compared to non-CGN group. However, unexpectedly, HFCC diet also exerted a similar effect in that HFCC differ considerably from the HFCN groups. The second study was conducted to determine if these two diets elicit similar

responses in the colonic mucosa at in the presence or absence of CGN and differ from those elicited by LFC diet group.

If one looks at the TACF distribution in the LFCN and HFCN groups, one can speculate that the HFC diet exerts a growth enhancing effect in the distal colon compared to the proximal colon. Regional differences in TACF exist because of varying sensitivity of colon regions to carcinogen. In the past, studies have shown that AOM treated colons have a higher number of TACF in the distal part compared to proximal regions (Bird, 1995; Shpitz et al., 1998;).

In the CGN-treated colon, the increase in the number of ACF seemed to be most pronounced in the 4-8 cm section in the LFC diet group. The number of TACF increased almost two-fold between the 4-8 cm and 8-12 cm sections. In contrast, all other diet groups showed a lower number of total aberrant crypts in the CGN treated groups compared to control groups. The fact that CGN treated high fat groups exhibited a lowering in the number of ACF throughout the colonic length suggests that the protective effect of high fat diet was general in nature. Based on these findings, using ACF as the disease end point, it can be proposed that CGN will enhance tumour outcome in the LFC but not in the HFCC or HFFC groups. A number of researchers have investigated the relationship between chronic inflammation and its carcinogenic effects (Kitano et al., 1982; Kuwuara et al.,1992). In fact, a recent study reported that the frequency of ACF in rats treated with DSS (a colitis causing agent) and AOM and fed a low fat diet was larger than that of AOM alone, signifying that colitis caused by DSS promoted ACF growth (Tanaka et al., 2001). The findings of our study support the fact that

CGN induces colon inflammation and increases ACF number when rats are fed LFC diets.

From the present study, we may presume that the interaction of CGN and fat may be at the biochemical level where, in CGN colitis model, the number of ACF increases in the low fat diet environment but not in the high fat diet.

CHAPTER 4

DIETARY LIPIDS MODULATE PROTEIN EXPRESSION OF CELL SIGNALING AND RELATED MOLECULES IN CARRAGEENAN TREATED COLONIC MUCOSA

4.1 Introduction

Inflammation is a complex process, and research is continuously trying to identify molecules that may manage its uncontrolled progression in disease such as ulcerative colitis and arthritis. Lipid compositions high in ω-3 fatty acids, such as fish oil and flaxseed oil, are believed to be affective in alleviating inflammation (Lorenz et al., 1989; McCall et al., 1989; Salmon et al., 1990; Stenson et al., 1992). The mechanism by which dietary lipids modulate the inflammatory process is evolving and expanding as more inflammatory mediators are being recognized. This study investigates the influence of dietary lipid composition on the proteins COX-1, COX-2, TGFβ-1, TGFβ-2 and MAP-K. These proteins were chosen because they are thought to have a role in the inflammatory process.

It has been stated that ω-3 fatty acids modulate inflammation by providing anti-inflammatory prostaglandins and suppressing the biosynthesis of pro-inflammatory prostaglandins (Caughey, 1996). The cycloxygenase enzymes are responsible for producing these inflammatory mediators, which in turn cause severe mucosal damage. COX-2 inhibitors are being used to treat inflammatory disorders (Ajeubor et al., 2000; Reuter et al., 1996;)

The transforming growth factors have been associated with uncontrolled inflammation and severe mucosal damage. These cytokines inhibit the proliferation of various immune cells and are therefore considered immunosuppressive (Taipale et al., 1998). TGFβ also inhibits the proliferation and growth of epithelial cells (Koli et al., 1996); this may slow down the repair of mucosal damage. It is not yet known whether lipid composition modulates TGFβ expression in epithelial cells. The interaction of dietary lipids, TGFβ and the COX's is summarized in Figure 11.

The MAP-Ks are central regulators of cellular response to growth factors, such as TGFβ, and inflammatory cytokines, such as prostaglandins. MAP-K has been associated with cell growth, differentiation and proliferation when stimulated by growth factors, and inflammation when stimulated by cytokines (Choi, 2000; Martin-Blanco, 2000). This process is summarized in Figure12.

The main objective of this study was to investigate if a high flax oil and corn oil diet would modulate the mucosal expression of proteins reported to be involved in inflammation (COX-1, COX-2, TGFβ-1, TGFβ-2, MAP-K p42/44). Researchers have proposed that n-3 fatty acids differ from n-6 fatty acids in modulating inflammation. N-3 fatty acids reduce inflammation and hence decrease the associated risk of developing cancer and are reported to act in an opposite manner to n-6 fatty acids(Kim and Mason, 1996). In the first study, as described in Chapter 3, we observed a different effect than that proposed by researchers in the past. Unexpectedly, both n-3 and n-6 fatty acids lowered ACF numbers in CGN treated tissue. If indeed fatty acid composition, especially n-3

Figure 11. A schematic representation of the interrelationship between dietary lipids, TGFβ, and the cycloxygenases. TGFβ inhibit proliferation or growth of epithelial cells. Dietary lipids may play a role in the modulation of growth factors. However, the exact mechanism remains unknown. Dietary lipids provide the cycloxygenases with a substrate, such as arachidonic acid or linolenic acid, from which the prostaglandins are formed. TGFβ has also been shown to magnify or induce COX-2 expression of intestinal epithelial cells.

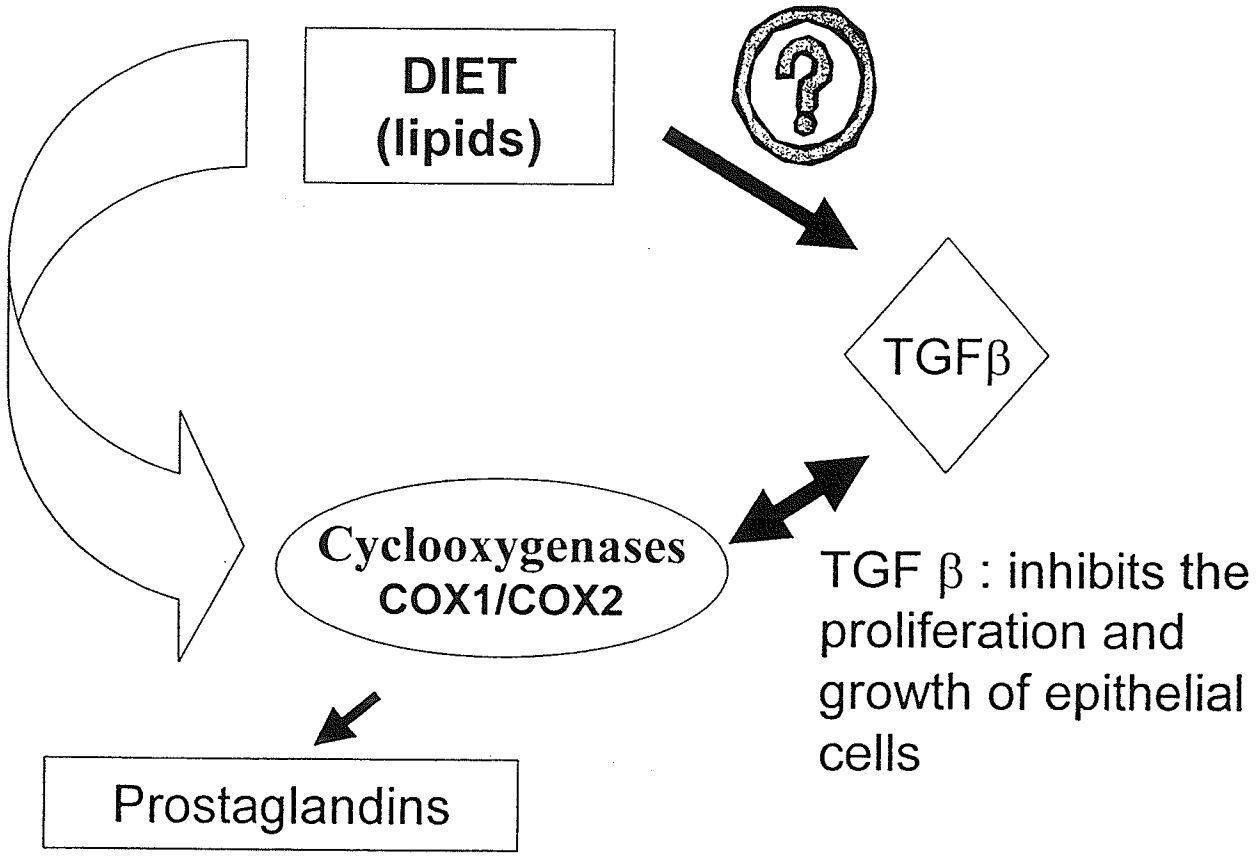
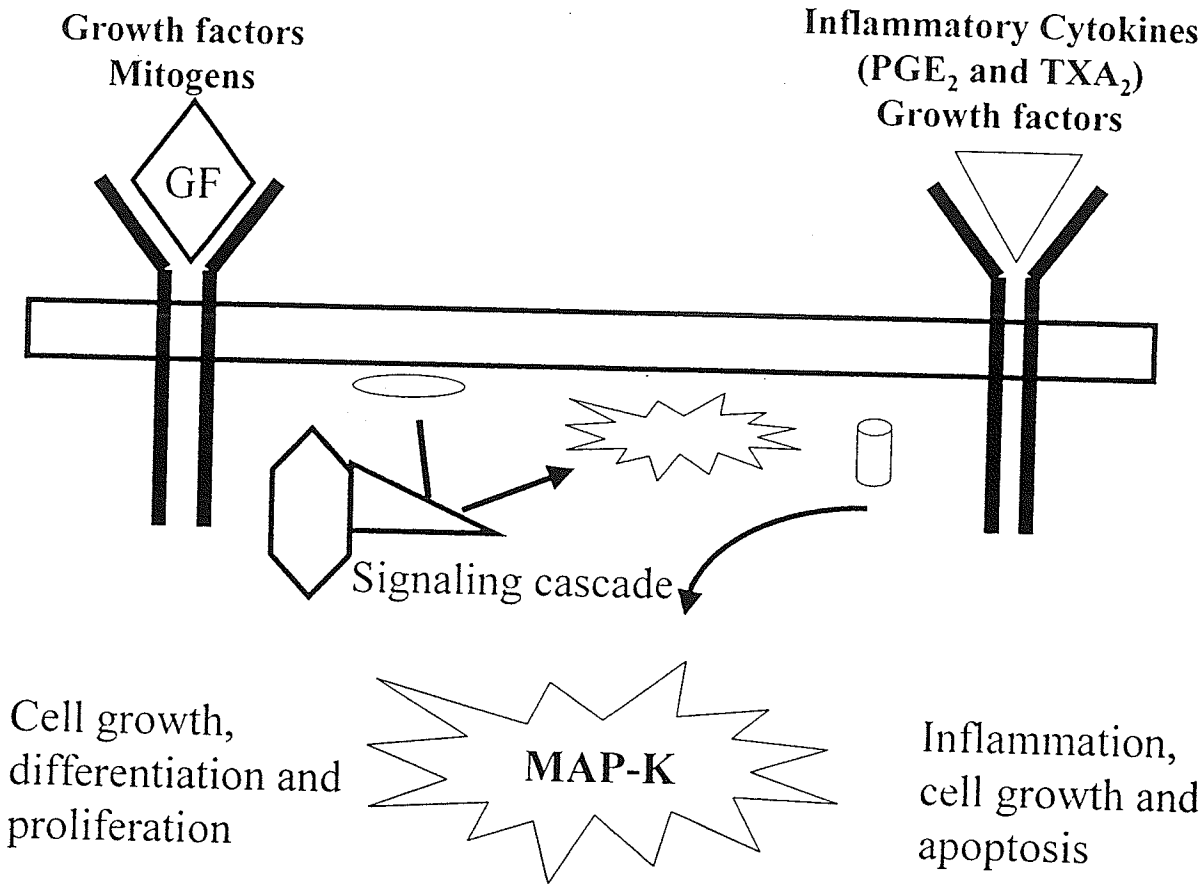


Figure 12. A schematic representation of the MAP-K signaling pathway. As the growth factors or cytokines attach to their receptors, a biological response is mediated through the MAP-K signaling cascade.



versus n-6, exerts opposite effects on inflammation, then how do we explain the identical effect they exhibited on ACF during inflammation?

We hypothesize that fatty acid level and composition of the dietary lipid play an important role in inflammation. ACF lowering effect of HFC and HFF noted in the previous study are mediated via different mechanisms; therefore, HFC and HFF will differ from each other in modulating proteins implicated in inflammation.

4.2 Materials and Methods

Animals

Weanling male Sprague-Dawley rats purchased from Charles River (Montreal, Canada) were used in all experiments. All procedures involving the animals were approved by the University of Manitoba Animal Care Committee and were conducted according to the guidelines of the Canadian Council of Animal Care (1993). Animals were housed in wire meshed stainless steel cages with sawdust bedding (two or three rats per cage) in a room under controlled environmental conditions (21° C and 50% humidity) and photoperiod (12-h light/dark cycle). Rats had free access to food and water *ad libitum*. Rats were allowed to acclimatize to conditions for several days before experimental diets and agents were introduced. Body weight of animals was measured using an electric, portable top loader balance at the start of the experiment, weekly, and at termination of animals. The mean weights of all animals at termination are provided in Table 6.

Table 6. Mean weights of non-injected animals.

Diet Group [1]	Animal Weights [2]
LFCN	444 ± 35
HFCN	458 ± 38
HFFN	465 ± 30
LFCC	423 ± 30
HFCC	467 ± 35
HFFC	455 ± 43

1. LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil. LFCN, low fat corn oil with no carrageenan treatment; LFCC, low fat corn oil with carrageenan treatment.
2. There were no significant differences in the weights of animals from different diet groups and treatments.

Diets

The experimental diets were prepared based on the AIN-76A diet (American Institute of Nutrition, 1977) with some alterations as described in Appendix A. Three diets were made with different fat compositions. The difference in fat content between the low fat and high fat diets was 18% fat by weight; this was compensated for using cornstarch (Appendix A). Furthermore, the amount of cellulose, vitamin mix, mineral mix and casein in the diets was adjusted to ensure that animals were fed isocaloric diets.

The low fat corn oil (LFC) diet was 5% corn oil by weight. The LFC diet was used as the control diet in the experiments. The high fat corn oil (HFC) diet was 23% corn oil by weight. The high fat flaxseed oil (HFF) diet was 14% corn oil and 9% flaxseed oil by weight. Flaxseed oil is roughly 73% unsaturated fatty acids (Appendix G: Flax Council of Canada); therefore, this oil needs to be stored carefully as it is at increased risk of rancidity. For this reason, the HFF diet's 23% fat composition was split into a 2:3 (flaxseed oil: corn oil) ratio. All diets were stored at 4 °C to prevent oxidation. Fresh diets were made weekly.

Cornstarch and dextrose were purchased from the University of Manitoba Food Services and Moonshiners (Winnipeg, MB), respectively. Flaxseed oil was purchased from Omega Nutrition (Vancouver, BC) and corn oil from Safeway (Mazola, Etioboke, ONT). All other dietary ingredients were purchased form Harlan Teklad (Madison, WI).

Carrageenan

The sulphated polysaccharide degraded λ- carrageenan (Sigma Chemical, Co., St. Louis, MO, USA) was used to induce colitis in the rats. Carrageenan was added to 2% by weight to the experimental diet.

Protein Extraction from Colon Scrapings

Animals were killed by CO_2 asphyxiation. The non-treated animals' colons were scraped and stored at –80 °C for future biochemical analyses.
Colon scrapings were later removed from –80 °C for protein extraction. One gram of colon scrapings from each animal was placed in a tube containing radioimmunoprecipitation (RIPA) buffer and protease inhibitors. The mixture was subsequently homogenized using the polytron homogenizer for 30 seconds at a setting of 5-7. 30 µl of phenylmethysulfonylflouride (PMSF) protease inhibitors were added next and homogenized for 3 seconds again. The homogenate was then centrifuged for 15 minutes at 10,000 RPM. The middle clear phase or protein phase was eventually carefully removed using a pipettor.

Protein Concentrations

Protein concentrations were determined using Bradford standard procedures. The Coomassie protein assay reagent was used (Pierce, Rockford, IL, USA). The assay depends on the fact that when certain dyes bind to proteins

they undergo a shift in the absorbance. By measuring the total absorbance at the shifted wavelength, one can readily estimate the concentration of the protein solution with respect to the standard protein. Bovine serum albumin was the standard used, and Spectra Max 3000 (Molecular Devices, Sunnyvale, CA, USA) was the program that analyzed the experimental and standard samples.

Western Blots

The samples were first prepared and kept on ice for Western blots. A final concentration of 0.1 % of sodium duodecyl sulphate (SDS)/ 100 µL of sample was prepared. All solutions used in this procedure are provided in Appendix D and E. The mixture was heated to 90 °C for at least 3-5 minutes prior to loading onto resolving gel. Standardized conditions for Western blots of the different analyzed proteins were used (Appendix F). Resolving gel concentrations were chosen according the molecular weight of the protein. The higher the molecular weight of the protein, the lower the concentration of the chosen running gel. The gel was placed in an electrophesis chamber system, and the samples were loaded into the gel's wells. A BenchMark Protein Ladder (Gibco BRL) was also loaded to track sample separation. Electrophesis was run at 170 volts for approximately 70 minutes, or until the dye ran off the gel.

Transfer of proteins from gel to membrane was performed in 20% methanol transfer buffer at 100 volts at 4 °C using a BIO-RAD Miniprotean cell (Mississauga, ON, Canada). Transfer time varied from one protein to the next (appendix). 0.45 micron nitrocellulose hybond-C (Amersham Life Sciences,

Arlington Heights, IL) membrane was soaked in distilled water for 1 hour, and in transfer buffer for 10 minutes prior to transfer. After transfer was completed, the membrane was blocked with 5% skim milk powder (SMP) in tri buffered saline plus tween (TBS-T) for 1 hour at room temperature. The membrane was then washed, by rocking, for approximately 30 minutes in TBS-T. The membrane was next incubated with primary antibody at a particular dilution (appendix) in 5% SMP/TBS-T at 4 °C overnight. After incubation, the membrane was washed again with TBS-T for 30 minutes. TBS-T was changed every 10 minutes while washing.

The membrane was incubated with secondary antibody, mouse anti-goat horseradish peroxidase (HRP) conjugated IgG at a dilution (appendix) for 1 hour at room temperature. The membrane was then re-washed one last time. Immunoreactivity was identified using an enhanced chemiluminescence kit (ECL, Amersham Life Science). Film (Kodak Scientific imaging) was exposed to illuminating membrane for 5 minutes. Densitometric analysis was completes using Scion Image 2.0 software for microcomputers. Western blots were performed for COX1, COX2, TGFβ-1/2, and MAPK (42/44).

Statistical Analysis

Statistical analysis of the data was performed using the statistics program primer of biostatistics, 4/E version 4.0 software in Windows. Analysis of variance and Duncan's Multiple Range Test at $p \leq 0.05$ were used to determined differences between the groups.

Experimental Design

The study's protocol is provided in Figure 13. Male weanling Sprague-Dawley rats were randomly allocated to 6 different groups. Three groups (LFCC, HFCC, HFFC) received carrageenan (CGN) in diets that contained different dietary lipid compositions. CGN was added at 2% of total weight of diet. The remaining three groups (LFCN, HFCN, HFFN) were control groups and were not given CGN in diets. After 6 weeks of feeding the experimental diets, animals were terminated with CO_2 asphyxiation and their colons were scraped and stored at –80 °C for biochemical analyses. The rats' livers were also removed and stored at –80 °C for biochemical analyses.

4.3 Results

Expression of COX-1

The expression of COX-1 protein in CGN treated and normal colonic mucosa as influenced by dietary lipids is presented in Figure 14. Corn oil fed animals (LFCN, LFCC, HFCN, HFCC) had lower levels than the high fat flax oil fed groups (HFFN and HFFC). The HFCC diet group had significantly less COX-1 protein expression than HFFN and HFFC groups. The colonic mucosa from the LFC and HFC groups had similar COX-1 expression (HFCN: 621.3 ± 57.2 pixels, HFCC: 480.7 ± 47.9 pixels).

Figure 13. Schematic presentation of experimental protocol. Animals were fed either carrageenan treated diets or non-treated diets for 6 weeks. LFCN, low fat corn oil diet with no carrageenan treatments, LFCC, low fat corn oil diet with carrageenan treatment; HFC, high fat corn oil diet, HFF, high fat flaxseed oil diet.

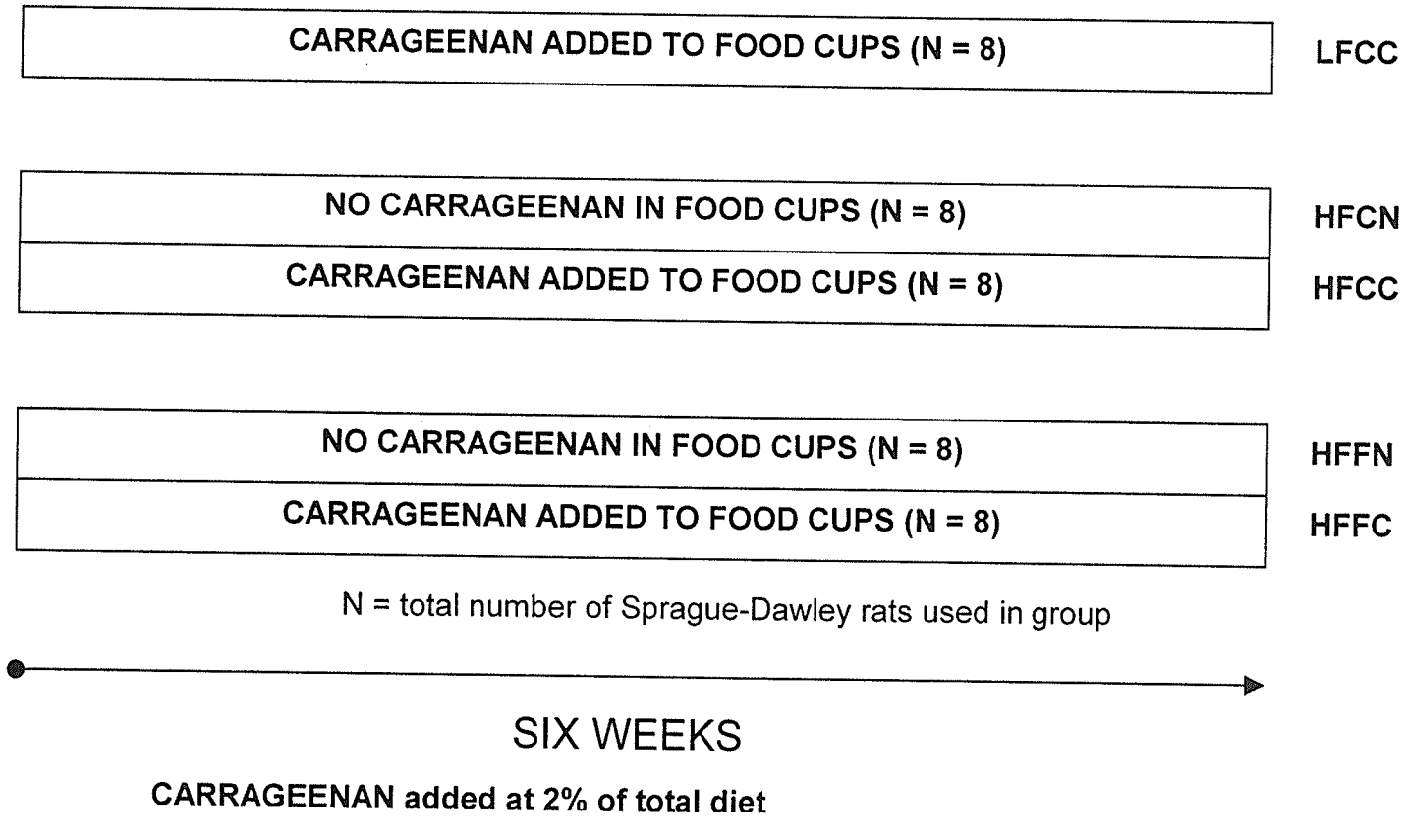
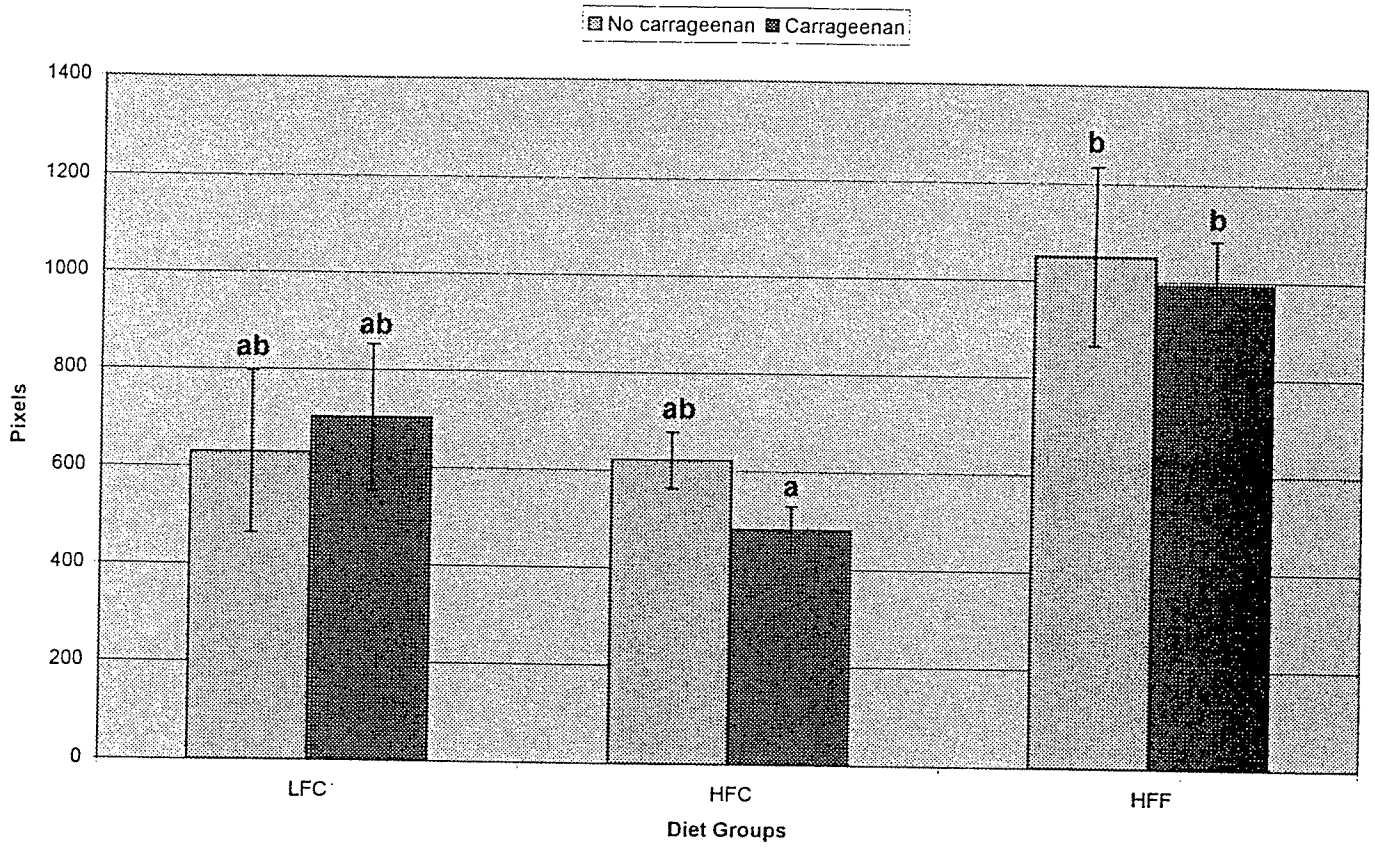


Figure 14. Density of COX-1 protein expression in normal colonic mucosa and carrageenan treated colonic mucosa from rats fed LFC, HFC and HFF diets for 6 weeks. Values are mean ± SEM (bars) expressed in pixels. Pixels are arbitrary units representing area of the Westerns' protein bands. There were no significant differences between these bars (ANOVA, $p \leq 0.05$).



Expression of COX-2

The expression of COX-2 in CGN-treated and normal colonic mucosa detected from a Western blot is shown in Figure 15. Among the non-CGN groups, it is apparent that the two high fat groups had elevated levels compared to the LFC group. CGN feeding significantly increased the COX-2 levels only in the low fat group; the LFCC values were significantly higher than the LFCN. In fact, the LFCC group showed a significantly higher COX-2 expression than to the 5 other groups. Among the normal groups fed the different diets, the LFCN was significantly lower than the HFCN and the HFCC ($p \leq 0.05$).

Expression of TGFβ-1

The expression of TGFβ-1 in CGN-treated and normal colonic mucosa is shown in Figure 16. TGFβ-1 expression was significantly higher in the low fat groups compared to the high fat groups. Among the non-CGN diet groups, LFC had the highest expression. Among the CGN-treated diet groups, LFC and HFC were both significantly higher in protein expression than HFF. CGN feeding significantly increased the level of the TGFβ-1 in the HFC group compared to the HFCN, HFFN and HFFC.

Expression of TGFβ-2

A Western blot of the expression of TGFβ-2 in normal and CGN-treated mucosa is illustrated in Figure 17. On the average, low fat groups

Figure 15. Density of COX-2 protein expression in normal colonic mucosa and carrageenan treated colonic mucosa from rats fed LFC, HFC and HFF diets for 6 weeks. Values are mean ± SEM (bars) expressed in pixels. Pixels are arbitrary units representing area of the Westerns' protein bands. Bars not sharing a common letter are significantly different ($p \leq 0.05$, ANOVA).

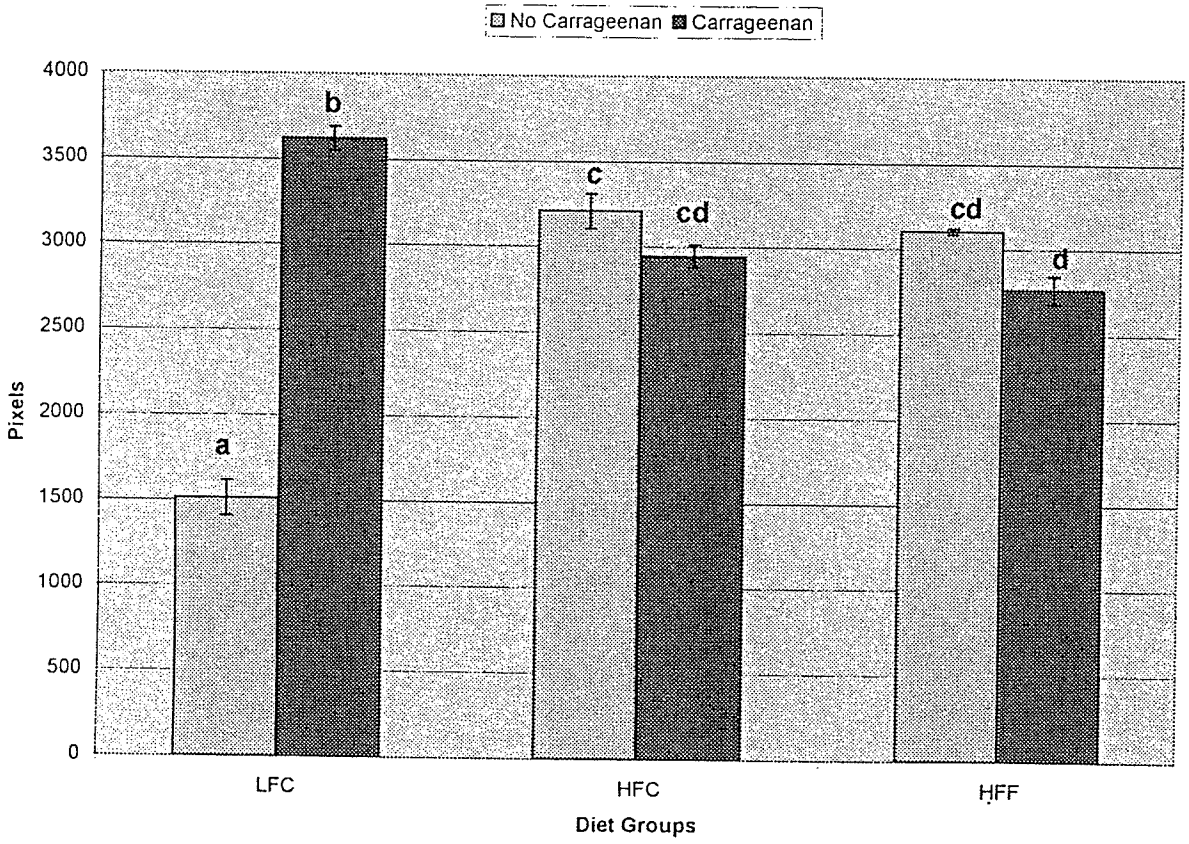


Figure 16. Density of TGFβ-1 protein expression in normal colonic mucosa and carrageenan treated colonic mucosa from rats fed LFC, HFC and HFF diets for 6 weeks. Values are mean ± SEM (bars) expressed in pixels. Pixels are arbitrary units representing area of the Westerns' protein bands. Bars not sharing a common letter are significantly different ($p \leq 0.05$, ANOVA).

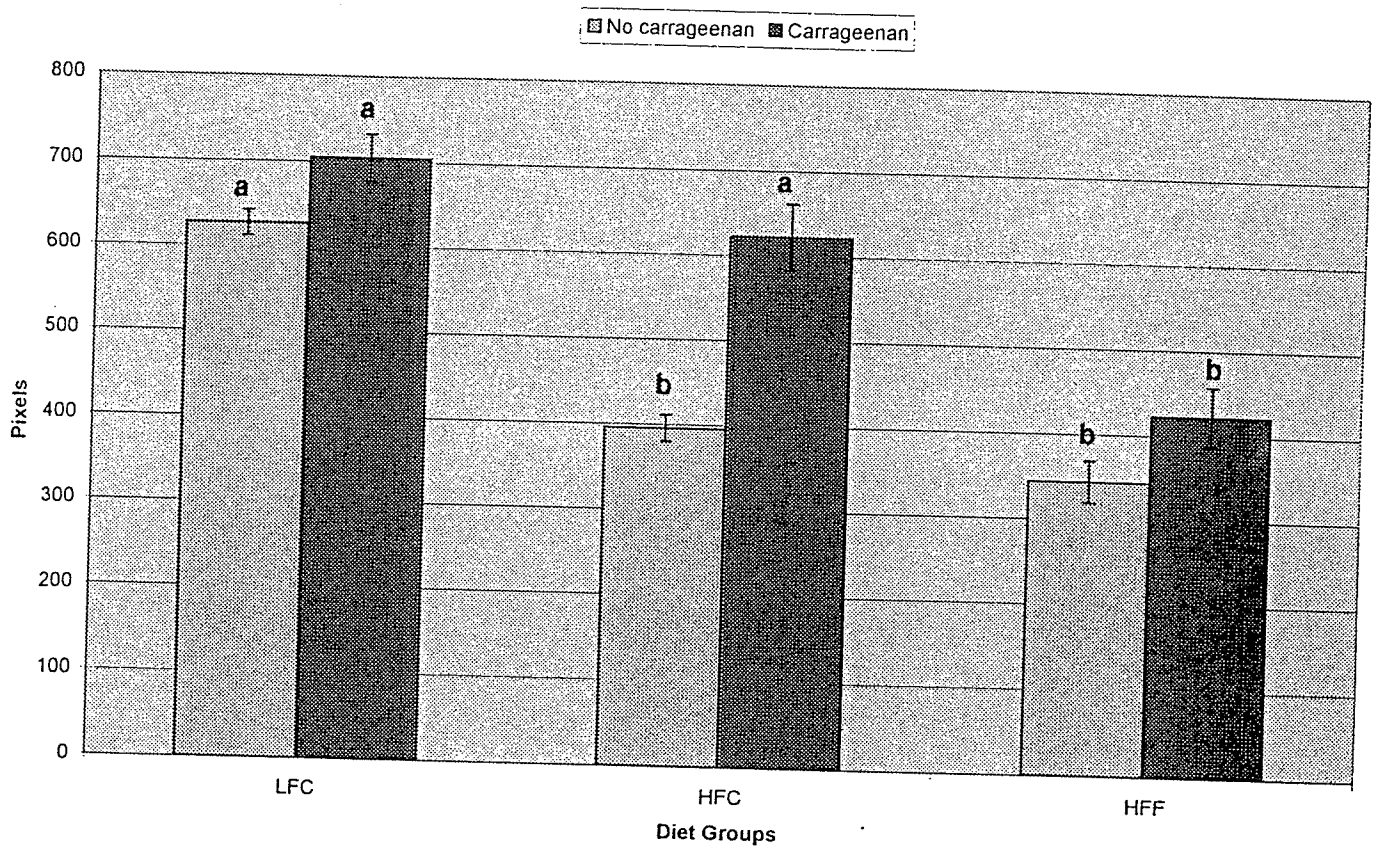
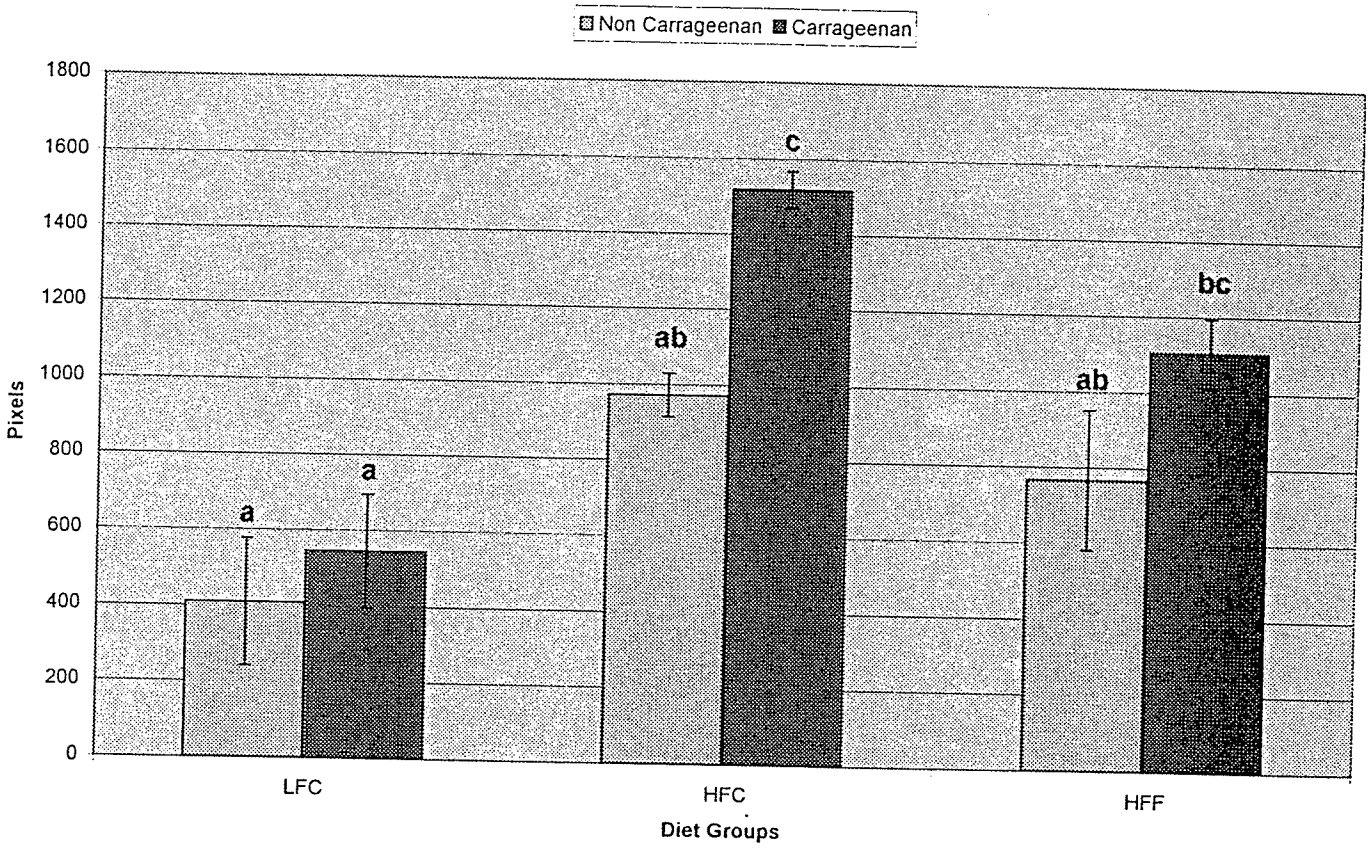


Figure 17. Density of TGFβ-2 protein expression in normal colonic mucosa and carrageenan treated colonic mucosa from rats fed LFC, HFC and HFF diets for 6 weeks. Values are mean ± SEM (bars) expressed in pixels. Pixels are arbitrary units representing area of the Westerns' protein bands. Bars not sharing a common letter are significantly different ($p \leq 0.05$, ANOVA).



(LFCN and LFCC) had lower expression than the high fat groups. CGN-treated tissue in the high fat groups, HFCC and HFFC, had higher TGFβ-2 expression than LFCC (1555.7 ± 53.1, 1519.0 ± 130.2, 545.1 ± 95.4 respectively).

Expression of MAP-K p42 in colonic mucosa

The expression of MAP-K p42 in normal and CGN-treated mucosa is shown in Figure 18. There were no significant differences in the protein expression of MAP-K between the different CGN-treated diet groups. However, rats fed HFCN and HFCC diets showed significantly higher MAP-K p42 expression in their colonic mucosa than rats fed HFFN diets (671.1 ± 51.9 pixels, 774.6 ± 50.9, 399.0 ± 101.4 pixels respectively).

Expression of MAP-K p44 in colonic mucosa

The expression of MAP-K p44 in normal and CGN-treated mucosa is shown in Figure 19. There were no significant differences in the expression of this protein between all 6 groups.

Figure 18. Density of MAP-K p42 protein expression in normal colonic mucosa and carrageenan treated colonic mucosa from rats fed LFC, HFC and HFF diets for 6 weeks. Values are mean ± SEM (bars) expressed in pixels. Pixels are arbitrary units representing area of the Westerns' protein bands. Bars not sharing a common letter are significantly different ($p \leq 0.05$, ANOVA).

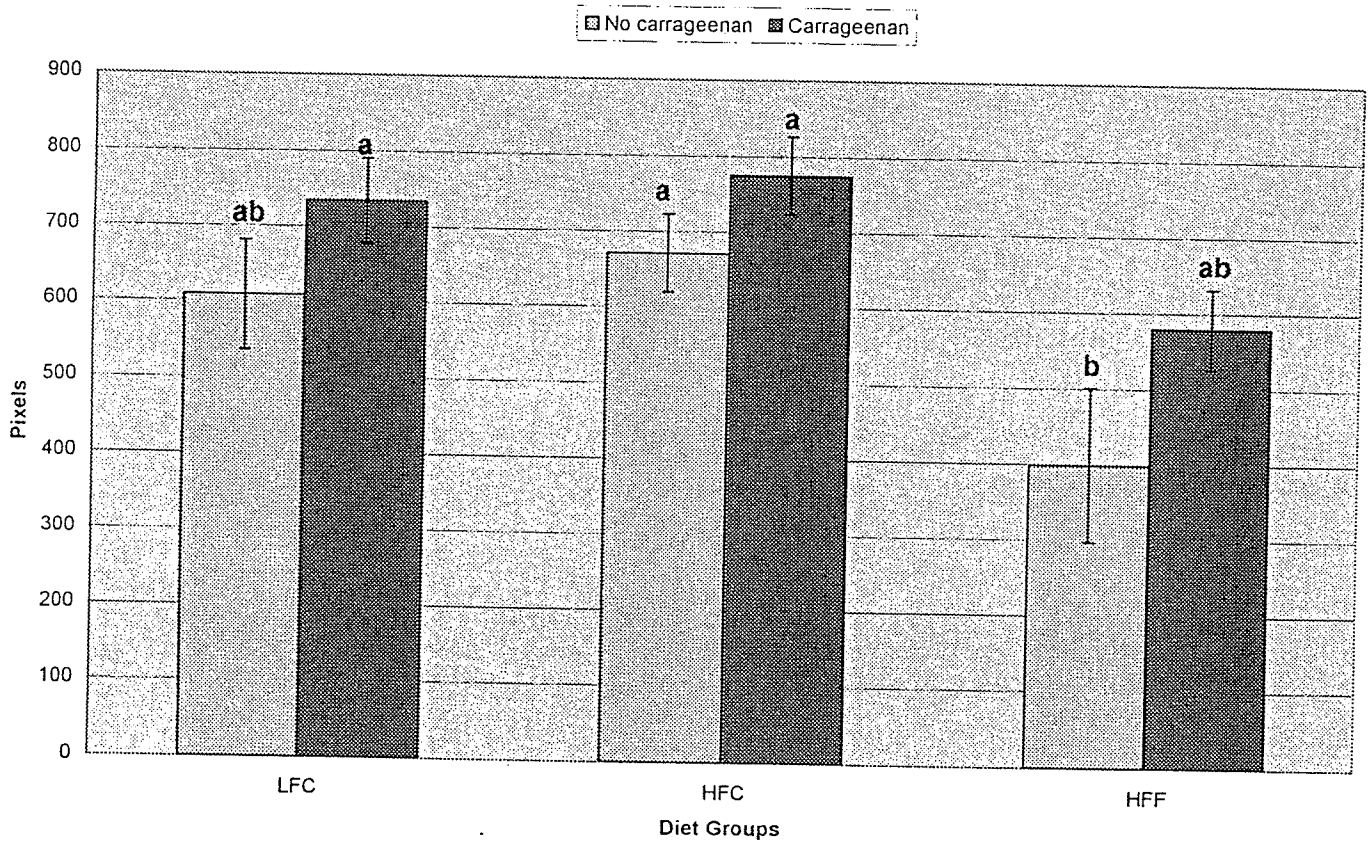
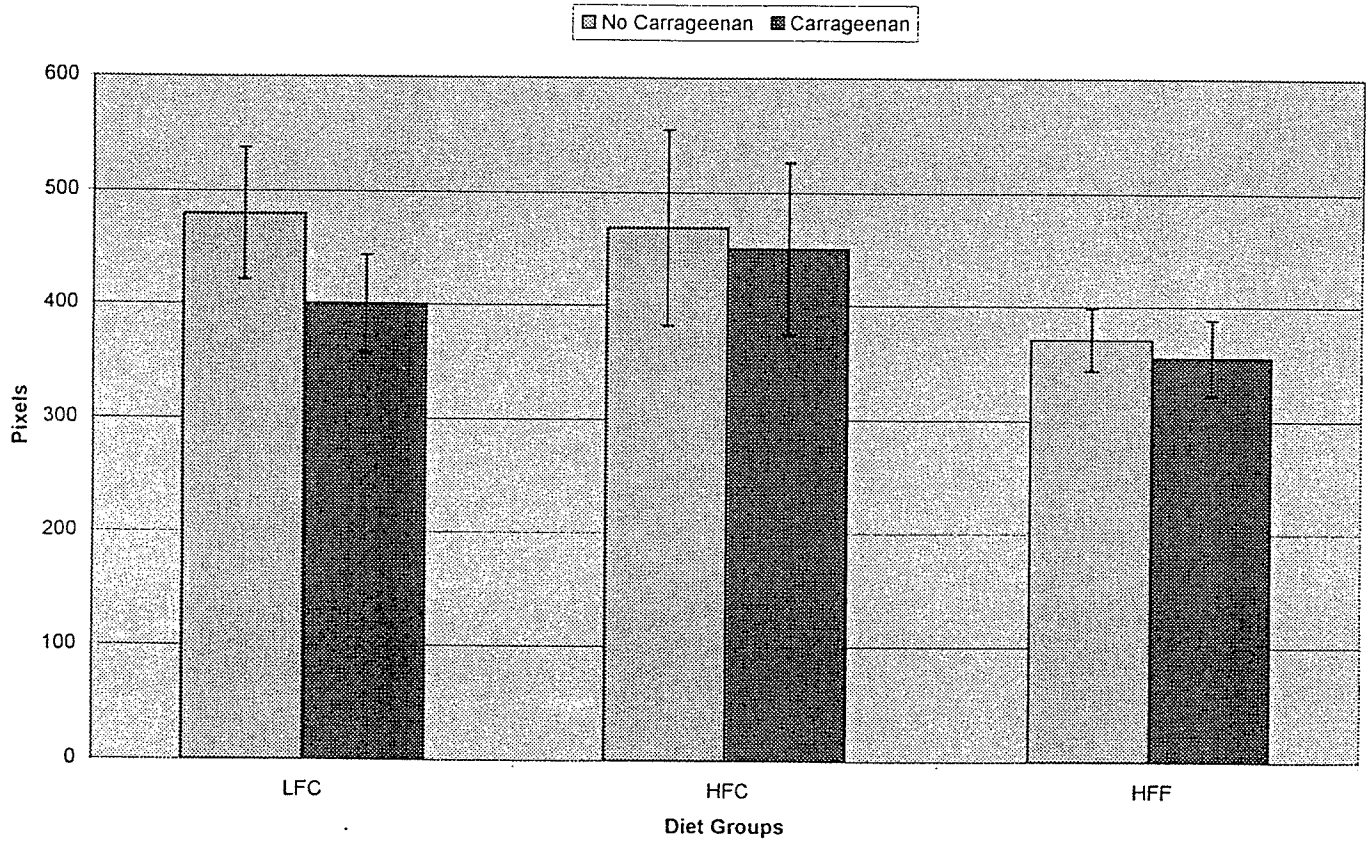


Figure 19. Density of MAP-K p44 protein expression in normal colonic mucosa and carrageenan treated colonic mucosa from rats fed LFC, HFC and HFF diets for 6 weeks. Values are mean ± SEM (bars) expressed in pixels. Pixels are arbitrary units representing area of the Westerns' protein bands. The groups were not significantly different ($p \leq 0.05$, ANOVA).



4.4 Discussion

The main objective of this study was to compare the levels of selected molecules in the normal and inflamed mucosa in rats fed dietary lipids. A comparison of the protein expression between the three different diet groups, normal or CGN-treated, provides insight as to which proteins are modulated by dietary lipids in normal and inflammatory states.

It is known that COX-1 is expressed constitutively and COX-2 is the inducible form (Singer et al., 2000). It was interesting to note that COX-1 appeared to be elevated in the HFF group compared to the other diet groups; in that COX-2 was elevated in high fat diet groups and was induced in inflamed mucosa only in the LFC group. This leads one to think whether COX-1 should also be considered an inducible form affected by dietary lipid. It also raises the question of whether unsaturation or long chain fatty acids are inducers of COX-1.

The activity of the cycloxygenase enzymes was not measured, and it would be important to do so in future studies. In view of the fact that COX-1 and COX-2 generate metabolites depending on the ratio and relative availability of n-3:n-6 (Badawi et al, 1998), it is possible that the activity will not be altered but the products may change depending on the membrane lipid composition. The fact that dietary fats altered protein levels is important, and it is suggested that dietary lipids are affecting changes at the post-transcriptional stages. The COX-1 and COX-2 metabolites, depending on the source of fatty acids, are known to affect cell growth, differentiation, tissue repair and immune system (Simon, 1999). The complex role of these metabolites does not allow one to speculate the

mechanism by which these diets may be affecting colonic mucosa in the present study, A metabolite of 20:4 n-6, PGE_2, is known to be immunosuppressive as well as angiogenic (Miyaunchi-Hashimoto et al, 2001). In contrast, lukotrienes produced from the same fatty acid through the lipoxygenase pathway are pro-inflammatory. COX-2 has been suggested to be the main enzyme producing PGE_2. Therefore, we propose that induction of COX-2 could be protective in nature during inflammation. This statement is supported in a study in rat model where specific COX-2 inhibitor heightened the symptoms of inflammation (Reuter et al., 1996).

The next family of molecules looked at in this study was TGFβ1 and TGFβ2. TGFβ1 has received more attention thanTGFβ2 and other isoforms. TGFβ is known to stimulate tissue repair probably by stimulating the growth of fibroblastic cells whereas they inhibit normal epithelial cells and immune cells (Gerard et al, 2000). These molecules cytokines are induced during inflammation. It was apparent that TGFβ1 level increased during inflammation in HFC only. These findings demonstrate that TGFβ1 is regulated by dietary lipids and by inflammation. It would appear in this case that the amount and type of lipid might be playing a critical role.

TGFβ2 has received less attention but certainly it appears to be more abundant in colonic tissue than TGFβ1 (Gerard et al., 2000). In this study, TGFβ1 acted somewhat opposite to TGFβ2. It should be noted that TGFβ1 levels were highest in the LFC group and TGFβ2 levels were lowest in this group compared to other groups. This demonstrated that dietary lipids regulate TGFβ1 and TGFβ2

differently. CGN feeding and presumably ensuing inflammation responses increased the level of TGFβ2 in all groups; however, the effect was only significant in the HFC group. The role of TGFβ isoforms in colon is unclear; yet, one can speculate that enhanced level of TGFβ2 during inflammation could be a protective response. The possibility that increased level of TGFβ2 in mucosa inhibits ACF growth is a possibility.

MAPK 42/44, from the family of map kinases, are implicated in normal and abnormal cell growth (Schaeffer and Weber, 1999). Dietary lipids, only in the normal mucosa, modulated MAP-K p42, but not p44. In this case, the dietary lipid composition appears to cause the difference in expression between the groups. The HFCN group demonstrated significantly higher MAP-K p42 expression than the HFFN group. In fact, when CGN and normal tissue protein expression levels were combined in each diet group, the HFF group had significantly less MAP-K 42 than HFC and LFC. It is the first time to our knowledge that these observations were made, and further research is required to fully understand the mechanism of how dietary lipids modulate this signaling pathway. It is interesting to further point out that MAP-K p42 expression when analyzed in the liver did not show a similar pattern (Appendix C), proving that this observation was organ specific. The metabolism of these tissues is different, and it may be that lipids have a more powerful effect on signaling pathways in colonic tissue than liver tissue.

Our hypothesis initially questioned whether high fat flax oil and corn oil might differ biochemically in eliciting their responses. COX-1 and MAPK42

expressions were different when comparing HFC and HFF groups. If one looks at TGFβ1 levels, between CGN and non-CGN groups, it is very clear that HCF behaved very differently than HFF only in the CGN treated groups. Therefore, these findings would allude to the possibility that the ACF lowering effect observed in the HFC and HFF groups are exerted by different mechanism and that the fatty acid composition of dietary lipids (possibly n-6 and n-3 fatty acids) affect colonic mucosa and molecules associated with growth, differentiation and inflammation differently.

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APPENDIX A
EXPERIMENTAL DIETS [1]

Ingredients	LF Corn Oil [2]	HF Corn Oil	HF Flax Oil
Corn Starch -	3120 [3]	2025	2025
Casein	1200	1380	1380
Dextrose	780	511.2	511.2
Cellufil	300	354	354
AIN-76 Mineral Mix	210	246.6	246.6
AIN-76 Vitamin Mix	60	70.8	70.8
Methionine	18	18	18
Choline bitartate	12	14.4	14.4
Corn Oil	300	1380	840
Test oil	0	0	540

1. Weekly 6 kg batches were prepared using the recipes in the table
2. Abbreviations are as follows: LFC, low fat corn oil; HFC, high fat corn oil; HFF, high fat flaxseed oil
3. All values in table are in grams

APPENDIX B
WESTERN BLOT: PROTEIN EXPRESSION RESULTS

Expression of Cycloxygenase-1 in colonic mucosa as modulated by dietary fats

Diet Groups	Mean ± SEM
LFCN	630.0 ± 166.7
LFCC	702.2 ± 149.9
HFCN	621.3 ± 57.2
HFCC	480.7 ± 47.9
HFFN	1049.7 ± 184.4
HFFC	992.0 ± 90.7

Expression of Transforming Growth Factor β-1 in colonic mucosa as modulated by dietary fats

Diet Groups	Mean ± SEM
LFCN	627.1 ± 14.9
LFCC	705.1 ± 27.8
HFCN	395.3 ± 15.2
HFCC	622.6 ± 38.9
HFFN	344.2 ± 24.5
HFFC	422.4 ± 34.6

Expression of Transforming Growth Factor β-2 in colonic mucosa as modulated by dietary fats

Diet Groups	Mean ± SEM
LFCN	409.3 ± 158.9
LFCC	545.1 ± 95.4
HFCN	971.3 ± 123.2
HFCC	1519.0 ± 127.9
HFFN	766.3 ± 38.5
HFFC	1104.2 ± 181.1

Expression of Mitogen Activating Protein Kinase 42 in colonic mucosa as modulated by dietary fats

Diet Groups	Mean ± SEM
LFCN	608.2 ± 72.7
LFCC	734.0 ± 56.0
HFCN	671.1 ± 51.9
HFCC	774.6 ± 50.9
HFFN	399.0 ± 101.4
HFFC	579.0 ± 52.7

Expression of Mitogen Activating Protein Kinase 44 in colonic mucosa as modulated by dietary fats

Diet Groups	Mean ± SEM
LFCN	479.4 ± 58.2
LFCC	400.3 ± 43.2
HFCN	468.4 ± 86.1
HFCC	450.3 ± 75.7
HFFN	371.0 ± 27.2
HFFC	355.1 ± 33.2

Expression of Mitogen Activating Protein Kinase 42 in liver as modulated by dietary fats

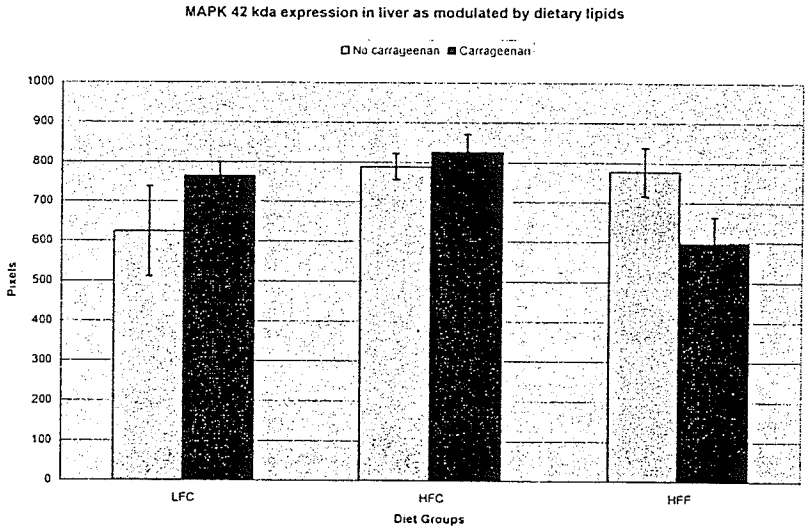
Diet Groups	Mean ± SEM
LFCN	624.2 ± 113.3
LFCC	764.0 ± 35.5
HFCN	789.2 ± 32.8
HFCC	826.8 ± 44.2
HFFN	777.0 ± 61.3
HFFC	595.5 ± 67.8

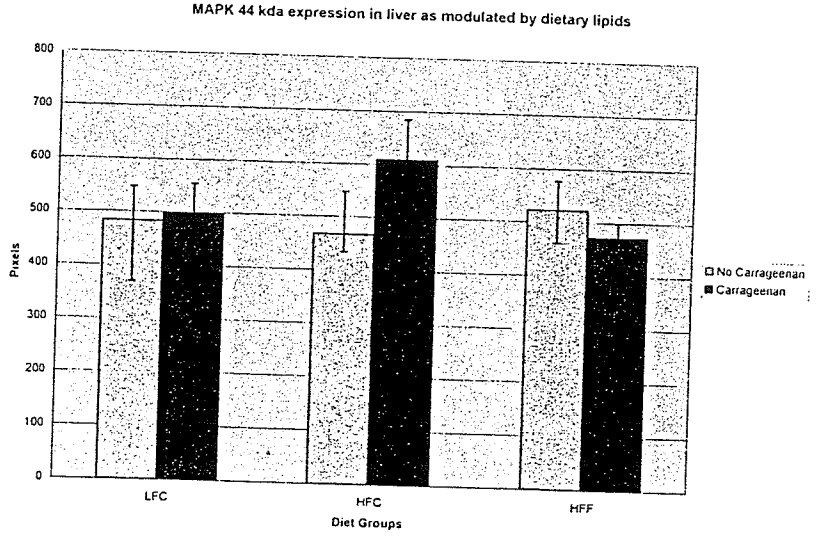
Expression of Mitogen Activating Protein Kinase 44 in liver as modulated by dietary fats

Diet Groups	Mean ± SEM
LFCN	483.4 ± 63.8
LFCC	498.3 ± 56.8
HFCN	466.8 ± 80.3
HFCC	609.2 ± 75.5
HFFN	522.2 ± 54.7
HFFC	471.7 ± 27.2

APPENDIX C
BAR CHARTS SHOWING MAPK 42/44 PROTEIN EXPRESSION IN LIVER

Density of MAP-K p42 and MAPK 44 protein expression in normal liver tissue and carrageenan treated liver tissue from rats fed LFC, HFC and HFF diets for 6 weeks. Values are mean ± SEM (bars) expressed in pixels. Bars not sharing a common letter are significantly different ($p \leq 0.05$, ANOVA).





APPENDIX D
PREPARATION OF ACRYLAMIDE

All reagents used were purchased from Bio-Rad.

10% Separating Gel[1]

H_2O	3.3 ml
30% Acrylamide Mix [2]	4.0 ml
1.5 M Tris HCL (pH 8.8)	2.5 ml
10% SDS [3]	0.1 ml
10% APA [4]	0.1 ml
TEMED [5]	0.004 ml

5% Stacking Gel

H_2O	3.4 ml
30% Acrylamide Mix	0.83 ml
0.5 M Tris HCL (pH 6.8)	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

1 Vary recipe with percentage of gel

2 Add 87.5 g acrylamide (29.2g/100 ml) and 2.4 g N'N-bis-methylene-acrylamide (0.8g/100ml) to water to make 300 ml

3 Sodium Dedocyl Sulphate: 10 g SDS in 90 ml H2O with stirring, volume is then increased to 100 ml

4 Ammonium Persulfate: 100 mg ammonium persulfate in 1 ml deionized water

5 N,N,N-teramethylethylenediamine

APPENDIX E
COMPOSITION FOR WESTERN BLOTTING BUFFERS

Sample Buffer

H_2O	3.8 ml
0.5 M Tris HCL (pH 6.8)	1.0 ml
Glycerol	0.80 ml
10% SDS	1.6 ml
2-mercaptoethanol	0.4 ml
1% (w/v) bromphenol blue	0.4 ml

5 x Electrode Running Buffer

Tris Base	**9 g**
Glycine	43.2 g
SDS	3 g

- Combined and made to 300 ml with water

Transfer Buffer

Tris Base	**3.03 g**
Glycine	14.4 g
SDS	200 ml

- Tris and glycine are mixed in 200 ml methanol. This mixture is made to 1000ml with water.

APPENDIX F
STANDARDIZED CONDITIONS FOR WESTERN BLOTTING PROCEDURES

Protein	Sample Protein Loaded[1] (μg)	Gel %	Transfer time (minutes)	Primary Antibody + Dilution	Molecular Weight (kDa)	Secondary Antibody	Positive Control
COX-1	50	10	150	COX-1 (murine) Cat# 160110 Cayman Chemical Co., Ann Arbor, MI 1:1000	70	Mouse HRP	COX-1 (ovine) Electrophesis Standard Cat# 360100 Cayman Chemical Co., Ann Arbor, MI
COX-2	50	10	150	COX-2 (murine) Cat# 160106 Cayman Chemical Co., Ann Arbor, MI 1:1000	72	Rabbit HRP	COX-2 (ovine) Electrophesis Standard Cat# 360120 Cayman Chemical Co., Ann Arbor, MI
TGFβ-1	50	15	120	TGFβ-1 Cat# sc-146 Santa Cruz Biotechnology CA, USA 1:100	12.5	Rabbit HRP	TGFβ-1 Cat# sc-4165WB Santa Cruz Biotechnology CA, USA
TGFβ-2	50	15	120	TGFβ-2 Cat# sc-90 Santa Crux Biotechnology CA, USA 1:100	12.5	Rabbit HRP	TGFβ-2 Cat# sc-4166WB Santa Cruz Biotechnology CA, USA
ERK1/2	15	12	120	Anti-MAP Kinase 1/2 Cat# 06-182 Upstate Biotechnology Lake Placid, NY 1:2000	44/42	Mouse HRP	NIH-3T3 cell lysate Cat# 06-182 Upstate Biotechnology Lake Placid, NY

1 Amount of protein loaded for both liver and mucosa samples were the same

APPENDIX G
PERCENTAGE FATTY ACID COMPOSITION OF HIGH FAT CORN OIL DIET AND HIGH FAT FLAXSEED OIL DIET

Fatty Acid	Percentage in Corn Oil	Percentage in Flaxseed Oil
Saturated Fatty Acids	11.7%	9%
Monounsaturated Fatty Acids	26.5%	18%
Polyunsaturated Fatty Acids (omega-6)	59.2%	16%
Polyunsaturated Fatty Acids (omega-3)	0.8%	57%

Source: Flaxseed Council of Canada/ Mazola Corn Oil

Fatty Acid	Percentage in HFC diet (23% corn oil)	Percentage in HFF diet (14% corn oil + 9% flaxseed oil)
Saturated Fatty Acids	12%	11%
Monounsaturated Fatty Acids	60%	42%
Polyunsaturated Fatty Acids (omega-6)	1%	24%
Polyunsaturated Fatty Acids (omega-3)	27%	23%