The potential of proton magnetic resonance spectroscopy (^{1}H MRS) in detecting early colonic inflammation and assessing the effect of various dietary fatty acids on modulation of inflammatory bowel disease in an animal model

By

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A thesis 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

Department of the Human Nutritional Sciences

University of Manitoba

Winnipeg

Defended on 31 Jan 2008

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ABSTRACT

The objectives of our study were to determine the potential of ^{1}H MRS in detecting (1) early colonic inflammation, (2) effects of various fatty acids on normal colon and (3) their effects on IBD. Sprague dawley rat fed with 2% carrageenan was used as a model of IBD. Flaxseed oil served as ω-3, corn oil as ω-6 and beef tallow as saturated fatty acid sources. Control group animals were fed 5% corn oil, whereas, those in high-fat diet groups received an additional 7% of the respective fatty acids. After 2 weeks, ^{1}H MRS and histology were conducted on excised colonic mucosa.

Statistical classification strategy (SCS) used for analyzing ^{1}H MRS data achieved an accuracy of 82 % in stage 1, 90-100% in stage 2 and 96-100% in stage 3. This implies that ^{1}H MRS is a sensitive tool to diagnose early IBD and the effects of dietary fat on IBD.

ACKNOWLEDGEMENT

This work is an epitome of constant inspiration by my parents, brother and my husband. I dedicate it to them and the almighty God**.** Realization of this dream would not have been possible without incredible support and guidance of my supervisor, Dr. Tedros Bezabeh. I could not have successfully passed through the twists and turns of this road without the support and encouragement of my co-supervisor, Dr. Michael Eskin. A figure who was involved with the intricacies of this project from its conception to the end and who provided invaluable intellectual and financial support all through this journey is, Dr. Ranjana Bird. I offer my sincere gratitude to Dr. Bezabeh, Dr. Eskin and Dr. Bird for being my mentors and a source of inspiration to me. I also want to thank my committee members, Dr. Asher Begleiter and Dr. Carla Taylor for their invaluable suggestions and guidance towards accomplishing the goals of this project.

I want to thank Saro Bascaramurty and Brion Dolenko, without whose technical support this project could not have been completed. In addition, special thanks to Dr. Omkar Ijare who provided very valuable input for data analysis. Last but certainly not the least; I want to thank the National Research Council, Winnipeg for providing state of the art facility and supportive environment for both intellectual and personal advancement.

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LIST OF ABBREVIATIONS

^{1}H MRS	Proton Magnetic Resonance Spectroscopy
^{13}C	Carbon atom with atomic mass 13
^{31}P	Phosphorus atom with atomic mass 31
DHA	Docosahexaenoic Acid
DSS	Dextran sulphate sodium
EPA	Eicosapentaenoic Acid
GABA	Gamma amino butyric acid
HLA B27	Human Leukocyte Antigen B27
IBD	Inflammatory Bowel Disease
IL	Interleukin
LT	Leukotriene
PAF	Platelet activation factor
PBS/D_2O	Phosphate buffered saline with deuterium oxide
PG	Prostaglandin
ppm	Parts Per Million
PUFA	Poly-unsaturated Fatty Acid
TNF	Tumor necrosis factor
SCS	Statistical classification strategy
TSP	3-(Trimethylsilyl)- propionic acid-D4, sodium salt
TX	Thromboxane

1 BACKGROUND

Inflammatory bowel disease (IBD) is a very common condition in industrialized countries including North America. It is estimated that about 1.4 million people in the United States and 2.2 million people in Europe suffer from IBD (Loftus, 2004). The exact cause and pathogenesis of IBD is not known, however, interplay of genetic, environmental and immune factors is considered to be involved in its causation. Many epidemiological and experimental studies have pointed out that there is a strong connection between diet and IBD. Diet particularly that is rich in omega-6 and saturated fats is one of the major environmental factors implicated in the etiology of IBD (Shoda et al., 1996). Conversely, omega-3 polyunsaturated fatty acids are considered to be anti-inflammatory and may have therapeutic benefit in IBD (Stenson et al., 1992). The focus of the first chapter of this thesis is to summarize the existing literature on epidemiology, pathogenesis and progression of IBD and describe the basic concepts of polyunsaturated fatty acids and their effects on inflammation.

IBD is not a point event, it's a continuum of disease process with remitting and relapsing course. People with longstanding IBD have a significant risk of developing colorectal cancer and this risk increases at the rate of 1% per year after 8-10 years of the disease (Eaden et al., 2001). As such, early diagnosis and intervention can help prevent the progression of IBD to colon cancer. Currently, the data from experimental or randomized trials on benefit of pharmacologic or dietary intervention on this window between IBD

and colon cancer is very limited. Some of the reasons for limited research in this area include lack of early diagnostic technique and appropriate animal model for long-term studies. Currently, colonoscopy with biopsy of grossly inflamed parts of the colon is the gold standard in diagnosing IBD. However, it is known that in diseased state, biochemical and physiological changes precede the histological manifestations of the disease and as such, the technique exploiting metabolic abnormalities may be suitable to aid in early diagnosis. One such technique that can provide extensive biochemical information from tissue samples is proton magnetic resonance spectroscopy (^{1}HMRS) (Bezabeh et al., 2001; Casciani et al., 2007). The MR spectra are generally very complex and require specialized analysis for comparing data from different groups. statistical classification strategy (SCS) is a very robust analytical method for MR data analysis (Nikulin et al., 1998; Somorjai et al., 2004). In the later half of the first chapter of this thesis, effort has been made to summarize the basic principles of ^{1}H MRS and statistical classification strategy. The following literature review of current state of knowledge of IBD, physiological effects of polyunsaturated fatty acids and the possibility of early diagnosis using ^{1}H MRS provided the final direction towards formulating the goals of my project.

The overall goal of this project was to assess the potential of ^{1}H MRS in detecting early colonic inflammation and its modulation by polyunsaturated fatty acids in animal model of IBD.

1.1 INFLAMMATORY BOWEL DISEASE (IBD)

Inflammatory bowel disease (IBD) is a remitting and relapsing disorder of the human intestine. It mainly comprises of 2 conditions, ulcerative colitis and Crohn's disease. IBD is shown to be more prevalent in developed and urban parts of the world including North America and Western Europe. A gradient has been observed between the northern and southern parts of Europe and the US in the incidence of IBD, with the northern parts having a higher prevalence (Shivananda et al., 1996; Sonnenberg et al., 1991). IBD has also been linked with urbanization. The prevalence has been reported to be lower in less urbanized and developing nations in Asia, Africa and South America. However, in recent years the incidence rates of IBD have been shown to be increasing in these regions with previously lower rates (Lakatos, 2006; Ouyang et al., 2005). According to the National Institute of Health data, prevalence of ulcerative colitis is 246 cases and that of Crohn's disease is 162 cases per 100,000 people per year in the United States (Loftus, 2004). In 1994, the prevalence of ulcerative colitis in Manitoba was reported to be 169.7 and that of Crohn's disease to be 198.5 per 100,000 population (Bernstein et al., 1999).

The age of onset of both these conditions is between 15-25 years of age. There is a 2nd peak of appearance of the disease particularly, ulcerative colitis around the age of 60. Recently, it has also been shown that the incidence of IBD in the paediatric population is increasing worldwide. A population-based Finnish study reported that the incidence of IBD doubled among children in Finland from 1987 to 2003 (Turunen et al., 2006). Besides direct effects of the disease, IBD can also lead to other complications in children. One such complication is low bone mineral density. Having low bone mineral density at

an early age results in low bone mass and subsequent problems in childhood and in adult life. The group of Sylvester et al. (2007) has reported that even with clinical improvement in IBD symptoms after therapy, the bone mineral density does not recover to the normal levels (Sylvester et al., 2007).

An important complication of IBD is colorectal cancer. The prevalence of colorectal cancer in IBD varies with the geographical location, severity and duration of inflammation. In a meta-analysis by Eaden et al. (2001), the incidence rates of colorectal cancer were reported to be 2% after 10 years, 8% after 20 years and 18% after 30 years of having IBD (Eaden et al., 2001). Social and economic burden of the disease has also been studied in recent years to understand its impact on the society. According to a National population health survey in Canada, the estimated cost related to work-loss due to IBD in 1998 was over 104 million dollars (Longobardi et al., 2003a). In the US, this cost was observed to be more than 3.6 billion dollars in the year 1998-1999 (Longobardi et al., 2003b). In addition, the non-gastrointestinal effects of IBD also increase the burden of disease on the community even more. In a large Canadian study, it was reported that the incidence of depression is three times higher in IBD patients as compared to the general population (Fuller-Thomson and Sulman, 2006).

1.1.1 ETIOLOGY AND RISK FACTORS OF IBD

The etiology of IBD remains unclear. There is an interplay of genetic, environmental and immune factors in its causation. The role of genetics in the causation of IBD has been studied for a long time and may partly explain the difference in the incidence among

various ethnic groups in the same region. It is being widely accepted that Ashkenazi Jews have two to four times higher risk for developing IBD compared to the general population. NOD2/CARD15 mutations have been specifically ascribed to the expression of Crohn's disease in the Jewish population (Karban et al., 2004). It is being postulated that the incidence of IBD is higher in monozygotic twins, 80% in siblings and 70% with parental affections (Karlinger et al., 2000). The genetic loci for susceptibility to IBD were initially identified to be present on chromosomes 3, 7 and 12 (Satsangi et al., 1996). Since then, based on family linkages and sex-affliction, many other regions on different chromosomes, particularly, chromosomes 6 and 16 have been shown to be involved in IBD (Brant et al., 2004).

About 50-70% of patients with IBD also show positive HLA-B27 association (Orchard et al, 1997). HLA-B27 is a known affliction in various auto-immune disorders, pointing to the fact that immune factors also play a role in the pathogenesis of IBD as suggested by several researchers. Saxon et al. (1990), have reported the presence of anti-neutrophil cytoplasmic antibody in over 80% of patients with ulcerative colitis (Saxon et al., 1990). Recently, Epstein-Barr virus has also been implicated as a trigger for immune response and pathogenesis of ulcerative colitis (Gehlert et al., 2004). The evidence, however, is not very clear and the association is currently being evaluated by elaborate research. It is possible that the iatrogenic suppression of immune response by steroids during the treatment of IBD may instead be the trigger for Epstein-Barr virus infection seen in IBD.

Environmental influences on IBD have been studied extensively. Environmental factors are further classified as enteral micro-environment and the external environment. It has been observed that there is an increased incidence of IBD among smokers (Mahid et al.,

2006). The results of various studies investigating smoking as a risk factor for IBD so far, however, have been inconsistent and have also suggested that smoking affects ulcerative colitis and Crohn's disease differently (Bridger et al., 2002).

Diet has emerged as another major environmental factor shown to be influencing the causation and progression of IBD. Diet rich in refined carbohydrates was shown to be associated with increased incidence of Crohn's disease by two separate groups of researchers; Martini and Brandes (1976) and Miller et al. (1976) (Martini and Brandes, 1976; Miller et al., 1976). To evaluate other dietary factors and to ascertain how these factors were related to IBD, many epidemiological studies have been conducted. A retrospective case-control study showed that high sucrose intake was associated with a high relative risk for developing Crohn's disease. The same study also showed that high fibre diet was protective against IBD. This group also noted high relative risk for developing both ulcerative colitis and Crohn's disease in people consuming fast food (Persson et al., 1992). A Japanese study further reported that ulcerative colitis is associated with excess consumption of hydrogenated fats like margarine (Kurata et al., 1994). To assess whether the dietary practices were present before the disease onset or were a consequence of the disease, Rief et al. (1997) conducted a study to look at the pre-illness dietary patterns of patients with IBD. They observed that high sucrose and total fat intake before the onset of disease were associated with ulcerative colitis (Reif et al., 1997). An extensive study by Geerling et al. (1999) reported that patients with Crohn's disease were recycling their cooking oil for significantly higher times than the control population (Geerling et al., 1999). This points to the fact that recycling of fats might be

generating some compounds to adversely affect gut defences. This can also explain the positive association between fast food consumption and IBD. An interesting study was conducted in Japan looking at the increase in the incidence of IBD and changes in dietary pattern of the population over a period of twenty years. This study reported that increase in daily intake of total fat and omega-6 polyunsaturated fatty acids was related to increase in incidence of IBD (Shoda et al., 1996). To further evaluate these findings of epidemiologic studies, many experimental studies are being conducted by researchers worldwide.

Before analyzing the present literature in the arena of the effects of polyunsaturated fatty acids (PUFAs) in IBD, the mechanism behind their role in inflammation is summarized below.

1.2 POLYUNSATURATED FATTY ACIDS (PUFAs)

The two main types of polyunsaturated fatty acids are omega-3 (ω-3) and omega-6 (ω-6) fatty acids. This classification is based on the position of the 1st double bond from the methyl terminus of the fatty acyl chain. Alpha-linolenic acid and linoleic acid are the essential ω-3 and ω-6 PUFAs, respectively, for the human body. They have to be provided in the diet as our system lacks enzymes to insert double bonds at these positions. These essential PUFAs can, however, undergo further elongation and desaturation to synthesise other ω-3 and 6 fatty acids in the body. The main PUFAs found and used in the human body are summarized below:

ω-3 fatty acids	# carbon atoms	# double bonds
Alpha-linolenic acid	18	3
Eicosapentaenoic acid (EPA)	20	5
Docosahexaenoic acid (DHA)	22	6
ω-6 fatty acids		
Linoleic acid	18	2
Dihomo γ-linolenic acid	20	3
Arachidonic acid	20	4

ω-6 fatty acids are more prevalent in the diet as compared to the ω-3 fatty acids. The main sources of ω-6 fatty acids in the diet are corn oil, sunflower seed oil and safflower oil. The oils with high ω-3 content include canola oil, flaxseed oil containing 40-60% ω-3 fatty acids and fish oil which contains about 37-45% ω-3 fatty acids (Lay and Dybing, 1989). The major ω-3 fatty acid in flaxseed oil is alpha-linoleic acid; while the main ω-3 fatty acids in fish oil are EPA and DHA. In a normal adult diet, PUFAs contribute approximately 19-22% of energy intake from fats. Of the total energy derived from PUFAs, linoleic acid contributes about 84-89% and alpha-linolenic acid about 9-11% (Kris-Etherton et al., 2000). The ratio of ω-6:ω-3 fatty acid in the human diet was believed to be 1 in early nomadic ages. In the United States, however, this ratio has increased presently to about 10.6 to 15.7. The current recommendation is to achieve a ω-6:ω-3 ratio of 2.3 in the North American diet.

1.2.1 ROLE OF PUFAs IN INFLAMMATION

PUFAs are important constituents of cell membranes. The main types of PUFAs incorporated in the cell membranes are arachidonic acid (ω-6), EPA (ω-3) and DHA (ω-3). Arachidonic acid is the major precursor of eicosanoids in the cells. Eicosanoids

include prostaglandins and leukotrienes, which are lipid-derived inflammatory mediators in the body. The biochemical pathway of generating eicosanoids from fatty acids is schematically shown in Figure 1.

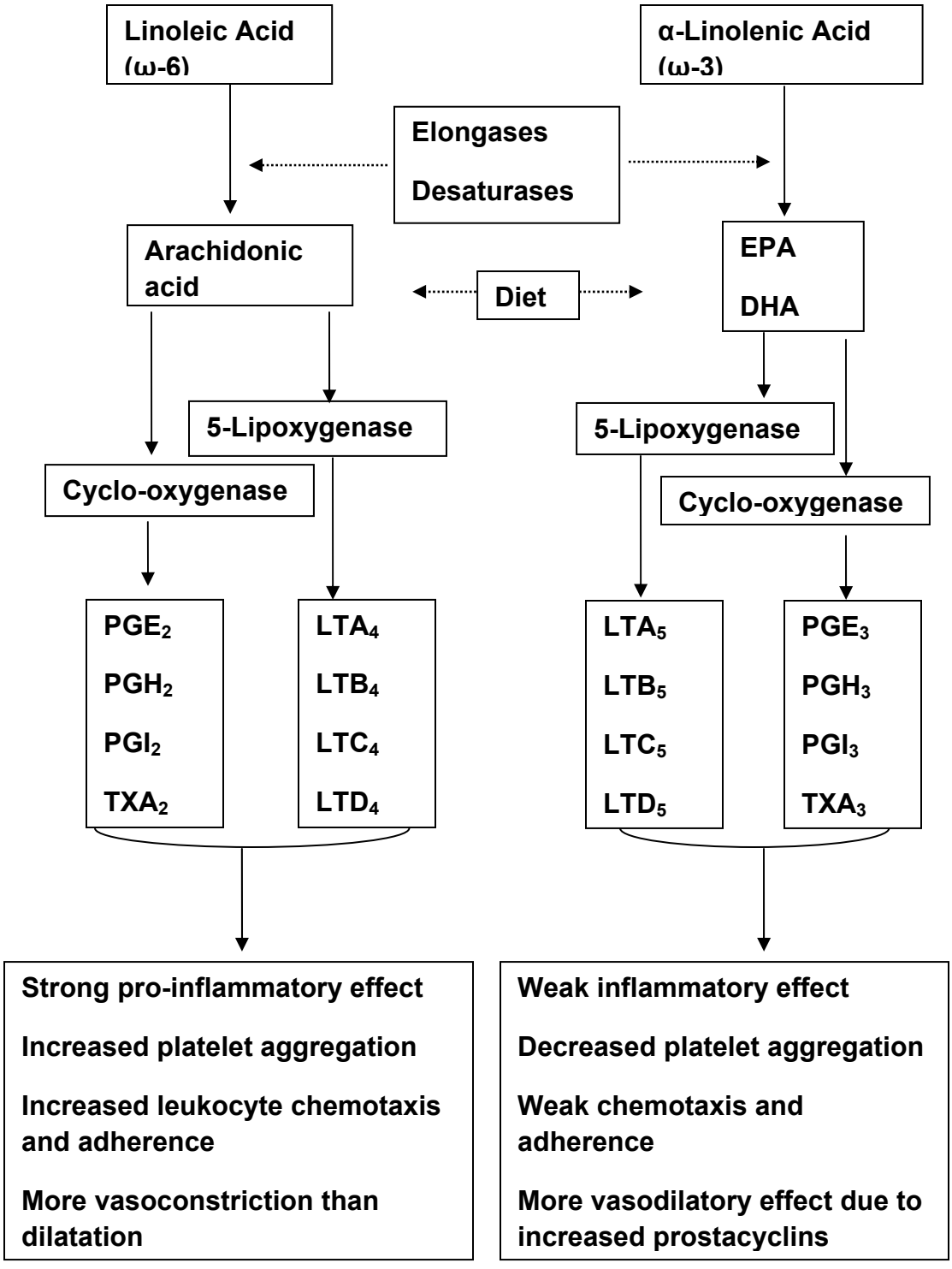


Figure 1: Oxidative metabolism of PUFAs

As shown in Figure 1, the same set of enzymes are involved in the metabolism of both ω-3 and ω-6 fatty acids. The concentration of ω-6 fatty acids is higher in the cell membrane, and so the enzymes are consumed in the ω-6 pathway to generate potent inflammatory mediators. When the concentration of ω-3 fatty acids is increased, however, there is competition between ω-3 and 6 pathways for the enzymes. The enzymes have greater affinity towards ω-3 fatty acids and thus, are preferentially diverted towards the ω-3 pathway when their concentration increases. Therefore, when the ω-3 pathway is activated, there is inhibition of the ω-6 pathway resulting in scarcity of inflammatory mediators (Simopoulos, 2002). In addition to their effects on promoting leukocytic inflammatory response, eicosanoids also stimulate various cytokines like interleukins IL-2, IL-4 and tumor necrosis factors TNF-α and γ. When ω-3 fatty acids are supplemented in the diet, there is inhibition of cytokine activation and thus, suppression of overall inflammatory response (Robinson et al., 1996).

In a crossover study by Stenson et al. (1992), fish oil supplementation for 4 months resulted in significant improvement in histology scores and a decrease in the levels of leukotriene B4 levels in patients with active ulcerative colitis (Stenson et al., 1992). During the fish oil supplementation period, the dose of steroid treatment required reduced by almost fifty percent. In a recent animal study, Whiting et al., (2005) reported similar findings including a reduction in histology scores and a decrease in the levels of interleukin-1β and 12, and tumor necrosis factor-α in colonic cells after supplementing ω-3 fatty acids in the diet (Whiting et al., 2005). The studies conducted so far are, however, controversial in terms of the effects of ω-3 fatty acids in IBD. Hawthorne et al. (1992), observed no benefit during maintenance phase and only moderate benefit of fish oil

supplementation in active phase of ulcerative colitis (Hawthorne et al., 1992). Some researchers found only a reduction in levels of markers of oxidative stress and no histological or immune benefits after ω-3 fatty acid supplementation (Aslan and Triadafilopoulos, 1992; Barbosa et al., 2003). One of the applications of research looking at the effect of ω-3 fatty acids on inflammation in IBD is to develop enteral formulas for patients with acute disease. Differential effects of supplementing ω-3 fatty acids in the enteral formula were seen for ulcerative colitis and Crohn's disease. The ω-3 fatty acids were seen to be more beneficial in ulcerative colitis than in Crohn's disease (Meister and Ghosh, 2005). The controversy in results from human studies looking at the effect of ω-3 fatty acids in IBD can be explained in part by the limitations conferred to human trials. Difficulty in recruiting patients, effect of baseline diet, and interaction with medical therapies for IBD are some of these limitations (MacLean et al., 2005). Another very important consideration in studies of this kind is the ω-6: ω-3 ratio. In human studies, the amount of ω-3 fatty acids is increased mainly at the expense of ω-6 fatty acids and the effects seen may be due to reduction in ω-6 fatty acids. To analyze the effect of ω-3 fatty acids and overcome some of the limitations of human studies, animal studies using a model to simulate human IBD are required.

1.3 ANIMAL MODEL OF IBD

As the knowledge about etiology of IBD keeps unfolding, many animal models are being designed to simulate human IBD. In addition to histological similarity of the lesions, the main criteria to classify colonic inflammation as being similar to human IBD include

chronic and relapsing nature of inflammation, and induction of T-helper cell-mediated immune response. The models can be experimental, wherein, IBD is induced either by externally administered agents or genetic modifications. On the other hand, they can also be spontaneous or natural models predisposed to IBD due to unknown etiology. Exogenous models involve induction of inflammation either by chemical agents such as dextran sulphate sodium (DSS) and carrageenan or by bacterial agents like peptidoglycan-polysaccharide from group-A streptococci (PG-APS) (Stadnicki and Colman 2003). Genetic models include IL-10 knock-out mouse models and T-cell receptor mutant model (Mombaerts et al., 1993). Genetic models target a specific pathway in the animals; however, many possible genetic changes have been identified in humans and differ by ethnicity, sex, type of IBD among other variables.

The nature of outcomes being studied is an important factor in choosing the model. Based on the criteria to classify the model as similar to human IBD and the nature of our study, we used a rodent model with carrageenan-induced inflammation. The carrageenan model is more suitable for the ultimate goal of our project to study chronic inflammation and effect of dietary agents on IBD-induced colon cancer. Other agents like DSS and PG-APS induce severe damage and acute colitis-like symptoms, including severe diarrhoea and bleeding. Carrageenan induces milder symptoms, and we found that it induced chronic sustained inflammation including crypt abscesses, villi blunting, epithelial necrosis and muscle thickening (unpublished observation) as has been noted by others. Hence, it is suitable for long term studies. In addition, carrageenan feeding also enhances the sensitivity of the colon to azoxymethane induced aberrant crypt foci and induces

increased production of TNF-alpha. We intend to induce colon cancer using azoxymethane in the setting of colonic inflammation for our future studies.

The carrageenan-induced colitis model was first designed by Marcus and Watt (Marcus, and Watt, 1969). Carrageenan is a polysaccharide extracted from red seaweed. It is used in various food and non-food products like yogurt, desserts and toothpastes because of its gelling property. Its concentration ranges from as low as 0.005% to as high as 3.0% in these products. There are 3 main types of carrageenan that are available in the market: kappa-carrageenan, iota-carrageenan and lambda-carrageenan. The molecular weight of the native carrageenan molecule is between 100,000 and 300,000 KDa. It is subjected to acid hydrolysis to produce degraded-carrageenan with a molecular weight between 30,000-50,000 KDa. The degraded carrageenan, with low molecular weight, can pass through some of the intestinal barriers and has been shown to cause harmful effects. It has been shown to cause intestinal ulceration, bleeding and colonic tumors in animals (Tobacman, 2001). The International Agency for Research on Cancer (IARC) has classified carrageenan as group-2B, indicating that it has sufficient evidence to be carcinogenic in animals and may be in humans too. The property of degraded carrageenan to cause intestinal ulceration is being exploited in animal studies on IBD.

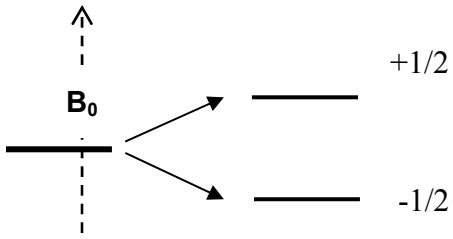
Moyana et al. (1990) administered lambda degraded carrageenan as an oral solution to Sprague-Dawley rats for 30 days and then evaluated their small intestine for the degree, extent and type of ulceration. They concluded that the inflammation seen in this rat model was similar to human IBD (Moyana and Lalonde, 1990). The changes in histopathologic features of carrageenan-induced inflammation and some of the molecular mechanisms involved in its induction were studied by Pricolo et al (Pricolo et al., 1996).

One of the proposed mechanisms for induction of ulceration by carrageenan is its ingestion by macrophages. After ingesting carrageenan, macrophages release lysosomal enzymes in the mucosa which causes inflammation (Abraham et al., 1974). Marcus et al., (1992) proposed that another mechanism by which carrageenan induces inflammation may be direct mucosal injury by acting as an antigen and penetrating the mucosa. In this study, infiltration of intestinal mucosa with inflammatory cells, crypt abscess and dilatation of crypts were observed very early even before gross or microscopic ulceration was visible (Marcus et al., 1992). In addition to triggering an immune response, carrageenan can also induce the growth of gram-negative anaerobic bacteria. These bacteria particularly, Bacteroides vulgates have been implicated in the pathogenesis of IBD (Breeling et al., 1988).

1.4 MAGNETIC RESONANCE SPECTROSCOPY (MRS)

Magnetic resonance spectroscopy (MRS) also known as nuclear magnetic resonance (NMR) spectroscopy is a widely used technique to analyze different chemical and biological samples. The atom of a molecule is comprised of a nucleus, which in turn contains protons and neutrons, and electrons revolving around the nucleus in their orbits. The technique of MRS exploits the magnetic property of the nuclei to generate data. The commonly used nuclei for MRS include proton (^{1}H), carbon (^{13}C) and phosphorus (^{31}P). The stable isotopes of these 3 elements are commonly present in most of the organic and inorganic compounds in biological systems and as such make the technique of MRS very sensitive (Khan et al., 2005).

All the subatomic particles spin about their axes. If the number of protons and neutrons is equal in a nucleus, then there is no net spin. For example, ^{12}C, an isotope of carbon that has 6 protons and 6 neutrons has no net spin on its nucleus. However, the nucleus of isotope ^{13}C has 6 protons and 7 neutrons, and thus, it has a net spin about it. A general rule is that if the sum of neutrons and protons in a nucleus is odd, then the net spin is ½ integer. Henceforth, the principle used in MRS will be explained using ^{1}H nucleus as an example. ^{1}H has 1 proton and no neutron and thus, its overall spin is ½. The overall spin determines the number of orientations in which the nucleus can exist and is calculated by the formula (2I + 1); where I is the overall spin. ^{1}H has ½ overall spin and so it can exist in 2 possible orientations of +1/2 and -1/2. Owing to the property of 'spin', these particles behave as tiny magnetic dipoles with north and south pole and a magnetic moment. In the absence of an external magnetic field, these tiny magnets are randomly oriented and are at equal energy. When an external magnetic field B_0 is applied, they align themselves parallel to the axis of the field either along or opposite to it. These particles can either be at low-energy state where they are aligned along the external field or higher energy state with an alignment opposite to the external field. Some nuclei stay in the lower level whereas some move to higher energy level. This phenomenon is depicted below:



Energy levels of proton nuclei after applying a magnetic field B_0

A significant point is that there are slightly higher numbers of nuclei that remain in the lower level than in the higher level of energy. This discrepancy is critical for the technique of MRS. By triggering the nuclei in lower energy level at appropriate frequency they can be moved to higher energy level. This frequency required for transition from low-energy state to high-energy state is called as resonance frequency or Larmor frequency.

In NMR experiments, an electromagnetic pulse with a frequency the same as the Larmor frequency is applied to trigger the nuclei in the lower energy state into the higher energy state. This absorption of energy causes spin-flip. When these particles return to their original energy state, they release energy which creates NMR signals. The original output signal is in the form of time-domain free induction decay. To make it easily comparable and to assign the peaks to various nuclei, it is converted into frequency-domain spectrum by Fourier transformation. The frequency-domain spectrum has a scale of parts per million (ppm). A signal in the spectrum is called a resonance and its position on the spectrum is denoted by chemical shift. The chemical shift of a nucleus is the difference between the resonance frequency of the nucleus and a standard (0 ppm). A reference or standard is a compound that is chemically inert so that it does not react with the sample and also has protons that can generate strong MR signals that do not interfere with common organic and inorganic samples. Some common standards used in proton MRS include, tetramethylsilane (TMS), 3-(trimethylsilyl)- propionic acid-D4, sodium salt (TSP) and 2,2-dimethyl-2-silapentane- 5-sulfonate sodium salt (DSS).

A sample spectrum from ethyl alcohol CH_3-CH_2-OH is shown below:

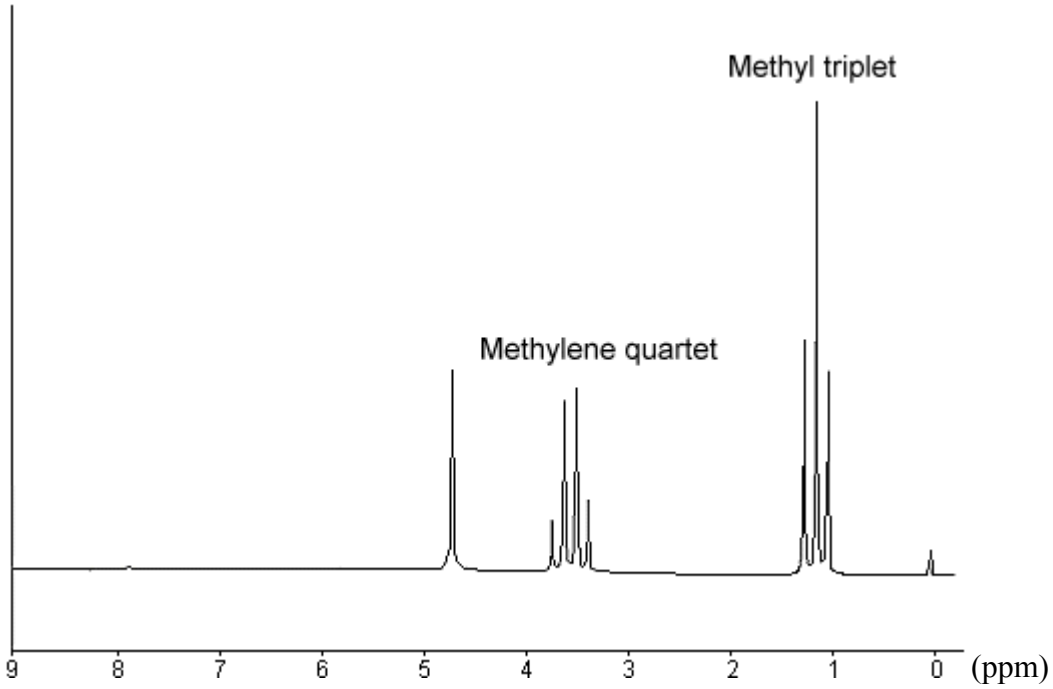


Figure 2: ^{1}H MR Spectrum of ethyl alcohol (unknown author, retrieved on 10 Nov 2007 from http://teaching.shu.ac.uk/hwb/chemistry/tutorials/molspec/nmr1.htm)

In this spectrum, the resonance (signal) due to methylene (CH_2) protons is at approximately 3.7 ppm and the resonance due to methyl (CH_3) protons is around 0.9 ppm. The resonance due to the reference added to the sample is at 0 ppm. Protons that reside in the same magnetic environment are termed chemically equivalent protons. For example, protons in CH_3 and CH_2 groups are usually equivalent. Symmetrical compounds, such as benzene also have equivalent protons. However, many other compounds are not symmetrical and have non-equivalent protons.

Protons that are different in any way absorb energy at different frequencies and hence, give a separate signal on the NMR spectra. NMR signals are not usually single peaks, but a complex pattern of split peaks labelled as doublets (2 peaks), triplets (3 peaks), quartets (4 peaks), etc. The distance between the split peaks is called coupling constants, denoted by J. Splitting is produced by the interaction between nearby protons when they orient themselves either parallel or anti-parallel to the external magnetic field (B0). This phenomenon, where the spin of the nucleus of one proton is close enough to affect the spin of another, is called spin-spin coupling. Essentially, splitting is always reciprocated between the protons, so if one proton is splitting another, the same proton will be split by the affected neighbouring proton. This phenomenon is very useful as it provides valuable information about the neighbours of a proton within the molecule. Generally, splitting follows the N+1 Rule, according to which, for a proton with N neighbours, its signal will be split into N+1 lines. Couplings can be either 1^{st} order or simple couplings where J (distance between split lines) is equal or 2^{nd} order which occurs when the proton experiences interaction from 2 or more different nuclei. The splits produced as a result of 2^{nd} order coupling are labelled as doublet of doublets or doublets of singlet etc. Based on these principles of coupling, the spectrum of ethyl alcohol has a quartet from the CH_2 group and a triplet from the CH_3 group. The two protons of CH_2 group couple with neighbouring protons of CH_3 group. Thus, it has 3+1=4 (N+1) splits in the signal. The protons from CH_3 group interact only with 2 protons of CH_2 group and so have 2+1=3 lines in the signal.

1.5 STATISTICAL CLASSIFICATION STRATEGY (SCS)

Statistical classification strategy, also known as multivariate analysis is a robust objective method used for comparing the complex MR spectra. In any biological tissue there are many metabolites. ^{1}H MRS is a very sensitive technique and receives signals from a large number of metabolites. This makes it very difficult to compare the spectra for differences in the metabolites. In addition, many metabolites are present in the tissue in both normal and diseased states. In such cases the differences in their intensity are more informative than just the presence/absence of these metabolites. The statistical classification strategy helps to resolve these challenges in the analysis and interpretation of MR spectra.

The strategy begins with spectral pre-processing. During pre-processing, all the spectra are aligned together to the reference peak and are normalized by dividing each data point in each spectrum by the spectrum's total area. The region of most significance is cut (for our analysis, we used the region between 0.5 to 4.5 ppm) and used for further analysis. A computer-based genetic algorithm for region subselection is run 100 times on the spectra. A histogram is generated identifying the number of times each region of the spectrum was selected, with linear discriminant analysis (LDA) classification accuracy as the selection criterion. The regions most often selected (5 for our analysis) are used for further analysis. The advantage of this process is that the original spectrum which had about 1100 points is now converted into 5 points after averaging the points within each selected region. This allows for simple classifiers to be used, such as LDA. The spectra in each group are then divided into test set and training set by 10,000 random splits. Each training set is used to develop a 'classifier' and the test sets are compared against it using

linear discriminant analysis (LDA) (Nikulin et al., 1998). Thus, we get 10,000 classifier coefficients. These classifier coefficients are ranked according to how well they did on the test set and their weighted average is used as a final single classifier. The classifier outcome is finally reported as ‘class probability’ (Somorjai et al., 2004).

2 STUDY RATIONALE

Based on the literature review, it is evident that there is sequential progression of IBD to dysplasia and subsequently, colon cancer. The genes associated with inflammation are elevated in both IBD as well as colon cancer. In addition, epidemiological studies have shown that aspirin and other aminosalicylates may prevent the development of sporadic colon cancer (Vinogradova et al., 2007). These observations point to the fact that there is an association between IBD and colon cancer; although the exact mechanism is not completely understood. There is a time lag of 8-10 years between the diagnosis of IBD and development of colon cancer. This gives a window of opportunity to control the transformation of inflammatory changes into malignancy. There are two basic requirements to achieve this goal. These include - a technique to detect early changes of inflammation, and a therapeutic intervention that can break or delay the sequence. To conduct a long-term study, we also need an animal model which can sustain chronic inflammation resembling human ulcerative colitis. As mentioned above, ^{1}H MRS is a technique that can detect biochemical changes that usually precede histological manifestations of a disease. Therefore, it may be suitable for detecting early inflammation before it becomes evident by histological analysis of a biopsy sample. As such, ^{1}H MRS may be a useful adjunct to histology to get the global picture of both biochemical and histological abnormalities in a biopsy sample. This led us to the first part of our hypothesis.

The second part of our hypothesis is based on the fact that anti-inflammatory effects of certain drugs may be preventative against colon cancer. Diet, particularly, ω-3 fatty acids have a net anti-inflammatory effect in the body as outlined in chapter 1 of this thesis. Studies have been conducted to demonstrate this effect either directly on colon cancer or on IBD. However, there is a lack of evidence on the benefit of ω-3 fatty acids on the window between IBD and cancer. As ω-6 fatty acids and saturated fats have adverse effect on cancer, we decided to use these diets as our comparison groups. These are other major components of modern diet and would give us opportunity to simulate the general dietary pattern of North American population. After setting these two hypotheses, we required a suitable model to conduct the study.

We used Sprague-dawley rats fed with 2% carrageenan by weight as model of IBD for our study. We used flaxseed oil as a source of ω-3, corn oil as ω-6 and beef tallow as a source of saturated fats. Fish oil is another good source of ω-3 fatty acids, however, we chose not to use it for our study. The reason behind this was that we wanted to design a study that can later be translated into human trials. Fish allergies are very common in North America and also there is some concern about contaminants, such as, mercury in fish products. This may pose some difficulties in conducting randomized trials in the future.

For our study, we fed the animals with either low fat diet or high fat diet. The low fat diet consisted of 5% corn oil and the high fat diet had 12% of the respective fats by weight. Studies have shown that 12% of ω-6 or saturated fat is enough to cause significant inflammation. In addition, 12% fat was also appropriate for human studies as there is a

limitation to the amount of fat we can add to the human diet. All these observations finally led us to developing the working hypotheses for our study.

3 WORKING HYPOTHESIS

This project was based on the following hypotheses:

- There are biochemical changes occurring in the colon during IBD and ^{1}H MRS in combination with multivariate analysis has the potential to detect those changes.

- Various dietary polyunsaturated fatty acids are assimilated in the colonic wall and cause discernible changes in the colon. It is also postulated that various PUFAs have differential modulatory effects on colonic inflammation in IBD with omega-3 PUFAs expected to reduce the inflammation as compared to the control samples. Such changes are reflected in the ^{1}H MR spectra.

4 OBJECTIVES

The study was accomplished in three stages. The objective of each stage was as follows:

Stage 1

Assess the potential of ^{1}H MRS and multivariate analysis in detecting early colonic inflammation using an animal model of IBD.

Stage 2

Study the potential of ^{1}H MRS combined with multivariate analysis in differentiating between the effects of various PUFAs on normal rat colon.

Stage 3

Validate the potential of ^{1}H MRS combined with multivariate analysis in analyzing the effects of various PUFAs on modulation of inflammation in an animal model of IBD.

5 STAGE 1

OBJECTIVE: Assess the potential of ^{1}H MRS and statistical classification strategy in detecting early colonic inflammation using animal model of IBD.

5.1 MATERIALS AND METHODS

5.1.1 SAMPLE COLLECTION

Thirty male Sprague Dawley rats were divided into the following 3 groups:

Control group – the animals were fed regular AIN-76 diet for 2 weeks

One week-treated group – the animals were fed 2% carrageenan by weight along with regular diet for the last 1 week of the study period

Two weeks-treated group – the animals were fed 2% carrageenan by weight mixed with regular diet for 2 weeks

All the animal housing, specialized feeding and sample collection procedures were undertaken at the animal facility in the department of Biology, University of Waterloo. At the end of 2 weeks, the animals were sacrificed by carbon dioxide inhalation method. The colon of each animal was excised after sacrificing the animal. Mucosal layer of the colon was scraped off using a glass slide by the method previously described by Briere et al (Briere et al., 1995). The mucosal samples were coded and stored in cryovials containing phosphate-buffered saline in deuterium oxide (PBS/D_2O) medium at -70º C. These coded cryovials were shipped on dry ice to the National Research Council of Canada, Winnipeg for MRS and histological assessment.

5.1.2 ^{1}H MRS EXPERIMENTS

For ^{1}H MRS experiments, the samples were thawed and cut into 5-7 mm long pieces along the longitudinal axis of the colon. This yielded 18-20 pieces from each colon sample. During the course of MR experiments, the pieces from the thawed sample were stored in a refrigerator at 4º C to avoid artefacts due to repeated freezing and thawing. To assess the differences in the spectra from the entire colon, ^{1}H MRS was conducted on all the specimens from one representative colon sample in each group. It took 1.5 – 2 days to conduct ^{1}H MRS experiments on every specimen (18-20 pieces) from one colon sample. There was a probability that keeping the specimens in the refrigerator at 4º C during MR experiments for extended period could have induced some histological artefacts and changes in the metabolites to confound the results. We observed that the ^{1}H MR spectra from closely adjacent pieces of the colon were very similar and therefore, we decided to conduct ^{1}H MRS experiments on alternate pieces from each sample. This yielded a total of 63 spectra from control, 60 spectra from 2 weeks-treated and 58 spectra from 1 week-treated group.

The sample preparation technique used for conducting ^{1}H MRS was similar to the technique described by Kuesel et al. (Kuesel et al., 1992). Briefly, the colon sample was placed in a glass capillary tube filled with Phosphate Buffered Saline (PBS)/D_2O with one end plugged. This capillary was inserted into the NMR tube containing 300 µl of PBS/ D_2O solution along with 5 µl of chemical shift reference, 3-Trimethylsilyl-Propionic acid-D4- sodium salt (TSP). ^{1}H MRS was conducted on these samples using a

Bruker Avance 360 MHz spectrometer at 25˚C with presaturation of the water signal. The acquisition parameters included: 90˚ pulse at 9.30μsec, number of scans = 256, spectral width = 4990.02 Hz, relaxation delay =3 sec and time domain data points = 8K. The samples were fixed in 10% neutral buffered formalin immediately after ^{1}H MRS experiments for histological assessment.

5.1.3 **HISTOLOGY**

Immediately after finishing the MR experiment, each specimen was fixed in 10% neutral buffered saline. Each specimen was coded to avoid analysis bias and embedded into paraffin blocks using the protocol in appendix-1a. The paraffin blocks were cut into 5 μm thin sections and collected on coated glass slides. The sections were stained with haematoxylin and eosin stain as per the protocol described in appendix-1b. A representative section was stained first and viewed under light microscope to assess the strength of staining. The optimum time period for each stain was decided based on that. Each slide was read under the microscope at 60X magnification. The criteria used for diagnosing inflammation included distortion of crypt architecture, presence of inflammatory cells, and fibrosis of the septa. The degree of inflammation was graded based on the proportion of total sample inflamed. If less than or equal to 30% of total sample was inflamed, it was graded as mild. Similarly, samples having 30-60% inflammation were graded as moderate and samples with over 60% inflamed area were graded as having severe inflammation. After charting the results of microscopic analysis,

the slides were decoded and assigned to the respective groups for further analysis. This ensured that error due to measurement bias was eliminated.

5.1.4 **STATISTICAL CLASSIFICATION STRATEGY**

The ^{1}H MR spectra were subjected to preprocessing before SCS analysis. For preprocessing, all the MR spectra were normalized by dividing each data point by the total area and then aligned on the reference peak due to TSP. Following this, the spectra were cut and only the region between 0.5-4.5 ppm consisting of 1184 points was used for multivariate analysis. This region was selected to avoid artefacts due to the water suppression peak at 4.7 ppm and the reference peak at 0 ppm. Multivariate analysis was carried out on these preprocessed spectra. For multivariate analysis, first derivatives were taken, and rank ordering was done on the resulting data to eliminate baseline differences between the spectra. For control vs. each of the treatment groups, random subsets of the data were used to train a genetic algorithm-based subregion selection method. The objective function was linear discriminant analysis (LDA) classification accuracy. A histogram was constructed and the regions that stood out as being selected the most often were used to develop the final classifier: linear discriminant analysis (LDA) with coefficients optimized using a bootstrapping method.

5.1.5 **BLOOD ANALYSIS**

Blood samples were also collected from animals in control (n=5) and 2 weeks-treated (n=6) groups to assess any systemic effects of inflammation. These samples were coded and sent to the laboratory services division at the University of Guelph for complete and differential cell count. Carrageenan has been shown to induce systemic response and we speculated that there may be an increase in the white cell count in the blood of treated animals (Moyana et al., 1994).

5.2 RESULTS

A representative MR spectrum of rat colon with labelled peaks showing most of the pertinent metabolites is shown in figure 3.

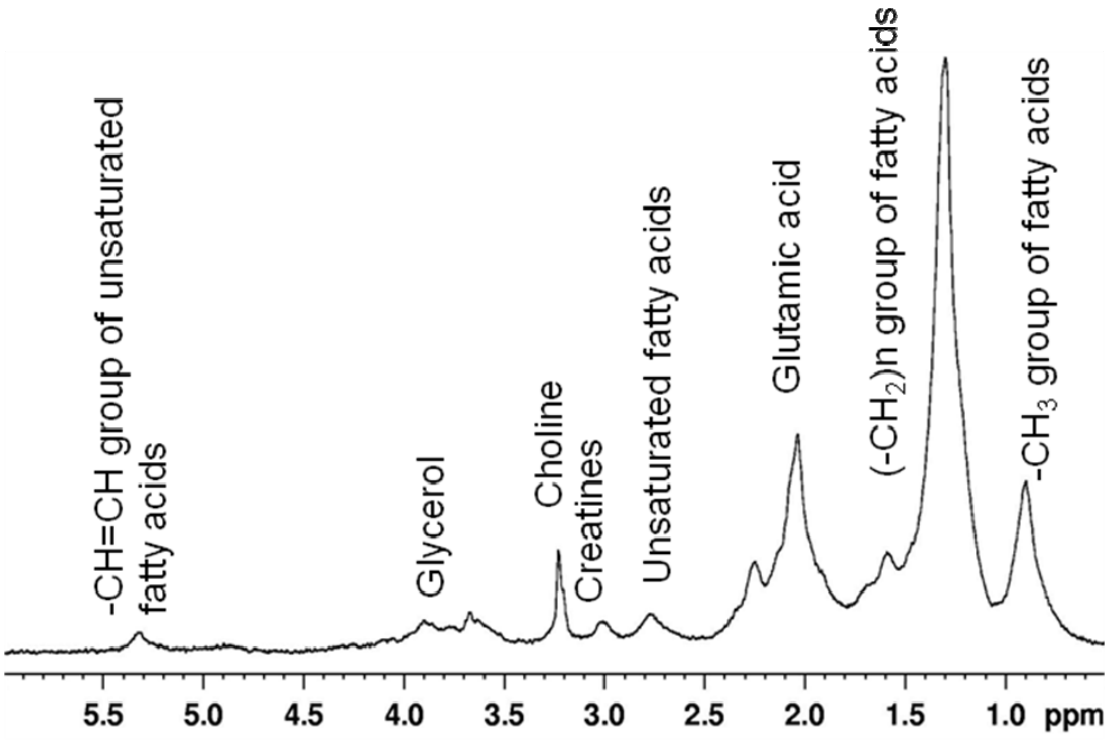
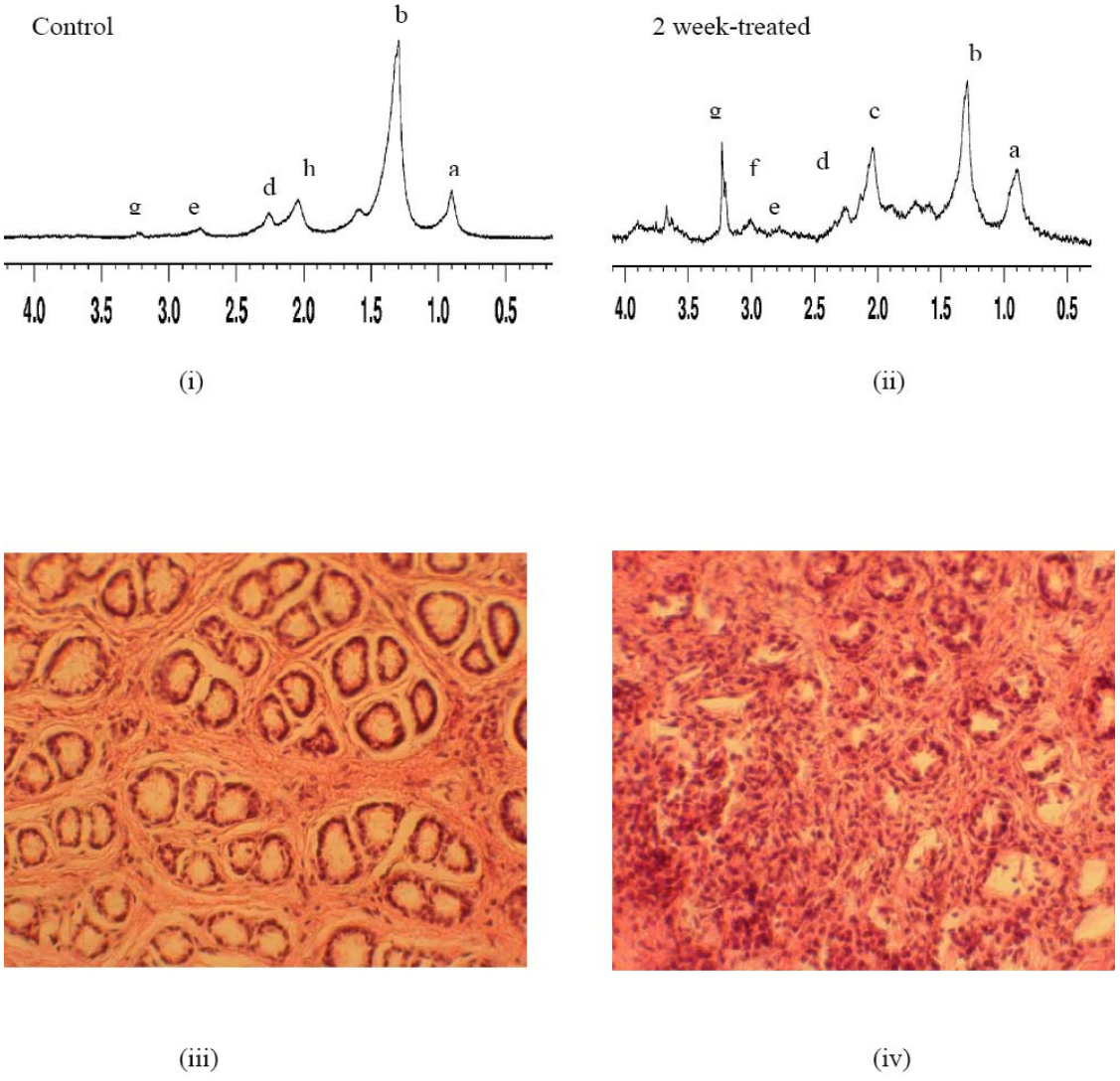


Figure 3: Representative MR spectrum of inflamed rat colon showing important metabolites seen in our study

SCS analysis was performed to compare the spectra from control group with both treatment groups. In performing the analysis, the spectral data of the 1-week treated group was found to overlap with both the control and the 2-week treated groups. This indicates that after a period of 1 week, there is some inflammation in the colon, but not significant enough to set it apart from the normal colon. Thus, we have focussed on the analysis of control and 2-week treated groups. The results of this analysis are shown in Table 1. In this table, all of the spectra (control, n=63; 2 weeks-treated group, n=60) were included in the analysis without correlating the data with histology.

After conducting SCS on the complete dataset, histological assessment was performed on these samples. The samples were coded before proceeding with histology to avoid analysis bias. Figure 4 shows representative spectra from the control group and the 2 week-treated group. The corresponding H & E stained sections from the same specimen are also shown in this figure.



MR spectrum of specimen from control (i) and 2 week-treated group (ii). H&E stained section of the same control (iii) and 2 week-treated group specimen (iv). The metabolites represented by the labelled peaks in MR spectra are as follows: (a) –C**H**$_3$ group of fatty acids in lipids, (b) C**H**$_2$ chain of fatty acids, (c) glutamic acid, d) -C**H**$_2$-COO group of fatty acids, (e) linoleic acid, (f) creatine, (g) total cholines, (h)-C**H**$_2$-CH= group of unsaturated fatty acids

Figure 4: ^{1}H MR spectra and corresponding histological sections from control and 2-week treated groups

In the histological assessment, it was observed that a few of the samples had significant contamination by muscle tissue from the deeper layers of the colonic wall. These samples (n=10) were excluded from the analysis. The remaining 113 samples were evaluated for the degree of inflammation. The histological analysis of these samples revealed that some of the samples from control group were actually inflamed and some samples from the 2-week treated group did not show any signs of inflammation. In addition, while conducting ^{1}H MRS on individual samples, it was noted that there were differences in some of the spectral features within the same group. This is probably due to the fact that some of these animals did not develop any significant inflammation. In addition, the inflammation can also be patchy and may not be present uniformly throughout the colon (Bernstein et al., 1995). However, we could not give a plausible explanation for colonic inflammation in the control group. Such samples (n=29) were removed from the full dataset (Table 1) and were analyzed separately. From now on, these spectra will be referred to as 'aberrant spectra'.

After correlating with histology and removing the samples contaminated with muscles, the remaining 113 spectra were subjected to multivariate analysis. It was necessary to train the classifier on 'pure' spectra so as to distinctly identify the characteristics of inflamed and non-inflamed samples. Therefore, the aberrant spectra from both control and 2 weeks-treated group (n=29) were not used to develop the classifier for the multivariate analysis. The classifier was developed using only the 'pure' spectra (n=84). The multivariate analysis of the main dataset (n=84) is shown in Table 2. The accuracy with which these samples were assigned to their respective groups increased, from 77.2% in the initial analysis to, 82%.

Table 1: SCS analysis results of all MR spectra without histological confirmation (n=123)

Control Vs. 2 weeks Carrageenan-treated group					
Desired Class	**N**	**Classification**		**Accuracy**	**Overall Accuracy**
		Correct	**False**		
Control	63	50	13	79.4%	77.2%
Treated	60	45	15	75.0%	

Table 2: Results of SCS analysis with histological confirmation (n=84)

Desired class	**N**	**Classification**		**Accuracy**	**Overall accuracy**
		Correct	**False**		
Control	38	31	7	81.6%	82.1%
Treated	46	38	8	82.6%	

The aberrant dataset (n=29) was separately submitted to the classifier developed using the 'pure' spectra. Out of the 16 'control' samples found to be inflamed on histological assessment, the SCS analysis classified 10 of them as '2 weeks-treated', i.e. inflamed. Similarly, out of the 13 carrageenan-treated samples that were found to be inflammation-free on histological assessment, the multivariate analysis classified 9 of them as 'control'. The 4 samples from this group that were classified by multivariate analysis as being inflamed, although they were regarded as 'not-inflamed' by histology, may imply that these samples had some pre-morphological inflammation. It also implies that these pre-morphological changes generated sufficient biochemical changes in the tissue so as to be detected by MRS.

Our region selection algorithm identified 4 regions of the spectra that were most discriminatory, i.e. were selected most often by the genetic algorithm. These included spectral resonances due to the =HC–C**H_2**–HC= group in fatty acyl chain of triglycerides (2.79-2.83 ppm), creatine (3.00 ppm), phosphatidylcholine (3.56-3.60 ppm) and glycerol backbone of lipids (4.03-4.05 ppm). The implications of these metabolites are discussed in the later part of my thesis.

To assess the systemic effects of colonic inflammation, we also analyzed blood samples from animals in the control (n=5) and 2 week-treated group (n=6). As IBD is a chronic inflammation, we compared the lymphocyte counts in these animals. In 10 out of these 11 animals, the histological assessment of inflammation agreed with their respective lymphocyte counts. In one animal, the colon specimens were classified as inflamed by histology whereas the lymphocyte count was not raised. This animal was our first sample, and in order to assess the homogeneity of spectra from the whole colon we performed ^{1}H

MRS on each piece of the tissue. As it was not possible to run all the pieces from this colon in one day, the experiments had to be done over a period of 2 days. The samples were not re-frozen and were kept in the refrigerator at 4°C. This may have created some artefact interfering with the histological analysis. ^{1}H MRS was performed on all the pieces from the rest of the other samples over the same day. Immediately after finishing ^{1}H MRS, the samples were fixed in neutral buffered formalin to avoid creating histological artefacts.

5.3 CONCLUSION

In stage-1 of our study, we have demonstrated that ^{1}H MRS combined with SCS analysis is a highly sensitive technique to distinguish between inflamed and non-inflamed colonic tissue. In addition, it also suggests that ^{1}H MRS can serve as a tool to study the sequential progression of IBD and explore the effect of various dietary and therapeutic agents on this process. The MR spectra and histological features are distinct in samples from carrageenan-treated vs. untreated group suggesting that this model is suitable for studying early IBD before the dysplastic changes of cancer manifest in the colon.

6 STAGE 2

OBJECTIVE: Study the potential of ^{1}H MRS combined with SCS analysis in differentiating between the effects of various PUFAs on normal rat colon.

6.1 MATERIALS AND METHODS

6.1.1 SAMPLE COLLECTION

Twenty male Sprague Dawley rats were divided into the following 4 groups:

Low fat corn oil (n=5) – the animals were fed regular AIN-76A diet containing 5% corn oil for 2 weeks.

All the high fat diets contained 12% fat by weight in their diet. The fat was added at the expense of carbohydrates in the diet.

High fat corn oil (n=5) – the animals were fed 12% corn oil for 2 weeks.

High fat flaxseed oil (n=5) – the animals were fed 5% corn oil + 7% flaxseed oil for 2 weeks.

High fat beef tallow (n=5) – the animals were fed 5% corn oil + 7% beef tallow for 2 weeks.

6.1.2 ^{1}H MRS EXPERIMENTS

The sample collection and transportation method was same as that for Stage 1 of our study. MR experiments and SCS analysis were conducted similar to Stage 1 protocol. Using the same sample preparation and data acquisition technique, a total of 127 spectra were obtained for Stage 2 of the study.

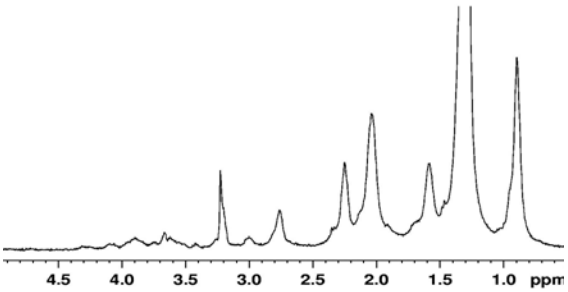
6.1.3 HISTOLOGICAL ANALYSIS

As we did not expect to see significant difference in the histology of colonic mucosal samples from the different groups, histology was done only on representative samples (2 samples from each animal - n=8) from each group.

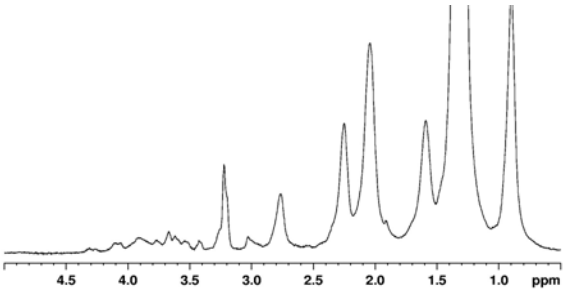
6.2 RESULTS

The ^{1}H MR spectra were analyzed using SCS analysis. Typical ^{1}H MR spectra from each group are shown in Figure 5.

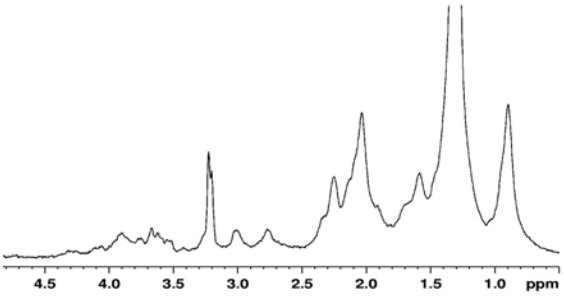
(i) Low fat corn oil



(ii) High fat corn oil



(iii) High fat flaxseed oil



(iv) High fat beef tallow

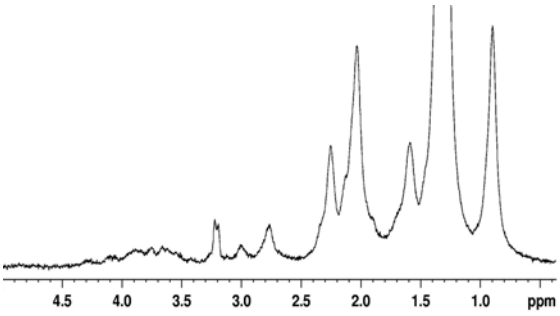


Figure 5: Representative ^{1}H MR spectra from different dietary groups in Stage 2

In the first part of SCS analysis, each of the three groups was compared separately to the control group as shown in Table 3. The classifier assigned the spectra to their respective groups with very high accuracy ranging from about 91 - 100%. The lowest accuracy of 91% was seen for the comparison between control and the beef tallow fed group. In this comparison, 4 out of 32 controls were misclassified and 2 out of 34 spectra from the beef tallow group were misclassified. The metabolites found to be discriminatory for the control vs. all the 3 groups were similar to those seen in Stage 1 of our study. These included resonances due to protons associated with unsaturated carbons and carboxyl terminal protons in the fatty acid chain, creatine and glycerol. In addition to comparing the specialized diet groups with control group, they were also compared amongst each other. The classification result for the comparison of various dietary groups is shown in Table 4. ^{1}H MRS combined with SCS analysis depicted an accuracy of 98-100% in classifying the spectra to their respective groups. In addition to the regions identified during comparison of each group to the control group, resonances due to glutamic acid, total cholines and glycerophospho-ethanolamine were also ascribed to be discriminatory in this analysis. To summarize the results, a combined 4-group classification was also performed as shown in Table 5.

Table 3: SCS analysis of control group vs. each dietary group in Stage 2 (n=127)

Control (low fat corn oil) Vs. high fat corn oil group				
Desired Class	**N**	**Overall Accuracy**	**Regions (ppm)**	**Metabolites**
Control	32	100%	**2.68-2.86**	=HC-C**H**$_2$-CH=
			1.95-2.0	- **H**$_2$C –CH=CH-
High fat corn oil	31		**1.59-1.61**	**-H**$_2$C- CH$_2$ -COO
			1.12-1.19	Unassigned
Control (low fat corn oil) Vs. flax seed oil group				
Control	32	98.4%	**3.02-3.05**	Creatine
Flax seed oil	30		**2.78-2.95**	=HC-C**H**$_2$-CH=
			2.66-2.68	=HC-C**H**$_2$-CH=
Control (low fat corn oil) Vs. beef tallow				
Control	32	90.9%	**3.75-3.80**	Glycerol
Beef tallow	34		**2.66-2.68**	=HC-C**H**$_2$-CH=
			2.20-2.27	**-**H$_2$C- C**H**$_2$ -COO

Table 4: SCS analysis of different dietary groups in Stage 2 to assess the differences between individual diets

High fat corn oil Vs. beef tallow				
Desired Class	**N**	**Overall Accuracy**	**Regions (ppm)**	**Metabolites**
High fat corn oil	31	100%	**3.73-3.80**	Glutamic acid
			2.58-2.71	=HC-C**H**$_2$-CH=
Beef tallow	34		**1.79-1.82**	Unassigned
High fat corn oil Vs. high fat flax seed oil group				
High fat corn oil	31	98.4%	**4.18-4.29**	Glycerol in triglycerides
			3.20-3.24	Cholines
High fat fax seed oil	30		**2.75-2.79**	=HC-C**H**$_2$-CH=
			1.49-1.54	**-H$_2$**C- CH$_2$ -COO
High fat flax seed oil Vs. beef tallow				
High fat flax seed oil	30	100%	**3.28**	Glycerophospho-ethanolamine
Beef tallow	34		**2.79-2.98**	=HC-C**H**$_2$-CH=
			2.31-2.35	Glutamic acid

Table 5: Final combined SCS analysis result for all the groups in Stage 2 to assess the overall efficacy of classification of samples in each group

Desired class	N	Classification		Accuracy
		Correct	False	
Low fat corn oil	32	29	3	90.6%
High fat corn oil	31	31	0	100%
Flax seed oil	30	29	1	96.7%
Beef tallow	34	32	2	94.1%

A final combined classification table (Table 5) was generated from all the analyses performed on Stage 2 samples which include comparison of each group with control group (Table 3) and comparison of different dietary groups with each other (Table 4). It shows the total number of samples in each group that were misclassified in the complete analysis. For example, the flaxseed oil group has 30 samples and only 1 sample was misclassified when comparing it to control as well as other dietary groups. This misclassified sample was placed into low fat corn oil group during SCS analysis. In the final combined classification, 3 out of 32 spectra in the low fat corn oil group were misclassified into beef tallow group; conversely, 2 out of 34 misclassified spectra from beef tallow group were classified into low fat corn oil group by the SCS analysis.

6.3 CONCLUSION

The composition of fatty acids in the diet modulates the concentration of these fatty acids in the colonic mucosa. Administering ω-3, ω-6 and saturated fatty acids in the diet of animals for 2 weeks induces differential accumulation of these fatty acids in the colonic mucosal cells. The sensitivity of ^{1}H MRS combined with SCS analysis approaches 91-100% in detecting these differences and can be used as a tool to study the effects of these PUFAs in modulating inflammation using an animal model of IBD.

7 STAGE 3

OBJECTIVE: Validate the potential of ^{1}H MRS combined with SCS analysis in analyzing the effects of various PUFAs on modulation of inflammation in an animal model of IBD.

7.1 MATERIALS AND METHODS

7.1.1 SAMPLE COLLECTION

Twenty male Sprague Dawley rats bought from Charles River, Canada were fed 2% carrageenan and one of the following diets for 2 weeks:

Low fat corn oil (n=5) – animals were fed AIN-76A diet containing 5% corn oil for 2 weeks.

High fat corn oil (n=5) – animals in this group were fed 12% corn oil for 2 weeks.

High fat flaxseed oil (n=5) – animals were fed 5% corn oil + 7% flaxseed oil for 2 weeks.

High fat beef tallow (n=5) – animals were fed 5% corn oil + 7% beef tallow for 2 weeks.

7.1.2 ^{1}H MRS EXPERIMENTS

The sample collection and transportation methods were similar to that used for Stage 1 of the study. MRS experiments conducted using procedures similar to Stage 1 of the study

yielded a total of 130 spectra. After pre-processing, SCS analysis was conducted using the same protocol as in Stage 1.

7.1.3 **HISTOLOGY**

Immediately after MR experiments, the samples were fixed in 10% neutral buffered formalin and histology was performed on the samples using the same methodology as mentioned in Stage 1 of our study.

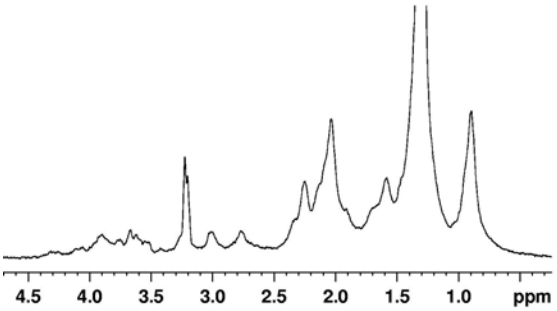
7.2 RESULTS

Typical ^{1}H MR spectra from each group are shown in Figure 6. The samples from each group were analyzed using H&E stain. Histological assessment identified 11 samples as heavily contaminated by submucosal muscle layer and so these samples were not included in the SCS analysis. The remaining 119 spectra were subjected to the SCS analysis. Initially, all the 3 dietary groups were compared to the control group using a classifier developed for each class as shown in Table 6. It was observed that there were significant differences in the metabolic profiles of dietary groups as compared to the control group. Based on these differences, MRS combined with SCS analysis gave an accuracy of about 97-100% in classifying the spectra. To further assess the sensitivity of SCS analysis and to identify whether the dietary groups were specifically different from each other, the dietary groups were compared amongst each other. The SCS analysis result for comparisons of dietary groups is shown in Table 7. In this analysis, as well, the

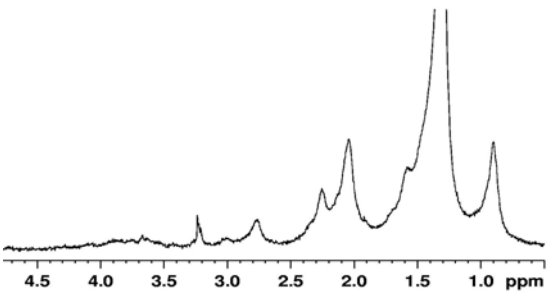
accuracy of SCS analysis approached 97-100%. To sum up the results of the analyses, a final combined classifier was developed for all the 4 groups, which is shown in Table 8.

Our region selection algorithm identified resonances from different parts of the fatty acid chain, and creatine to be discriminatory as enlisted in Tables 6 and 7. We were not able to assign any specific metabolite to the region between 1.10-1.22 ppm. The implications of these metabolites will be discussed together with all the stages in the subsequent parts of my thesis.

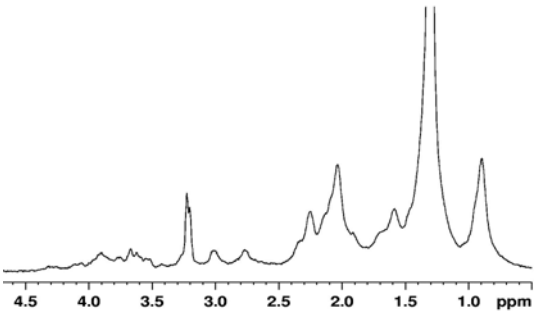
(ii) Low fat corn oil



(i) High fat corn oil



(iv) High fat flaxseed oil



(iii) High fat beef tallow

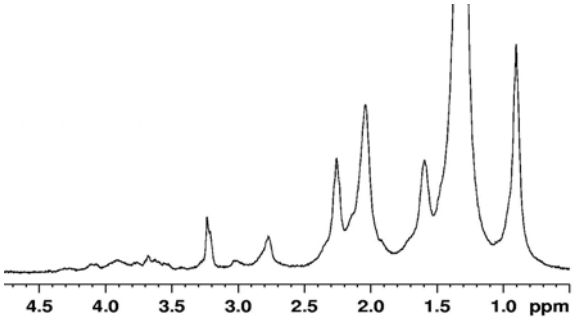


Figure 6: Representative ^{1}H MR spectra from carrageenan-treated dietary groups in Stage 3 of the study

Table 6: Stage 3 SCS analysis of control group (low fat corn oil) vs. each dietary group (n=119)

Class	**N**	**Accuracy**	**Regions (ppm)**	**Metabolites**
Low fat corn oil Vs. High fat corn oil				
Control	24	96.6%	**2.69-2.79**	=HC-C**H**$_2$-CH=
			2.05-2.11	-H$_2$C-C**H**=C**H**-
High fat corn oil	35		**1.35-1.4**	**(CH$_2$) Chain**
			1.1-1.22	Unassigned
Low fat corn oil Vs. High fat flaxseed oil				
Control	24	98.2%	**3.0-3.08**	**Creatine**
			2.69-2.83	=HC-C**H**$_2$-CH=
High fat flax oil	32		**1.15-1.22**	Unassigned
			0.87-0.94	**-CH$_3$**
Low fat corn oil Vs. High fat beef tallow				
Control	24	100%	**3.0-3.05**	**Creatine**
			2.76-2.9	=HC-C**H**$_2$-CH=
High fat beef tallow	28		**2.04-2.08**	-H$_2$C-C**H**=C**H**-

Table 7: Stage 3 SCS analysis of different dietary groups against each other to assess the differences between these diets in addition to differentiating them from the control group

Class	**N**	**Accuracy**	**Regions (ppm)**	**Metabolites**
High fat corn oil vs. Flaxseed oil				
High fat corn oil	35	97%	**2.90-3.10** **2.58-2.78** **1.95-1.98**	**Creatine** =HC-C**H**$_2$-CH= -H$_2$C-C**H=CH**- -C**H**$_3$
Flax seed oil	32		**0.96 – 0.98**	
High fat corn oil vs. Beef tallow				
High fat corn oil	35	98.4%	**2.99-3.04** **2.69-2.77** **1.94-2.00**	**Creatine** =HC-C**H**$_2$-CH= -H$_2$C-C**H=CH**-
Beef tallow	28			
Flaxseed oil vs. Beef tallow				
Flax seed oil	32	100%	**2.88-3.05** **2.57-2.68**	**Creatine** =HC-C**H**$_2$-CH=
Beef tallow	28		**1.59-1.61**	**H$_2$**C-CH$_2$-COO

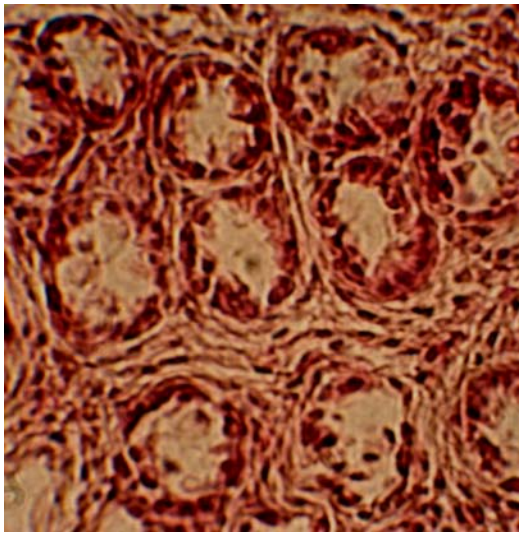
Table 8: Final combined SCS analysis result for all the groups

Desired class	N	Classification		Accuracy
		Correct	False	
Low fat corn oil	24	23	1	95.8%
High fat corn oil	35	31	4	88.6%
Flax seed oil	32	30	2	93.8%
Beef tallow	28	28	0	100%

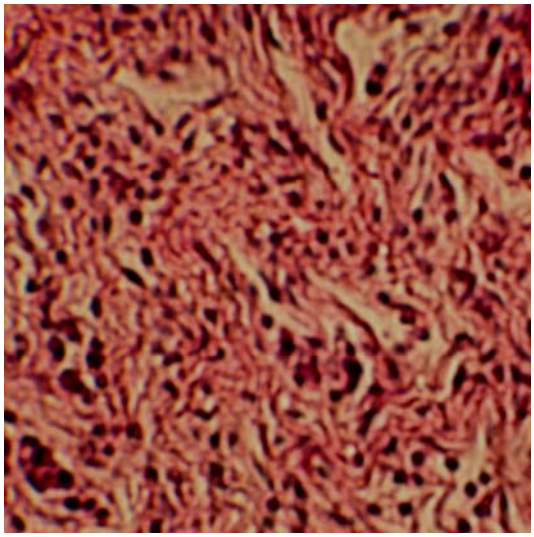
In the combined SCS analysis (Table 8), 1 out of 24 spectra in the low fat corn oil group was misclassified into the flaxseed oil group. Out of 4 misclassified spectra in the high fat corn oil group, 3 were classified into the low fat corn oil group whereas 1 was classified into the flaxseed oil group. Out of the 2 misclassified spectra in the flaxseed oil group, 1 was classified into the low fat corn oil group and the other was classified into high fat corn oil group. Histologically, both the low fat corn oil group and the flaxseed oil group had minimal inflammation and this may be the reason for some overlap among the spectra in these groups. Although, the spectra in the high fat corn oil group were characterized by moderate to severe inflammation, there was some variation in the degree of inflammation and this may be responsible for the misclassification of 3 spectra from this group.

In the histological assessment, out of 23 blocks analyzed from the flaxseed oil 21 blocks were either not inflamed or had very mild inflammation. Only 2 blocks were observed to have moderate to severe inflammation. Similarly, out of 21 blocks from the low fat corn oil group only 1 block had severe inflammation. On the other hand, all of the blocks from beef tallow (n=20) and high fat corn oil (n=23) group showed moderate to severe inflammation. The inflammation was graded based on the approximate area of the sections that was inflamed. It was classified as mild if less that 30% area showed inflammatory changes, moderate when 30-60% area was inflamed and severe when more than 60% area of the section was inflamed. The representative histological images from each group are shown in Figure 7.

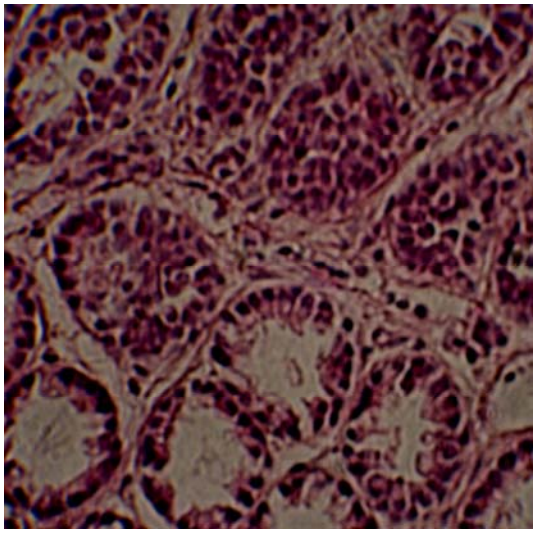
(i) Low fat corn oil



(ii) High fat corn oil



(iii) High fat flax seed oil



(iv) High fat beef tallow

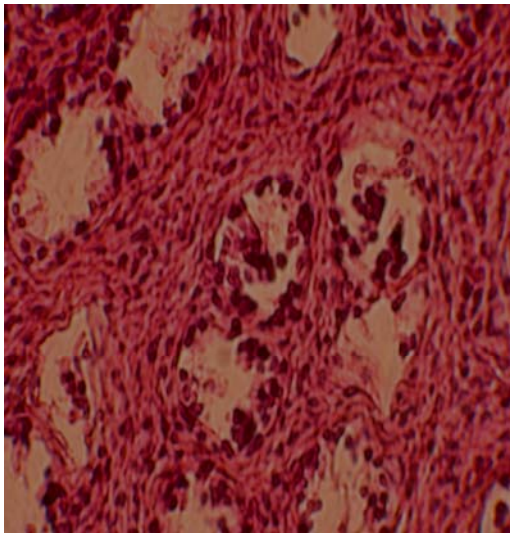


Figure 7: Histological images from carrageenan-treated dietary groups in Stage 3 corresponding to the ^{1}H MR spectra shown above

7.2.1 **COMPARISON BETWEEN THE GROUPS IN STAGE 2 Vs. STAGE 3**

The animals in different groups in Stage 2 of our study were fed the same diet as those in the corresponding groups in Stage 3. In addition, the animals in Stage 3 were also administered 2% carrageenan by weight in their diet to induce colonic inflammation. To test our hypothesis that ^{1}H MRS is suitable for studying the effects of various dietary fatty acids in colonic inflammation, the spectra from the groups in Stage 2 were compared with the spectra from the corresponding groups in Stage 3 using SCS analysis. As shown in Table 9, SCS analysis was highly accurate in classifying the spectra from each group in Stage 2 versus the same group in Stage 3. The region selection algorithm identified resonances due to total cholines, creatine, glycerol, methyl group protons and olefinic protons of fatty acid chain. We could not assign any specific metabolite to the region around 1.2 ppm.

Table 9: Results of comparative SCS analysis between Stage 2 vs. Stage 3

Class	**Accuracy**	**Regions (ppm)**	**Metabolites**
Low fat corn oil	100%	**4.18-4.23**	Glycerol
		3.64-3.67	Phosphatidyl choline
		0.89-0.93	-C**H**$_3$
High fat corn oil	97.0%	**2.85-2.93**	=HC-C**H**$_2$-CH=
		2.63-2.67	=HC-C**H**$_2$-CH=
		1.21-1.25	Unassigned
High fat flax seed oil	100%	**3.20-3.24**	t-Choline
		0.87-0.93	-C**H**$_3$
High fat beef tallow	100%	**3.22-3.24**	t-Choline
		2.95-3.04	Creatine
		0.85-0.92	-C**H**$_3$

8 DISCUSSION

^{1}H MRS combined with SCS analysis has attained very high accuracy in classifying the spectra to their respective groups in all the 3 stages of the study. Furthermore, it has depicted very high accuracy in the comparison of groups in Stage 2 against the same groups in Stage 3 of our study. This denotes that ^{1}H MRS can not only assess the effect of various PUFAs on normal colon, but also accurately differentiate between the effects of these PUFAs on inflamed colon.

There are 4 regions that are shown to be discriminatory by our region selection algorithm in Stage 1 SCS analysis. These included spectral resonances due to the =HC–C**H$_2$**–HC= group in fatty acyl chain of triglycerides (2.79-2.83 ppm), creatine (3.00 ppm), phosphatidylcholine (3.56-3.60 ppm) and glycerol backbone of lipids (4.03-4.05 ppm). Recently, Martin et al. (2007) have characterized the metabolic profile of rodent intestinal tissue including proximal and distal colon using high resolution ^{1}H MRS (Martin et al., 2007). They observed significant levels of phosphatidylcholine, creatine, glycerol and triglycerides in the normal colon. Phosphorylation of choline in the presence of the enzyme choline kinase is an important step in the formation of phosphatidylcholine. Phosphatidylcholine is the predominant phospholipid present in the lipid bilayer of cell membranes (Cullis and Hope, 1992) and in colonic mucosal lining (White, 1973). In addition, it is an important constituent of the inflammatory mediator platelet activating factor (PAF). PAF is a critical mediator of inflammation and necessary signal for steps in the inflammatory cascade such as platelet activation and increase in vascular permeability. The active species of PAF have been shown to increase significantly in

mucosal biopsies from ulcerative colitis patients (Thyssen et al., 1996). Levels of PAF in stool are even considered to be diagnostic and indicative of severity in patients with ulcerative colitis (Hocke et al., 1999). The increased levels of phosphatidylcholine seen in our study may be because of increased levels of PAF in colonic inflammation. As there is increased apoptosis and cell turnover in IBD, there is increased requirement of phosphatidylcholine to repair and regenerate the cells in colonic mucosa. This may have also contributed to the increased levels of phosphatidylcholine seen in our spectra.

Creatine is a metabolite that is found in normal tissues and serves as an energy reservoir. It is converted into phosphocreatine by adding a high energy phosphate bond in the presence of the enzyme creatine kinase. This is a reversible reaction and whenever energy is needed phosphocreatine is broken down to creatine while releasing the energy as ATP. Significant concentrations of creatine have previously been detected in human colonic mucosa (Ende et al., 1996). The BB isoenzyme of creatine kinase has been isolated from normal (Jockers-Wretou et al., 1977) and infracted colonic mucosa (Graeber et al., 1994). As colonic inflammation causes cellular damage and increased requirement for energy, the creatine concentrations may be elevated in these cells.

The region around 2.8 ppm denotes the peak from protons in polyunsaturated fatty acids (Adosraku et al., 1994). Polyunsaturated fatty acids (PUFA) are critical for maintaining the membrane structure and function of various cells in the body (Clandinin et al., 1991). Linoleic acid is an abundant PUFA in the cell membrane and a precursor of arachidonic acid. Arachidonic acid is the precursor for the synthesis of many inflammatory mediators such as the products of lipoxygenase pathway LT B4 and LT C4. These inflammatory markers are increased in many disorders in the human body including inflammatory

bowel disease (Simopoulos, 2002). Nishida et al. (1987) observed an increase in arachidonic acid concentration in the colonic mucosa of patients with ulcerative colitis (Nishida et al., 1987). Nieto et al. (1998) also observed an increase in linoleic acid concentration in the colonic mucosa of rats 2 weeks after inducing experimental colitis (Nieto et al., 1998). The linoleic acid incorporated into colonic cells may be used to synthesize arachidonic acid and inflammatory mediators. These changes in plasma and colonic mucosal lipid profile are similar in human ulcerative colitis as well as experimental colitis induced in animals. Increase in glycerol resonance in our samples may be reflective of an increase in the total lipids in the colonic mucosa during inflammation.

In Stage 2 experiments, we did not induce any inflammation but were looking at the effect of various PUFAs on normal rat colon. The regions identified as being discriminatory when comparing control (low fat corn oil) group with the specialized diet groups included resonances due to creatine, glycerol and $=H_2C$-$\mathbf{H_2}$C-CH= , -$\mathbf{H_2}$C-CH=CH-, -C$\mathbf{H_2}$-CH_2-COO and CH_2-C$\mathbf{H_2}$-COO groups of fatty acyl chain. This denotes that when rats are fed a diet rich in fats they are accumulated in the colonic mucosa. This was a very important finding to prove that the dietary treatment given to rats was causing a change in the targeted tissue i.e. colonic mucosa. Hawthorne et al conducted a randomised control trial of fish oil supplementation in ulcerative colitis patients. Using a gas chromatography technique, they observed that the levels of unsaturated fatty acids, EPA and DHA increased in rectal mucosa after ω-3 PUFA supplementation (Hawthorne et al., 1992). A similar effect on colonic fatty acid profile was seen by Bartoli et al. (2000) in Sprague-Dawley rats after ω-3 supplementation for 12 weeks (Bartoli et al.,

2000). Again, the increase in total lipid content of the colonic mucosa may have resulted in the peak due to glycerol as being discriminatory. When comparing the different dietary groups amongst each other, the resonances due to glutamic acid, choline, glycerophospho-ethanolamine, glycerol and =HC-C**H$_2$**-CH= and, -C**H$_2$**-CH$_2$-COO groups of fatty acyl chain were found to be discriminatory. We can not, however, determine which specific polyunsaturated fatty acid contributed the peak due to unsaturated carbons based solely on these spectra. We deduced that the predominant polyunsaturated fatty acid in a particular dietary group must have been responsible for the peak due to unsaturated carbons in the spectra from that group. Glutamine is the major fuel for intestinal cells. Significant levels of glutamine were observed both in human colon (James et al., 1998a) and rat colonic mucosa (James et al., 1998b). Glutamate acts as a excitatory neurotransmitter in the intestinal neuronal cells. Intense glutamate staining was observed in the neural plexuses of human colon. It is being postulated that glutamate regulates acetylcholine release and motility in colon (Giaroni et al., 2003). Glutamic acid is converted to γ-amino butyric acid (GABA) by glutamic acid decarboxylase enzyme. Recently, it was shown that glutamic acid decarboxylase and GABA are present in the lamina propria of colonic mucosa of rats and may be involved in maturation and differentiation of colonic epithelial cells (Wang et al., 2006).

In Stage 3 of our study when comparing the dietary groups with the control group, resonances due to creatine and -C**H**$_3$, (CH2)n, =HC-**H$_2$**C-CH= and, -**H$_2$**C-CH=CH- groups of fatty acyl chain were observed to be discriminatory. Additional resonance due -C**H$_2$**-CH$_2$-COO was discriminatory during the comparison of various dietary groups against each other.

The histological assessment of Stage 3 samples showed that both corn oil in low concentration and high fat flaxseed oil may be protective against inflammation. The high sensitivity attained in SCS analysis when comparing these 2 groups (98.2%) possibly is due to the difference in concentration of arachidonic acid and EPA accumulated in the colonic cells after supplementation with ω-6 and ω-3 PUFAs respectively as explained earlier. It also points to the fact that both the amount and type of fatty acids supplemented may be important in modulating colonic inflammation. High fat corn oil and high fat beef tallow groups showed significant inflammation in our study. High fat corn oil diet supplied a high concentration of ω-6 fatty acids, which are pro-inflammatory and may have caused severe inflammation in those samples. Similarly, high amounts of saturated fats supplied by high fat beef tallow diet may have caused severe inflammation. On the contrary, low fat corn oil diet, although, mainly provided ω-6 PUFAs, the concentration of these PUFAs wasn't high enough to manifest severe pro-inflammatory effects. In a randomized control trial on patients with IBD, Bamba et al. (2003) observed that low fat diet induced remission in 80% patients as compared to only 25% in the high fat diet group (Bamba et al., 2003). Interestingly, the fat they supplemented in the diet was soybean oil, which has significant amount of ω-3 PUFAs. The same phenomenon was observed by Reddy et al. in a study on colon cancer. They showed that rats fed with 20% corn oil had significantly higher incidence of colon cancer than rats fed with 5% corn oil for the same duration (Reddy et al, 1976). More recently, Rao et al. (2001) reported that rats fed with high fat fish oil (ω-3) and low fat corn oil (ω-6) exerted similar protective effect on colon tumour development as compared to high saturated fat diet (Rao et al.,

2001). In addition, the duration for which these PUFAs are administered is also important in regulating the outcome of the therapy. A differential effect of ω-3 fatty acids after different time periods on various stages of colon cancer was observed in a study by Good et al (Good et al., 1998). In this study, the beneficial effect of ω-3 fatty acid supplemented in the diet of male Fischer 344 rats was evident only after prolonged treatment. The effect of ω-3 and 6 fatty acids on IBD need to be studied in long-term studies. It also needs to be assessed whether the effect of these fatty acids on IBD is reversible and that relapse and remission rates are higher for patients who discontinue the use of these PUFAs.

9 CONCLUSION

In this study we have demonstrated that ^{1}H MRS combined with SCS analysis is a highly sensitive technique to distinguish between inflamed and non-inflamed colonic tissue. Furthermore, the technique is very sensitive in analyzing the effect of various PUFAs on normal as well as inflamed colon. This finding expands the scope of MRS and SCS analysis in further studies looking at different stages of IBD.

10 LIMITATIONS OF THE STUDY

Because of the broad scope of this study, there were some limitations in the experimental design and methodology. The number of animals used in Stage 2 and Stage 3 of the study was small, even though we examined 6 samples from each animal. For further studies, larger sample size should be used so that more data can be generated and our hypothesis can be strongly validated. We observed a lot of variation in the degree of inflammation even in the samples from the same animal. Using larger sample size will eliminate any confounding role of this variation.

The samples were stored at -70ºC before conducting ^{1}H MRS and subsequently histology. The samples had to be thawed to room temperature before and while conducting ^{1}H MRS. This process may have caused some changes in the biochemical characteristics of the sample. However, the time frame was similar for all the samples and we expected similar changes to have occurred in all the samples. We used a glass slide to strip-off the mucosal layer from the colon. The shear and tear from this process may have caused some distortion of the tissue that could mimic inflammatory change. Effort was made to minimize the tissue strain and the samples were handled very carefully. In addition, only one person carried out the process of separating the mucosal layer and so, we expect that all the samples were manoeuvred in the same way.

We could not assign specific metabolites to some of the regions that were found to be discriminatory in the ^{1}H MRS analysis. Doing advanced spectroscopic experiments such as two-dimensional correlation spectroscopy (COSY) may provide more information on

the specific metabolites that may represent these regions. However, the time required to run these experiments can range from 12 to 24 hours. Given the nature of our study, keeping samples at room temperature for extended periods of time would have caused some tissue damage and confounded the histology results. We conducted COSY on some samples to get more details about the metabolites present and used this information to assign metabolites to various discriminatory regions. These samples were discarded after ^{1}H MRS and were not used subsequently for histology.

11 FUTURE DIRECTIONS

Our future goal is to conduct long-term study using the same dietary groups to assess the effect of fatty acids on natural progression of IBD to colon cancer. Studies have shown that colon cancer can be induced in experimental models using azoxymethane, which is a colon-specific carcinogen. As a subsequent step, the effect of these diets will be studied on the progression of IBD to colon cancer using azoxymethane as a carcinogen. The benefits of ω-3 fatty acids at various stages of cancer including pre-induction, induction and propagation stages will be studied in future projects.

In our study, we compared the effect of different diets on IBD. The logical next step would be to compare the effect of ω-3 fatty acids with the current medical therapies like anti-inflammatory agents and corticosteroids in animal model. We used histological changes as definitive evidence of inflammation in our study to compare ^{1}H MRS results. In subsequent studies testing for molecular markers of inflammation such as interleukin levels can be used to provide corroborative biochemical evidence of inflammation and response to the therapy.

The ultimate step after testing the study hypothesis in animal model is to design a similar study in humans with dietary supplementation of ω-3 fatty acids and collection of biopsy samples to validate the potential of ^{1}H MRS in detecting the effects of diet on IBD. Eventually, randomized control trial can be conducted by supplementing the regular diet with flaxseed oil to assess the efficacy of diet modification.

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13 APPENDICES

APPENDIX 1: PROTOCOL FOR PARAFFIN EMBEDDING OF THE SAMPLES

1. **Preparing the cassettes:**

   1.1. Cut 2x1 inch pieces of thin filter paper to place the sample in. Cut about 1x1 inch pieces of sponge to hold the tissue in the cassette.

   1.2. Label the cassettes with pencil on 2 sides for sample identification.

   1.3. Take the sample out of formalin and flatten it on a board.

   1.4. Place the flat piece of tissue on the blotting paper and fold it in half. Do not let the edges perfectly overlap each other so that its easier to unfold the paper to take the tissue out after processing.

   1.5. Place the tissue in filter paper in between 2 pieces of sponge in the cassette and put the cassettes in a container with formalin for further processing.

2. **Processing of the tissue:** Before the tissue can be mounted on paraffin blocks, it needs to be fixed and dehydrated. This process is carried out in an automated Histotek tissue processor and it takes 38 hours to finish processing of the samples. The time periods for which the samples are placed in different solutions is shown in the table at the end of this appendix.

3. **Mounting:** It is done on a paraffin embedding station with separate hot working area and cold plate.

   3.1. Take the cassettes out of the automated processor and place them in the wax-bath in the embedding station

   3.2. Oil the stainless steel cassette holders and place them in the warm chamber of the embedding station

   3.3. Open the cassette and take the tissue out of the folded filter paper on the hot working area of the embedding station.

3.4. Fill steel cassette holder with molten wax up to half the height and place the tissue in it.

3.5. Place the cassette over top of the steel holder and fill remaining of the holder with molten wax.

3.6. Place this complete unit on the cold plate (-5°C) to solidify. Repeat the process for each sample.

3.7. After the wax is solidified, remove the steel holder and place the paraffin blocks in the fridge before sectioning.

Processing times of tissue in various solutions in the processor as mentioned in step 2

Station #	**Solution**	**Time period**
1	10% Buffered Formalin	8hr
2	70% ethanol	14hr
3	80% ethanol	6hr
4	95% ethanol	6hr
5	100% ethanol	30min
6	100% ethanol	30min
7	100% ethanol	1hr
8	100% ethanol	1hr
9	Xylene	1hr
10	Xylene	2hr
11	Paraffin Paraplast plus	1hr 30min
12	Paraffin Paraplast plus	1hr 30min

APPENDIX 2: PROTOCOL FOR H&E STAINING OF PARAFFIN EMBEDDED SECTIONS

1. **Introduction:** Haematoxylin and Eosin are used to stain nuclei blue and cytoplasm of cells red. When staining paraffin embedded sections, six steps are followed:

    a. Deparaffinization

    b. Hydration

    c. Staining

    d. Dehydration

    e. Clearing

    f. Cover-slipping or mounting

2. Arrange the labelled slides on a slide holder and place the slides in the appropriate solutions for the specified times given below (steps 2.1-2.5). Mount slides as described in step 2.6.

    2.1 **Deparaffinization:** To remove the paraffin wax from the tissue and the surrounding area on the slide, dip slides in the following solvents:

2.1.1	Xylene I	- 3 min.
2.1.2	Xylene II	- 3 min.

    2.2 **Hydration:** The purpose of this process is to prepare the tissue for staining with an aqueous dye such as haematoxylin. It is a gradual process that uses a series of decreasing alcohol concentrations and then water to rehydrate the sections as follows:

2.2.1	Absolute alcohol I	- 10 dips
2.2.2	Absolute alcohol II	- 10 dips
2.2.3	Absolute alcohol III	- 10 dips
2.2.4	95% alcohol	- 15 dips
2.2.5	70% alcohol	- 10 dips
2.2.6	Tap water	- until sections are clear

2.3 Staining:

2.3.1. Stain in Mayer's Haematoxylin for ~ 7-10 min. (Filter the solution before each use).The time in the stain depends on the strength of the solution and the thickness and processing of the section. **N.B.** First run entire staining procedure on a test slide. Examine the section under a microscope for blue staining of the nuclei and pink staining of the cytoplasm to confirm the time in Haematoxylin or Eosin are appropriate for that batch.

2.3.2 Wash in tap water.

2.3.3 Blue in Ammonia water (5-6 dips).

2.3.4 Wash well in tap water until sections are blue (5 minutes or less).

2.3.5 Wash in 95% alcohol.

2.3.6 Stain in Eosin for 3 min.

2.4. **Dehydration:** After staining sections are commonly dehydrated by successive changes of graded alcohols.

2.4.1 Differentiate in 95% alcohol, to remove the excess eosin.

2.4.2 Dehydrate in 3 changes of absolute alcohol (5-6 dips each).

2.5. **Clearing:** The dehydrating agent needs to be removed using clearing agents such as xylene.

2.5.1 Clear in 2 changes of xylene.

2.6. **Coverslipping:** Mount with coverslip to preserve the stained tissue section for subsequent handling and microscopic examination.

2.6.1 Drain the excess xylene from the slide and wipe the back of the slide with a lint-free cloth. Place amount of mounting medium required to cover the slide or cover slip using a Pasteur pipette. Once mounted, allow the medium to dry before viewing the slides under a microscope.