EFFECTS OF DIETARY FAT AND FIBER ON THE OXIDATIVE

STATUS OF THE SMALL INTESTINE AND COLON OF RATS

A Dissertation

by

LISA MERLE SANDERS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Nutrition

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Approved as to style and content by:

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May 2005

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ABSTRACT

Effects of Dietary Fat and Fiber on the Oxidative Status of the Small Intestine and Colon

of Rats. (May 2005)

Lisa Merle Sanders, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Joanne R. Lupton

Colon cancer is one of the most commonly diagnosed cancers in the US, yet small intestine cancer is a rare event. While there are many similarities between these two tissues, inherent differences such as redox status, may contribute to the variation in cancer occurrence. We examined the difference in reactive oxygen species (ROS) generation, antioxidant enzyme activity and oxidative DNA damage in the small and large intestine of rats under normal conditions and following exposure to exogenous oxidative stress. Basal ROS and antioxidant enzyme activities were greater in the colon than the small intestine, and the balance of ROS to antioxidant enzymes in the colon was more pro-oxidant than in the small intestine. During oxidative stress, ROS and oxidative DNA damage were greater in the colon than the small intestine. Thus the colon responds to oxidative stress less effectively than the small intestine, possibly contributing to increased cancer incidence at this site. We next wanted to understand how diets containing a combination of fish or corn oil and pectin or cellulose may alter the redox environment of the colon. ROS, oxidative DNA damage, antioxidant enzyme activity and apoptosis were measured in colonocytes of rats fed one of four diets containing either corn oil or fish oil and cellulose or pectin. Measurements were made

in rats untreated with carcinogen and rats exposed to a chemical carcinogen and radiation.   In rats not treated with a carcinogen, fish oil enhanced ROS, and fish oil/pectin suppressed antioxidant enzymes as compared to corn oil/cellulose. Oxidative DNA damage was inversely related to ROS in the fish oil/pectin diet and apoptosis was enhanced relative to other diets. In carcinogen treated and irradiated rats, a similar protective effect was seen with fish oil/pectin as evidenced by a reduction in oxidative DNA damage and enhancement of apoptosis. This suggests that a diet containing fish oil/pectin may protect against colon carcinogenesis by modulation of the redox environment to promote apoptosis and minimize oxidative DNA damage.

to The Potter

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Intestinal Physiology and Carcinogenesis

Cancer statistics. One of the greatest public health concerns in developed

countries, including the United States, is cancer. Of all cancers, colon cancer is the third

most commonly diagnosed and it is estimated that almost 57,000 Americans will die

from the disease this year. Furthermore, colon cancer diagnosis and mortality rates are

almost equal for men and women, unlike breast and prostate cancer, which are gender

specific (1). Worldwide, colon cancer incidence varies widely. Incident rates in the

United Sates and northern Europe are almost 10-fold more than southern Europe, Asia

and Africa. Furthermore, individuals migrating from low to high-incidence areas show

increased rates of colon cancer, indicating the strong influence of environmental factors

on colon cancer occurrence (2).

In the United States, for every 28 cases of colorectal cancer reported, there is

only one case of small intestine cancer (1). Nevertheless, epidemiological evidence

suggests a strong correlation between colon cancer and small intestine cancer incidence

(3). While investigations into the etiology of small intestine cancer are few due to the

rarity of the disease, it does appear that the same environmental factors which influence

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colon cancer incidence also impact small intestine cancer incidence and to a similar magnitude (4).

Physiology of the small and large intestine. The small and large intestine comprise approximately 8 m of the 9 m-long alimentary canal (5). The majority of this length (6 m) is the small intestine which begins at the pyloric sphincter of the stomach and coils within the abdominal cavity until it joins the large intestine at the ileocecal valve. The large intestine, so named as it is larger in diameter than the small intestine, is the remaining 1.5-2 m.

The small intestine is divided into three major sections: the duodenum, the jejunum and the ileum. The duodenum is the first section of the small intestine immediately following the stomach. The majority of absorption as well as the introduction of secretions from the pancreas and liver occur within this 25 cm region. The duodenum is followed by the jejunum and the ileum which show little structural change besides a thinning of the intestinal wall. Absorption continues throughout these portions so much that there is very little absorbable material that passes from the ileum into the large intestine.

The large intestine consists of the cecum, colon, rectum, and anal canal. Of these sections, the colon makes up the majority of the length and surface area. Compared to the small intestine, minimal digestion occurs in the colon and absorption is primarily limited to water and electrolytes. Additionally, the colon contains microflora which can digest and/or ferment materials, such as fiber, that are unable to be broken down by

intestinal enzymes. Products of this microbial degradation include vitamins, such as vitamin K, and short chain fatty acids, such as butyrate, which can then be absorbed for utilization by the body or the colon cells.

The walls of the intestine are made up of four distinct layers, each with their own unique function. The serosal layer is the outermost covering of the intestine which protects the intestine from the peritoneal environment and lubricates the surface so as to ease movement against neighboring organs. The muscular layer is responsible for the movement of the intestinal walls which aids in transit and digestion of the luminal contents. In the small intestine, circular fibers are used to contract the diameter of the intestine, while longitudinal fibers shorten the length. The colon contains only longitudinal fibers arranged in bands which give the colon its pouch-like appearance. The submucosa consists primarily of loose connective tissue, but also contains glands, lymphatic vessels and blood vessels responsible for nourishing the intestinal wall as well as removing absorbed materials. The innermost layer of the intestinal wall is the mucosal layer, which actually consists of sub-layers of smooth muscle, connective tissue (lamina propria) and epithelium. It is within this layer that most enzyme and mucous secretion as well as absorption occur (5).

The epithelium is organized into small invaginations referred to as crypts. The crypt formation allows for increased surface area to enhance absorption. As the small intestine is the primary site for absorption of nutrients contained in food, the epithelium of the small intestine also contains villi. These finger-like projections extend from the crypt into the lumen, further increasing surface area. Lining the crypts and villi is a

single layer of epithelial cells, responsible for most of the secretory, absorptive and barrier functions of the intestine. These epithelial cells are self-renewing and maintain a highly-controlled balance of cell proliferation and cell death (6). It is the perturbation of this balance that can lead to malignant transformation and tumorigenesis.

Carcinogenic transformation of intestinal epithelial cells. Carcinogenesis in humans is a multi-step process, evolving over many years, that results in the culmination of multiple genetic mutations which transform normal intestinal epithelial cells to a malignant phenotype. This malignant phenotype is characterized initially by the cell's ability to proliferate indefinitely, generate its own growth signals, resist anti-growth signals and evade apoptosis (programmed cell death). At later stages, tumor cells acquire the ability to induce and sustain vascularization and eventually succeed in tissue invasion and metastasis (7). The genetic alterations leading to this carcinogenic transformation involve the mutational activation of oncogenes as well as the loss of function of tumor suppressor genes (8). Mutations in several of these genes are required to fully induce the malignant phenotype and in colon cancer these mutations generally accumulate sequentially through three distinct stages: initiation, promotion and progression.

During initiation, DNA can suffer several forms of damage ranging from base modification by oxidation or alkylation to single or double strand breaks. Modification of DNA bases can lead to mismatches during gene expression and DNA replication, which if left unrepaired, can lead to mutations (9). The consequences of single and double strand breaks may even be more severe as the repair process is extremely error-

prone (10). The DNA mutations sustained during the initiation stage are often the genetic alterations responsible for the hyperproliferation and resistance to apoptosis seen in the promotion stage of colon carcinogenesis. At this stage, microscopic changes in crypt morphology become apparent in the formation of aberrant crypts. Often these aberrant crypts are seen clustered together in the colon, and these areas have been considered by some to be precursor lesions to colon tumors (11,12). Evidence suggests that only a fraction of aberrant crypts will continue on to form tumors (12) and this may be due to the continued accumulation of genetic mutations which offer a growth advantage toward the tumor progression stage. During progression, tumor formation is apparent as is evidence of malignancy, such as increased angiogenesis. Once the tumor begins invasion of the submucosa of the intestine, it is termed a carcinoma (8). At this point, multiple genetic mutations have occurred, giving rise to the characteristics indicative of cancer: limitless cellular replication, resistance to apoptosis, self-sustained growth and ability to ignore anti-growth signals, enhanced vascularization and tissue invasion (7).

In colon cancer, there are a number of genes which are frequently found to be mutated. One of the most common mutations, generally found early in the carcinogenic sequence, occurs in the adenomatous polyposis coli (APC) tumor suppressor gene. This gene functions as a regulator of cell proliferation, but may also play roles in apoptosis and cell migration (12). Mutation of this gene product results in epithelial cell hyperproliferation and the formation of adenomas which have the potential to progress into tumors. Other mutations frequently found later in the carcinogenic process involve

the genes for K-ras, cyclin D1, and p53 (8,13). Several of these same gene mutations are also observed in cases of small intestine cancer (14).

Differences in cancer susceptibility between the small and large intestine.

Despite anatomical similarities between the small and large intestine as well as the similar genetic alterations that occur during carcinogenesis, the small intestine remains highly resistant to tumorigenesis. Over the past several decades, a number of environmental and intrinsic differences between the small and large intestine have been proposed and evaluated in an attempt to explain the difference in cancer susceptibility between these two tissues. The fluid nature of the luminal contents in the small intestine may infer protection as oral carcinogens would be more dilute. Furthermore, the rapid transit through the small intestine may also minimize exposure time (3). The environment of the small intestine is also relatively sterile compared to the large intestine. It is well-documented that bacteria are required for the activation of methylazoxymethanol (MAM) an experimental, colon-specific carcinogen and the colonic microflora have also been implicated in the formation of tumor promoting secondary bile acids (3). However, the relevance of environmental differences between the small and large intestine was seriously questioned following a study by Gennaro et al. (15) in 1973. In this study, segments of the small intestine were transposed into the colon and segments of the colon were transposed into the small intestine in rats. Following treatment with a chemical carcinogen, tumors were found throughout the colon but not within the transposed small intestine segment. Furthermore, very few tumors were found in the small intestine with the exception of the transposed segment of

colon which contained a number of adenocarcinomas. This investigation suggested that something intrinsic to the small intestine tissue was responsible for its apparent tumor resistance and prompted a more thorough examination of the inherent differences between the small and large intestine tissue.

Cell turnover, or the rate of cell proliferation and cell death, is much more rapid in the small intestine and proved a reasonable argument for tumor resistance. However, in the colon, it is widely accepted that increased rates of cell proliferation are a risk factor for carcinogenesis (16,17). The small intestine also contains benzpyrene hydrolase, a detoxifying enzyme absent in the colon that is responsible for degradation of the potent carcinogen, benzpyrene. While other detoxification enzymes may exist, none have been well characterized (14). One highly plausible intrinsic difference, but in need of further investigation, is the presence of a strong, localized immune system in the small intestine. High levels of immunoglobulin A (IgA), an important defense against viruses, are found in the small intestine and patients with a deficiency in this immunoglobulin display an increased risk of cancer (14). Another major finding by Potten and colleagues (17,18), is the evidence that apoptosis, in response to a carcinogen or cytotoxic insult in the small intestine of mice, is targeted to the stem cell population. Stem cells are a group of progenitor cells located near the base of the crypt which are responsible for maintaining the population of epithelial cells. If stem cells become damaged or suffer mutations and continue to proliferate, the potential exists to generate a large number of epithelial cells with the same mutations. In the colon, apoptosis was not found to be targeted to the stem cell population (17,19). Thus the small intestine may be

more protected against cancer by targeted elimination of damaged and potentially mutated stem cells.

   Chemical and radiation carcinogenesis. To study colon carcinogenesis in animal models requires the use of chemical carcinogens as most animals (with the exception of those with engineered genetic modifications) do not spontaneously develop colon cancer within their normal life cycle. Mice and rats remain the species of choice in dietary studies on colon cancer prevention and the alkylating chemical carcinogens, 1,2-dimethylhydrazine (DMH), methylazoxymethanol acetate (MAM) and azoxymethane (AOM) are the most frequently used as they specifically target the colon. These carcinogens are metabolically related, with DMH being converted to AOM in the liver followed by conversion to MAM. The continued metabolism of MAM leads to the ultimate formation of a methylcarbonium ion, the biologically active carcinogen responsible for the alkylation of DNA (20).

   Enhancement of colonic epithelial cell proliferation is characteristic of administration of this family of chemical carcinogens and a hallmark of carcinogenesis. Hyperproliferation leads to aberrations in crypt formation that are often considered pre-neoplastic lesions and are similar to changes in the crypt architecture of human colon tissue adjacent to colon tumors (11). Of critical importance in the use of this animal model is the response to dietary changes. This chemically-induced carcinogenesis model is responsive to dietary changes, especially changes in fat, which corresponds to epidemiological studies showing an increase in tumor formation with increased dietary fat intake (21).

In addition to chemical carcinogens, ionizing radiation has been shown, in as early as 1902, to induce cancer. Since that time, ionizing radiation has gained the reputation of a "universal carcinogen," in that it can cause cancer in most tissues, in most species of animal, at any stage of the life cycle (10). As compared to chemical agents, radiation is generally considered a weak carcinogen, but this is dependent on the dose received and duration of exposure. Additionally, radiation is able to avoid most of the cellular barriers presented to chemical carcinogens and can directly penetrate cells to damage DNA (10). The type of DNA damage caused by radiation can be quite diverse and differ from that caused by chemical agents. It is generally accepted that the primary DNA lesion responsible for the mutagenic results of ionizing radiation is the double strand break (DSB). Repair of this form of DNA damage is highly error-prone and thus responsible for large-scale chromosomal abnormalities and multiple genetic mutations (22). The DSB is a direct result of the interaction of radiation with DNA. However, radiation can also indirectly cause DNA damage by the generation of oxidative stress within a cell, leading to oxidation of DNA bases. This type of DNA damage is not as immediate as a DSB, but it still may play an important role in carcinogenesis as the oxidative stress resulting from radiation has been shown to continue into later generations of daughter cells (23,24).

Environmental Factors in Colon Cancer

Epidemiological investigations. Although the role of genetic factors in colon cancer is well established, epidemiological studies of diverse and migrant populations suggest that environmental factors, especially dietary patterns, play an equally critical

role (2). Studies among the Japanese in the 1960s prompted Wynder et al. (25) to propose a strong association between fat intake and colon cancer. Just a few years later, Dr. Denis Burkitt, a medical missionary in Africa, reported fewer deaths from colon cancer in native Africans consuming a high-fiber, low-fat diet (26). Since these important observations, dietary fat and fiber have emerged as two of the most important and widely investigated dietary factors related to colon carcinogenesis. While early epidemiological studies focused primarily on the amount of fat and fiber in the diet, more recent investigations have determined that not only the amount, but the type of fat and fiber in the diet may affect colon cancer risk.

Dietary fat and colon cancer. Dietary fat has been considered by many experts to be the most important nutritional modulator of colon cancer risk (27,28). This is not surprising considering that the majority of epidemiological and case-control studies over the past few decades have shown a positive association between high-fat diets and risk of colon cancer (29-34). In addition to the amount of fat in the diet, the type of fat also has been shown to affect colon cancer risk, with the greatest risk found among individuals with elevated intakes of saturated or animal fat (31,32,34,35). Interestingly, the only beneficial effect of a high-fat diet was seen when the diet was rich in polyunsaturated fatty acids (PUFAs), particularly those found in fish oil (35,36). Similarly, Blot et al. (37) observed that diets rich in marine animals and fish, common among Alaska natives, was associated with a decreased risk of colon cancer as compared to other North Americans.

Animal studies have further supported the link between the type of dietary fat and colon cancer risk as well as suggesting possible mechanisms of action (38-44). Reddy et al. (42) found that rats fed diets containing high levels of fish oil had fewer experimentally induced colon tumors than rats fed diets high in corn or safflower oil. Another investigation found that a high-fat fish oil diet was more protective against tumor formation than a low-fat corn oil diet (43). Fish oil and corn oil differ in their fatty acid composition, with fish oil being rich in the omega-3 fatty acids eicosapetaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and corn oil being rich in omega-6 fatty acids such as linoleic acid (LA, 18:2n-6). It is suggested that this unique composition of fatty acids determines the ability of these dietary lipids to differentially influence colon tumorigenesis.

The wealth of research on the role of fatty acids in colon cancer focuses on their ability to modulate phospholipid turnover and composition and prostaglandin synthesis (45,46). The omega-3 fatty acids EPA and DHA, found in fish oil, have been shown to compete with LA (and its derivative arachidonic acid, AA) for incorporation into phospholipids. Phospholipids play a critical role in signaling pathways for cell proliferation and differentiation, thus alteration of their fatty acid content may influence their signaling ability (47,48). EPA also competes with AA for conversion to prostaglandins by cyclooxygenase enzymes. Cyclooxygenase-2 (COX-2), an inducible form, is up-regulated in colon cancer but is suppressed by fish oil diets (46). This is a particularly promising and potent mechanism of fish oil as pharmacological COX-2 inhibitors have shown considerable effectiveness in colon cancer prevention(46).

Omega-3 fatty acids found in fish oil may also protect against colon cancer by mechanisms other than their ability to compete with omega-6 fatty acids. Chang et al. (49) showed that the ability of fish oil to protect against colon tumorigenesis was due to an increase in apoptosis. However, the mechanism by which this diet induces apoptosis is not yet clear. A recent investigation suggests that the highly unsaturated fatty acids in fish oil are readily incorporated into the mitochondrial membrane making it more susceptible to reactive oxygen species (ROS) generation and propagation which may initiate apoptosis (50).

Dietary fiber and colon cancer. Along with dietary fat, dietary fiber has received considerable attention for its potential to protect against colon cancer. Population based studies have found strong associations between fiber intake and reduced risk of colon cancer (26,51,52). Prospective studies have not been as conclusive (34,53,54), although one of the most recent and well-designed investigations, the European Prospective Investigation into Cancer and Nutrition (EPIC) study, found that doubling fiber intake from foods may decrease the risk of colon cancer by 40% (55). While many case-control studies have found a protective effect of fiber, specifically the fiber found in fruits and vegetables, several have also found no association between intake of fiber and colon cancer (27). The inconsistency of these studies has created a fiber controversy among experts and left the public confused. Some of the discrepancies may be attributed to the type of fiber investigated, the stage at which intervention began and the interaction of fiber with other dietary components.

Dietary fiber has only recently been defined as "non-digestible carbohydrates and lignan that are intrinsic and intact in plants (56)." Within this broad definition exists many different fibers which can be classified primarily by their fermentability. Fermentation is accomplished by the microflora of the colon, with the major products being H_2, methane, CO_2, and short chain fatty acids. The fermentability of a fiber is likely the determining characteristic in its ability to protect against colon cancer (57), but this remains the subject of much debate since highly fermentable and poorly fermentable fibers both show chemoprotective effects. Poorly fermentable fibers, such as cellulose and wheat bran, have been considered protective due to their ability to dilute possible carcinogens and accelerate transit time. However, the fermentation products of fiber, specifically the short chain fatty acid, butyrate, have also been shown to possess chemopreventive qualities (57,58). Butyrate's primary protective effects include inhibition of cell proliferation and enhancement of cell differentiation and apoptosis in colon cancer cell lines (59,60). Yet controversy remains regarding the efficacy of butyrate as a chemopreventive agent due to the lack of supporting data in vivo. The few studies which have examined the effects of butyrate in the colon of experimental animals have not found a protective effect of butyrate on aberrant crypt formation or colon tumor development (61-63).

Interaction of dietary fat and fiber. While a number of potential explanations exist for the differential effects of dietary fiber and butyrate on colon cancer development, of particular interest is the interaction of fiber with other dietary components, such as fat. A study by Chang et al. (49) demonstrated that the fermentable

fiber pectin is protective against colon tumorigenesis when combined with fish oil in the diet. Thus, it appears that the fatty acid composition of the diet may alter the action of the fiber. Recent studies continue to support this finding and have suggested the mechanism of action of this dietary combination may be enhancement of apoptosis (50,64).

Oxidative Stress and Carcinogenesis

Reactive oxygen species in the carcinogenic process. Reactive oxygen species (ROS) have been shown to play somewhat paradoxical roles in the carcinogenic process, serving as both promoters and protectors against tumorigenesis. ROS are cellular oxidants which include free radicals such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$) and peroxyl ($RO_2^{\cdot}$), as well as non-radical species such as hydrogen peroxide (H_2O_2). These oxidizing species are a common by-product of several cellular processes including aerobic metabolism, fatty acid oxidation, cytochrome P450 activity, and the respiratory burst of immune cells (9). There also are a number of exogenous sources of ROS, especially in the colon, including oxidized food particles, toxins and transition metals (65). Radiation exposure can also lead to the formation of cellular ROS (24,66).

ROS have been implicated in the carcinogenic process by their ability to oxidize and damage DNA and other macromolecules, including proteins and lipids (9,67). Damage to DNA can lead to mutations in the genome, while oxidation of proteins and lipids may cause functional alterations or modifications in cell signaling that trigger undesirable responses such as enhanced proliferation (9). Lipid oxidation results in the formation of lipid peroxides which can further oxidize other macromolecules including

other fatty acids within membranes (68). Polyunsaturated fatty acids are frequent targets

of ROS and their susceptibility to oxidation increases with their degree of unsaturation

(69). Thus long chain PUFAs, such as EPA and DHA, with five to six double bonds are

at greater risk of oxidation than PUFAs with fewer double bonds, such as linoleic acid.

Lipid peroxidation is of particular concern within cellular membranes when peroxide

propagation is not terminated by antioxidants, which can result in large-scale damage to

the membrane.

Despite the apparent harm that may be caused by ROS, these oxidants also play a

critical role in a number of important biological processes. As mentioned previously, the

respiratory burst of immune cells, particularly phagocytes, generates a large amount of

ROS. The ROS generated enable the immune cell to destroy engulfed bacteria as well as

potential cancer cells (70). In addition, cytochrome P450 activity is necessary for the

detoxification of toxic substances and drugs for removal from the body. Much of this

detoxification process requires the generation of ROS. Finally, accumulating evidence is

revealing ROS to be critical initiators and mediators of apoptosis during carcinogenesis

(69-74). Even lipid peroxides have been shown to promote apoptosis (75). Thus ROS,

while generally perceived as harmful and potentially carcinogenic by their ability to

damage macromolecules, may actually be beneficial in their ability to trigger apoptosis

in potential cancer cells.

As ROS are short-lived molecules and can only be measured in living cells,

currently the most suitable method of detection is fluorescence microscopy. Fluorescent

dyes can be taken up by live cells and are able to emit fluorescence upon oxidation

which can then be monitored using digital microscopy. There are a number of vital dyes available, some specific for the detection of a particular species, however dichlorofluorescein diacetate (DCFDA) and its derivatives are frequently used because of their ability to detect a variety of ROS. The chloromethyl derivative of DCFDA (CM-H_2DCFDA) shows exceptional retention in live cells due to the reaction of thiols with the chloromethyl group which "traps" the dichlorofluorescein in the cell. Oxidation by ROS yields a fluorescent dichlorofluorescein which is detectible and measurable using digital fluorescence microscopy.

Role of oxidative DNA damage in carcinogenesis. It is proposed that the primary mutagenic potential of ROS is their ability to damage DNA. The most common form of ROS-induced DNA damage is oxidation of nucleotide bases, which can lead to a number of alterations in DNA from gross chromosomal rearrangements to specific point mutations (9). These mutations can result in activation of an oncogene or inactivation of a tumor suppressor gene, both of which are characteristic of cancer development.

Although a number of oxidative DNA adducts have been identified, the one most frequently linked to genetic mutations is 8-hydroxy-2'-deoxyguanosine (8OHdG). This DNA lesion is generated by the attack of a hydroxyl radical on a guanosine nucleotide which alters its hydrogen binding specificity such that the modified base preferentially binds with adenine (A) instead of cytosine (C) during DNA replication. The cytosine will then bind with thymidine (T) in the next replication cycle, achieving a complete guanosine (G) to thymidine transversion (9). The oxidation of guanosine by ROS and

the resulting transversion has been suggested to be the point of mutation for several genes related to colon cancer development, including K-ras and p53 (9).

Due to the classification of 8OHdG as a major pre-mutagenic lesion, it is frequently utilized as a biomarker of oxidative DNA damage (9,76,77). However, accurate measurement of 8OHdG has posed many difficulties due to the creation of artifacts during the DNA isolation process. High pressure liquid chromatography has been the most widely used technique for detection of 8OHdG adducts. However, there is often large variability among similar experiments and it is suggested that this variability is due to the generation of artifactual adducts (76). Thus, newer technologies were developed in an attempt to more accurately determine cellular levels of 8OHdG. Two of these technologies examine 8OHdG levels in intact cells or nuclei, thereby eliminating the need to isolate DNA and minimizing the generation of artifactual adducts.

The single cell gel electrophoresis or comet assay has been frequently used to determine levels of DNA damage (primarily single and double strand breaks) within an individual cell (76,78,79). The procedure involves isolation of intact nuclei from single cells followed by alkaline unwinding of the DNA. Subsequent exposure of the nuclei to electrophoresis will result in the movement of fragmented DNA, indicative of damage, to form a comet-like structure that is visible under the microscope when the DNA is stained. Collins et al. (80) proposed an additional enzyme digestion step within the comet assay. The enzyme utilized was *E. coli* formamido pyrimidine glycosylase (fpg), which induces single strand breaks in DNA at the location of 8OHdG adducts. Addition

of this enzyme to the comet assay increased the sensitivity of the assay for oxidative DNA damage as well as the specificity for the potentially mutagenic adduct, 8OHdG. This procedure, now termed the FLARE (fragment length analysis using repair enzymes) assay, has be used by several others (78,81-84), although debate has continued as to the appropriate method of quantitiation by image analysis. While various quantitative and semi-quantitative methods, such as visual scoring, % DNA in the tail, and migration distance have been used, the method regarded as the best index of damage is the relative tail moment (85). This measurement has gained respect due to its consideration of the total amount of DNA present within the cell which can differ based upon the stage of the cell cycle. The relative tail moment evaluates the amount of DNA in the tail relative to the amount of DNA remaining in the nuclei (or head of the comet), unlike other methods which only examine the DNA contained in the tail.

An alternative method for determining 8OHdG within intact cells is by immunohistochemistry using an antibody specific for the 8OHdG adduct. Toyokuni et al. (86,87) have developed a monoclonal antibody with very low cross-reactivity to other oxidized bases, a concern with previously developed 8OHdG antibodies. Similar to the FLARE assay, this method also eliminates the need for DNA isolation, thereby preventing the possible creation of artifactual adducts. However, an additional advantage of this procedure is the ability to visualize cellular 8OHdG adduct staining while maintaining the crypt architecture of the colon.

Role of antioxidant defenses in carcinogenesis. To protect against the constant generation of ROS, cells posses a number of antioxidant defense mechanisms. These

include non-enzymatic scavengers such as glutathione, uric acid, ascorbic acid and α-tocopherol, as well as the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Additionally, proteins such as transferrin, metallothionein and ceruloplasmin may act indirectly as antioxidants by sequestering transition metals responsible for the generation of hydroxyl radicals (70,88). These antioxidant defenses do not completely remove all ROS from the cell, indicating that ROS may play an important role in cellular functions and that antioxidants exist primarily to maintain a steady state of ROS (70). In the event that ROS greatly exceed the antioxidant capabilities of the cell, either by increased generation of ROS or decreased capability of antioxidant defenses, a condition termed oxidative stress results (89). Oxidative stress can cause a variety of cellular responses including changes in gene expression, stimulation of proliferation, growth-arrest and apoptosis. Another possible consequence of oxidative stress is an increase in DNA damage.

As oxidative damage to DNA can lead to mutagenesis, antioxidants can play a critical role in the prevention of mutations that may lead to cancer. In support of this claim are epidemiological findings that increased intake of fruits and vegetables (rich in antioxidants) is associated with a decreased risk of certain cancers (9,90). Additionally, high intake of certain antioxidant vitamins, such as vitamin E, has been shown to reduce the risk of colon cancer (91). However, the chemoprotective effect of fruits and vegetables has not been conclusively linked to their antioxidant content or their ability to prevent DNA damage and mutation. In fact, it has been suggested that the anti-cancer effects of fruits and vegetables may also be attributed to other protective substances such

as fibers and phytates or the finding that most diets high in fruits and vegetables are also low in fat which has been closely linked to cancer incidence (9).

While antioxidants are generally perceived as beneficial by their ability to quench ROS, they have not always proven to be protective in chronic disease states, especially cancer. Two of the most well known trials to find an adverse effect of antioxidant supplementation are the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) trial and the Carotene and Retinol Efficacy Trial (CARET). Both of these studies found an increased incidence of lung cancer and mortality in smokers supplemented with the antioxidant, β-carotene, compared to their non-supplemented, smoking counterparts (92,93). In fact, the CARET trial was terminated 21 months prematurely due to these findings. Other clinical investigations into antioxidant enzyme defenses have shown that elevation of SOD activity in human colon carcinomas is associated with a poor 5 y survival rate (94). Furthermore, studies in cell lines and animals have shown overexpression of antioxidant enzymes promotes tumor survival (95), while depletion of antioxidants prevents tumor growth and enhances tumor cell death (96). In agreement with these findings is a recent study that found high doses of β-carotene to enhance aberrant crypt formation in an AOM model of colon carcinogenesis in rats (97). The common hypothesis that many of these studies propose as to the harmful effects of antioxidants is their ability of quench ROS that may be necessary for stress-mediated pathways such as apoptosis.

The role of ROS in apoptosis. There have been a number of ROS-mediated pathways identified which influence cell growth and survival (98), but one of the most

intriguing and widely investigated is apoptosis. Apoptosis is a programmed mechanism of cell death which selectively eliminates damaged cells that have the potential to form cancer cells. The biochemical pathways characteristic of apoptosis have been well characterized and are highly conserved among different species (74). These pathways include disruption of the mitochondrial membrane, activation of caspases and changes in gene expression, each of which are influenced by ROS.

Alterations in the mitochondria occur early in the apoptotic sequence, with the most obvious change being loss of mitochondrial membrane potential (Ψ_m). While the loss of membrane potential alone can induce oxidative stress, ROS can also oxidize the mitochondrial membrane lipids, damaging the membrane and leading to altered Ψ_m (74). The greater content of long chain PUFAs in the membrane, the more susceptible the mitochondria is to damage by ROS (50). Severe oxidative stress can lead to opening of the permeability transition pore, which in addition to disturbing the membrane potential, also releases large molecules into the cytosol. Several of these molecules, such as cytochrome c and apoptosis-inducing factor, continue the downstream events of apoptosis (99).

Cytochrome c has been shown to activate cytosolic caspases, enzymes that are responsible for continuing the process of apoptosis by degrading proteins critical to cell survival. Caspases are cysteine proteases and thus are sensitive to the oxidative status of the cell. In fact, investigations show that a redox shift in a cell towards a more oxidized environment is a necessary occurrence prior to caspase activation (99,100).

Furthermore, in cell culture experiments, the addition of antioxidants blocked apoptosis in a similar fashion to caspase inhibitors (101,102).

Several investigations have suggested that ROS *per se* are not the actual initiators of apoptosis, but rather an oxidative shift in the cellular environment which generates an apoptotic signal (75,103-105). Modulations in the cellular redox environment can be determined by changes in the ratio of glutathione (GSH) to its oxidized counterpart, glutathione disulfide (GSSG). The GSH redox cycle is highly sensitive to changes in the oxidative environment due to the rapid formation of GSSG from 2 molecules of GSH in the presence of ROS, in combination with the rapid enzymatic recycling back to GSH. In fact, Wang et al. (75) was able to detect significant changes in GSH/GSSG within 15 min of an oxidative challenge which correlated with later activation of caspases. Another investigation determined that oxidation of GSH precedes DNA fragmentation, a hallmark of apoptosis (105). Therefore, using the GSH redox cycle to measure changes in the oxidative environment may reveal greater insight into the mechanisms of ROS-induced apoptosis than the measurement of absolute levels of ROS.

Summary and Purpose

For several decades the variation in cancer susceptibility between the small intestine and colon has remained unclear. Environmental and intrinsic differences between these two tissues have been investigated, but still provide only an incomplete explanation. One inherent difference which may play a critical role in the tumorigenic process that has not been well investigated is the oxidative environment of these two tissues. The cellular redox environment is determined by the balance of ROS generation

and antioxidant defenses. Differences in the steady state redox balance may alter the levels of oxidative DNA damage as well as the ability of the tissue to respond to additional oxidative stress. These differences may contribute to the variation in cancer susceptibility at these two intestinal sites.

Colon cancer is also one of the most preventable cancers by dietary intervention and fat and fiber are two of the most widely investigated dietary components in colon cancer prevention. There is substantial evidence that diets rich in n-3 PUFAs, such as those found in fish oil (EPA and DHA) protect against colon carcinogenesis. However, the chemopreventive abilities of fiber, fermentable and non-fermentable, have been the subject of much debate and have shown varied results in intervention trials and epidemiological studies. It has been proposed that the inconsistent effect seen with fiber may be somewhat attributable to the influence of fat in the diet. Specifically, our laboratory has shown that the fermentable fiber, pectin, in combination with fish oil has a synergistic, protective effect on multiple stages of colon cancer primarily through the enhancement of apoptosis, a form of programmed cell death (49,64). Evidence suggests these dietary constituents may enhance apoptosis through alterations of the cellular redox environment. Dietary fat and to a lesser extent dietary fiber, have been shown to alter cellular ROS and lipid peroxidation, while other studies have shown its impact on antioxidant enzyme expression and activity (106-109).

This study will evaluate the differences in ROS generation, response to exogenous ROS, antioxidant defenses and oxidative DNA adducts in the small intestine and colon in an effort to explain, in part, the dramatic difference in cancer occurrence at

these two similar organ sites. Additionally, this investigation will evaluate the ability of dietary lipid and fiber to alter the oxidative status of colonocytes in a manner to infer protection against colon carcinogenesis.

Hypotheses

1) The colon will have a more prooxidant environment as compared to the small intestine and therefore respond unfavorably to additional oxidative stress.

2) The colonic redox environment will be altered by dietary fat and fiber such that fish oil and pectin will be protective prior to and during the initiation stage of radiation enhanced, chemically-induced colon cancer by minimizing oxidative DNA damage and promoting ROS-induced apoptosis.

Specific Aims

1) Determine the steady-state levels of ROS, antioxidant enzyme activity, and oxidative DNA damage in the small intestine and colon of rats.

2) Determine changes in ROS and oxidative DNA damage in the small intestine and colon of rats when exposed to an exogenous source of ROS.

3) Determine the effect of fat and fiber on ROS generation, oxidative DNA damage, antioxidant enzyme activity and apoptosis in the colon prior to exposure to carcinogen or ionizing radiation.

4) Determine the effect of fat and fiber on GSH/GSSG, oxidative DNA damage and apoptosis in colonocytes of rats exposed to AOM and ionizing radiation.

CHAPTER II

PRO-OXIDANT ENVIRONMENT OF THE COLON COMPARED TO THE SMALL INTESTINE MAY CONTRIBUTE TO GREATER CANCER SUSCEPTIBILITY[*]

It is predicted that the cases of colorectal cancer this year will outnumber small intestine cancer cases 28 to 1 (110), despite the fact that the small intestine has over 3 times the surface area of the colon. While the variation in cancer occurrence between these two intestinal sites is not completely understood, environmental differences such as the presence or absence of bacterial microflora, and intrinsic differences such as the rate of cell turnover have been examined (3,17). Yet, these differences provide an incomplete explanation for the variation in cancer susceptibility. For example, cell turnover involves the proliferation of cells as well as cell death. The more rapid rate of cell death in the small intestine as compared to the colon should infer protection against cancer development as potentially tumorigenic cells could be quickly eliminated. However, the small intestine also has a greater rate of cell proliferation which may promote tumorigenesis as transformed cells must propagate for tumors to develop (16).

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[*] Reprinted from Cancer Letters, v. 208(2), Sanders, L. M., Henderson, C. E., Hong, M. Y., Barhoumi, R., Burghardt, R. C., Carroll, R. J., Turner, N. D., Chapkin, R. S., & Lupton, J. R. Pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility. pp. 155-161, 2004, with permission from Elsevier.

One inherent difference which may play a critical role in the tumorigenic process that has not been well investigated is the oxidative environment of these two tissues.

Damage to DNA from ROS is a consequence of oxidative stress, and several oxidative DNA adducts, including 8-hydroxy-deoxyguanosine (8OHdG), have been implicated in the tumorigenic process (9,68,76,111). Oxidative stress exists when pro-oxidants such as ROS exceed antioxidant capabilities. This environment can result from increased generation of ROS as well as impaired removal of ROS by antioxidant defenses such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzyme systems (Figure 1). Differences in ROS generation or antioxidant enzyme activities between the small intestine and colon may alter the levels of oxidative DNA damage, thus contributing to the variation in cancer susceptibility at these two intestinal sites.

The current study evaluated the differences in ROS generation, antioxidant enzyme activities and oxidative DNA adducts in the small intestine and colon in an effort to explain, in part, the dramatic difference in cancer occurrence at these two similar organ sites.

H_2O

O_2

GPx/CAT

$O_2 \longrightarrow O_2^{\bullet-}$ — SOD — H_2O_2

Fe^{2+}/Cu^+

Fe^{3+}/Cu^{2+}

$\cdot OH$

DNA damage
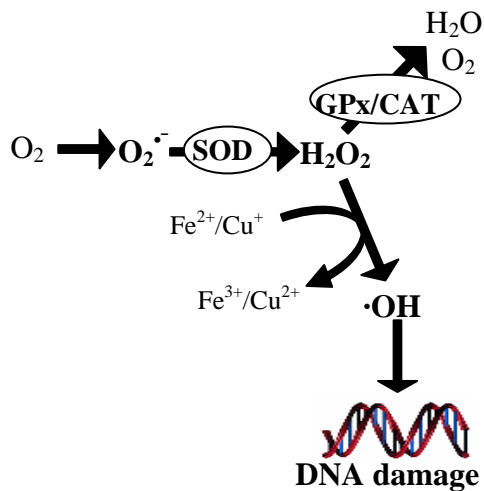
FIGURE 1 Cellular antioxidant enzyme defenses against reactive oxygen species. Superoxide radicals ($O_2^{\bullet-}$) can be generated from O_2 via cellular oxidases or mitochondrial electron transport. Superoxide dismutase (SOD) converts superoxide radicals to the non-radical reactive species, H_2O_2. H_2O_2 can be removed enzymatically by glutathione peroxidase (GPx) or catalase (CAT) to form water and/or O_2. Alternatively, H_2O_2 can react with iron (Fe) or copper (Cu) via a Fenton reaction to form hydroxyl radicals ($OH^{\bullet}$) that can directly damage DNA. The balance of the activity of these enzymes in the generation and removal of H_2O_2 is crucial in maintaining the oxidative status of the cell.

Materials and Methods

Animals and diets. Animal protocols were approved by the University Animal Care Committee of Texas A&M University and conformed to the National Institutes of Health guidelines (NRC 1985). Sixty male weanling (28-d old) and 40 9-mo old Sprague-Dawley rats (Harlan Sprague Dawley, Houston, Texas) were housed individually in raised wire cages and maintained in a temperature- and humidity-controlled animal facility with a daily photoperiod of 12 h light and 12 h dark. The rats were stratified by body weight and assigned to one of four defined diets (25 rats/diet), which were consumed for 2 wk.

Cell isolation. Enterocytes and colonocytes were isolated based on a procedure by Zoran et al. (112). After rats were euthanized, the colon and small intestine were

removed and flushed with warm Ca^{2+}- and Mg^{2+}-free PBS (GibcoBRL, Grand Island,

NY). The first 10 cm of small intestine and last half of the colon were taken as

duodenum and distal colon, respectively. Each segment was cut longitudinally to expose

the lumen and placed in warm (37°C) Ca and Mg free Hank's Balanced Salt Solution

(HBSS), 30 mM EDTA, 5 mM dithiothreitol (DTT), 0.1% fatty acid free BSA, 1 mM

glutamine and 1 mM butyrate (pH 7.4). Following a 15 min shaking incubation, the

mucosal side of each segment was gently scraped with a rubber policeman to remove

surface epithelial cells as well as intact intestinal crypts (113). Removal of crypts and

surface cells was confirmed by histological examination of remaining intestinal tissue

following the scraping procedure. The isolated epithelial cells were washed in warm

HBSS containing Ca^{2+} and Mg^{2+}, 0.1% BSA, 1 mM glutamine, and 1 mM butyrate.

Two aliquots of cells from the duodenum and distal colon were taken for antioxidant

enzyme analysis and Fragment Length Analysis using Repair Enzymes (FLARE)

analysis (114). The remaining cell suspensions were used for ROS analysis as described

below.

   Detection of reactive oxygen species. Isolated enterocytes and colonocytes

(maintained at 37°C) were divided into two treatment groups, which received either an

oxidative challenge of 50 μM H_2O_2 for 5 min or no treatment (basal). This

concentration of H_2O_2 was chosen as it was sufficient to create an oxidative stress

without being toxic to the cells. Exposure time was necessarily limited due to the short-

term viability of coloncytes and enterocytes *ex vivo.* Samples were prepared in duplicate

and incubated for 15 min with CM-H_2DCFDA (Molecular Probes, Eugene, OR), a

fluorescence probe sensitive to such cellular oxidants as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and peroxyl radicals (OOH•). This probe passively diffuses into cells and upon oxidation by ROS forms a fluorescent adduct which remains trapped in the cell. Fluorescence was monitored on a Meridian Ultima confocal microscope (Meridian Instruments, Okemos, MI) with a 530 nm barrier filter and laser excitation at 488 nm, as previously described (115). Intensity of fluorescence is used as an indirect measure of prevalence of ROS. Data for each sample were collected from 15 fields/treatment/rat. Viability of the cells used for analysis was determined after each treatment by staining with ethidium homodimer–1. Mean viability was $81 \pm 4.5\%$.

Measurement of oxidative DNA damage using the FLARE assay. FLARE kits were purchased from Trevigen (Gaithersburg, MD). This assay is a modification of the single cell gel electrophoresis (comet) assay, which uses fpg to introduce DNA strand breaks at 8OHdG, a prevalent and potentially mutagenic oxidative adduct. This process allows measurement of 8OHdG on a single cell level in intact nuclei thus reducing the confounding factor of artifactual adducts (114). Isolated surface epithelial cells and crypts from the duodenum and distal colon were divided into two treatment groups receiving either an oxidative challenge of 50 μM H_2O_2 for 5 min or no treatment (basal). Intact crypts were broken into single cells by aspiration through 27 gauge needle and plated with agarose in duplicate on comet slides. Slides were then exposed to a lysis buffer, followed by immersion in 1X FLARE buffer according to the kit protocol. Slides were treated with fpg enzyme diluted 1:50 with reaction buffer and a control slide received reaction buffer without fpg enzyme. Following treatment with alkali solution,

the slides were exposed to electrophoresis (1 V/cm, 20 min), immersed briefly in 70%

ethanol and stored horizontally. Nuclei were viewed by epifluorescence microscopy

using SYBR green staining. Quantitation of the relative tail moment (tail moment/(tail

moment + head moment) x 100) (78) was measured using the Metamorph Imaging

System (v.4.6r3, Universal Imaging Corp., Downington, PA) and a macro designed by

Nikon. One hundred randomly selected cells were analyzed per treatment group for

each rat.

Measurement of antioxidant enzyme activity. Activities of CAT, GPx, and SOD

in isolated enterocytes and colonocytes during basal condition (no H_2O_2 treatment) were

measured spectrophotometrically using assay kits from Calbiochem (San Diego, CA).

Cell lysates were prepared by homogenization of cells in 50 mM potassium phosphate

buffer (250 mM sucrose/1 mM EDTA/1 mM DTT/0.1% Triton X-100) followed by

centrifugation for 3 min at 10,000 x g. Supernatant was used for enzyme assays

following protocols provided in the kit. Briefly, SOD activity was determined by

measuring the rate of generation of a chromophore at 525 nm. CAT activity was

determined by measuring absorbance of quinoneimene dye at 520 nm. GPx was

determined indirectly by oxidation of NADPH to $NADP^+$ measured at 340 nm. (Due to

use of cell lysates H_2O_2 was the preferred substrate over tert-butyl hydroperoxide as this

compound is also a substrate for some glutathione transferases (116). Sodium azide

(NaN_3) was used to inhibit catalase competition for H_2O_2.) Samples were assayed in

triplicate in 96 well microplates with standards provided in kits or purchased separately.

Microplates were read on a Spectra Max 250 microtiter plate reader using SoftMax Pro

v.1.2 software (Molecular Devices, Sunnyvale, CA). Activity was normalized to protein

concentration as determined by Coomassie Blue assay kits (Pierce Biotechnologies,

Rockford, IL).

     Statistical analysis. Analysis of ROS, antioxidant enzyme activity and oxidative

DNA damage data was performed by analysis of variance (ANOVA) using SAS 8.0

(SAS Institute, Inc). Results were considered significant at $p < 0.05$. The results

presented here were not affected by the diet or age of the animal.

Results

     Generation of reactive oxygen species. The basal levels of ROS, as determined

by indirect fluorescence microscopy with the probe CMH_2DCFDA, in the epithelial cells

of the colon were significantly greater than the ROS levels found in the small intestine

($p < 0.0001$) (Figure 2). When exposed to an oxidative challenge of 50 μM H_2O_2, the

colon continued to maintain greater levels of ROS as compared to the small intestine

($p < 0.0001$). Both tissues experienced a significant increase in ROS over basal levels

during oxidative stress ($p < 0.02$).
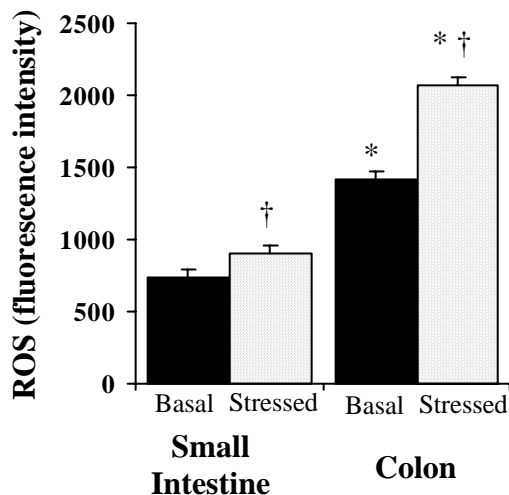
FIGURE 2 Levels of ROS are greater in the colon than the small intestine. Isolated epithelial cells from the intestine were analyzed for ROS by fluorescence microscopy. Intensity of fluorescence was used as an indirect measurement of prevalence of ROS. All samples were prepared in duplicate. Data are means ± SEM from 80 rats with 15 readings/treatment. * Represents mean significantly greater than the same measurement in the small intestine (p<0.0001). † Represents mean significantly greater than the unstressed levels in the same tissue (p<0.02).

Oxidative DNA damage. Levels of 8OHdG as measured by the FLARE assay were used as a marker of oxidative DNA damage. During basal conditions (without H_2O_2 treatment), the level of oxidative DNA damage did not differ between the small intestine and colon (Figure 3). However, in an oxidatively stressful environment, the difference in damage between the colon and small intestine was enhanced. During exogenous stress with 50 μM H_2O_2, the colon exhibited significantly greater oxidative DNA damage compared to the small intestine (p<0.038). Both tissues exhibited a significant increase in oxidative DNA damage over basal levels with the addition of an exogenous oxidant stress (p<0.0001).

Antioxidant enzyme activity. Antioxidant enzyme activities for SOD, CAT and GPx were greater in the colon as compared to the small intestine (p<0.008, p<0.006, p<0.0001 respectively) (Figure 4). CAT and SOD displayed 20-35% greater activity in the colon than the small intestine, while GPx activity was 72% greater in the colon.
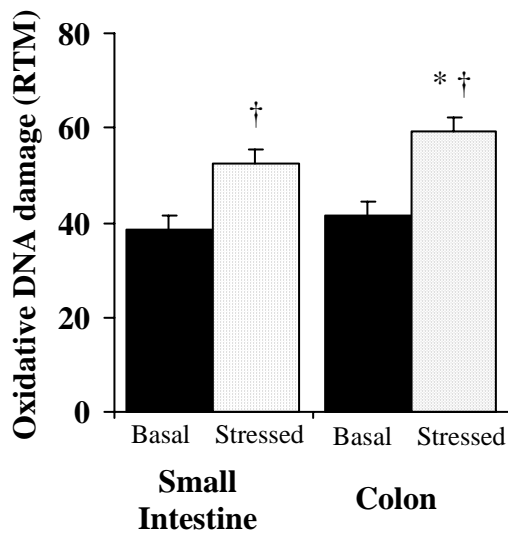
FIGURE 3 During oxidative stress the colon exhibits significantly more oxidative DNA damage than the small intestine. Isolated intestinal epithelial cells were analyzed for oxidative DNA damage with the FLARE assay with relative tail moment (RTM) as the quantitative measurement. Experiments were conducted in duplicate. Data are means ± SEM from 40 rats with 100 cells/tissue/rat analyzed. * Represents mean significantly greater than the same measurement in the small intestine (p<0.038). † Represents mean significantly greater than the unstressed levels in the same tissue (p<0.0001).
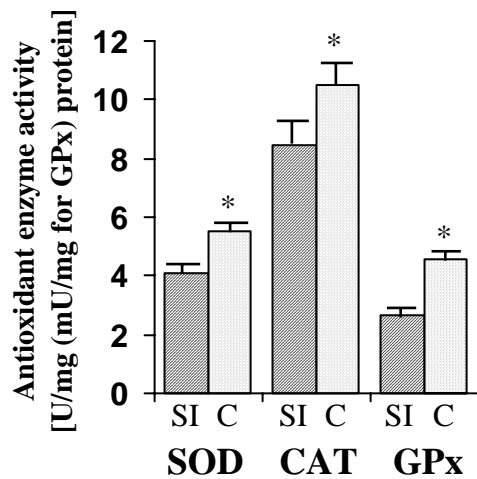
FIGURE 4 The activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) was greater in the colon (C) than the small intestine (SI). Isolated intestinal epithelial cells were analyzed for antioxidant enzyme activity by spectrophotometry. Data are means ± SEM from 40 rats with 3 readings/tissue. * Represents mean significantly greater than the mean for the small intestine ($p<0.008$).

Discussion

Limited research is available on small intestine cancer due to the rarity of this disease. However, there are abundant investigations into the etiology and cause of colon cancer, the second leading cause of cancer death in the US. While these anatomical sites share structural and functional similarities, there also exist inherent differences which may contribute to the variation in cancer incidence. In this investigation we propose that intrinsic differences in redox status and response to oxidative stress contribute to differences in cancer occurrence in the small intestine and colon.

ROS are frequently implicated as key players in tumorigenesis primarily by their potentially mutagenic oxidation of DNA. Our current data show the colon to have significantly greater basal levels of ROS than the small intestine, thereby creating an environment for more extensive oxidative DNA damage. While many exogenous

sources of ROS in the intestine have been determined, endogenous sources have not been well investigated. There is considerable evidence that a key producer of endogenous ROS is mitochondrial oxidative phosphorylation (71,117,118). When this process operates inefficiently, superoxide radicals ($O_2^{\bullet-}$) are produced that, if not quenched by antioxidant defenses, can lead to the formation of other damaging oxidants including hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). There is compelling evidence that oxidative metabolism in the colon is less efficient than in the small intestine. Wu et al. (119) has shown the oxidation of butyrate (the primary energy substrate for colonocytes) yields 4.4 ATP/O_2 while the oxidation of glutamine (the primary energy substrate for enterocytes) yields 5.3 ATP/O_2. This strongly suggests less efficient mitochondrial respiration exists in the colon, which could lead to increased production of ROS.

Despite these elevations of endogenous ROS in the colon, levels of basal 8OHdG did not differ significantly between the two intestinal sites. The resistance of the colon to oxidative DNA damage may be partially explained by enhanced protection against ROS via increases in the activity of antioxidant enzymes found at this site. Indeed, overexpression of certain antioxidant enzymes has been shown to decrease urinary excretion of 8OHdG adducts (120) while elimination of specific antioxidant enzymes in knockout mice have resulted in enhanced cellular oxidative damage (121,122). While our findings reveal antioxidant enzyme activity to be greater in the colon, other investigations have shown varied results. One report found no difference in GPx and SOD in the mucosa of rat small intestine and colon (123), while another investigation

found GPx activity to be slightly greater in the colon and rectum (124). Yet another study found CAT and SOD to be greater in the small intestine than the colon (65). However, this study included in its measurements the submucosal layer of the intestine, which contains immune cells and possibly vascular endothelial and blood cells (125) rich in antioxidant enzymes. The present study examined enzyme activity exclusively in the epithelial cells of the intestine, as these are the cells that undergo the process of malignant transformation as well as directly endure the exogenous stressors contained in the lumen.

Other mechanisms besides antioxidant enzymes may also be at work to control oxidative DNA damage. There are several DNA repair enzymes responsible for the removal of oxidative DNA adducts, including the excision repair enzyme oxoguanine glycosylase (OGG-1), specific for 8OHdG repair in eukaryotes. However, the contribution of these repair enzymes in situations of elevated ROS may be minimal considering that even in basal conditions, the amount of DNA damage exceeds the repair capacity of these enzymes (126). While comparisons of repair enzymes in the small intestine and colon has not been well characterized, findings in our laboratory indicate expression of the repair enzyme OGG-1 in the colon is not greater than in the small intestine despite enhanced generation of ROS in the colon (114). Therefore, differences in DNA repair may not completely explain the resistance of the colon to oxidative DNA damage in the presence of elevated ROS.

It is demonstrated here that while antioxidant enzyme activity is elevated in the colon, there is no significant enhancement of protection against oxidative DNA damage

when compared with the small intestine. Furthermore, basal ROS levels continue to be elevated despite enhanced enzyme activity. This suggests that under basal conditions in the colon, antioxidant enzymes are fully functional, which appears only sufficient to maintain, not reduce, oxidative DNA damage in the presence of elevated ROS. As a result, there exists a rather precarious pro-oxidant basal environment in the colon that when challenged with an exogenous oxidant, predisposes the colonocytes for more severe oxidative stress and increased oxidative DNA damage over the small intestine. This is evidenced by the dramatic rise in ROS and 8OHdG adducts in the colon following brief exposure to 50 μM H_2O_2. Specifically, the colon experienced a 50% increase in ROS from the basal level and 8OHdG adducts increased by 43%. Exposure of the small intestine to the same exogenous stress yielded only a 24% increase in ROS and subsequent 36% increase in oxidative DNA damage.

The inefficiency of the colon in managing exogenous oxidative stress is critical as the colon experiences greater oxidant exposure compared to the small intestine. Prolonged transit time in the colon allows for longer exposure to oxidized food particles, toxins and redox active minerals. In addition, bacterial microflora consistently generate reactive metabolites (127). This poses a significant hazard to the colon since oxidative stress is involved in the development of inflammatory bowel diseases (128) and has been shown to induce malignant transformation (129,130). The inability of the colon to manage oxidative stress especially in the presence of chronic oxidant exposure may partially explain the predisposition of this site for inflammatory bowel diseases and cancer over the small intestine.

Overall, these data indicate that the colon generates more endogenous ROS than the small intestine which persists despite enhanced antioxidant enzyme function. This seemingly pro-oxidant environment of the colon may lead to this tissue's inability to handle oxidative stress as effectively as the small intestine. As a result, oxidative DNA damage in the colon is greater than in the small intestine during times of exogenous oxidant stress. As oxidative damage has been implicated in the carcinogenic process, these results may help to explain the difference in cancer incidence between the small intestine and colon. However, further functional studies of biological endpoints, such as tumor formation, are necessary to establish a definitive link between altered oxidative status and cancer susceptibility. This is the first study to report ROS and oxidative DNA damage differences in the small intestine and colon. Continued investigation of the inherent differences in these two similar tissues and their relation to cancer susceptibility can contribute to the understanding of the pathogenesis and possibly the future prevention of colon cancer.

CHAPTER III

AN INCREASE IN REACTIVE OXYGEN SPECIES BY DIETARY FISH OIL COUPLED WITH THE ATTENUATION OF ANTIOXIDANT ENZYME DEFENSES BY DIETARY PECTIN ENHANCES RAT COLONOCYTE APOPTOSIS[*]

Colorectal cancer is anticipated to be the third most frequently diagnosed cancer in the US this year and it is predicted that almost half of individuals diagnosed will die from the disease within 5 y (1). Yet many of these cases could be prevented by appropriate diet and lifestyle modifications. Dietary fat and fiber are two of the most widely investigated dietary components with respect to colon cancer prevention (27,131). There is substantial evidence that diets rich in (n-3) polyunsaturated fatty acids (PUFAs), such as those found in fish oil (eicosapentaenoic acid and docosahexaenoic acid) protect against colon carcinogenesis (49,132), while diets rich in (n-6) PUFAs, such as those found in corn oil appear to promote cancer development in the colon. However, the chemopreventive abilities of fiber, fermentable and non-fermentable, have been the subject of much debate and have shown varied results in intervention trials and epidemiological studies (133,134). Poorly fermented fibers, such as cellulose, have been considered protective in their ability to dilute putative

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[*] Reprinted with permission from Sanders, L.M., Henderson, C. E., Hong, M.Y., Barhoumi, R., Burghardt, R. C., Wang, N., Spinka, C. M., Carroll, R. J., Turner, N. D., Chapkin, R. S. & Lupton, J. R. (2004) An increase in reactive oxygen species by dietary fish oil coupled to the attenuation of antioxidant defenses by dietary pectin enhances rat colonocyte apoptosis. Journal of Nutrition 134: 3233-3238.

carcinogens that may be present in the fecal stream. Yet, the products of highly

fermentable fibers (i.e., butyrate) have been shown, at least in vitro to possess

chemopreventive qualities (58). We propose that the inconsistent effect seen with fiber

may be attributable to the composition of fat in the diet (57). Specifically, we have

shown that the fermentable fiber, pectin, in combination with fish oil has a protective

effect in multiple stages of colon cancer (49,135).

The protective effect of this diet has been shown to be primarily through

enhancement of apoptosis, a form of programmed cell death (49,64). However, the

mechanism by which the fish oil and pectin diet induces apoptosis has not been clearly

elucidated. Recent evidence suggests an important mediator of apoptosis is reactive

oxygen species (ROS) (reviewed in 69). Considering the degree of unsaturation of (n-3)

PUFAs in combination with the rapid colonocyte oxidation of butyrate, this protective

diet may alter cellular ROS in a manner sufficient to induce apoptosis in the colonocyte.

Yet ROS can also damage and potentially mutate DNA (76); therefore, cells employ

several defenses against ROS including antioxidant enzymes such as superoxide

dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). While these

enzymes are key players in preventing cellular damage caused by endogenous ROS

(88,136), overexpression or addition of these enzymes to tissue systems has been shown

to enhance tumorigenesis and block the action of several chemotherapeutic drugs by

suppressing ROS-induced apoptosis (95,137). The ability of diet to modulate antioxidant

enzyme expression and activity has been documented (107,108). However the ability of

diet to simultaneously influence additional redox factors, such as ROS generation, in the

colon has not been characterized. This investigation evaluates the ability of dietary lipid and fiber to alter the oxidative status of rat colonocytes, via ROS generation and modulation of antioxidant enzyme activity, thus creating an environment permissive for apoptosis.

Materials and Methods

    Animals and diets. Animal use was approved by the University Animal Care Committee of Texas A&M University and conforms to National Institutes of Health guidelines (NRC 1985). Sixty male weanling (28-d old) Sprague-Dawley rats (Harlan Sprague Dawley, Houston, Texas) were housed individually in raised wire cages to diminish coprophagy and maintained in a temperature- and humidity-controlled animal facility with a daily photoperiod of 12 h light and 12 h dark. The rats were stratified by body weight and assigned to one of four experimental diets (15 rats/diet) as previously described (138). Experimental diets were consumed for 2 wk. This study represents a 2 x 2 factorial design with two types of fat (corn oil or fish oil) and two types of fiber (cellulose or pectin). Dietary composition included 15% dietary fat by weight (30% kcals from fat) and 6% dietary fiber by weight (equivalent to 30 g fiber/d in a human diet). The fish oil diet contained 3.5 g of corn oil/100 g diet to prevent essential fatty acid deficiency(48). The types of fiber were chosen based on their fermentability with cellulose being poorly fermentable and pectin being highly fermentable (112). Corn oil and fish oil were analyzed for peroxide value (3.3 mEq/kg corn oil, 3.4 mEq/kg fish oil), fatty acid composition and antioxidant composition. To ensure equal antioxidant content

in all diets, fish oil was supplemented with α-tocopherol, γ-tocopherol and tert-butyl hydroquinone equal to the levels found in corn oil. Food and water were freely available. To minimize fatty acid oxidation, diets were stored at -80°C and fresh food was provided every 24 h. Food intake and body weights were measured weekly.

Tissue collection and cell isolation.   After rats were killed by CO_2 asphyxiation and cervical dislocation, the colon was removed and flushed with warm Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) (GibcoBRL, Grand Island, NY).   For each rat, the last half of the colon was taken as distal colon. The last centimeter of the distal colon was taken for histology and fixed in 4% paraformaldehyde for 4 h, followed by sequential washings in 50% and 70% ethanol. The remaining colon segment was cut longitudinally to expose the lumen and placed in warm Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS), 30 mmol/L EDTA, 5 mmol/L dithiothreitol (DTT), 0.1% fatty acid free BSA (w/v), 1 mmol/L glutamine and 1 mmol/L butyrate (pH 7.4). Following a 15 min shaking incubation, the mucosal side was gently scraped with a rubber policeman. This procedure is designed to remove intact crypts and surface cells leaving behind the lamina propria (113). Removal of crypts and surface cells was confirmed by histological examination of remaining intestinal tissue following the scraping procedure. The isolated crypts were then centrifuged at 100 x g and washed twice in warm HBSS containing Ca^{2+}, Mg^{2+}, 0.1% BSA (w/v), 1 mmol/L glutamine, and 1 mmol/L butyrate. An aliquot of cells from the distal colon was taken for antioxidant enzyme analysis and Fragment Length Analysis using Repair Enzymes (FLARE).

Measurement of apoptosis using the TUNEL assay. Paraffin sections of the 4% paraformaldehyde fixed tissues were utilized for in situ measurement of apoptosis using ApopTag kits (Intergen, Purchase, NY) as previously described (139). This technology is based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) technique. Intestinal crypts were scored according to positive staining by DAB and morphological criteria previously described by Kerr et al. (140). Apoptotic index for each crypt was determined by dividing the number of apoptotic cells in a crypt column by the crypt column height (# of cells). The mean apoptotic index of 25 crypts within a tissue was used as the apoptotic index for that tissue.

Measurement of antioxidant enzyme activity. Activities of CAT, GPx, and SOD in isolated colonocytes were measured spectrophotometrically using commercial assay kits (Calbiochem, San Diego, CA). Cell lysates were prepared by homogenization of cells in 50 mmol/L potassium phosphate buffer (250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1% Triton X-100 (v/v)) followed by centrifugation for 3 min at 10,000 x g. Supernatant was used for enzyme assays following protocols provided in each kit. Briefly, SOD activity was determined by measuring the rate of generation of a chromophore at 525 nm. CAT activity was determined by measuring absorbance of quinoneimene dye at 520 nm. GPx was determined indirectly by oxidation of NADPH to $NADP^+$ measured at 340 nm using H_2O_2 as the preferred substrate. Sodium azide (NaN_3) was used to inhibit catalase competition for H_2O_2. Samples were analyzed in triplicate in 96 well microplates with standards provided in kits or purchased separately from Calbiochem. Microplates were read on a Spectra Max 250 microtiter plate reader

using SoftMax Pro v.1.2 software (Molecular Devices, Sunnyvale, CA). Activity was normalized to protein concentration as determined by Commassie Blue assay (Pierce, Rockford, IL).

Detection of reactive oxygen species. Samples of isolated colonocytes (maintained at 37°C) were prepared in duplicate and incubated for 15 min with chloromethyl -2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, Eugene, OR), a fluorescence probe sensitive to such cellular oxidants as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), and peroxyl radicals (OOH•). This probe passively diffuses into cells and upon oxidation by ROS forms a fluorescent adduct, which remains trapped in the cell. Fluorescence was monitored on a Meridian Ultima confocal microscope (Meridian Instruments, Okemos, MI) with a 530 nm barrier filter and laser excitation at 488 nm, as previously described (115). Fluorescence intensity was used as an indirect measure of ROS prevalence. Data for each sample were collected from 15 fields/treatment for each rat. Viability of the cells used for analysis was determined after each treatment by staining with ethidium homodimer–1 (Molecular Probes). Mean viability was 81 ± 4.5% (n=60).

Measurement of oxidative DNA damage using the FLARE assay. This assay is a modification of single cell gel electrophoresis which uses *E. Coli* formamidopyrimidine-DNA glycosylase (fpg) to introduce DNA strand breaks specifically at 8-hydroxydeoxyguanosine (8-OHdG) adducts (79,141), a prevalent and potentially mutagenic oxidative DNA adduct (77). This process measures the levels of 8-OHdG on a single cell level in intact nuclei (114). 8-OHdG adducts were quantified

using a comet assay kit (Trevigen, Gaithersburg, MD). After isolation, crypts were kept at 4°C for the entire procedure to minimize DNA repair and were dispersed into single cells by repeated aspiration through a 27-gauge needle and plated with agarose in duplicate on comet slides. Slides were exposed to lysis buffer (1% sodium lauryl sarcosinate (v/v), 2.5 mol/L NaCl, 100 mmol/L EDTA, 1% Triton X-100 (v/v), 10 mmol/L Tris base, pH 10) to remove the outer cell membrane leaving only intact nuclei. Slides were then immersed in 1X FLARE buffer (10 mmol/L HEPES-KOH, 100 mmol/L KCl, pH 7.4) followed by the addition of the fpg enzyme (Trevigen) diluted 1:50 with reaction buffer (25X FLARE buffer, 100X BSA). Control slides received only reaction buffer without the fpg enzyme. Following treatment with alkali solution (1 mmol/L EDTA, pH 12.5) to denature DNA strands, slides were exposed to electrophoresis (1 V/cm, 20 min) and immersed briefly in 70% ethanol (v/v). Nuclei were viewed by epifluorescence microscopy using SYBR green staining (Molecular Probes). Quantitation of the relative tail moment (tail moment/(tail moment + head moment)) (78) was measured using Metamorph software (Nikon, Garden City, NY). One hundred randomly selected cells were analyzed per treatment group for each rat.

Statistical analysis. Analysis of ROS, antioxidant enzyme activity and oxidative DNA damage was performed by mixed model analysis of variance (ANOVA) using SAS 8.0 (SAS Institute, Inc.). Apoptotic indices were analyzed with Poisson regression using Proc Genmod. The relationship between antioxidant enzymes and ROS was determined by regression analysis in SPSS. The relationship between ROS and oxidative DNA damage was examined by regression analysis using the generalized

estimation equation approach with an identity link (142). The covariates considered in the model included the combinations of oil and fiber as well as oxidative DNA damage nested within these treatment combinations. To explain the relationship of ROS and apoptotic index, the linear relationship between the log-transformed apoptotic index and ROS levels was modeled. To reduce the influence from potential outlying observations, a robust regression using Huber's weight function was performed (143). Results were considered significant at $p<0.05$.

Results

  Food intake and body weight gain. There were no significant differences in food intake or body weight gain among the experimental groups (results not shown).

  Apoptosis. The combination of dietary fish oil and pectin yielded a greater apoptotic index as compared to the other experimental diets ($p<0.008$) (Figure 5). These results are in agreement with previous findings from our laboratory in which a diet of fish oil and pectin enhanced apoptosis during experimentally induced colon cancer (49,64).
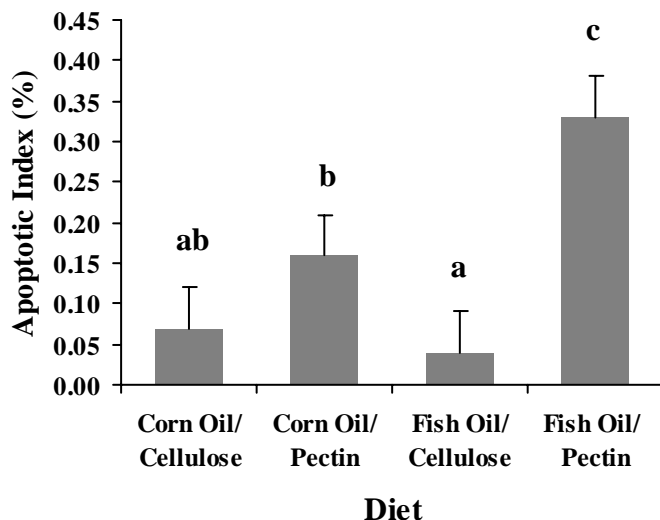
FIGURE 5 Rats fed fish oil and pectin displayed a greater apoptotic index as compared to other dietary treatments of corn oil/cellulose, corn oil/pectin and fish oil/cellulose. Data are means ± SEM from 25 crypts for n=15 rats/diet. Apoptotic index represents the total number of apoptotic cells in a crypt column/total number of cells in the crypt column. Bars with different letters are significantly different (p<0.008).

Antioxidant enzyme activity. The activity of CAT in colonocytes was lower

(p<0.006) in diets containing pectin as the fiber source as compared to cellulose (results

not shown). Furthermore, the combination of fish oil with pectin resulted in 75% less

CAT activity (p<0.003) and 35% less SOD activity (p<0.05) than a diet containing corn

oil and cellulose (Figure 6A). There was no diet effect on GPx. Since SOD and CAT

act sequentially in a pathway of ROS elimination, we expressed the data as enzyme

activity ratios. In diets containing pectin as the fiber source, the mean ratio of

SOD/CAT was greater relative to the cellulose containing diets (SOD/CAT = 1.7 and

0.7, respectively) (p<0.02). A similar trend (p=0.06) was seen with respect to dietary

fish oil (SOD/CAT = 1.6) and corn oil (SOD/CAT = 1.0). When the combination of

lipid and fiber was considered, as expected, the combination of fish oil and pectin

yielded a greater SOD/CAT ratio (2.2) than the combination of corn oil and cellulose

(0.6) and fish oil and cellulose (0.9) (p<0.02) (Figure 6B).
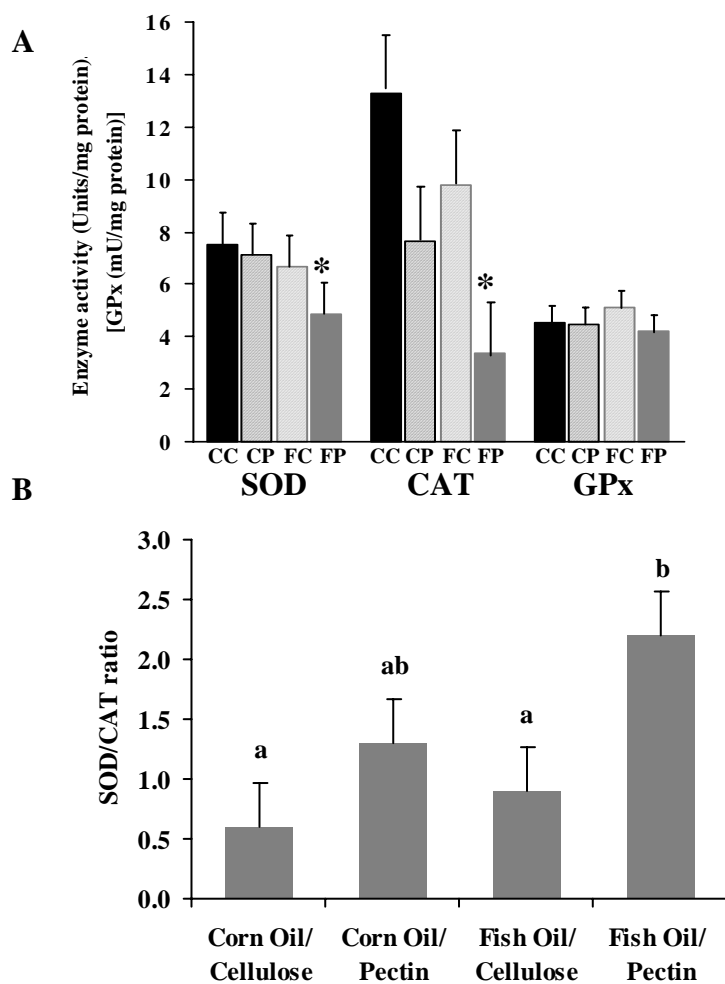
FIGURE 6 CAT and SOD activity are diminished and SOD/CAT is elevated in rat colonocytes by dietary fish oil in combination with pectin. Data are means ± SEM from n=10 rats/diet with samples read in triplicate. * in panel A represents enzyme activity significantly less than the corn oil and cellulose diet (SOD p<0.05; CAT p<0.003). Bars with different letters in panel B are significantly different (p<0.02). Abbreviations: CC=Corn oil/Cellulose, CP=Corn oil/Pectin, FC=Fish oil/Cellulose, FP=Fish oil/Pectin.

Reactive oxygen species. ROS levels in colonocytes were greater in rats consuming fish oil compared to corn oil (p<0.02, Figure 7). Dietary fiber did not significantly alter ROS levels, nor did the combination of oil and fiber.
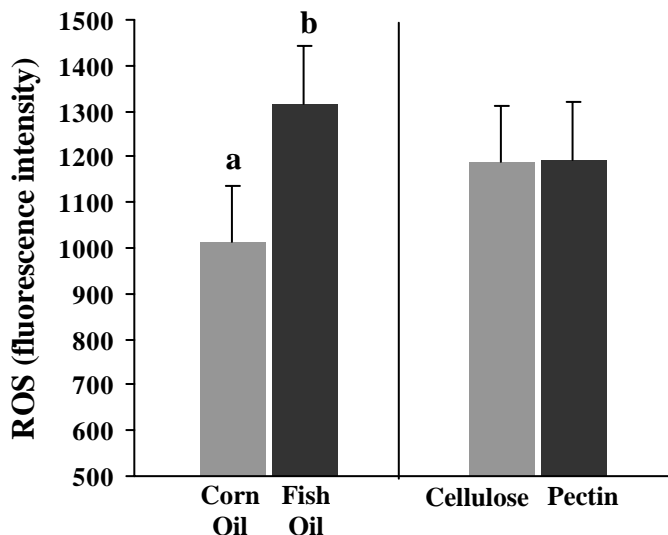
FIGURE 7 Dietary fish oil elevates ROS generation in rat colonocytes. All samples were prepared in duplicate. Data are main effect means ± SEM from n=15 rats/oil or fiber group with 15 fluorescence images captured/rat. Bars with different letters are significantly different within that panel (p<0.05).

Relationship between ROS, apoptosis and oxidative DNA damage. Regardless of diet, there was an exponential relationship between ROS and apoptosis (Figure 8). As the levels of ROS rise in rat colonocytes, the apoptotic index rises exponentially (p<0.005). Across all diets, there was no difference in overall levels of oxidative DNA damage as determined by quantification of 8-OHdG adducts. However, upon examination of the relationship of oxidative DNA damage to ROS levels within a rat, there were distinct diet differences (Figure 9). In rats consuming the fish oil and pectin diet, oxidative DNA damage was inversely related to ROS level (p<0.0001), indicating

that as the level of ROS increased, there was a decrease in the level of oxidative DNA

damage. In contrast, rats consuming the other experimental diets did not exhibit this

inverse relationship. In fact, the relationship of ROS and oxidative DNA damage in the

corn oil and cellulose diet was significantly different from the fish oil and pectin diet

($p<0.002$).
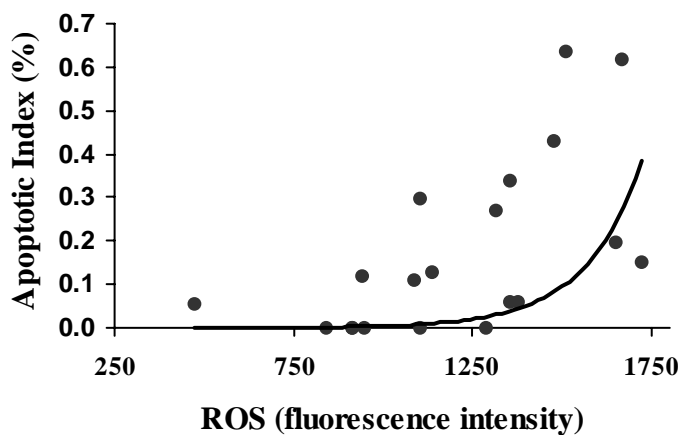
FIGURE 8 Elevation in ROS corresponds to the exponential increase in apoptotic index in rat colonocytes. Data are from n=20 rats (10 fish oil/pectin, 10 corn oil/cellulose) with 15 fluorescence images captured/rat for ROS and 25 crypts/rat for apoptosis. Apoptotic index was calculated as total number of apoptotic cells in a crypt column/total number of cells in the crypt column. Equation for the exponential relationship is $y = \exp(-1.9611 + 0.0011 \times ROS) - 0.25$.
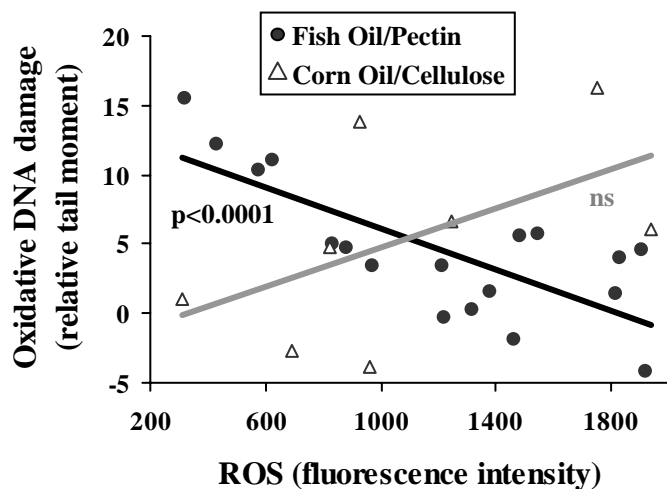
FIGURE 9 Oxidative DNA damage was inversely associated with ROS production in colonocytes from rats receiving the fish oil/pectin diet. Data are from n=26 rats with 100 cells/rat analyzed for oxidative DNA damage and 25 crypts/rat for apoptosis. Apoptotic index was calculated as total number of apoptotic cells in a crypt column/total number of cells in the crypt column. The fish oil pectin correlation was significant (p<0.0001, r=0.75). The corn oil cellulose correlation was not significant (ns, r=0.53). The slopes of the two lines are significantly different (p<0.002).

Discussion

Apoptosis has been shown to be one of the most critical control processes in cancer prevention and treatment (144). In fact, induction of apoptosis is the primary mode of action for most chemotherapeutic drugs and radiation. Therefore, the ability of dietary fish oil in combination with pectin to enhance apoptosis, as shown here and in previous studies (49,132), may be a critical mechanism by which this diet is able to prevent colon cancer. The specific pro-apoptotic actions of dietary fish oil and pectin are not well characterized; however, the initiation and regulation of apoptosis appears to be intimately associated with modifications in the oxidative environment (9,69). The balance of ROS generation and antioxidant capacity within the cell determines the

oxidative environment. When ROS exceed the antioxidant capacity of the cell, oxidative

stress results. Oxidative stress can initiate and/or mediate a number of signaling

cascades, including apoptosis. Thus a potential mechanism whereby fish oil and pectin

may initiate apoptosis is via alterations in the cellular redox balance. We have

previously shown dietary fish oil to enhance ROS generation in colonocytes (50);

however, we did not consider the antioxidant response. The intent of this study was to

use primary cultures from an *in vivo* rat model to determine the ability of dietary fish oil

and pectin to modulate cellular ROS and antioxidant capacity to promote apoptosis.

Important determinants of cellular antioxidant capacity are the enzymes SOD,

CAT and GPx which are responsible for the elimination of ROS. As these enzymes act

sequentially to remove ROS, the balance of the activity of these enzymes may be as

critical in the defense against ROS as the activity of the enzymes alone (145). Data from

this experiment show the activity of these enzymes, especially CAT, to be influenced by

dietary fiber. Specifically, antioxidant enzyme activity in colonocytes from rats

receiving a pectin diet was less than that observed in the cellulose diets. Furthermore,

the combination of fish oil with pectin in the diet resulted in even lower activity for CAT

and SOD. Interestingly, the difference in CAT activity was greater than the change seen

with SOD, suggesting a possible enzyme activity imbalance. SOD converts superoxide

$(O_2^{\bullet-})$ to H_2O_2, which is then converted to water and/or O_2 by CAT & GPx. Thus,

dramatically diminished CAT activity coupled to only a subtle reduction in SOD (an

increase in SOD/CAT) may yield a system that can no longer eliminate H_2O_2 at the rate

it is formed. Indeed, dietary pectin and to a lesser extent dietary fish oil elevate the

SOD/CAT ratio compared to cellulose and corn oil, respectively. Furthermore, the greatest enhancement in SOD/CAT was seen when fish oil and pectin were combined in the diet. Similar experiments in rat colonocytes have found dietary fish oil to reduce antioxidant enzyme activity (107) and a recent dietary intervention trial showed diets high in fiber and (n-3) PUFAs are capable of reducing antioxidant enzyme activity in humans (108). Although the mechanism by which these dietary constituents achieve a reduction in enzyme activity is unclear, these results suggest that dietary lipid and dietary fiber, specifically fish oil and pectin, work coordinately to alter antioxidant enzyme activity and balance in a manner that may create a pro-oxidant environment in the colonocytes.

ROS measurements further suggest that dietary fish oil may create a more oxidative environment in the colonocytes as compared to a corn oil diet. Diets with fish oil as the lipid source enhanced ROS generation in the colonocytes. This is not unexpected considering the high degree of unsaturation found in the long chain (n-3) PUFAs in fish oil. The primary (n-3) fatty acids in fish oil, eicosapentaenoic acid (20:5(n-3)) and docosahexaenoic acid (22:6(n-3)) have up to three times as many double bonds per molecule than the (n-6) fatty acids found in corn oil, such as linoleic acid (18:2(n-6)). This increases the opportunity for oxidant attack and can contribute to the propagation of ROS. Furthermore, we have shown dietary (n-3) PUFAs to be readily incorporated into the mitochondrial membrane (146), predisposing the mitochondria to enhanced lipid peroxidation and membrane damage and contributing to the propagation of ROS generated by the mitochondrial electron transport system (50). It is likely that

many of the fatty acids consumed in these experimental diets maintained a high degree of unsaturation as the diets were kept at -80°C to prevent fatty acid oxidation. However, these conditions may not be practical outside of the laboratory setting and less than optimal storage may enhance the degree of fatty acid oxidation prior to consumption. This may have a significant impact on the physiological effects of dietary lipid and definitely warrants further attention.

Although dietary fish oil and pectin alter the antioxidant capacity and ROS generation of colonocytes to favor a pro-oxidant environment, the outcome of these diet-induced cellular modifications are critical. An oxidative environment may favor apoptosis, or alternatively, may increase the potentially mutagenic event of damaging DNA. Therefore, it was important to determine apoptosis and oxidative DNA damage in the same animals in which the diet-induced changes in ROS and antioxidant enzyme activity were measured. In the fish oil/pectin diet, apoptosis was enhanced and the oxidative environment created by this diet was associated with a decline in oxidative DNA damage. Alternatively, in the corn oil/cellulose diet, apoptosis was less than in the fish oil/pectin diet and as ROS increased, oxidative damage did not decrease as seen in the fish oil/pectin diet. Thus, the suppression of oxidant protection systems and enhancement of ROS generation by dietary fish oil and pectin appears to protect the colon against oxidative DNA damage by promoting ROS-mediated apoptosis. However, continued investigation is needed to further elucidate the multi-faceted relationship between cellular redox status and apoptosis. Additional investigation is also necessary to determine if these dietary modifications of the redox environment and the resulting

increase in apoptosis continues during the initiation and progression stages of colon

carcinogenesis. While we have previously shown apoptosis to be enhanced by dietary

fish oil and pectin during the initiation (64) and progression (49) of colon

carcinogenesis, whether or not the mechanism is alteration of the redox environment

remains to be determined.

In summary, dietary fish oil and pectin work coordinately to enhance colonocyte

apoptosis by modulation of the cellular redox environment. In this ex vivo model, we

showed dietary fish oil to enhance ROS in colonocytes while the effects of dietary pectin

were more clearly seen in the attenuation of antioxidant enzyme activity. Therefore, it is

not surprising that the combination of these dietary constituents work in concert to create

an environment permissive for apoptosis, thereby protecting cells from severe and

possibly mutagenic DNA damage. This study reinforces the importance of diet for the

prevention of cancer and strengthens the growing realization that the effects of

individual diet components may not be as significant as the combination of foods that

are consumed in the diet. Further investigations should evaluate the influence of other

components of the diet matrix as well as the impact of alterations to diet components

(e.g., fatty acid oxidation prior to consumption).

CHAPTER IV

DIETARY FISH OIL AND PECTIN SUPPRESS OXIDATIVE DNA DAMAGE AND ENHANCE APOPTOSIS TO PROTECT AGAINST RADIATION-ENHANCED COLON CARCINOGENESIS IN THE INITIATION STAGE

One of the primary goals of NASA's vision for space exploration is to "extend human presence across the solar system (147)." However, there are many risks associated with extended time in space, one of the most serious being exposure to cosmic radiation. Prolonged exposure to high energy cosmic radiation, such as would be found in outer space, can cause severe and possibly irreparable physiological damage including damage to DNA that can lead to mutation and cancer (10). Therefore, countermeasures to radiation damage must be found before long-term space flight is safely achievable. Dietary modification is one reasonably attainable countermeasure.

A wealth of evidence has shown that modification of dietary fat and fiber can significantly alter the risk of colon cancer (27), one of the leading causes of cancer death in the US (1) and one of the most preventable by diet (2). We have shown a diet rich in n-3 PUFAs (such as those found in fish oil) in combination with the fermentable fiber, pectin, to be protective against an experimental model of colon carcinogenesis in rats (49). Furthermore, this protective effect is consistent through each stage of carcinogenesis, from initiation to tumor formation (135,139). In contrast, diets rich in n-

6 fatty acids and the poorly fermentable fiber, cellulose, appear to promote colon tumorigenesis. Recently, Hong et al. demonstrated that the dietary combination of fish oil and pectin is protective in the initiation stage of colon carcinogenesis by minimizing DNA damage and enhancing apoptosis, a form of programmed cell death (64). It has been further suggested that this enhancement of apoptosis may be due to the ability of dietary fish oil and pectin to modulate the oxidative environment of the colon (148).

Radiation exposure also modulates the cellular oxidative environment. Radiation is considered a carcinogen by its ability to cause genetic mutations primarily through DNA single and double strand breaks. However, radiation exposure can also result in the generation of reactive oxygen species which creates a secondary mechanism by which radiation may damage DNA (10). This secondary mechanism is not as well characterized as DNA strand breaks, even though it may play a critical role in carcinogenesis. Furthermore, the ability of diet to protect against radiation-induced DNA damage is not widely investigated. We have shown dietary fish oil and pectin to protect against DNA damage caused by a chemical carcinogen via modulation of the colonocyte redox environment to enhance apoptosis (64). This dietary combination may also be able to protect against radiation-induced DNA damage by a similar mechanism. In addition, it is reasonable to expect that astronauts may not only be exposed to radiation, but also to chemical carcinogens upon their return to earth and that the combined effect may be more severe than either carcinogen alone. Thus, it is also critical to investigate the ability of diet to protect against the combination of radiation and chemical carcinogen.

As we have previously shown the combination of dietary fish oil and pectin to protect against a chemical carcinogen in the initiation stage of colon carcinogenesis, we hypothesized that this diet will also protect against the added insult of high energy radiation exposure. In addition, we proposed that the protective mechanism of this diet is through modification of the cellular redox environment to minimize oxidative DNA damage and enhance apoptosis.

Materials and Methods

Animals and diets. Animal use was approved by the University Animal Care Committee of Texas A&M University and conforms to National Institutes of Health guidelines (NRC 1985). One hundred sixty male weanling (28-d old) Sprague-Dawley rats (Harlan Sprague Dawley, Houston, Texas) were housed individually in raised wire cages to diminish coprophagy and maintained in a temperature- and humidity-controlled animal facility with a daily photoperiod of 12 h light and 12 h dark. The rats were stratified by body weight and assigned to one of four experimental diets (40 rats/diet), which were consumed for 31 d. This study represents a 2 x 2 x 2 factorial design with two treatments (irradiated and non-irradiated), two types of fat (corn oil or fish oil) and two types of fiber (cellulose or pectin). Dietary composition included 15% dietary fat by weight (30% kcals from fat) and 6% dietary fiber by weight (equivalent to 30 g fiber/d in a human diet). The fish oil diet contained 3.5 g of corn oil/100 g diet to prevent essential fatty acid deficiency (48). The types of fiber were chosen based on their fermentability with cellulose being poorly fermentable and pectin being highly fermentable (112). To

ensure equal antioxidant content in all diets, fish oil was supplemented with α-tocopherol, γ-tocopherol and tert-butyl hydroquinone equal to the levels found in corn oil. Food and water were freely available. To minimize fatty acid oxidation, diets were stored at -80°C and fresh food was provided every 24 h. Food intake over 48 h and body weights were measured prior to carcinogen injection.

After 21 d of receiving experimental diet, 60 rats were exposed to 1 Gy of 1 GeV/nucleon Fe ion radiation at the AGS facility of Brookhaven National Laboratory (Upton, New York). The beam was targeted to the abdomen of the rats to include irradiation of the small and large intestine. Ten days post-irradiation the rats received subcutaneous injections of the colon specific carcinogen, azoxymethane (AOM), at 15 mg/kg body weight. The remaining rats (n=100) were not exposed to irradiation but were injected with AOM at the same time point (day 31). Rats were then killed by CO_2 asphyxiation and cervical dislocation at 0, 6, 9, 12 and 24 h post-AOM injection (n=20 non-irradiated rats/timepoint, n=12 irradiated rats/timepoint). The zero hour timepoint was used as a negative control as these rats were not injected with AOM.

Cell isolation. Following termination by CO_2 asphyxiation and cervical dislocation, the colon was removed (cecum to rectum) and cut longitudinally to expose the lumen. Two centimeters of the most distal colon were fixed in 4% PFA or 70% ethanol (1 cm/fixation). The mucosal layer of the remaining colon was removed by scraping with a glass slide and a small amount (5-20 mg) was used for glutathione (GSH) analysis.

Measurement of apoptosis using the TUNEL assay. Paraffin sections of the 4% paraformaldehyde fixed tissue were utilized for *in situ* measurement of apoptosis using ApopTag technology (Intergen, Purchase, NY) as previously described (139). Colon crypts were scored according to positive staining by DAB and morphological criteria previously described by Kerr et al. (140). Apoptosis was measured in rats not receiving AOM (0 h timepoint) and at 12 h post AOM injection, as this is the peak time for apoptosis following this carcinogen insult. Apoptotic index for each crypt was determined by dividing the number of apoptotic cells in a crypt column by the crypt column height (# of cells). The mean apoptotic index of 25 well-oriented crypts was used as the apoptotic index for that rat.

Determination of GSH/GSSG. The ratio of glutathione to glutathione disulfide (GSH/GSSG), a highly sensitive indicator of cellular redox status (103), was determined by HPLC using fluorescence detection of dansyl derivatives from samples of scraped mucosa based on a protocol from Jones et al. (149). Briefly, the tissue (and a 50 µmol/L GSH and GSSG standard) was homogenized in preservation solution (100 mmol/L boric acid, 100 mmol/L sodium tetraborate, 10 mg/ml L-serine, 1 mmol/L BPDS, 10 mmol/L iodoacetic acid) and an equal volume of perchloric acid solution (10% w/v) followed by centrifugation at 10,000 x g for 1 min. Derivatization was completed by exposure of the supernatant to dansyl chloride for 18-24 h. Following choloroform extraction, 20 µL of the clear upper layer was injected into the autosampler and separated by passing through a 3-aminopropyl column (5 µm; 4.6 mm X 25 cm; Custom LC, Houston, Texas) at a flow rate of 1 ml/min. A linear gradient of 80% (v/v) methanol and acetate buffer

(pH=4.6) was run over 45 min to achieve separation. Dansyl derivatives were detected by fluorescence monitoring with 335 nm excitation and 610 nm emission. GSH and GSSG were measured at each of the five timepoints in the initiation timeframe.

Immunohistochemical analysis of 8-hydroxy-deoxyguanosine (8OHdG) adducts. Paraffin sections of the 70% ethanol fixed tissue were utilized for in situ measurement of 8OHdG adducts using anti-8OHdG from Oxis International and a protocol adapted from Tanaka et al. (86). This antibody has a high specificity for the 8OHdG adduct and low cross reactivity with other modified bases (87). Following deparaffinization with xylene and ethanol, slides were incubated with Dnase-free RNAse (Ambion, Austin, TX) at 37°C to eliminate RNA adduct interference. DNA was denatured by incubating in 4 mol/L HCl and non-specific binding of the secondary antibody was blocked with 10% rabbit serum. Anti-8OHdG (1:20 in 1% rabbit serum) was applied to the slides and incubated at 4°C overnight. Biotinylated rabbit anti-mouse (Jackson Immunoresearch, West Grove, PA) (1:800 in 1% rabbit serum) was used as the secondary antibody. Endogenous peroxidases were quenched and the slides then incubated with avidin-biotin horseradish peroxidase (ABC-HRP) (Vector Laboratories, Burlingame, CA). DAB with H_2O_2 was used for detection of adducts. 8OHdG adducts were measured in rats not receiving AOM (0 h timepoint) and at 12 h post AOM injection in correspondence with apoptosis measurements. The staining intensity of each cell within a crypt column was determined using NIH Image software. The mean stain intensity was determined for each crypt column and fifteen crypt columns were analyzed in each rat.

Statistical analysis. Analysis of intake, weight change, oxidative DNA damage, apoptosis, GSH, GSSG and GSH/GSSG was performed by mixed model analysis of variance (ANOVA) using SAS 8.0 (Sas Institute, Inc.). A log transformation was used in the analysis of GSH/GSSG. A Poisson regression was used to analyze the difference in apoptosis prior to and after AOM treatment. Results were considered significant at $p < 0.05$.

Results

Food intake and body weight gain. There were no differences in weight gain between irradiated and non-irradiated animals or among the diet groups. Intake was also similar among the diet groups, but interestingly, irradiated rats consumed significantly more than non-irradiated rats ($p < 0.001$) (results not shown).

Apoptosis. As expected, apoptotic index increased 12 hours after administration of AOM regardless of radiation or diet treatment ($p < 0.0001$). Radiation alone (as measured at time 0) was not sufficient to enhance apoptosis. When radiation was combined with AOM, apoptosis was enhanced compared to non-irradiated, AOM injected rats, but only in rats consuming pectin diets ($p < 0.04$) (Figure 10). Additionally, pectin fed rats exposed to radiation and AOM had significantly greater apoptosis than cellulose fed rats within the same treatment group ($p < 0.03$). There was no effect of dietary fat on apoptotic index. Thus, irradiated rats fed corn oil & pectin had a similar apoptotic index as rats fed fish oil & pectin.
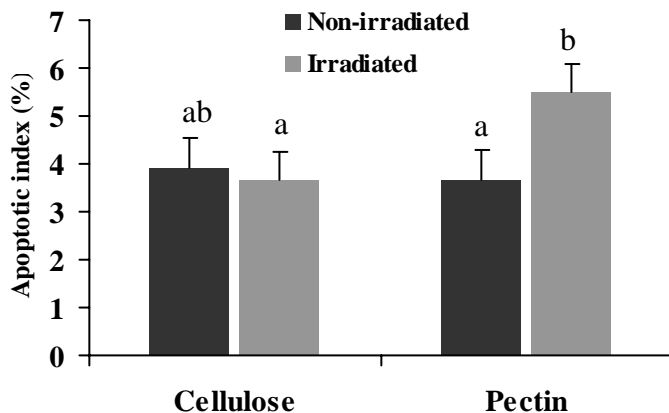
FIGURE 10 Irradiated rats fed pectin have greater apoptosis than non-irradiated pectin fed rats and irradiated cellulose fed rats at 12 h post AOM. Data are means ± SEM for n=10 rats/fiber in the non-irradiated group and n=6 rats/fiber in the irradiated group). Apoptotic index was measured at 12 h post AOM injection. Bars with different letters are significantly different (p<0.04).

GSH and GSSG. In response to radiation and prior to chemical carcinogen treatment, fish oil fed rats displayed an increase in GSH/GSSG which was significant in the fish oil and pectin diet (p=0.05) (Figure 11A). This change in the redox environment was not seen in the corn oil diets. After treatment with a chemical carcinogen (all timepoints considered), the irradiated rats fed fish oil and pectin continued to maintain elevated GSH/GSSG, compared to the other diet groups (p<0.05) (Figure 11B). This elevation in GSH/GSSG may be due, in part, to a decrease in GSSG levels (p<0.05) since there was no change in GSH (Figure 12). No diet effects in GSH/GSSG or GSSG were observed in non-irradiated animals.

A

B
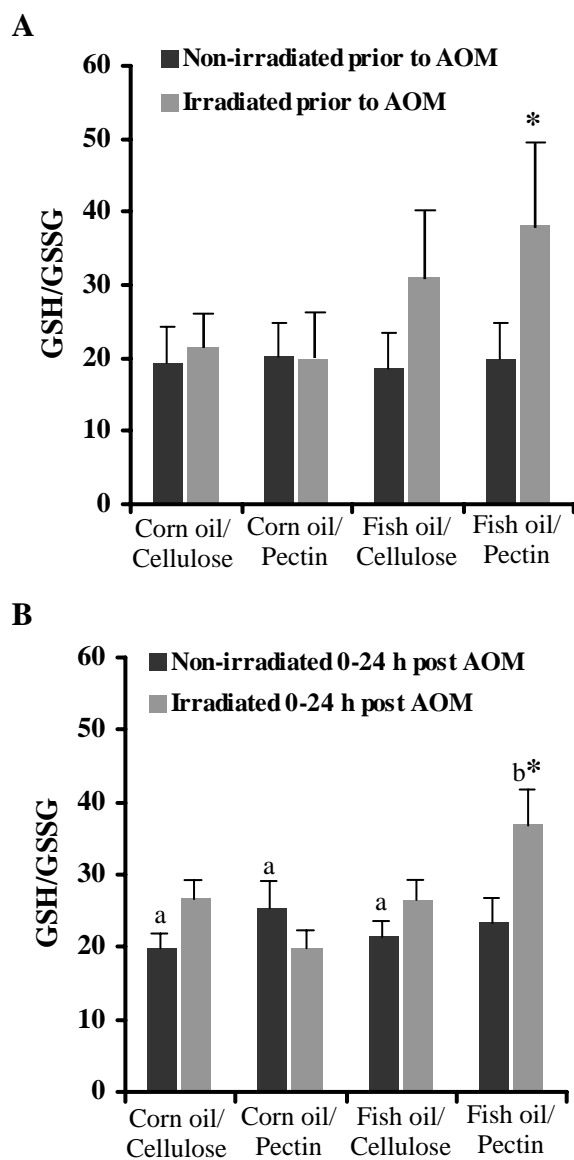
FIGURE 11 Fish oil and pectin fed rats displayed a more reduced environment (increase in GSH/GSSG) in response to radiation (Panel A) and the combination of radiation and AOM (Panel B). Data in panel A are geometric means ± SEM for 32 rats (n=3/diet in the irradiated group and n=5/diet in the non-irradiated group). Data in panel B are geometric means ± SEM for 160 rats (n=15/diet in the irradiated group and n=25/diet in the non-irradiated group). * represents significant difference from non-irradiated within the same diet group (p≤0.05). Bars with different letters are significantly different from irradiated rats in other diet groups (p<0.05).

A

B
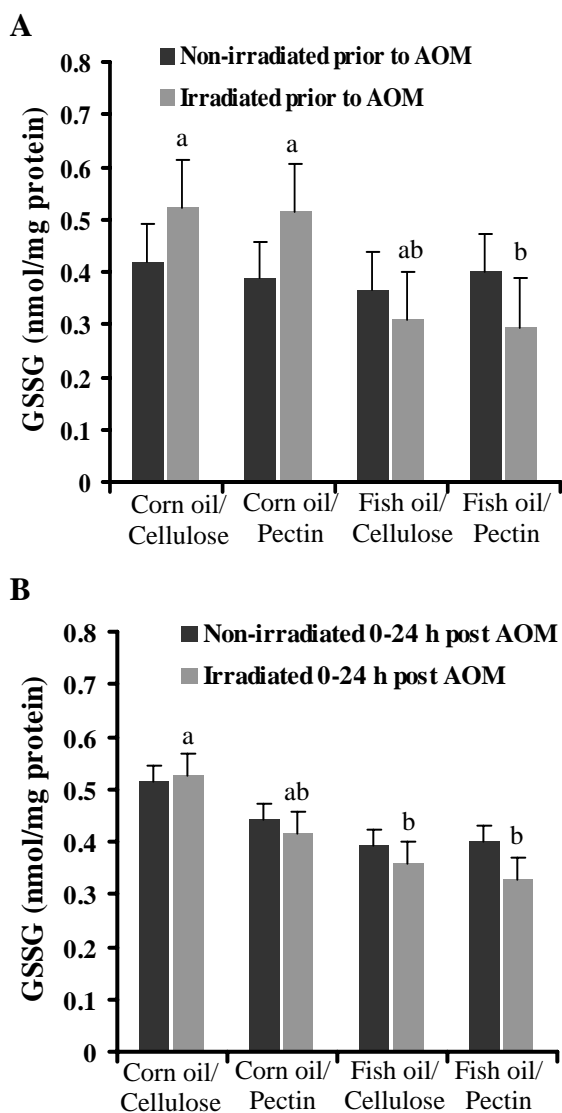
FIGURE 12 Irradiated rats fed fish oil and pectin had lower levels of GSSG prior to AOM treatment compared to corn oil diets (Panel A) and maintained lower levels following AOM treatment (Panel B). Data in panel A are means ± SEM for 32 rats (n=3/diet in the irradiated group and n=5/diet in the non-irradiated group). Data in panel B are means ± SEM for 160 rats (n=15/diet in the irradiated group and n=25/diet in the non-irradiated group). Bars with different letters are significantly different from irradiated rats in other diet groups (p<0.05).

Oxidative DNA damage. Radiation alone did not increase 8OHdG DNA adducts

in rat colonocytes as determined by immunohistochemistry. AOM treatment did

increase the formation of 8OHdG adducts by 12 h post AOM in non-irradiated rats

(p<0.02) (Figure 13A). However, in irradiated rats, the increase in 8OHdG from AOM

administration was only significant in corn oil and pectin fed animals (p<0.009) (Figure

13B).
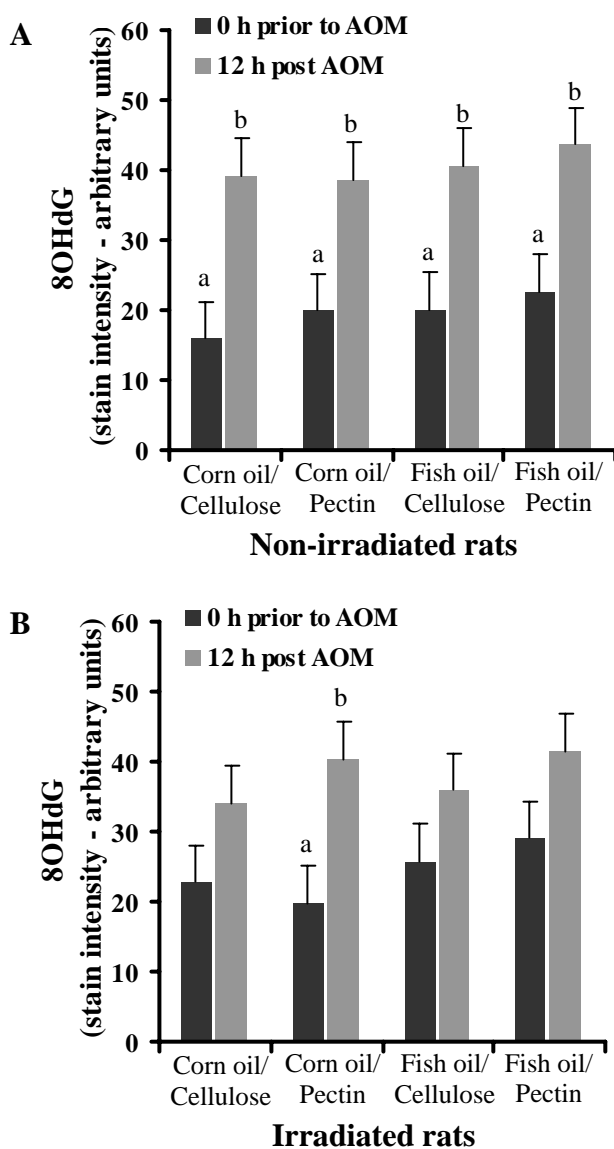
FIGURE 13 Irradiated rats fed corn oil and pectin experience greater levels of 8OHdG 12 h post AOM injection compared to prior to AOM. Panel A displays the effect of AOM on 8OHdG in non-irradiated rats. Data are means ± SEM for n=5 rats/timepoint/diet. Bars with different letters are significantly different within that diet (p<0.02). Panel B displays the effect of AOM on 8OHdG in irradiated rats. Data are means ± SEM for n=3 rats/timepoint/diet. Bars with different letters are significantly different within that diet (p<0.009).

Discussion

The classification of high energy radiation as a carcinogen is based on its ability to severely damage DNA (10). While the most common form of damage to DNA from radiation is the formation of double strand breaks, there are also more indirect and lasting effects that may be just as potentially mutagenic. One of these indirect effects is the generation of reactive oxygen species leading to cellular oxidative stress and enhancement of oxidative DNA damage (23). Oxidative DNA damage, specifically the 8OHdG DNA adduct investigated in this study, has been implicated in the carcinogenic process (9). In fact, 8OHdG has been cited as the possible source of commonly found mutations in ras and p53 present in colorectal cancer (9). Several investigations have shown enhanced urinary excretion of 8OHdG immediately following space flight, indicating an increase in oxidative DNA damage following exposure to high energy radiation (150). In this investigation, we did not find 8OHdG to be enhanced at 10 d post irradiation, suggesting that cellular mechanisms are in place to repair or remove damaged DNA bases soon after a radiation insult. A similar finding was seen by Stein et al. who showed an initial non-significant increase in 8OHdG following radiation exposure, but a return of 8OHdG to pre-radiation exposure levels at 12-14 d post irradiation (150).

One mechanism by which radiation-induced DNA damage may be removed is apoptosis. This form of programmed cell death occurs fairly rapidly in colonocytes following an insult, such that the normal rate of cell turnover is restored approximately 24 h after radiation exposure (6). This rapid response to radiation injury and early

elimination of severely damaged cells is most likely why there was not an elevation in apoptosis observed in these rats at 10 d post irradiation.

Although there was no change in oxidative DNA damage or apoptosis following irradiation and prior to AOM, there was a dietary modification of the redox environment in response to radiation as demonstrated by a change in GSH/GSSG. Other investigators have also shown redox changes to persist in the progeny of irradiated cells, contributing to a genomic instability and increasing the likelihood of future mutations (23,151). Glutathione (GSH), a low molecular weight thiol, is highly sensitive to changes in the oxidative environment, and changes in the ratio of GSH to its oxidized form (GSSG) is often used as an indicator of cellular redox status (103). The environment in fish oil fed rats was more reduced (greater GSH/GSSG) following irradiation than non-irradiated rats and this was most pronounced in the fish oil/pectin diet. Furthermore, elevations in GSH/GSSG were maintained in rats fed a fish oil and pectin diet even after treatment with AOM. This change in GSH/GSSG seen in the fish oil diets appears to be due to a reduction in the levels of cellular GSSG rather than an increase in GSH. As a more reduced environment is better able to endure an oxidative stress without severe damage to the cell, colonocytes from rats fed fish oil and pectin may be better prepared for future oxidative attacks.

This is of critical importance as astronauts, upon their return to earth, will likely be exposed to numerous carcinogens in the environment and previous radiation exposure may make their cells more susceptible to damage by chemical carcinogens. Indeed we demonstrate here that treatment with AOM triggered an increase in 8OHdG adducts. Yet

in irradiated rats, this AOM-induced increase in 8OHdG adducts is only significant in rats fed corn oil and pectin. The combination of fish oil with pectin did not elicit this significant increase in oxidative damage and this may be due to the more reduced redox environment (elevated GSH/GSSG) present among irradiated rats fed this diet. Nevertheless, dietary pectin was able to enhance colonocyte apoptosis in rats receiving either lipid source regardless of the differences in the oxidative environment. Apoptosis was enhanced in irradiated rats fed pectin compared to non-irradiated rats also receiving pectin as a fiber source. Furthermore, irradiated rats fed pectin experienced more apoptosis than cellulose fed rats. Yet, the enhancement of apoptosis in the fish oil and pectin diet was not coupled with an increase in oxidative DNA damage as was seen in the corn oil and pectin diet. Thus, the combination of fish oil with pectin is able to enhance colonocyte apoptosis while simultaneously maintaining a more protective (i.e. more reduced) cellular environment to promote less oxidative DNA damage.

Therefore, it appears that the more reduced environment created by fish oil in response to radiation is better prepared for the additional stress of a subsequent carcinogen insult and thereby more effectively attenuates the increase in oxidative DNA damage. Additionally, the more oxidative environment in corn oil fed rats may predispose the colonocytes to more severe increases in oxidative DNA damage in response to a chemical carcinogen. Another investigation in cell culture using free radical scavengers to promote a less oxidatively stressful environment during irradiation also found a reduction in oxidative DNA damage from ROS as well as a reduction in

chromosomal instability (66). We show here, the ability of dietary modification to achieve a similar protection from oxidative DNA damage in colonocytes.

In summary, this investigation demonstrates the ability of diet to minimize oxidative DNA damage and enhance apoptosis during the initiation stage of radiation-enhanced colon carcinogenesis. Dietary pectin elevates colonocyte apoptosis in irradiated rats as compared to cellulose fed rats. However, when pectin is combined with corn oil in the diet, there is a significant enhancement of oxidative DNA damage that is not seen when pectin is combined with fish oil. This protective effect may be due in part to the ability of fish oil and pectin to promote a more reduced environment in response to radiation thereby preparing the colonocytes for subsequent attack by other carcinogens. While several studies have examined colonocyte responses to radiation and chemical carcinogen individually (49,152), this is the first investigation to describe the combinatorial effects of these two distinct carcinogens as well as the ability of diet to protect against this dual insult. These findings demonstrate the importance of diet as a potential countermeasure to the risks associated with radiation exposure in space flight. However, further investigation is needed in animal and human studies before recommendations can be made. Not only can these investigations increase the safety of current and future space travelers, but they also may increase our understanding of diet's role in the prevention of cancer.

CHAPTER V

SUMMARY AND CONCLUSION

For several decades, the variation in cancer susceptibility between the small and large intestine has intrigued scientists and prompted some to investigate the environmental and intrinsic differences in these two similar tissues (4,17). A clear understanding of the relative resistance of the small intestine to tumorigenesis may offer insight into potential mechanisms of chemoprevention in the colon. Yet, due to the rarity of small intestine cancer, research is limited and several of the proposed differences provide only an incomplete explanation (3). In this investigation, we proposed that intrinsic differences in redox status and response to oxidative stress contribute to differences in cancer occurrence in the small intestine and colon.

Indeed, this study showed that basal levels of ROS were greater in the colon than the small intestine, and that this persisted despite elevated activity of antioxidant enzymes. Considering this seemingly pro-oxidant environment, it was not surprising that exposure to an exogenous oxidative stress resulted in more oxidative DNA damage in the colon compared to the small intestine. Exogenous stress is of greater concern for the large intestine which endures prolonged exposure to oxidized food particles or toxins in the fecal stream as well as the oxidants generated by microbial fermentation (127). Inability to manage exogenous stressors and the resulting increases in oxidative DNA damage likely contributes to the etiology of inflammatory bowel diseases and possibly

colon cancer (9). Thus, these findings may contribute to the explanation of the difference in cancer susceptibility between the small and large intestine.

While ROS generation may enhance oxidative DNA damage, as shown in the comparison of the small and large intestine, there is also considerable evidence that ROS are critical signaling molecules in apoptosis (69-71,73-75,137). Our laboratory has previously shown the combination of dietary fish oil and pectin to enhance apoptosis in the colon of rats exposed to a carcinogen. One potential mechanism by which this diet may induce apoptosis is via elevations in ROS. This would not be unexpected considering the degree of unsaturation of n-3 PUFAs in fish oil and the rapid colonocyte metabolism of butyrate, a fermentation product of pectin, both of which can generate substantial ROS. In this investigation, we evaluated the ability of dietary fish oil and pectin to modulate the colonocyte redox balance to promote apoptosis.

As the time at which dietary interventions are initiated may influence the ability of the diet to protect against colon cancer (58), it was desirable to examine the effects of diet prior to and following a carcinogen insult. The first portion of the experiment examined ROS generation, antioxidant enzyme defenses, oxidative DNA damage and apoptosis in a primary culture from an in vivo rat model prior to a carcinogen insult. Dietary fish oil and pectin did work coordinately to create a more oxidative cellular environment, with fish oil primarily enhancing cellular ROS while pectin attenuated antioxidant enzyme activity. Despite the creation of this oxidative environment, oxidative DNA damage declined in the fish oil and pectin diet but apoptosis was enhanced. In fact, apoptosis increased exponentially as ROS increased. These findings

suggest that the combination of dietary fish oil and pectin work coordinately to create an environment permissive for apoptosis, thereby protecting against potentially mutagenic oxidative DNA damage that could lead to colon cancer.

The next portion of this investigation examined the ability of fish oil and pectin to alter the colonoctye oxidative environment to promote apoptosis following exposure to carcinogens. Two unique carcinogens, ionizing radiation and AOM, were used in combination. Radiation is considered a carcinogen due to its ability to directly (via strand breaks) and indirectly (via generation of oxidative stress) damage DNA which is a particular concern for the space program. One of the greatest limitations to long term space flight is exposure to high energy, cosmic radiation. Thus, countermeasures to radiation damage must be found before extended time in space flight is safely achievable. In addition, individuals returning from space flight will most likely encounter chemical carcinogens upon their return to the earth and the combined effect of radiation and chemical carcinogen may be more severe than either carcinogen alone. For this reason, we combined an ionizing radiation insult with our well-characterized model of AOM-induced colon cancer. As we have previously shown dietary fish oil and pectin to protect in the initiation stages of AOM-induced colon carcinogenesis (49,64), we proposed that this diet would also protect against the added insult of high energy radiation. Furthermore, the protective mechanism of this diet would be reflected in its ability to modulate the colonocyte redox environment to promote apoptosis.

Dietary fish oil and pectin did protect against colon carcinogenesis during the initiation stage of radiation enhanced AOM-induced colon cancer by promoting

apoptosis and attenuating oxidative DNA damage. Dietary pectin was able to enhance apoptosis regardless of the oxidative environment. However, only when pectin was combined with fish oil was there also a less of an increase in AOM-induced DNA damage as compared to the combination of corn oil and pectin. Unlike the previous experiment which measured ROS at only one timepoint, this study examined the change in the redox environment over a 24 h period using GSH/GSSG, a sensitive indicator of oxidative status. In this investigation, we demonstrate that in response to radiation, the combination of dietary fish oil and pectin promotes a more reduced cellular environment (elevated GSH/GSSG) as compared to the other experimental diets. This reduced environment enables the coloncytes to endure an additional stress of a chemical carcinogen without a significant increase in oxidative DNA damage.

In summary, the oxidative environment of intestinal epithelial cells is an important determinant in the resistance or susceptibility of a tissue to cancer development and the ability of dietary fish oil and pectin to favorably modulate this environment implicates it as an important environmental factor in colon cancer prevention. While the redox environment of the small and large intestine may explain, in part, the difference in cancer susceptibility, it is most likely not the only determining factor. Many differences have been discovered and others remain to be determined, all of which will continue to provide insight into the carcinogenic process in both of these tissues. The findings presented here also show promise in the ability of dietary fish oil and pectin to protect against colon carcinogenesis. Yet, further animal and human studies are necessary before recommendations can be made. These investigations are

also unique in that they examine the interaction of two dietary factors rather than an individual dietary component. Further studies into interactions of other diet matrix components may also yield more compelling evidence of the role of diet in the prevention of colon cancer.

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APPENDIX A

TABLES OF RESULTS

Table A1
GSH levels (nmol/mg protein)
Oil, radiation, time effects

	Corn oil					Fish oil				
	0 h	6 h	9 h	12 h	24 h	0 h	6 h	9 h	12 h	24 h
Non-irradiated	7.93^a $\pm$ 1.58	16.01^b $\pm$ 1.58	12.47^{bc} $\pm$ 1.58	9.00^{ac} $\pm$ 1.58	11.23^{ac} $\pm$ 1.68	7.54^a $\pm$ 1.68	7.79^a $\pm$ 1.58	9.51^{ac} $\pm$ 1.58	10.65^{ac} $\pm$ 1.58	9.79^{ac} $\pm$ 1.58
Irradiated	10.96^a $\pm$ 2.04	14.71^a $\pm$ 2.04	20.71^{b*} $\pm$ 2.28	9.33^a $\pm$ 2.04	10.43^a $\pm$ 2.28	11.19^a $\pm$ 2.04	18.49^{b*} $\pm$ 2.04	11.25^a $\pm$ 2.04	10.24^a $\pm$ 2.04	8.23^a $\pm$ 2.04

Values are means $\pm$ SEM for n=10 non-irradiated rats/timepoint/oil group and n=6 irradiated rats/timepoint/oil group.
In each row, means not sharing a common superscript are significantly different (p<0.05). In each column, means with a * are significantly different than the other mean in the column (p<0.05).
Oil*radiation*time interaction is significant (p=0.0121)
All p-values are two-sided.

Table A2
GSSG levels (nmol/mg protein)
Oil effect in both irradiated and non-irradiated rats

	Main effect	0 h	6 h	9 h	12 h	24 h
Corn oil	0.47 $\pm$ 0.02	0.46^{ab} $\pm$ 0.04	0.36^a $\pm$ 0.04	0.57^b $\pm$ 0.04	0.49^b $\pm$ 0.04	0.49^b $\pm$ 0.05
Fish oil	0.37* $\pm$ 0.02	0.34^a $\pm$ 0.04	0.36^a $\pm$ 0.04	0.39^{a*} $\pm$ 0.04	0.42^a $\pm$ 0.04	0.34^{a*} $\pm$ 0.04

Values are means $\pm$ SEM for n=16 rats/timepoint/oil group.
In each time point row, means not sharing a common superscript are significantly different (p<0.05). In each column, means with a * are significantly different than the other mean in the column (p<0.05). Main effect of oil (p=0.0002).
All p-values are 2 sided.

Table A3
GSH/GSSG
Diet, radiation, time effects

	Non-irradiated					Irradiated			
	Corn oil/ Cellulose	Corn oil/ Pectin	Fish oil/ Cellulose	Fish oil/ Pectin		Corn oil/ Cellulose	Corn oil/ Pectin	Fish oil/ Cellulose	Fish oil/ Pectin
0 h	19.38[a12] ± 4.46	20.13[a1] ± 4.64	18.68[a1] ± 4.30	20.02[a1] ± 5.15		21.61[a12] ± 6.43	19.99[a1] ± 5.94	31.04[a1] ± 9.23	38.24[a1] ± 11.38
6 h	29.67[ab1] ± 6.83	39.46[a2] ± 9.09	22.70[ab1] ± 5.23	19.87[b1] ± 4.58		36.21[ab12] ± 10.77	21.27[a1] ± 7.75	42.96[ab1] ± 12.77	54.57[b1]* ± 16.23
9 h	17.47[a12] ± 4.02	26.11[a12] ± 6.01	19.83[a1] ± 4.57	24.77[a1] ± 5.70		39.43[a2]* ± 14.36	16.51[b1] ± 4.91	24.66[ab12] ± 7.33	37.78[a1] ± 11.24
12 h	14.22[a2] ± 3.27	20.64[a1] ± 4.75	23.46[a1] ± 5.40	24.58[a1] ± 5.66		21.23[a12] ± 6.31	17.97[a1] ± 5.35	28.30[a12] ± 8.42	26.75[a1] ± 7.96
24 h	19.37[a12] ± 4.99	24.22[a12] ± 5.58	23.53[a1] ± 5.42	28.73[a1] ± 6.62		17.42[ab1] ± 5.18	26.24[ab1] ± 9.56	13.86[a2] ± 4.12	31.93[b1] ± 9.50

These values are geometric means ± SEM.

In each row, means not sharing a common letter superscript are significantly different (p≤0.05) within the irradiated or non-irradiated group. In each column, means not sharing a common numerical superscript are significantly different (p≤0.05).

* indicates mean is significantly different from non-irradiated group at same timepoint and diet. All p-values are two-sided.

Table A4
Apoptotic Index
Diet and radiation effects at 12 h post AOM

	Corn oil/ Cellulose	Corn oil/ Pectin	Fish oil/ Cellulose	Fish oil/ Pectin
Non-irradiated	4.34^a ± 0.90	3.23^a ± 0.90	3.47^a ± 0.90	4.09^a ± 0.90
Irradiated	4.42^{ab} ± 0.82	$6.00^{a}*$ ± 0.82	2.89^b ± 0.82	5.01^a ± 0.82

In each row, means not sharing a common superscript are significantly different (p<0.04). In each column, means with a * are significantly different than other means in the column (p<0.02).

Table A5
48-hour food intake (g)
Radiation effects

	Non-irradiated	Irradiated
Intake	36.94^a ± 0.39	39.09^b ± 0.37

Means not sharing a common superscript are significantly different (p<0.0001).

APPENDIX B

EXPERIMENTAL PROTOCOLS

Preparation of a Modified AIN-76A Diet
15% fat, 6% fiber

Equipment and Supplies:

1. The following ingredients are stored in Room 001L:
    Dextrose   Cellulose
    Casein   Mineral Mix
    Pectin

2. The following ingredients are stored in Dr. Lupton's or Dr. Chapkin's lab at room temperature:
    DL-methionine
    Choline Bitartrate (with desiccant)

3. The following ingredients are stored in a 4°C refrigerator in Dr. Lupton's or Dr. Chapkin's lab:
    Vitamin Mix   Tenox GT-1 (tocopherols)
    Vitamin E   Corn oil (under N_2)
    Tenox 20A (TBHQ)

4. The following ingredients are stored in the -20°C walk-in cooler in Dr. Chapkin's lab:
    Fish oil (under N_2)
    **REMEMBER TO REMOVE THE OIL EARLY AND ALLOW TO THAW

5. The two mixing bowls and beaters for the Hobart mixer can be obtained from Dr. Lupton's lab, and should be color coded with tape: yellow and orange for corn oil diets and green and blue for fish oil diets. The top loading scale from Dr. Lupton's lab can be carried downstairs and used to measure smaller quantities of nutrients. For larger quantities the large scale in Dr. Chapkin's lab may be used. All ingredients (except the oils) are weighed in the basement where the mixing will occur.

6. Obtain 50 1-2 gallon Ziploc plastic bags (12 bags for each diet, 1kg of diet per bag). Write the diet on each bag with a Sharpie and use a piece of colored tape for color-coding.   Yellow=Corn/Cellulose;   Orange=Corn/Pectin; Green=Fish/Cellulose; Blue=Fish/Pectin.

7. Supplies to bring to the basement:
   2 large mixing bowls and beaters (color coded) for the Hobart mixer
   1 top loading scale
   1 large scale
   12 large plastic containers with lids
   20 small plastic containers with lids
   50 Ziploc bags (1-2 gallon)
   4 spatulas (2 color coded for corn oil & fish oil diets)
   1 large metal spoon
   several scoops (at least one metal)
   several weigh spatulas (large)
   colored tape (yellow, orange, green, blue)
   paper towels, scissors, gloves, and sharpies

Weighing Ingredients:

**Make sure all equipment is clean before use.
Weigh out the ingredients to prepare each diet (12kg/diet).

Dr. Chapkin's lab:
Bring 4 extra large beakers (4000 ml), 2 medium beakers (1000 ml), vitamin E, Tenox GT-1, Tenox 20A and 4-5 long sterile pipets.

1. Before the large scale is moved, weigh out the amount of oil needed for each diet in separate beakers (extra large beakers-2 for corn oil & 2 for fish oil, medium beakers-corn oil for fish oil diet).

2. After weighing, add the small amount of corn oil needed for the fish oil diets to the fish oil.

3. There should now be four beakers with the appropriate weight of oils for each diet. Place one of the four beakers on the large scale and tare. Add vitamin E 5-67, Tenox GT-1, and Tenox 20A directly to the oil until the appropriate weight is achieved. Repeat this for the other beakers of oil.

4. Using a large sterile pipet (one for each oil), stir the oils for about one minute or until mixed well.

5. Keep the oils in a tightly covered container and on ice during the transfer to the basement and until they are ready to be added to the diet mixture.

6. Flush original stock containers of oil with nitrogen and return them to the refrigerator or freezer.

To the Basement:

Bring equipment listed in #5 of equipment and supplies.
Refer to tables attached for weights.

1. Place one of the large plastic containers on the large scale and tare. Using the plastic scoop, add dextrose to the container until the appropriate weight is reached. Remove from the scale, set aside and keep covered. Repeat this procedure until there are four separate containers of dextrose (one for each diet). **If there are not enough containers, weigh out ingredients for only one diet. After mixing, wipe out the containers and reuse for other diets.

2. Using the same procedure as above, weigh out casein and fiber (cellulose or pectin) for each diet.

3. Place a small plastic container on the top loading scale and tare. Using a weigh spatula, add mineral mix to the container until the appropriate weight is reached. Remove from the scale, set aside and keep covered. Repeat this procedure until there are four separate containers of mineral mix (one for each diet).

4. Using the same procedure as in #3, weigh out vitamin mix, DL-methionine, and choline bitartrate.

Mixing the Ingredients:

**IMPORTANT—PREPARE THE CORN OIL DIETS FIRST

1. Place the large container of casein on a stable surface. Add the following pre-weighed ingredients to the casein in the order listed, stirring briefly between each addition with the metal scoop.

   DL-methionine
   Choline bitartrate
   Vitamin Mix
   Mineral Mix

   Mix the above ingredients well, making sure to get rid of clumps (mineral mix tends to clump)

2. Using the large bowl for the Hobart mixer (not attached to the mixer yet), slowly pour in one of the containers of pre-weighed fiber (cellulose or pectin).

3. On top of the fiber, slowly pour in one of the containers of pre-weighed dextrose. Using clean gloves and the metal scoop, mix these two ingredients until fairly well blended.

4. On top of the dextrose/fiber mixture, slowly pour in the container of casein (mixed with smaller ingredients). Mix again with metal scoop until fairly well blended.

5. Make sure the Hobart mixer is clean and dust free.

6. Place the mixing bowl on the stand and latch it down on both sides. Attach the color-coded mixing beater, and raise the mixing stand until it is at its highest point.

7. Set the mixer speed to (1), the slowest speed. Set the timer for 2 minutes and press the start button. The machine will begin mixing.

8. After 2 minutes, check the bottom of the bowl to ensure that all the ingredients are well mixed. If not use the scoop to reach the bottom and stir slightly. Turn on the mixer again for 1 minute or until the ingredients are well mixed.

9. Obtain the appropriate oil for the diet being mixed. With the mixer speed still on (1), press the start button. Slowly pour about 1/5 of the oil into the dry ingredients staying in the area between the beater and the side of the bowl. It may be easier to stop and start the mixer for each addition to avoid pouring oil on the beater.

10. Once the oil initially poured appears to be well blended, add another 1/5 of the oil mixture and allow it to mix. Repeat this step until all of the oil mixture has been added.

11. Set the timer for 5 minutes and allow the mixer to blend on low speed (1).

12. After five minutes, use clean gloves and a color-coded spatula to clean the beater bar and the sides of the mixing bowl. Try to get as much of the mixture off of the spatula as possible.

13. Increase the mixer speed to (2), and blend for another 10 minutes to assure complete mixing.

14. After storing the prepared diet and cleaning, follow these same steps to prepare the other 3 diets.

Storage of Diets:

1. Remove the bowl from the mixer, being sure to scrape as much off of the beater bar as possible.

2. Using a clean plastic scoop, place a small amount of the diet mixture into a small ziploc bag labeled with the diet and date. This will be kept on ice and returned to Dr. Chapkin's lab for analysis.

3. Scoop the diet into the color-coded storage bags. Using the large scale, place approximately 1 kg in each bag and seal (remove as much air from the bag as possible).

4. Place the bags of diet in a box and store in Dr. Chapkin's –80°C walk-in freezer.

Clean-up:

1. Clean the large bowl and beater before making the next diet. **Remember to use a separate bowl and beater for the corn oil and fish oil diets.

2. Use detergent and the large sink in the equipment room to wash the large bowls. Make sure they are thoroughly clean. They can be sprayed with 70% ethanol and dried with a paper towel between each diet preparation. The smaller equipment can be washed there or in the lab.

3. Leftover dry ingredients should be closed and sealed in the box. Date the box for future reference and return it to Room 001L.

4. Date and seal other ingredients used and return them to their original locations (i.e. refrigerator, freezer).

5. Sweep and mop the floor of the equipment room.

6. Return all equipment to the appropriate place.

Ingredients for 12kg of
Corn Oil/Cellulose Diet

Ingredients	Weight in grams
Dextrose[1]	6127
Casein[2]	2682
Cellulose[4]	720
Mineral mix[5]	469
Vitamin mix[6]	134
DL-methionine[7]	41
Choline bitartrate[8]	26
Tenox GT-1[9]	3.6
Tenox 20A[10]	1.4
Corn Oil[11]	1800
Vitamin E[12]	0

Ingredients for 12kg of
Corn Oil/Pectin **Diet**

Ingredients	Weight in grams
Dextrose	6127
Casein	2682
Pectin[3]	720
Mineral mix	469
Vitamin mix	134
DL-methionine	41
Choline bitartrate	26
Tenox GT-1	3.6
Tenox 20A	1.4
Corn Oil	1800
Vitamin E	0

1   Bio-Serv 3400; technical grade
2   Bio-Serv 1100V; vitamin free
3   Danisco GRINDSTED Pectin 1100; from citrus peel
4   Bio-Serv 3425
5   Bio-Serv F8505; AIN-76A rodent diet
6   Harlan 40077; AIN-76A rodent diet
7   Bio-Serv 1340
8   Harlan 30190
9   ADM; 80mg/g D-α-tocopherol, 528mg/g total tocopherol

Ingredients for 12kg of
Fish Oil/Cellulose Diet

Ingredients	Weight in grams
Dextrose	6127
Casein	2682
Cellulose	720
Mineral mix	469
Vitamin mix	134
DL-methionine	41
Choline bitartrate	26
Tenox GT-1	0.81
Tenox 20A	0.35
Fish Oil[13]	1380
Corn Oil	420
Vitamin E	0.33

Ingredients for 12kg of
Fish Oil/Pectin Diet

Ingredients	Weight in grams
Dextrose	6127
Casein	2682
Pectin	720
Mineral mix	469
Vitamin mix	134
DL-methionine	41
Choline bitartrate	26
Tenox GT-1	0.81
Tenox 20A	0.35
Fish Oil	1380
Corn Oil	420
Vitamin E	0.33

10 ADM; 20% tert-butyl hydroquinone(TBHQ)

11 Traco Labs (provided by Sid Tracy)

12 ADM 410217; vit E 5-67, 672 mg α-tocopherol/gram

13 Menhaden oil; vacuum deodorized; provided by NIH
    Fish Oil Test Material Program

Isolation of Intestinal Cells for ROS test

Preparation-Several days before:

1. Prepare and aliquot stock solutions of BSA (10%), Glutamine (200X), Butyrate (200X) and EDTA (50X). Store EDTA at 4°C. Store BSA, Glutamine and Butyrate at -80°C.
   BSA: 100 X=10 g BSA / 100 ml HBSS-CaMg
   Gln: purchased from GibcoBRL at 200 mM (200 X)
   Butyrate: 200 X=2.2 g Butyrate / 100 ml HBSS-CaMg
   EDTA: 50 X=56 g EDTA / 100 ml HBSS-CaMg

2. Prepare RNase-free PBS, 70% ethanol, 50% ethanol, methacarn, and 4% paraformaldehyde.
   **See attached precautions to ensure RNase-free environment.
   PBS (for 4% paraformaldehyde):
   Mix 1 vial of Dulbecco's Phosphate Buffered Saline (w/o CaMg) into 990 ml RNase-free water until dissolved. Store at room temperature.
   This will make 10X PBS.
   Dissolve 20 ml of 10X PBS into 80 ml of RNase-free water to make 2X PBS.
   70% and 50% ethanol:
   1400 mL 100% ethanol + 600 mL RNase-free water = 2 liters of 70% ethanol
   Make 1.2 L / rat
   1000 mL 100% ethanol + 1000 mL RNase-free water = 2 liters of 50% ethanol
   Make 1 L / rat
   4% paraformaldehyde:
   **Do not breathe paraformaldehyde. Wear mask when weighing and perform other steps in a hood. Paraformaldehyde must be prepared fresh every week (100 mL/rat).
   Add 24 g of PFA to 300 mL RNase-free water.
   Stir and heat gently to 60°C (setting 2 on hot plate in Chapkin lab) for 5 minutes.
   Solution will be cloudy. Add 6-30 drops of 1 M NaOH until solution clears. Let cool.
   Add 300 mL 2X RNase-free PBS.
   Store at 4°C for up to 1 week.

Preparation-Day before:

1. Bake necessary equipment @ 180°C:
    50ml flasks (for shaking incubation) (1 / tissue)
    1000ml bottle (for PBS)
    1000ml bottles or flasks (for HBSS solutions)
    large magnetic stir bars (1 / HBSS solution)
    spatulas
    large glass graduated cylinders (for measuring HBSS & PBS)

2. Autoclave and RNase-zap rubber policeman for scraping tissue (1 / tissue).

3. Prepare 1X PBS if necessary
    Mix 1 vial of Dulbecco's Phosphate Buffered Saline (w/o CaMg) into 990 ml
    RNase-free H_2O until dissolved. Store at room temperature.
    This will make 10X PBS.
    Dissolve 100 ml of 10X PBS into 900 ml of RNase-free H_2O to make 1X
    PBS.

4. Set out equipment listed above and pre-labeled instruments and supplies:
    Surgical equipment (blunt and sharp scissors, forceps)

50ml centrifuge tubes	labeled RNase-free cassettes
petri dishes	25ml sterile pipets
pipet tips	sterile transfer pipets
2 well chamber slides	18x18 cover slips
50ml test tube racks	gauze pads
large and small weigh boats	biohazard bags
specimen cups	

Preparation-Day of Experiment

1. Arrive approximately 2 hours before scheduled time of kill. Turn on shaking water bath and bench top water bath. Warm to 37°C.

2. Remove BSA and Glutamine aliquots from -20°C freezer and Butyrate aliquots from -80°C freezer. Allow to thaw at room temperature.

3. Measure out amounts of buffers needed for procedure:
   1000ml 1X PBS
   980 ml HBSS+CaMg (1000 after additives)—generally 5 ml/cm tissue
   480ml HBSS-CaMg (500 after additives)—generally 5 ml/cm tissue
   Warm solutions to 37°C in water bath and bubble with $95\% O_2/5\% CO_2$ for 30 minutes. Solutions will remain at 37°C for the remainder of the procedure.

4. Prepare H_2O_2 and DPI solutions.
   H_2O_2:
   0.57 mL 30% H_2O_2 + 4.43 mL ddH_2O = 1 M H_2O_2
   0.25 mL of 1 M H_2O_2 + 4.75 mL ddH_2O = 50 mM H_2O_2 (wrap tube with foil)
   DPI:
   15.9 mg DPI + 10 mL ddH_2O = 5 mM DPI

5. After solutions are bubbled, add the previously prepared stock solutions to the appropriate buffer solution as described in the table below. Use RNase-free stir bars and spatulas.

	Amount to add in ml				Amount to add in mg
	EDTA 50X	Glutamine 200X	Butyrate 200X	BSA 10% (100X)	DTT (add as powder)
HBSS+CaMg (1000ml)	--	5	5	10	--
HBSS-CaMg (500ml)	10	2.5	2.5	5	385
Final Conc.	**30mM**	**1mM**	**1mM**	**0.1%**	**5mM**

6. Adjust HBSS solutions pH to 7.4.

7. Pour 70% ethanol, methacarn and PFA into specimen cups (put methacarn and PFA on ice) for fixations. **Remember RNase-free.

Crypt Isolation Procedure
**This procedure describes the isolation of all 4 tissues (duodenum, ileum, proximal colon and distal colon). However, only 2 tissues at a time will be isolated.

1. Euthanize rat by CO_2 asphyxiation (3 min) and cervical dislocation. Open abdomen with a ventral midline incision and perforate the diaphragm.

2. Cut the large intestine at the rectum. Cut one centimeter of most distal colon for cassetting. Measure six centimeters and cut again (distal colon). Continue to remove the large intestine up to the cecum. Cut intestine at cecum and take one centimeter from most proximal colon for cassetting. Measure six centimeters for the proximal colon. Place each six centimeter tissue segment in separate weigh boats of warm PBS.

3. Dispose of cecum. Cut the next seven centimeters of small intestine (ileum) and cut the most distal centimeter for cassetting. Continue to remove the small intestine up to the stomach. Cut the first six centimeters of small intestine (duodenum) and cut the most proximal centimeter for cassetting. Place each six centimeter tissue in separate weigh boats of warm PBS.

4. Flush duodenum from distal to proximal end with a sterile syringe and warm PBS. Cut open duodenum, ileum, proximal and distal colon to expose lumen and rinse in warm PBS. Place each tissue in separate 50ml flasks containing 30ml (5ml/cm of tissue) of HBSS-CaMg+EDTA+DTT buffer. Incubate in the shaking water bath for 15 minutes. Set the shaker to level #6.

5. After 7-8 minutes of incubation, aspirate the incubation solution of the duodenum and place in a 50ml sterile centrifuge tube. Add another 30ml of HBSS-CaMg+EDTA+DTT buffer to the tissue in the flask. Continue the incubation for another 7-8 minutes with #6 shaking.

6. Centrifuge the incubation solution from the duodenum at 100xg (800RPM) for 3 minutes at room temperature. Remove supernatant and resuspend in 30ml HBSS+CaMg to wash cells. Centrifuge again for 3 minutes, remove supernatant and resuspend in 15ml HBSS+CaMg. Keep the suspension at 37°C.

7. After the shaking incubations are completed pour the contents of each flask into separate sterile petri dishes. Gently scrape the mucosal side of the tissue with a RNase-free rubber policeman.

8. Using a sterile pipet, transfer the contents of the petri dish into a 50ml centrifuge tube and place in centrifuge.

9. Centrifuge the four cell suspensions at 100xg (800RPM) for 3 minutes at room temperature. Remove supernatant and resuspend in 30ml HBSS+CaMg to wash cells. Centrifuge again for 3 minutes, remove supernatant and resuspend duodenum cells in 15ml HBSS+CaMg. Resuspend the ileum, proximal colon and distal colon in 30ml HBSS+CaMg.

10. Combine each 15ml suspension of the duodenum for a total volume of 30ml. Keep all cell suspensions at 37°C and take to Image Lab.

At the Image Lab

Don't forget to bring:
warm cell suspensions	chamber slides
1.5ml microcentrifuge tubes	18x18 cover slips
H_2O_2	pipet tips and pipets
DPI	leftover HBSS+CaMg buffer

centrifuge and microcentrifuge tube racks for incubator
time sheet
extra timers if necessary

Testing for ROS

1. Hold all cells in 37°C incubator in cell culture room until needed.

2. Add 20 μL of DMSO to vial of CMH_2DCFDA (stored in freezer) and pipet up and down slowly to mix.

3. Aliquot necessary amount of viability dye, Ethidium Homodimer, (1 μL / tube) into microcentrifuge tube covered with foil.

4. Add 1 ml of HBSS+CaMg buffer and 25 μl of the appropriate cell pellet to the labeled microcentrifuge tubes. (We had duplicate tubes for each treatment.)

5. After adding treatment (according to time chart), invert microcentrifuge tubes to mix cells and incubate the suspensions for the appropriate time at 37°C. Cells will settle to the bottom of the tube.

6. Load 1μl of CMH$_2$DCFDA to each eppi tube of cells 15 minutes before the completion of the incubation time. Invert tube to mix cells.

7. When the incubation and loading are complete, transfer 2μl of the cell pellet to a 2 well chamber slide. Gently cover with an 18x18 cover slip (try to avoid smashing cells). Give to Rola for ROS analysis on Meridian Ultima.

8. After Rola has completed her analysis of a treatment, load 1 μL of Ethidium Homodimer to the cells remaining in the tube, invert tube and incubate at 37°C for 5 minutes.

9. After incubation with the viability dye is completed, transfer 2 μL of the cell pellet to a glass microscope slide. Cover with and 18x18 cover slip. At the Scanalytics microscope, place a drop of oil on the lens and place the microscope slide *upside down* on the stage. Using UV, select 10 different frames of cells and estimate the percentage that are green (viable). Red cells are not viable.

Apoptosis - ApopTag Kit

Note: To be performed on 4%PFA fixed tissue.

reagent	company	catalog #
Apotag Kit:	Chemicon	S7101
Proteinase K	Ambion	2546
PBS	Life Technologies	21600-069

***Put 200 ml PBS for Prot. K in 37° C oven and begin bleach rinse.

1. Deparaffinize and rehydrate tissue:
   Xylene, 3X, 5 min
   let xylene just dry, circle sections w/ PAP pen, dry 1 min
   100% EtOH, 2X, 5 min
   95% EtOH, 1X, 3 min
   70% EtOH, 1X, 3 min
   PBS, 1X, 5 min
   (Get Equilibration Buffer and Reaction Buffer out of freezer-put on ice)

2. Pretreat tissue – 3 min, in 37°
   Proteinase K (10 μg/ml PBS) = 0.1 ml Proteinase K (Ambion # 2546) in
   200 ml PBS.

3. Wash in dH$_2$O, 2x, 2 min

4. Quench Endogenous Peroxidase: 0.3% H$_2$O$_2$ in 100% Methanol:
   3.0 ml 30% H$_2$O$_2$ in 297 ml 100% Methanol or 2.0 ml in 198 ml(add fresh H$_2$O$_2$
   immediately before quenching). 30 min, RT

5. Wash in dH$_2$O, 2x, 5 min

6. Wash all slides in PBS 5 min.

7. Gently tap off PBS and carefully blot around sections. (Do this step and following
   step one slide at a time to avoid drying out sections.)

8. Apply EQUILIBRATION BUFFER to all sections: incubate in humidified chamber
   for 15 sec to 1 hr @ RT.
   (# of slides X 150 μl) (9 slides X 150μl = 1.35 ml)

9. Tap off equilibration buffer and immediately apply REACTION BUFFER (-controls) or working strength TdT Enzyme with dilution ratio 1/30 (enzyme /reaction buffer). (Get TdT directly from freezer & keep on ice)

Apply only reaction buffer to – control sections:

___(# sections) X 40μl

For normal sample sections (# sections X 40μl):
1080 μl reaction buffer (for 9 slides)
36 μl TdT enzyme (for 9 slides)

Incubate in a humidified chamber at 37°C, 1 hr
(Prepare Stop/Wash so it can warm to RT.)

12. Put slides in coplin jar with Working Strength Stop/Wash Buffer (1ml + 34 ml dH₂O). Agitate for 15 sec; incubate 10 min, RT.
Take aliquot of ANTI-DIGOXIGENIN PEROXIDASE (# slides X 125 μl) and allow to warm to room temperature (9 slides x 125μl = 1.125ml)

14. Wash slides in PBS, 3X, 1min

15. Blot dry the slides quickly (do one slide at a time) and apply ANTI-DIGOXIGENIN PEROXIDASE to all sections; incubate 30 min. in humidity chamber @ RT.

16. Wash in PBS 4X, 2min
Prepare DAB peroxidase (1:50, substrate:dilution buffer) (#slides x 150μl) and warm to room temperature. Protect from light. (9 slides X 150μl = 1350μl = 27μl substrate:1323μl dilution buffer)

17. Blot dry the slides quickly (do one slide at a time) and stain sections with DAB until light brown color shows up (≤ 1 min).

18. Wash in dH₂O, 3X, 1 min
Leave in 4ᵗʰ for 5 min

19. Counterstain w/ Methyl Green (reusable):
Dip quickly into Methyl green
Rinse in dH₂O 5X; dip 1x in the 1ˢᵗ 2 changes and briefly agitate
Dip 10 x in 3ʳᵈ & leave ~ 30 sec.
Leave in the last 2 for 1 min w/o agitation

20.Dehydrate: ALL FRESH
     70% EtOH, 1X, 1 min
     95% EtOH, 1X, 1 min
     100% EtOH, 1X, 1 min
     Xylene: 3X, 2 min (dip 10 times/ea)

21. Wet mount w/ Permount (80:20, Permount:Xylene) – leave overnight to dry

Apoptosis Scoring and Quantitation

Selection of crypts and villi
1. Examine positive and negative controls from each assay run to ensure the assay worked correctly every time.

2. Beginning with the right side of the slide, read sections from top to bottom.

3. Use the following criteria to determine scorable crypts:
   a. base of the crypt must touch or be very near the muscularis layer.
   b. crypts must be open to the luminal surface.
   c. crypt height must be easily determined by being able to count the cells on at least one side of the crypt as a continuous line.

4. Use the following criteria to determine scorable villi:
   a. villi base must be in contact with a crypt (crypt does not have to meet scorable crypt criteria)
   b. at least one side of the villi must be completely intact
   NOTE: if unable to obtain 25 intact villi columns, villi that are missing some surface cells but the upper curve is still apparent may be scored

   c. the top of the crypt and the bottom of the villi must be discernable by the slight outward curve on at least one side (the outward curve on the opposite side may be used as a reference)

Scoring crypts and villi
   1. Colon crypts which have met the scoring criteria are counted under 40X magnification and the following guidelines are used:
   a. the crypt is divided down the middle with an imaginary line and the cells along one or both sides of the crypt are counted (this is called the crypt column); cell number 1 is the first complete cell to the side of the imaginary line

   b. if an apoptotic cell is present, it is marked on the scoring sheet according to its location (see attached scoring sheet)
   c. cells are only counted to the point where the crypt begins to curve out
   d. if desired, 3-5 surface cells immediately following the top cell of the crypt may be scored (ensure that these cells are not beginning to curve into the neighboring crypt)
   e. if both sides of the crypt are counted, note this on the scoring sheet
   f. a total of 20-25 crypt columns/tissue are scored

2. Small intestine crypts are also counted under 20X or 40X magnification by the same method as the colon crypts. The only exception is that there are no surface cells since the location where the crypt curves out is the beginning of the villi.

3. Small intestine villi that have met the scoring criteria are viewed under 20X magnification and an image of the villi is captured using NIH image.

a. if the crypt leading to the villi was also scored, type the crypt number (from scoring sheet) over the crypt in the image and save; also mark on the crypt scoring sheet that the villi attached to that crypt was scored

b. when saving images, use a numbering system that ensures you will score the villi in the order that they were seen on the slide (left to right, top to bottom)
NOTE: This means that villi images must be captured at the same time crypts are being scored!! The crypt is scored immediately and the image of the villi is taken for scoring at a later time.

4. After images are collected, villi are scored in NIH image using the following guidelines:

a. divide the villi longitudinally down the center with an imaginary line as was done with the crypt and one or both of the sides (columns) will be used to determine villi height

b. to measure the height of a villi column, pixels must be converted to microns (see additional instructions below)

c. using the measuring line in NIH image toolbox, draw a line from the top of the villi to the crypt top (segmented line may be used to accommodate for curves in the villi); if some of the villi surface cells are missing, begin the line where the first cell in the column appears (this will be considered the top of the villi)

d. under the "Analyze" pull-down menu, select "Measure" and then select "Show Results" to display the length of the line in microns

e. divide the length of the line into thirds and using the line drawing tool, divide the villi into tertiles (the measuring line may have to be used again to determine the appropriate points at which to divide the villi)

f. count the number of apoptotic cells within each tertile and record this on the villi scoring sheet (see attached scoring sheet)

g. a total of 20-25 villi columns/tissue are captured and scored

Calculations

Apoptotic Index is used to normalize the number of apoptotic cells in a crypt column to the number of cells actually in a crypt column.

1. Apoptotic index for crypts:
a. for each crypt column
the number of apoptotic cells in a crypt column

the total number of cells in a crypt column
  b. for a tissue or rat
     average of apoptotic indices for crypt columns
2. Apoptotic index for surface cells:
  a. for each crypt column
     the number of apoptotic cells scored on the surface
         the total number of scored surface cells
  b. for a tissue or rat
     average of apoptotic indices for surface cells
3. Apoptotic index for villi:
  a. for each villi column
     number of apoptotic cells in a villi column
             height of villi in microns
  b. for a tissue or rat
     average of apoptotic indices for villi columns

NOTE: DO NOT calculate the rat or tissue apoptotic index with the following equation:
       (Total number of apoptotic cells/total number of crypt columns)
                           average crypt ht
       where the "average crypt ht" = total number of cells in a crypt column
                                      total number of crypt columns

Additional instructions:
    Measuring in microns
    1. use a stage micrometer to determine the number of pixels in a micrometer (be
       sure to use the correct magnification)
       10X   1μm = 0.5 pixels
       20X   1μm = 1 pixel
       40X   1μm = 2 pixels
    2. in NIH image, under the "Analyze" pull-down menu, select "Set Scale"
    3. change the units to microns and set how many pixels are in a micron
    4. step 3 needs to be done every time you open NIH image as it will reset back
       to pixels after it is closed

Other general information:
➢ These instructions are for NIH image software. Metamorph can also be used but the
  instructions will differ.
➢ The average time needed to completely score one colon slide is 1 hour.
➢ The average time to completely score one small intestine slide (crypts and villi) is 2-
  1/2 hours.

Preparation of cells for protein and antioxidant enzyme assays

Background: Cells were prepared using protocol for isolation of intact crypts. Cells were spun down in HBSS+Ca/Mg and the supernatant removed. Cells were frozen at –80.

Equipment:
pH meter (calibrated)
Microcentrifuge (4°C)
Sonicator (Chapkin Lab)

Reagents:
K_2HPO_4	Sigma
KH_2PO_4	Sigma
Sucrose	Sigma S7903
EDTA (disodium)	
Protease Inhibitor Cocktail	Sigma P8340
Triton X-100	Calbiochem 648464
Dithiothreitol (DTT)	Sigma D9779

Preparation of homogenization buffer (adapted from Dr. Wu's assay for enzyme extraction)

1. Prepare 50mM Potassium Phosphate buffer
   --50mM K_2HPO_4: Dissolve 4.35g of K_2HPO_4 in 500ml of deionized H_2O
   --50mM KH_2PO_4: Dissolve 3.4g of KH_2PO_4 in 500ml of deionized H_2O (use 1L bottle)
   --50mM potassium phosphate buffer (pH 7.2): Add 50mM K_2HPO_4 to 50mM KH_2PO_4 until pH is 7.2

2. Prepare 250mM Sucrose/1mM EDTA solution by dissolving 42.8g of sucrose and 186mg of disodium EDTA in 500ml of potassium phosphate buffer. pH again to 7.2 (use KOH or HCl)

3. Prepare homogenization buffer (250mM sucrose/1mM EDTA/1mM DTT in 50mM potassium phosphate buffer):
   5ml - 250mM sucrose/1mM EDTA solution
   200μl – protease inhibitor cocktail
   5μl – Triton X-100 (0.1%)
   5μl – 1M DTT solution (made fresh)
   0.3858 mg DTT in 2.5mL Phosphate Buffer

   Homogenization buffer should be prepared fresh each day.

Cell homogenization

1. Thaw cell pellet in appropriate amount of homogenization buffer. Aspirate cell pellet through 27 gauge needle several times. Sonicate cells briefly (2-3 seconds) at 20-second intervals for about 1 minute (distal colon may need >1minute).
   4-5cm Distal colon - 150μl
   4cm Duodenum – 200 μl
   8cm Duodenum – 350 μl
2. Transfer crypt suspension to microcentrifuge tube.
3. Centrifuge cells for 3 minutes @ 10000 x g (4°C).
4. Transfer supernatant to clean microcentrifuge tube. (Pellet may be discarded or examined with Trypan Blue to determine efficiency of lysis process.)
5. Supernatant can be aliquoted appropriately for assays:
   a. 10μl for protein
   b. remainder for antioxidant enzyme assays
6. Aliquots of homogenization buffer will need to be saved for protein assay and aliquots may be needed for dilution of samples for assays.

Coomassie Protein Assay

Equipment:

Microtiter plate reader (A595)

Reagents:

Coomassie Plus Protein Assay Kit   Pierce 23236
        contains Coomassie Blue stain
                BSA standards (2mg/ml)

Procedure:

1. Prepare BSA standards:
   2μg/μl (in kit)
   500μl of 2μg/μl + 500μl ddH$_2$O = 1μg/μl
   125μl of 2μg/μl + 1000μl ddH$_2$O = 0.25μg/μl
   (this is sufficient for only one set of standards)
2. Prepare microcentrifuge tubes of standards and samples **in triplicate.** (Add Coomassie to all tubes last.)

Standards:

μg protein	0.25μg/μl BSA	1μg/μl BSA	2μg/μl BSA	Water	Homog. Buffer	Coomassie Reagent
0	0μl	-	-	497.5μl	2.5μl	500μl
1	4μl	-	-	493.5μl		
2	-	2μl	-	495.5μl		
4	-	4μl	-	493.5μl		
10	-	10μl	-	487.5μl		
20	-	-	10μl	487.5μl	↓	↓

Samples:

Amt. of sample	Water	Coomassie Reagent
2.5μl	497.5μl	500μl

3. Incubate samples in Coomassie at RT for 10 minutes.
4. Transfer 300μl of each tube to the appropriate well on a microtiter plate.
5. Read absorbance (A595) on microtiter plate reader.
   (Absorbances for standards generally range from 0.3 to 1.0.)
6. Plot standard curve (absorbance vs. μg protein). Most plate readers will do this for you.
7. Use readout of "unknowns" to determine protein concentration of samples.

Antioxidant Enzyme Assays
Catalase, Glutathione Peroxidase and Superoxide Dismutase

Background: Protein was determined by Pierce Coomassie Assay.

Equipment:
Microtiter plate reader (Bio-Tek)
Microtiter plates (Fisher, flat bottomed, 360ul)

Reagents:
Catalase Assay Kit   Calbiochem 219263
Glutathione Peroxidase Kit   Calbiochem 354104
Superoxide Dismutase Kit   Calbiochem 574600
Superoxide Dismutase   Calbiochem 574594
Sodium Azide (NaN$_3$)   Sigma S8032
30% H$_2$O$_2$ Sigma H1009

Catalase
Based on kit instructions. Protein levels must be determined prior to assay (Coomassie Blue.)

Prepare CAT standard and dilutions:
1. Add appropriate amount of deionized water to Standard vial (volume recommended on vial or by tech support – generally 1.1 ml)
2. Prepare dilution aliquots by combining the indicated volumes of standard as described in the table below.

Standard, µL	0	26.4	56.8	133.6	240	400
Sample Diluent, µL	400	400	400	400	400	400
Final Activity, U/mL	0	10	20	40	60	80

Freeze 25µL aliquots at –20°C. Stable for 1 month.

Prepare Reagents:
1. Add 1 volume of HRP to 1000 volumes of Chromogen reagent. Stable for one month if stored sealed and protected from light at 4°C. If reagent develops a slight pink color it has deteriorated and must be replaced.
2. Add 1 volume of 30% H_2O_2 to 1000 volumes of Substrate diluent. Stable for one week if store sealed and protected from light at 4°C.
Reagents must be brought to room temperature before use.

Prepare microplate reader (Lupton Lab – BioTek) by opening protocol and preparing for read. Allow reader to calibrate before use. Only do, at the most, 6 samples (in triplicate) at one time.

Assay:
1. Dilute samples with homogenization buffer according to protein content (approx 3µg/µL).
2. Prepare **triplicate** 1.7ml microcentrifuge tubes and add 3µL of the following:
   a. 0 U CAT std (Sample diluent)
   b. 10 U CAT std
   c. 20 U CAT std
   d. 40 U CAT std
   e. 60 U CAT std
   f. 80 U CAT std
   g. Sample
3. Add 3µL of protein buffer to standard tubes. Add 3µL of sample diluent to sample tubes.
4. Add 50µL of 10mM H_2O_2 to each tube and incubate at RT for 1 min. (Can do 18 tubes during this minute. Have Stop Reagent ready immediately.)
5. Add 50µL of Stop Reagent. Mix well but do not vortex.
6. Add 3µL of reaction mixture to wells.

7. Add 300µL of HRP/Chromogen Reagent to microplate wells. Mix well.
8. Incubate in the dark at RT for 10 minutes.
9. Read absorbance at 520nm.

Glutathione Peroxidase

Based on kit protocol with modifications - use NaN_3 and H_2O_2 instead of tert-butyl hydroperoxide.[1]

Prepare Reagents:
1. Add 7.5mL of assay buffer to vial of NADPH. Mix well. Keep on ice or refrigerated and protected from light. Reagent is only good for 24 hours. Do not refreeze.
2. Prepare 700μM H_2O_2. Add 1.134mL of 30% H_2O_2 to 8.866mL H_2O to make 1M H_2O_2. Add 7μL of 1M H_2O_2 to 9.993mL H_2O to make 700μM H_2O_2. Store at 4°C and protect from light. Stable for 1 week.
3. Add 0.325g of NaN_3 to 5mL of phosphate buffer to make 100mM NaN_3. Aliquot if needed and keep refrigerated until use.
   All reagents must be brought to RT before use.

Prepare microplate reader by opening protocol and preparing for read. Allow reader to calibrate before use.

Assay:
1. Dilute samples with homogenization buffer according to protein content (>7μg/μL pro).
2. Prepare **triplicate** microplate wells of each column by adding the following reagents in the order listed:

Assay Buffer, μL	75	75
NADPH, μL	75	75
NaN_3, μL	2.4	2.4
Sample, μL	--	15
Buffer, μL	15	--

3. Place plate in tray of the reader. Add 75μL of 700μM H_2O_2, mix well on plate reader plate shaker (intensity 2 for 15 seconds) and read absorbance at 340nm as often as possible for three minutes.

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[1] Tert-butyl hydroperoxide is a substrate for some glutathione S-transferases. Therefore, H_2O_2 is the preferred substrate in cell lysates and tissue homogenates. However, catalase must also be inhibited which is accomplished by the addition of 1mM sodium azide (NaN_3) (Flohe and Gunzler, Methods in Enzymology, 1984).

Superoxide Dismutase
Based on kit protocol.

All reagents and microplate reader must be at 37°C before use.

Prepare microplate reader by opening the protocol entering the template and setup.

Assay:
1. Dilute samples with homogenization buffer according to protein content ($>6\mu g/\mu L$).
2. Prepare **triplicate** microplate wells by adding the following reagents in the order listed:

Buffer, µL	225
Sample, µL	10
R2, µL	7.5

3. Mix well after addition of R2 and incubate samples for at least 1 minute at 37°C.
4. Add 7.5µL of R1 and mix well. (Have computer ready to read and plate sitting in reader tray.)
5. Immediately read absorbance at 525nm as frequently as possible for 3 minutes.

Helpful tip: Put R1 reagent in wells of an empty plate – draw it out with a multi-tip pipettor and add to samples.

Immunohistochemistry of 8-hydroxy-2'-deoxyguanosine

Note: For 70%EtOH fixed tissue.

<u>Day 1</u>

___**1. Deparaffinize slides and rehydrate tissue:**
    ___3 x 5 min Xylene
    ___Let slide just dry, circle sections with PAP pen, dry 1 min
    ___2 x 2 min 100 % ETOH
    ___2 x 2 min 95 % ETOH
    ___1 x 2 min 70 % ETOH
    ___1 x 5 min H_2O

___**2. Wash in TBS for 2 min x 2.** Gently tap off TBS and blot around sections. (Do this step and following step one slide at a time to avoid drying out sections.) (Prepare RNase dilution.)

___**3. Treat sections with RNase** (100 μg/ml Tris buffer) **for 1 h at 37 °C in humidity chamber.**
(Tris buffer recipe and RNase prep listed on separate sheet.)

___**4. Wash in TBS for 2 min x 3.**

___**7. Denature DNA by placing slides in 4 N HCl for 7 min at RT.**
(HCl recipe on separate page.)

___**8. Neutralize with 50 mM Tris base for 5 min at RT.**
(Tris base recipe on separate page.)

___**9. Wash in TBS for 5 min x 2.** Gently tap off TBS and blot around sections. (Do this step and following step one slide at a time to avoid drying out sections.) (Prepare 10% serum.)

___**10. Incubate sections with 10% rabbit serum** (Jackson #011-000-120) **in TBS 1 h at RT in humidity chamber.**
(Prepare primary Ab dilution.)

___**11. Incubate sections with primary Ab** (1:20 in TBS w/ 1% rabbit serum (50mM Tris)) (Oxis #24325) **at 4 °C in humidity chamber overnight.** (Recipe for TBS with 50mM Tris on separate page.)

Day 2

___**12. Wash in TBS for 5 min x 3.** Gently tap off TBS and blot around sections. (Do this step and following step one slide at a time to avoid drying out sections.) (Prepare secondary ab dilution.)

___**13. Incubate sections with biotinylated rabbit anti-mouse** (1:800 in TBS w/ 1% rabbit serum (remember already diluted 1:1 in glycerol)) (Jackson #315-065-045) **45 min at RT in humidity chamber.**

___**14. Wash in TBS for 5 min x 3.**

___**15. Apply 3% H$_2$O$_2$** (Sigma #H-1009) **in methanol for 30 min** to quench endogenous peroxidase.
(Add 1ml H$_2$O$_2$ for every 10 ml of methanol. Add H$_2$O$_2$ immediately before quenching.)
(Prepare ABC-HRP and let stand for 30 min.)

___**16. Wash in TBS for 5 min x 3.** Gently tap off TBS and blot around sections. (Do this step and following step one slide at a time to avoid drying out sections.)

___**17. Incubate with ABC-HRP kit (Vector #PK-6100) for 1 h at RT in humidity chamber.**
1) 5 ml 1X TBS
2) Add 2 drops of reagent A and mix well
3) Add 2 drops of reagent B and mix well

___**18. Wash in TBS for 5 min x 3.** Gently tap off TBS and blot around sections. (Do this step and following step one slide at a time to avoid drying out sections.)
(Prepare DAB and water rinse.)

___**19. Apply DAB** (Vector #SK-4100) **stain for 1 min** (or until brown stain shows up). Tap off DAB, rinse briefly with ddH$_2$O and place in ddH$_2$O. Finish one slide before moving to next slide.
1) 5 ml dH$_2$O.
2) 2 drops of buffer, mix.
3) 4 drops of DAB, mix.
4) 2 drops of H$_2$O$_2$, mix.

___**20. Wash in ddH$_2$O for 1 min x 2.**

___**21. Dehydrate slides:**
1 x 1 min 70 % ETOH.

1 x 1 min 95 % ETOH.
1 x 1 min 100 % ETOH.
1 x 2 min Xylene.

___**22. Mount cover glass with Permount** (Fisher #SP15-500).
    (Permount diluted - 20% xylene, 80% permount.)

Recipes for 8OHdG

TBS (20mM Tris, 0.9% NaCl, pH 7.5) (Sigma) – all washes and dilutions except primary ab
As received: 10X Concentrate
Dilution: 1X with ddH_2O

Adjust pH to 7.5 with HCl. Prepare as needed.

TBS (50mM Tris, 0.9% NaCl, pH 7.5) – for primary ab dilution only
121.1g/mol x 0.05mol/L =   6.055 g/L Tris base (Sigma #T-1503)
0.9% NaCl x 1000ml =   9g NaCl (Sigma)
                            1 L ddH_2O

Adjust pH to 7.5 with HCl. Prepare as needed.

Tris Buffer (10mM Tris, 1mM EDTA, 0.4M NaCl, pH 7.5) – for RNase dilution
121.1g/mol x 0.01mol/L =   1.211 g/L Tris base (Sigma #T-1503)
380.2g/mol x 0.001mol/L = 0.3802 g/L (Sigma # ED4SS)
58.44g/mol x 0.4mol/L =   23.38 g/L (Sigma #BP358-1)
                            1L ddH_2O

Adjust pH to 7.5 with HCl. Prepare as needed.

Tris base (50mM)
121.1g/mol x 0.05mol/L =   6.055g/L
                            1L ddH_2O

Do not pH. Prepare as needed.

RNase (Ambion #2272)
As received: 1ml [1mg/ml]
Dilution: 100ug/ml Tris buffer

100ul stock + 900ul Tris buffer = 1ml of 100ug/ml

Remove aliquot on day of assay. Add Tris buffer to appropriate dilution.

4N HCl
As received: 30M solution 9 (EM Science #HX0603-3)
Dilution: 4M solution

Normality = molarity x number of H+ released by acid
4N HCl = 4M HCl

4M/30M = 0.13
130ml of 30M HCl
870ml of ddH$_2$O

Prepare as needed.

Rabbit Serum (Jackson #011-000-120)
As received: Freeze dried
Reconstitution: Add 5ml ddH$_2$O (100% serum) – good for 6 weeks at 4°
Dilution: 10% serum – good for 1 day

1ml 100% serum
9ml TBS

Remove aliquot on day of assay. Add TBS to appropriate dilution.

Primary Antibody (Oxis # 24325)
As received: Freeze dried   20 ug vial   100 ug vial
Reconstitution: 100ug/ml   0.2 ml ddH$_2$O   1ml ddH$_2$O   --good
1month at 4°
Dilution: 5-10ug/ml with TBS (50mM Tris)

Remove aliquot on day of assay. Add TBS to appropriate dilution.

Secondary Antibody (Rabbit anti-mouse: Jackson #315-065-045)

As received: Freeze dried

Reconstitution: Add 1ml ddH$_2$O – good for 6 weeks at 4°

                               Add equal portion of glycerol (1ml) – store in -20°

Dilution: 1:600 in TBS (1:300 if in glycerol)

2ul antibody / 4ul antibody (in glycerol)
1200ul TBS

Remove aliquot on day of assay. Add TBS to appropriate dilution.

3% H$_2$O$_2$ in methanol

30% H$_2$O$_2$ (Sigma H-1009)
100% Methanol

Add 1ml of 30% H$_2$O$_2$ to every 10ml of MeOH for 3% H$_2$O$_2$.

Determination of GSH and GSSG
by high-performance liquid chromatography with fluorescence
detection of dansyl derivatives

Obtained from Dr. Wu & performed in his lab. Original protocol from Jones et al.[2]

***Protocol performed on scraped mucosa frozen in liquid nitrogen and stored at -80°C.

Chemicals & supplies needed:

Name	Supplier & Catalog #
Sodium Heparin	Sigma H4784
Bathophenanthroline disulfonate sodium salt (BPDS)	Sigma B1375
Iodoacetic acid	Sigma I2512
Dansyl chloride	Sigma 39220 (Fluka)
L-serine	Sigma S4500
GSH standard	Sigma G6529
GSSG standard	Sigma G6654
Sodium acetate trihydrate	Sigma S9513
Boric acid	Sigma B0394
Sodium tetraborate	Sigma B0127
70% Perchloric acid	Fisher A469
r-glutamylglutamate (r-glu-glu)	Sigma G3640
HPLC grade water	Fisher W7
Potassium hydroxide (KOH)	Sigma P5958
Potassium tetraborate tetrahydrate	Sigma P5754
Acetone	Fisher A949
Chloroform	Fisher C606
Glacial acetic acid	Bio/Bio
Methanol (HPLC grade)	Fisher A452
Helium gas	in lab
3-aminopropyl column	CEL Associates #132-204 Ph# 800-537-9339 Pearland, TX

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[2] Jones, DP et al. Glutathione measurement in human plasma: evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. *Clinica Chimica Acta* 275, 175-184 (1998).

Other supplies & equipment needed:

balance	tweezers
1.5 ml eppi tubes (2/sample)	ice & ice chest
vortex	mini vortexer
mini centrifuge	HPLC glass vials with springs, inserts & lids
pipet and tips	calculator
HPLC vial racks	

Preparation of Reagents:

A. Prepare in advance and store at room temp (except preservation & perchloric acid solution – store at 4°C).

1. 100 mM boric acid stock solution:
   Add 0.62 g boric acid into 100 ml HPLC water.

2. 100 mM sodium tetraborate stock solution:
   Add 2.0 g sodium tetraborate to 100 ml HPLC water. May need to stir several hours to get into solution – may not completely go into solution.

3. Preservation solution:
   8 ml of 100 mM boric acid
   2 ml of 100 mM sodium tetraborate
   105 mg L-serine
   5 mg sodium heparin
   10 mg BPDS
   20 mg iodoacetic acid
   gas with helium for 30-45 min, store at 4°C

4. Perchloric acid solution (10% (w/v), 0.2 M boric acid, 10 µM r-glu-glu):
   300 ml HPLC water
   6.2 g boric acid
   1.38 g r-glu-glu
   71 ml of 70% perchloric acid
   adjust to 500 ml total volume with HPLC water
   gas with helium for 30-45 min, store at 4°C

5. KOH/tetrahydroborate solution:
   5.6 g KOH
   50 g Potassium tetraborate tetrahydrate ($K_2B_4O_7 \cdot 4\ H_2O$)
   100 ml HPLC water

    **Stir for a few hours. This is a supersaturated solution and will not completely dissolve. Allow solute to settle before using. Only use liquid layer.

6. HPLC solvents
    Acetate stock:
        272 g sodium acetate trihydrate
        122 ml HPLC water
        378 ml glacial acetic acid
    Solvent A / Acetate buffer (pH=4.6):
        640 ml methanol
        200 ml acetate stock
        125 ml glacial acetic acid
        50 ml HPLC water
    Solvent B / 80% (v/v) Methanol
        800 ml methanol
        200 ml HPLC water

B. Prepare weekly and keep at 4°C.

1. GSH & GSSG standard (50 µM or nmol/ml)

for 5 mM GSH solution:	for 5mM GSSG solution:
1.51 mg GSH	3.1 mg GSSG
500 µL preservation solution	500 µL preservation solution
500 µL perchloric acid solution	500 µL perchloric acid solution

    for 50 µM GSH & GSSG working standards:
        10 µL of 5 mM GSH or GSSG
        445 µL preservation solution
        445 µL perchloric acid solution
    for 50 µM mixed GSH/GSSG working standard:
        10 µL of 5 mM GSH
        10 µL of 5 mM GSSG
        440 µL preservation solution
        440 µL perchloric acid solution

C. Prepare fresh on day of derivitization and keep at room temp.

1. Iodoacetic acid solution:
    2 ml HPLC water
    14.8 mg iodoacetic acid

2. Dansyl chloride solution:
    40 mg dansyl chloride
    2 ml acetone - must prepare in glass tube (acetone will eat through plastic),
        keep in the dark.

Procedure:

A. Preparing samples for homogenization.

1. Remove cryo tubes with sample from -80°C and keep on ice.
2. Wipe excess moisture from cryo tube and weigh on balance. Record wt.
3. Remove tissue from inside cryo tube and place in 1.5 ml eppi tube – close eppi tube.
4. Weigh empty cryo tube and record wt. Subtract empty tube wt from tube wt with sample to determine the wt of the sample.
5. Immediately add 100 µL of preservation solution and 100 µL of perchloric acid solution for every 10 mg of tissue (e.g. 20 mg tissue – 200 µL preservation solution + 200 µL perchloric acid solution). Make sure tissue is covered with solution, close eppi tube and place back on ice. Move to next sample.

B. Homogenization and preparation for derivitization.

1. Using mini vortexer, homogenize tissue samples in preservation/perchloric acid solution. Be careful not to splash any solution out.
2. After each sample is homogenized, add the same volume of preservation solution and perchloric acid solution as was added prior to homogenization. (e.g. 20 mg tissue – homogenized in 400 µL of equal parts preservation & perchloric acid solution – add another 200 µL preservation solution + 200 µL perchloric acid solution. Final volume = 800 µL). Vortex on standing vortexer (not mini vortexer) to mix well.
3. Centrifuge tubes at 10,000 x g for 1 minute.
4. Transfer 150 µL supernatant to a clean eppi tube. Store remainder at 4°C. Transferred supernatant may stay at room temperature.
5. Prepare standards and blank for derivitization.
   GSH standard: 150 µL
   GSSG standard: 150 µL
   mixed standard: 150 µL
   blank: 75 µL preservation solution + 75 µL perchloric acid solution

C. Derivitization

Day 1
1. To the 150 µL supernatant (or standard/blank) add the following:
   30 µL iodoacetic acid
   100 µL KOH/tetraborate solution
   vortex and incubate 20 min at RT. (A precipitate will form.)
2. Add 150 µL dansyl chloride solution, vortex and keep at RT in the dark 18-26 hours.

<u>Day 2</u>
3. Add 250 µL chloroform to extract unreacted dansyl chloride. (Samples can be stored at this point – as is with perchlorate precipitate and chloroform - at 4°C until assayed by HPLC)
4. Top layer is used for HPLC analysis.

D. HPLC analysis

1. Install column and prep column according to manufacturer's instructions.
2. Open Glutathione protocol* on HPLC computer. Make sure autosampler and detector are on and programmed correctly (Dr. Wu's laboratory personnel can help). Make sure plenty of Solvent A & B are present.
3. Pipet 50-150 µL of top layer of sample/standard/blank into an HPLC vial. Screw on lid and vortex to remove air bubbles and ensure sample gets to the base of the vial.
4. Insert vial(s) into the autosampler and start run on computer and autosampler.
5. Each sample runs for 45 minutes. Do not load more than 13 or 14 samples to minimize oxidation that may occur with extended time at room temperature.

*Protocol details:

Detector:	fluorescence, 335 nm excitation, 610 nm emission, gain 1000	
Injection volume:	10 µL	
Flow rate:	1.0 ml/min	

Gradient:

Min	Solvent A	Solvent B
0	20	80
10	20	80
30	80	20
35	0	100
38	20	80
45	20	80

Peak times: GSH 27-28 min
GSSG 32-33 min

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Education

Institution	Degree	Discipline	Graduation Date
Texas A&M Univ.	B.S. (honors)	Nutritional Science	August 1998
Texas A&M Univ.	Ph.D.	Nutritional Science	May 2005

Societies & Professional Activities

Registered Dietitian
ASNS, SEBM, Sigma-Xi Scientific Honor Society
Graduate Student Council Representative
Mentor – NSF/ONR undergraduate summer research program
Judge – SEBM travel grant awards
      Texas Junior Science & Humanities Symposium
Instructor – Senior nutrition seminar (NUTR 481)
Teaching Asssitant – Intro to Nutr (NUTR 203), Physiology of Nutrition (NUTR 470)
Search Committee – Dietetic Internship Director

Honors & Awards

Scholarships – Board of Regent's fellowship, IFN scholarship, IFSE scholarship
Travel Awards – IFN travel grant, SEBM travel grant
Finalist – ASNS/Proctor & Gamble Student Oral Competition (EB 2002, EB2003)
Recipient of Sigma-Xi Grant-in-aid of research

Publications (abstracts excluded)

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   MY Hong, RS Chapkin, R Barhoumi, RC Burghardt, ND Turner, CE Henderson, LM Sanders, YY Fan, LA Davidson, ME Murphy, CM Spinka, RJ Carroll, and JR Lupton. Fish oil increases mitochondrial phospholipids unsaturation, upregulating reactive oxygen species and apoptosis in rat colonocytes. (2002) Carcinogenesis 23:1919-1925.

   LM Sanders, CE Henderson, MY Hong, R Barhoumi, RC Burghardt, RJ Carroll, ND Turner, RS Chapkin, and JR Lupton. Pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility. (2004) Cancer Letters 208(2):155-161.

   LM Sanders, CE Henderson, MY Hong, R Barhoumi, RC Burghardt, N Wang, CM Spinka, RJ Carroll, ND Turner, RS Chapkin and JR Lupton. An increase in reactive oxygen species by dietary fish oil coupled to the attenuation of antioxidant defenses by dietary pectin enhances rat colonocyte apoptosis. (2004) Journal of Nutrition 134: 3233-3238.