QUERCETIN AND DIETARY LIPIDS ALTER THE CELLULAR REDOX ENVIRONMENT OF THE COLONOCYTE IN THE PROMOTION STAGE OF COLON CARCINOGENESIS

A Thesis

by

KIMBERLY JONES PAULHILL

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

MASTER OF SCIENCE

August 2008

Major Subject: Nutrition

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Approved by:

Chair of Committee,	Nancy D. Turner
Committee Members,	Joanne R. Lupton
	Robert S. Chapkin
	Edward D. Harris
Chair of Nutrition Faculty	Stephen B. Smith

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ABSTRACT

Quercetin and Dietary Lipids Alter the Cellular Redox Environment of the Colonocyte in the Promotion Stage of Colon Carcinogenesis. (August 2008)

Kimberly Jones Paulhill, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Nancy D. Turner

Quercetin (Q), a water-soluble flavonoid that is ubiquitous to foods of plant origin is postulated to protect against colon cancer due to its antioxidant activity. In contrast, we have shown that a dietary combination of fish oil (FO; n-3 fatty acids) and pectin may protect against colon cancer by decreasing endogenous antioxidant enzyme activities leading to increased reactive oxygen species (ROS), an inducer of apoptosis. We hypothesized that adding an antioxidant to a FO diet may negate the beneficial effects of FO by counteracting FO effects on colonocyte redox status. To test this, we provided 40 rats with FO or CO (fiber = pectin) diets with Q being 0 or 0.45% of the diet for 10 wk. All rats were injected with azoxymethane (AOM) on d 21 and 28. Measurements included: aberrant crypt (AC) enumeration (colon cancer marker); apoptosis (TUNEL assay); catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities; reduced and oxidized glutathione concentrations (GSH/GSSG); and oxidative DNA damage (8-OHdG adducts). AC numbers were lower in FO vs CO rats ($p<0.0001$), but tended to increase for FO diets containing Q ($P<0.098$). The apoptotic index was higher ($p<0.0001$) when Q was added to the FO and CO diets. Total SOD (lipid main effect, $p=0.0136$) and GPX activity ($p=0.0025$) was elevated in CO rats. CAT activity was higher (p=0.0204) in FO rats, however Q diminished this effect. GSH was not affected by diet; yet, GSSG accumulated (p=0.0554) in CO rats with Q as compared to CO rats without Q. The GSH/GSSG ratio was lower (p=0.0314) in CO rats than in FO rats. There was no difference in 8-OHdG adduct levels in FO vs CO rats, however, Q decreased 8-OHdG adducts in CO rats (p=0.0428). Despite increasing apoptosis, Q did not significantly lower AC formation. These data suggest that the distinct effects of the CO/Q and FO/Q combinations are functioning through different mechanisms to induce apoptosis. The long-term consequences of adding antioxidants such as Q to a diet thought to exert its anticancer effect through a pro-oxidant mechanism are unknown and deserve further study.

DEDICATION

In honor of my parents,

Michael and Janet Jones,

Who taught me to live my dreams and always keep God first.

Thank you to my husband,

DuWayne Paulhill,

For your unending support, friendship, and love.

And to my children,

Faith, DuWayne Jr., and Vivica,

For sharing me with the world.

You bring such joy, happiness, and purpose to my life.

Thank you all for supporting and allowing me to be the best person that I can be.

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CHAPTER I

INTRODUCTION

Colon cancer is one of the leading causes of cancer related deaths in the United States. Colon cancer is a multi-step process progressing to an invasive stage over many years. Oxidative DNA damage is one possible contributor to cancer or tumor formation in the intestine (1-3). Studies have revealed that a sustained increase over time in reactive oxygen species (ROS) correlates positively with an increase in oxidative DNA damage initiating malignant cells (4,5).

Colon cancer has been shown in previous studies to be influenced by diet, and several studies have revealed that the level of consumption and type of dietary fat and fiber are two of the most significant dietary determinants for the risk of colorectal cancer (6-12). A diet rich in n-3 PUFAs, such as those found in fish oil, in combination with the fermentable fiber, pectin, is shown to be protective against an experimental model of colon carcinogenesis in rats at each stage of carcinogenesis from initiation to tumor formation (7,9,13). In contrast the dietary combination of corn oil and cellulose has been shown to be promotive of tumor formation (7). The combination of dietary fish oil and pectin resulted in an increase in apoptosis (a form of programmed cell death) along with a decrease in the level of cellular DNA damage, which proved to be beneficial in suppressing tumor formation (14,15). One proposed mechanism for the effectiveness of the dietary combination of fish oil and pectin is an increase in ROS inducing more

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apoptosis. This increase in apoptosis leads to a decrease in the number of damaged cells (14,16-18). Another mechanism postulated to support the effectiveness of a fish oil pectin diet is not only the significant increase in ROS but also a concomitant decrease in antioxidant enzyme capacity (9). These changes occurring in colonocytes should also contribute towards a decrease in aberrant crypt formation (pre-neoplastic lesions), and eventual colon tumor development.

Other epidemiologic studies have shown that the proportion of the population with the lowest intake of fruits and vegetables has nearly twice the incidence of cancer of epithelial origin than those with the highest intake of fruits and vegetables (19-21). Quercetin, a water-soluble flavonoid that is ubiquitous to foods of plant origin has been postulated to decrease the number of aberrant crypt foci (ACF) by inhibiting the growth of malignant cells through down regulation of cell proliferation and increasing apoptosis. These responses have been, in part, attributed to quercetin's antioxidant capacity (22-32).

Salganik et al. (33), however, demonstrated that feeding antioxidant depleted diets devoid of vitamins A and E inhibited tumor growth in mice by enhancing apoptosis, whereas an antioxidant-rich diet (with vitamins E and A supplemented at twice the level of a standard diet) had relatively little effect on tumor growth. Thus, if quercetin is functioning as a strong antioxidant, then it is possible that the protective, pro-apoptotic environment that normally exist in the colon caused by the combination of fish oil and pectin could be compromised due to elevated levels of dietary antioxidants such as quercetin (34).

We hypothesized that the addition of an antioxidant (quercetin) to a fish oil and pectin diet would alter the redox status of the rat colonic epithelial environment, which would counteract the pro-apoptotic protective effects of fish oil and pectin in the promotion stage of colon carcinogenesis. The purpose of this study was to determine whether elevated levels of quercetin influence the redox status of rat colonocytes through changes in antioxidant enzyme activities and shifts in GSH/GSSG cellular levels. These changes should lead to changes in apoptosis, oxidative DNA damage, and ACF numbers.

CHAPTER II

LITERATURE REVIEW

Research Justification

Initiation of malignant colonocytes. The intestinal colonic epithelium is organized into small invaginations called crypts that open into the lumen. These crypts allow for increased surface area to enhance nutrient absorption. A single layer of epithelial cells line the crypts and are responsible for the secretory, absorptive, and barrier functions of the large intestine. The epithelial cells maintain a highly-controlled balance of cell growth (proliferation) and programmed cell death (apoptosis), thus any perturbation of this balance may reflect a malignant transformation that may lead to tumorigenesis (35-39). Cells differentiate and move up the crypt as they age and lose their ability to proliferate. Eventually these differentiated colonocytes undergo apoptosis and/or are exfoliated into the fecal stream (40,41).

Carcinogenesis in humans is a multi-step process, involving multiple genetic mutations that transform normal cells into a malignant phenotype. Cancer is typically defined in three stages: initiation, promotion, and progression, with each stage possessing distinct morphological characteristics. The initiation stage of colon carcinogenesis is characterized by the production of DNA lesions or adducts that can be caused by oxidative stress generated by excess ROS. These lesions cause changes in DNA structure that can lead to genetic alterations such as mutational activation of oncogenes and the silencing of tumor suppressor genes

(35-39,42). These alterations could cause cells to hyperproliferate and evade apoptosis (42-47), and are considered initiated or precancerous cells. However, these mutations can usually be repaired during DNA synthesis. Whenever DNA repair or apoptotic removal fails to eliminate mutated colonocytes, then the early preneoplastic lesions of colon cancer (aberrant crypts) develop, which can eventually lead to polyp and tumor formation.

Aberrant crypts. The promotion stage of colon carcinogenesis involves clonal expansion of initiated cells that accumulate more DNA damage or are "misrepaired" thereby gaining a selective growth advantage. The crypts become larger and abnormally shaped compared to normal crypts. An accumulation of cells of the malignant phenotype then results. Groupings of these abnormal crypts are called aberrant crypt foci (ACF). Evidence suggests that foci incorporating large numbers of aberrant crypts (AC) are preneoplastic lesions of colon cancer, and are common biomarkers which have been identified in human colons (42,45). Studies reveal that colonocytes lining ACF have an increase in proliferation (48-50) and a resistance to apoptosis (48,49,51) which provide further evidence that ACF are preneoplastic lesions and the ACF assay is a reliable intermediate biomarker that can be used to evaluate the development of colon carcinogenesis in experimental animal models. Though events in the promotion stage are reversible, if damage continues and is not repaired correctly or transformed cells eliminated, accumulating cells can progress to tumors. This suggests that the promotion stage of tumorigenesis is an ideal target for colon cancer prevention strategies (52,53).

Chemoprotective agents that decrease the proliferation of malignant cells while enhancing the elimination of precancerous cells through apoptosis can alter the progression of malignant cells and lead to a decrease in the selective growth advantage of putative preneoplastic lesions (29).

Apoptosis. Apoptosis is one form of cell death that can eliminate damaged cells with the potential to form cancerous cells. Apoptosis is initiated through signaling pathways that have been well characterized and are highly conserved among species (54). These pathways are influenced by ROS and include disruption of the mitochondrial membrane, activation of caspases, and changes in gene expression. The loss of mitochondrial membrane potential (MMP) is the most obvious change that alters the mitochondria early in the apoptotic sequence. ROS can also oxidize the mitochondrial membrane lipids causing damage to the lipids and thus altering MMP (54). The mitochondria are more susceptible to damage by ROS when there is a greater content of long chain polyunsaturated fatty acids in the membrane (16). If severe oxidative stress occurs in the mitochondria, the permeability transition pores can be opened, which in addition to altering the MMP, can release large molecules into the cytosol such as cytochrome c and apoptotic inducing factor. These molecules continue the downstream events of apoptosis (55). A cascade of cell-signaling and caspase-mediated events that regulate pro- and anti-apoptotic proteins such as the Bcl-2 family members then results (56). In response to appropriate signals, these proteins can be modified post-translationally by kinases (44,55,57). ROS have been shown to play a critical role as a second messenger in cell signaling (58) and as an inducer of apoptosis. Furthermore an increase in ROS leading to apoptosis has been suggested to suppress tumorigenesis (9).

Reactive oxygen species. Reactive oxygen species (ROS) are a group of transient, highly active molecules (some of which contain an oxygen radical) that can be generated by both exogenous and endogenous sources such as those derived as a byproduct of normal cellular metabolism (36,59). ROS include superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) (2,4,5,60). Likely endogenous origins of ROS include oxidative phosphorylation, p450 metabolism, peroxisomes, and inflammatory cell activation.

The production of ATP through electron transport reactions in which O_2 accepts electrons and H^+ and is reduced to water is the basis of cellular metabolism. However, leakage of a single electron being transferred can occur resulting in O_2^- production. During mitochondrial oxidative metabolism, most oxygen consumed is reduced to water; however on average 1 to 5% of molecular oxygen is converted to ROS, essentially superoxide anion, making mitochondria a major site of ROS production (36-38,61). The endoplasmic reticulum is another site of electron transport. Here the leakage of electrons from NADPH cytochrome p450 reductase also generates O_2^- (61).

Under normal physiological conditions excess ROS formation is prevented by the cell's endogenous antioxidant defense systems. However, in highly oxidative environments, oxidative stress can occur. Oxidative stress is an imbalance of oxidants verses antioxidants in favor of the oxidants. This results in an overall increase of cellular levels of ROS (36).

Antioxidant enzyme capacity. The precise modulation of ROS levels are needed to allow for normal cellular function or to induce apoptosis of precancerous or transformed cells. ROS levels can exceed the antioxidant capabilities of the cell's antioxidant defense systems, which include removal of ROS by superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes as well as non-enzymatic redox compounds such as glutathione, thioredoxin, glutaredoxins, nicotinamide adenine dinucleotide phosphate (NADP), and exogenously absorbed micronutrients and vitamins (36,38,39,62,63). There is a balance that is essential for the survival and health of an organism between both the activities and the intracellular level of these antioxidants (38).

The enzymes work together as an antioxidant defense system such that when superoxide anion ($O_2^{-\cdot}$) appears in the environment, the anion is scavenged by SOD and produces H_2O_2 and water. From this point GPx decomposes H_2O_2 and reduced glutathione (GSH) is oxidized (GSSG). CAT competes with GPx to decompose excess H_2O_2 to water and molecular oxygen (64).

There are three isoforms of SOD in humans, cytosolic (Cu/Zn-SOD), mitochondrial (Mn-SOD), and extracellular SOD (EC-SOD). Cu/Zn-SOD is a homodimer with a molecular weight of about 32 kDa that specifically catalyzes the dismutation of the superoxide anion to oxygen and water. Each subunit contains a dinuclear metal cluster of copper and zinc as the active site. Mitochondrial Mn-

SOD is a tetramer containing one manganese molecule per subunit. The respiratory chain in the mitochondria is a major source of oxygen radicals and Mn-SOD is one of the most effective antioxidant enzymes and has been postulated to have anti-tumor activity (65). EC-SOD is a tetrameric, copper and zinc containing, secretory glycoprotein, and is regulated more by cytokines rather than by responses of individual cells to oxidants (39).

CAT is found in peroxisomes, which are organelles in the cells of plants, animals, and aerobic bacteria. CAT protects the cell from hydrogen peroxide produced within the cell, and water and molecular oxygen are formed when CAT efficiently reacts with H_2O_2. Although CAT plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response, CAT is not essential for some cell type under normal physiologic conditions (65).

GPx, a selenium containing peroxidase, catalyzes the reduction of hydroperoxides at the expense of GSH and therefore protects the cell against oxidative damage. GPx has five isoenzymes expressed ubiquitously in mammals and their antioxidant properties allow them to eliminate peroxides as possible substrates for the Fenton reaction. GPx enzymes add two electrons to reduce hydrogen peroxide to water while oxidizing GSH at the same time. Although GPx shares hydrogen peroxide as a substrate with CAT, GPx can react effectively alone with lipid and other organic peroxides, and is the major source of protection against low levels of oxidant stress.

Reduced and oxidized glutathione. The tripeptide, glutathione (GSH) is multifunctional and highly abundant in the cytosol, nucleus, and mitochondria of the cell, as well as the major thiol, nonenzymatic soluble antioxidant in these same cell compartments (38). GSH has many protective roles against oxidative stress such as detoxifying hydrogen and lipid peroxides by catalytic action of GPx, scavenging of hydroxyl radical and singlet oxygen, regeneration of important antioxidants to their active form, and acting as a cofactor of several detoxifying enzymes. Oxidized glutathione (GSSG) is a disulphide. The redox state of critical protein sulphydryls necessary for DNA repair and expression are maintained in the nucleus by GSH (38,39). Because GSSG is accumulated in the cell, the ratio of GSH/GSSG is a good measure of oxidative stress in the organism as well as the capacity of glutathione to regenerate the most important antioxidants (66,67). Many enzymes can be oxidatively damaged at higher than normal levels of GSSG. ROS are therefore critical determinants of cellular redox status, which is in part determined by the proportional amount of reduced and oxidized forms of the GSH redox system pair, and thus constitute a regulatory mechanism in the cell through the modification of protein conformation and functional signal transduction (38,39). Several studies suggest that an oxidative shift in the cellular environment is critical to generating the apoptotic signal, not ROS alone (68-71). Therefore changes in the ratio of GSH/GSSG can determine modulations in the cellular redox environment. In fact, Wang et al. (70) detected significant changes in GSH/GSSG within 15 min of an oxidative challenge, which correlated with later caspase activation. Esteve et al. (69) observed that GSH oxidation preceded DNA fragmentation, a characteristic of apoptosis. Therefore, measurement of changes in the oxidative environment using the GSH redox cycle may provide better insights into the mechanisms of ROS-mediated apoptosis than just the measurement of ROS alone.

Oxidative DNA damage. DNA can suffer different types of damage from base modification by oxidation or alkylation, to single or double strand breaks. During gene expression and DNA replication, base modifications can lead to mismatches and if left un-repaired can lead to mutations. Oxidative DNA damage is produced by oxidative stress generated from ROS and is one possible initiator of tumorigenesis (19). DNA and other macromolecules can be attacked by ROS, resulting in oxidative adducts that can then lead to mutations, which can cause transformation of cells and subsequent tumor formation (2,5). ROS can contribute to each stage of carcinogenesis. During this initial stage of carcinogenesis, sustained DNA mutations are often the genetic alterations responsible for the hyper-proliferation and resistance to apoptosis seen in later stages of colon carcinogenesis (36,38,39,63).

The oxidative DNA adduct 8-hydroxy-deoxyguanosine (8-OHdG) is commonly used as a biomarker for oxidative damage. 8-OHdG is one of the most abundant and highly mutagenic forms of oxidative DNA damage (3,72), and has been implicated in the tumorigenic process (73). This lesion can induce GC → TA transversions during DNA replication, which are found in mutated oncogenes and tumor suppressor genes, if not repaired and/or removed by apoptosis (74). The proliferation of these altered cells can lead to the malignant transformation of colonic mucosa.

Fish oil and pectin. Extensive research has been conducted to evaluate the role of dietary fiber in colon cancer with ambiguous results. The fermentable fiber pectin has been shown to have chemoprotective effects (16). Butyrate a fermentation by-product of pectin is preferentially used by colonocytes as an energy source. Butyrate has been found to be an inducer of apoptosis, specifically in transformed cells (75), and in vitro as well as in vivo studies have found butyrate to be antiproliferative and pro-apoptotic (11,40,76). The mechanism by which butyrate induces apoptosis has yet to be elucidated. Chapkin et al. (40) suggest that butyrate acts through induction of a Fas death receptor pathway (extrinsic) and Ruemmele et al. (76) also suggest that butyrate induces apoptosis via a mitochondria-mediated pathway (intrinsic). Yet, another in vitro study proposes that butyrate-induced apoptosis is activated through either intrinsic or extrinsic pathways in varying types of colon cancers (77). In vitro and in vivo studies have shown that butyrate uptake by colonocytes results in enhanced mitochondrial function and increased ROS production (16,78). However, evidence appears to support the fact that the protective role of fiber depends on type of fiber (fermentable or unfermentable), type of fat present in the diet, and the stage of tumorigenesis of the colonocyte (79). In fact a recent study by Kolar et al. (8) substantiates that the combination of fish oil and pectin work coordinately to protect against colon tumorigenesis. This protective effect is proposed to be in part due to increasing apoptosis rather than decreasing proliferation (7,15,49). In the rat experimental model of colon cancer a diet enriched with fish oil and pectin as the fiber source has been shown to be protective against colon cancer by increasing apoptosis, and decreasing proliferation, and decreasing ACF formation (13,16,44,80).

Antioxidant properties of quercetin. Epidemiologic studies have shown that the proportion of the population with the lowest level of fruit and vegetable intake has nearly two times the incidence of cancer of epithelial origin than those with the highest level of fruit and vegetable intake (19-21). Quercetin, a water-soluble flavonoid ubiquitous to foods of plant origin is found in at least 80% of higher order plants making it the most commonly ingested compound of the flavonol subclass of flavonoids (81). Quercetin has been shown to inhibit the growth of malignant cells by down regulation of cell proliferation and an increase in apoptosis in a variety of experimental models, including azoxymethane-induced colorectal carcinogenesis in F344 rats, in CF1 mice, and in many cell culture lines (26,28-30,82,83). Because these responses have been, in part, attributed to quercetin's antioxidant capacity (22-31), the question has been raised as to whether or not a pro-oxidative or anti-oxidative cellular redox environment is preferred in apoptosis induction of malignant colonocytes. Salganik et al. (33) demonstrated that tumor growth is inhibited in mice fed an antioxidant-depleted diet (devoid of vitamins E and A) due to ROS-enhanced apoptosis, whereas an antioxidant-rich diet (vitamins E and A doubled as compared to standard diet) had relatively no impact on tumor growth. Thus, if quercetin is only functioning via an antioxidant pathway, then it is possible that the protective, pro-apoptotic environment found in the colon of rats caused by the combination of fish oil and pectin may be negated by elevated levels of dietary antioxidants such as quercetin.

Antioxidant supplementation. Because the human diet is a complex mixture of oxidants and antioxidants, and the gastrointestinal tract is thought to be a major site of antioxidant action, the question of whether antioxidant supplementation might protect against cancer has been a subject of debate (84). Salganik et al. (33) demonstrated that feeding antioxidant depleted diets devoid of vitamins A and E inhibited tumor growth in mice by enhancing apoptosis, whereas an antioxidant-rich diet (with vitamins E and A supplemented at twice the level of a standard diet) had relatively little effect on tumor growth. Results of randomized trials with one or more selected antioxidants supplemented to the diet and reviewing the possible preventative effects on disease states have been contradictory (84). Thus far convincing evidence has not been found that antioxidant supplementation can prevent gastrointestinal cancers; but to the contrary may increase mortality (84,85). Increased mortality also seems to be the case in trials using beta carotene, vitamin A, and vitamin E treatments for different disease states such as gastrointestinal cancer (84) and lung cancer (86), although the potential roles for vitamin C and selenium need further study (85).

Purpose of study. This study evaluated the effect of the addition of an antioxidant (quercetin) to fish oil and pectin enriched diets on AOM exposed rat colonocytes. This study also determined whether quercetin influenced the cellular redox environment through changes in antioxidant enzyme activities, and thus apoptosis caused by diets containing fish oil and pectin.

Hypothesis. Addition of the antioxidant quercetin to a fish oil pectin diet will alter the redox status of rat colonocytes, thus possibly inhibiting the beneficial effects that a fish oil/pectin diet has on the induction of colonocyte apoptosis, antioxidant enzyme activities, and oxidative DNA damage.

Specific aims. The objectives of this study are to:

1. Determine if quercetin has an effect on ACF formation in rats consuming a fish oil and pectin or corn oil and pectin diet.
2. Determine if rat colonocyte apoptosis induction is altered by the addition of quercetin to fish oil and pectin or corn oil and pectin diets.
3. Determine if quercetin alone or in combination with fish oil and pectin or corn oil and pectin diets change SOD, CAT, and GPx activity and therefore cellular redox status (reduced/oxidized glutathione levels and ratios).
4. And lastly determine if the net effect of these changes lead to a difference in the amount of oxidative DNA damage.

CHAPTER III

DIETARY LIPIDS AND QUERCETIN SUPPRESS COLON CARCINOGENESIS THROUGH DIFFERENTIAL EFFECTS ON APOPTOSIS AND COLONOCYTE REDOX BALANCE

Introduction

The “antioxidant hypothesis” of disease prevention emerged during the 1980s from studies showing that people whose diets where rich in fruits and vegetables had a lower incidence of diabetes, stroke, heart disease, dementia, and certain types of cancer. These diseases are all associated with free radical damage (87). Since fruits and vegetables are rich sources of antioxidants and thus have the power to quench free radicals by donating electrons, scientists assumed that taking antioxidants as supplements or fortifying foods with antioxidants should decrease oxidative damage and diminish disease. And thus the hypothesis that dietary antioxidants are protective against disease caused by oxidative damage was born.

Our laboratory has shown in previous studies that the combination of fish oil (high in n-3 fatty acids) and pectin (a butyrate producing fermentable fiber) is protective against colorectal cancer. Chang et al. (7) demonstrated that a fish oil pectin diet reduced the incidence of tumorigenesis by increasing colonocyte apoptosis in the experimentally induced rat model of colon carcinogenesis. Hong et al. (16) suggested that the increase of apoptosis in the colonocytes of these fish oil and pectin enriched diet fed rats may be due in part to fish oil priming the colonocytes for butyrate induced apoptosis. Because fish oil enhances the unsaturation of mitochondrial phospholipids, an increase in cellular reactive oxygen species (ROS) levels likely occurs. Mitochondrial function becomes impaired whenever the membrane is no longer able to maintain the required potential, which occurs in fish oil pectin rats. Loss of mitochondrial membrane potential leads to the initiation of the apoptotic cascade (37,38,65,88).

Sanders et al. (9) went on to demonstrate in rats not injected with a colon specific carcinogen, that ROS levels in the colonocytes of rats consuming a combination of fish oil and pectin was indeed greater ($p< 0.02$) than ROS levels of rats consuming corn oil and cellulose. This increased level of ROS was inversely related to oxidative DNA damage. An exponential increase of the apoptotic index, as well as a concomitant decrease in the enzyme activity levels of superoxide dismutase and catalase also resulted in the fish oil and pectin fed rats. Conversely, rats consuming the corn oil cellulose diet did not exhibit these relationships.

From the results of these aforementioned studies we can infer that a fish oil pectin diet protects against colorectal cancer by generating a permissive environment such that colonocytes are more receptive to pro-apoptotic signals, especially those generated by ROS. Thus, the elimination of pre-cancerous cells would be more likely in rats consuming diets enriched in fish oil and pectin.

Our experimental results suggesting that a certain degree of pro-oxidant environment may be protective are being reflected by human studies showing that antioxidant supplementation may in fact be counterproductive. Randomized clinical trials using antioxidants to prevent several diseases have shown that antioxidant supplementation can increase cancer incidence and mortality

(84,85,89). Since adding significant amounts of antioxidants to the diet should lower cellular ROS, the ROS-dependent pathways of apoptosis induction could be depressed allowing more potentially cancerous cells to spread and increase tumor growth. We hypothesize that adding an antioxidant to a pro-oxidant diet (fish oil and pectin) could negate the chemoprotective effect of the pro-oxidant diet (fish oil and pectin) counteracting the effects on colonocyte redox balance.

Quercetin, a water-soluble flavonoid ubiquitous to foods of plant origin was our antioxidant of choice. Quercetin is found in at least 80% of higher order plants making it the most commonly ingested compound of the flavonol subclass of flavonoids (81). Dietary quercetin is postulated to contribute to the chemoprotective activities of fruits and vegetables through its antioxidant properties (22,23,25,26,28-31,90). The purpose of our study was to determine if the addition of quercetin to an experimental diet of fish oil and pectin would decrease apoptosis due to modifications in cellular redox status, thus increasing aberrant crypt formation.

Materials and Methods

Experimental design. Animal protocols used in this study were approved by the University Animal Care Committee of Texas A&M University, and conform to the National Institutes of Health guidelines. Forty male weanling (21-d old) Sprague-Dawley rats (Harlan Sprague Dawley, Houston, Texas), were separately housed in raised wire cages to reduce coprophagy and access to bedding. The rats were maintained in a temperature (18-26° C) and humidity controlled animal facility with a 12 h daily light/dark cycle.

The rats were stratified by initial weight and randomized to one of four experimental diets (10 rats/diet), which were consumed for 70 d after an initial 5-d acclimation period. This study employed a 2x2 factorial design with two levels of quercetin (0% quercetin or 0.45% quercetin) and two types of oil (fish oil and corn oil). Experimental diets and water were freely available. All animals were injected with azoxymethane (AOM; 15 mg/kg body weight, Midwest Research Institute, Kansas City, Missouri), a colon specific carcinogen, 3 wk after starting the experimental diets. A second injection was administered 1 wk later. Food intake (48 h) and body weight were recorded for each animal on days 18, 56, and 67 after starting the experimental diet. Termination occurred on day 70 for each animal.

Experimental diets. Rats were provided with fresh diet in clean bowels daily. Each experimental diet contained pectin (6 g/100 g) as the fiber source, and either fish oil, rich in n-3 fatty acids, or corn oil, rich in n-6 fatty acids, as a lipid source (15 g/100 g) with or without quercetin. Experimental diets containing fish oil were fortified with 3.5 g of corn oil/100 g diet to provide essential fatty acids. Antioxidants were also added to each experimental diet to assure equivalent levels in the lipid component of the diet (**Table 1**).

TABLE 1 Composition of Experimental Diets

Ingredients	**g/100g**	**g/100g**
Dextrose Monohydrate[1]	51.06	50.61
Casein[1]	22.35	22.35
DL-Methionine[1]	0.34	0.34
Pectin[1]	6.00	6.00
Corn Oil[3]	15.00	15.00
Or Fish Oil[4]/Corn Oil[3]	11.50/3.50	11.50/3.50
Mineral Mix AIN-76A[1]	3.91	3.91
Vitamin Mix AIN-76A[1]	1.12	1.12
Choline Bitartrate[1]	0.22	0.22
Quercetin Dihydrate[2]	0.00	0.45

[1] Harlan Teklad, Madison, WI.
[2] Sigma, St. Louis, MO.
[3] Dyets, Bethlehem, PA.
[4] Degussa BioActive, Champaign, IL.

Tissue sample collection. Following termination by CO_2 asphyxiation and cervical dislocation, the colon was removed, cut longitudinally to expose the lumen, and washed in 1% PBS. Two centimeters of the most distal colon was fixed in 4% PFA (1 cm) and 70% ethanol (1 cm). The remaining section of the colon was divided in half. One half was used for aberrant crypt foci enumeration, and the other half was scraped with a glass slide to remove the mucosal layer which was used for antioxidant enzyme activity and glutathione analyses.

Aberrant crypt foci. Aberrant crypt foci number and multiplicity were determined on one half of the colon using the procedure of Vanamala et al. (91). The tissue was protected using RNAse free filter paper at the time of termination and fixed in 70% ethanol for 24 h. Each colon section was then stained with 0.5% methylene blue and examined microscopically (40x). The mucosal surface was used to quantify the total number of aberrant crypts and high multiplicity aberrant crypt foci (four or more aberrant crypts per focus).

Apoptosis. Non-serial sections cut from 1 cm of the most distal colon, fixed in 4% paraformaldehyde (PFA) and embedded in paraffin were utilized for in situ measurement of apoptosis using ApopTag (Chemicon, Temecula, CA) technology as previously described by Chang et al. (35). Cells stained by diaminobenzidine tetrachloride (DAB) and having the appropriate morphological criteria were scored as apoptotic and the apoptotic index determined. The apoptotic index for each crypt column was determined by dividing the number of apoptotic cells in a crypt column by the number of cells in the crypt column. The mean apoptotic index of 50 crypt columns was used as the apoptotic index for each rat.

Antioxidant enzyme assays. Cell lysates were prepared by homogenization of scraped mucosal cells in a potassium phosphate buffer as described in Appendix A, followed by centrifugation for 30 min at 15,000 x g (4°C) and aliquots were stored at -80°C. The supernatant was used for enzyme assays and protocols from the kits were followed. Total superoxide dismutase (SOD) as well as manganese superoxide dismutase (Mn-SOD/mitochondrial) activity was determined by measuring the rate of chromophore generation at 450 nm (Cayman Chemicals, Ann Arbor, MI) (92). The Mn-SOD levels were determined by adding potassium cyanide to inhibit copper/zinc SOD activity (65). Catalase (CAT) activity was determined by measuring formaldehyde generation at 540 nm (Calbiochem, San Diego, CA) (93). Glutathione peroxidase (GPx) activity was determined by measuring the oxidation of NADPH to $NADP^+$ at 340 nm (Cayman Chemicals, Ann Arbor, MI) (94). Samples were analyzed in triplicate in 96 well microplates, and measurements made using a Spectra Max 250 microtiter plate reader with SoftMax Pro, v.1.2 software (Molecular Devices, Sunnyvale, CA) for all assays. Enzyme activity was normalized to protein concentration as determined by Commassie Blue assay (Pierce Biotechnologies, Rockford, IL).

In vivo measurement of oxidative DNA damage. Tissue samples fixed in 70% ethanol and embedded in paraffin were used for *in situ* measurement of 8-OHdG adducts using a mouse monoclonal antibody for 8-OHdG (Oxis, Portland, Oregon, see Appendix A) and a protocol adapted from Hong et al. (16). Tissue sections were deparaffinized, rehydrated, treated with RNase (100 µg/ml) in Tris buffer (pH 7.5, 10 mM Trizma base, 1 mM EDTA, 0.4 M NaCl), and incubated in humidified chamber at 37˚C for 1 h. DNA was then denatured in 4N HCl for 7 min and neutralized with 50 mM Trizma base for 5 min. Tissue sections were incubated with 10% normal rabbit serum (Jackson, West Grove, PA) to block non-specific background staining followed by an overnight incubation in primary antibody (1:40 dilution) at 4˚C. Tissue sections were then incubated with biotinylated rabbit anti-mouse IgG (1:800 dilution, Jackson, West Grove, PA) as a secondary antibody followed by incubation in 3% H_2O_2/methanol to quench endogenous peroxidase. Slides were then incubated using an ABC kit (Vector Laboratories, Inc., Burlingame, CA), and the entire complex was visualized with DAB. Omission of primary antibody was used as a negative control and 6% DSS treated rat colonic tissue sections were used as a positive control. Intensity of staining in each cell within a crypt column was measured using NIH Image software. The mean stain intensity minus the average of background staining was determined for each nucleus in a crypt column, and 20 crypt columns per rat were analyzed.

Determination of GSH/GSSG. Colonic GSH and GSSG were measured using an HPLC method of Jones et al. (66,67) with modifications. Snap frozen mucosal tissue (~50 mg) was homogenized in 0.5 ml Solution A (1.05% L-serine, 0.1 mM sodium heparin, 2 mM bathophenanthroline disulfonate sodium salt, 11 mM iodoacetic acid, 80 mM boric acid, and 20 mM sodium tetraborate) plus 0.5 ml Solution B (1.2 mM perchloric acid and 200 mM boric acid). The homogenizer was rinsed with 0.1 ml Solution A and 0.1 ml Solution B. The combined homogenates were centrifuged at 10,000 g for 1 min. The supernatant (150 μl) or 150 μl of GSH and GSSG standards (0, 50, 200, and 500 μM each) were mixed with 30 μl of 40 mM iodoacetic acid and 100 μl of 1 M KOH/1.6 M potassium tetraborate (pH ~9.0), followed by addition of 150 μl of 75 mM dansyl chloride. The solutions were vortexed and kept in the dark at room temperature for 16 h, followed by addition of 250 μl chloroform. The mixture was centrifuged at

10,000 g for 1 min, and 100 µl of the supernatant fluid (dansyl derivatives) was transferred to a micro-insert tube in a brown vial, with 25 µl injected into a 3-aminopropyl column (5 µm; 4.6 x 250 mm; Custom LC, Houston, TX). GSH and GSSG were eluted from the column using Solvent A (0.8 M sodium acetate, 27% glacial acetic acid, and 63% methanol; pH 4.6) and Solvent B (80% Methanol) at the combined flow rate of 1.0 ml/min and the following gradient (0-10 min, 20% Solvent A; 30-33 min, 80% Solvent A; 33.1-38 min, 20% Solvent A). Fluorescence detection (Waters 2475 Multi-λ Fluorescence Detector) was set at 590 nm excitation and 610 nm emission (0.0 to 7.5 min) to eliminate the appearance of amino acid peaks and at 335 nm excitation and 610 nm emission (7.5 to 38 min) for GSH and GSSG detection. Detector gain was set at 100 (0 to 32.2 min) for GSH detection and at 1000 (32.2 to 38 min) for GSSG detection. GSH and GSSG were quantified on the basis of authentic standards (Sigma Chemicals, St. Louis, MO) using the Millennium™-32 Software and workstation. Data was expressed relative to protein concentration as determined by Coommassie Blue assay (Pierce Biotechnology, Rockford, IL).

Statistical analysis. Analysis of data acquired by the TUNEL, enzymatic activity assays, glutathione concentration and ratio, as well as 8-OHdG DNA adduct quantification were performed by mixed model analysis of variance (ANOVA) using SAS 9.1 (SAS Institute, Inc.). Differences among the treatments were considered significant at $p<0.05$. Sample outliers were removed if normalization of the sample set did not correct for skewedness. Analysis of data acquired by the aberrant crypt foci assay was measured using the nonparametric Wilcoxon Ranks Sums test.

Results

Food intake and body weight gain. Because of the potential for differences in weight gain to affect the outcome, food intake (48 h) and body weight gain were recorded and analyzed for each animal on days 18, 56, and 67 after starting the experimental diets. There were no significant differences in food intake or body weight gain among the experimental groups at the time of termination (see Appendix B).

Apoptosis. Inhibition of programmed cell death or apoptosis plays a pivotal role in tumorigenesis. Therefore non-serial sections cut from 1 cm of the most distal colon, fixed in 4% PFA and embedded in paraffin were utilized for in situ measurement of apoptosis using the TUNEL assay. There was no significant difference in the apoptotic index between the fish oil and corn oil animals (**Fig. 1**). However, rats whose diets were supplemented with quercetin showed a significant increase in the apoptotic index (p=0.0001).

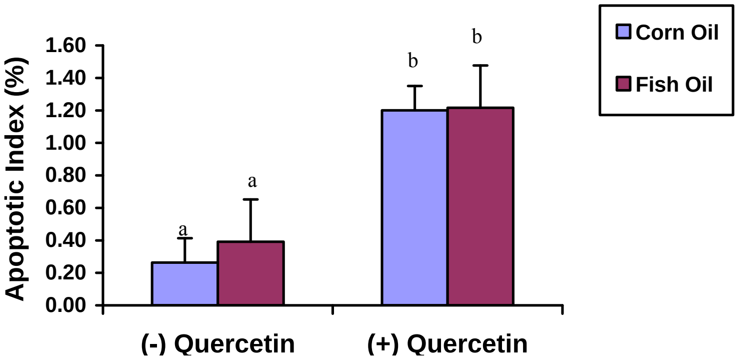

FIGURE 1 Rats fed a corn oil or fish oil diet supplemented with quercetin displayed a greater apoptotic index as compared to rats fed corn oil or fish oil without quercetin supplementation. Data are means ± SEM from 50 crypts for n=10 rats/diet. The apoptotic index represents the total number of apoptotic cells in a crypt column/total number of cells in the crypt column. Bars not sharing a letter differ, p=0.0001.

Antioxidant enzyme activity. When pro-oxidants such as ROS exceed antioxidant capabilities, oxidative stress exists. This oxidatively stressed environment can result from increased generation of ROS as well as impaired removal of ROS by antioxidant defense systems such as SOD, CAT, and GPx enzymes. To test for any variations in antioxidant capacity among the experimental diet groups SOD, CAT, and GPx activities were measured. We observed a consistent numerical reduction in the activity of total superoxide dismutase in fish oil fed animals as compared to corn oil fed animals when the main effect of lipid was analyzed (**Fig. 2**, p=0.0136). However, there was no difference observed in mitochondrial SOD activity among the diet groups (see Appendix B). Furthermore, CAT activity was elevated (p=0.0119) in fish oil fed rats without quercetin supplementation (**Fig. 3**). Because these enzymes work sequentially the ratio of SOD/CAT was analyzed.

SOD/CAT was higher in corn oil fed rats in comparison to fish oil fed rats (**Fig. 4**). GPx activity was higher in corn oil rats (**Fig. 5**).

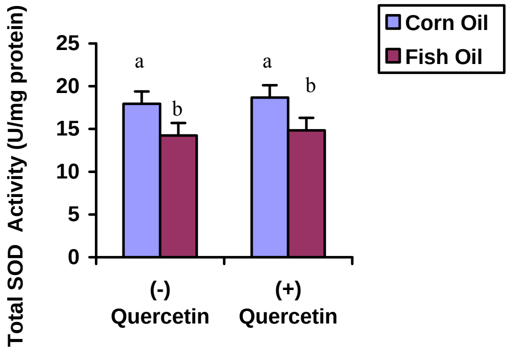

FIGURE 2 Rats fed a corn oil diet (n=20) had higher levels of SOD activity as compared to rats fed a fish oil diet (n=20, p=0.0136). Data are means ± SEM for n=10 rats/diet.

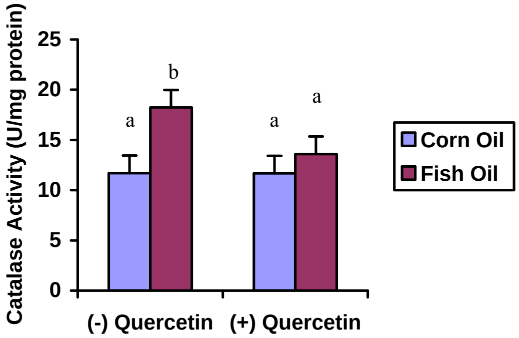

FIGURE 3 Rats fed a corn oil diet (n=20) had lower levels of CAT activity as compared to rats fed a fish oil diet (n=20, p=0.0204). There was a significant difference in CAT activity between corn oil and fish oil fed rats not supplemented with quercetin (n=10 rats/diet, p=0.0119), however when quercetin was added to the diet the difference in CAT activity was no longer observed. Data are means ± SEM for n=10 rats/diet.

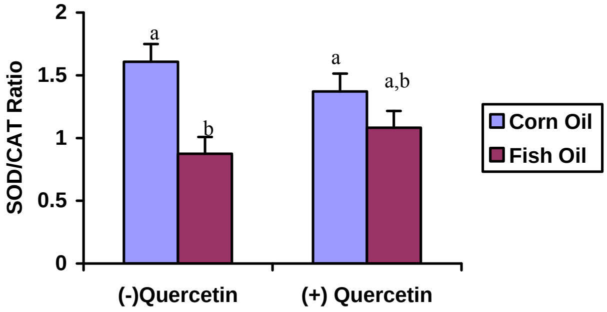

FIGURE 4 AOM injected rats fed a corn oil diet had a higher SOD/CAT ratio (n=18 rats/diet, p=0.0006) as compared to fish oil fed rats (n=19 rats). There was a significant difference in SOD/CAT ratio in corn oil vs. fish oil fed rats when the diet did not contain quercetin (p=0.0003). Upon the addition of quercetin to the diet, the difference between the ratios was diminished. Data are means ± SEM for n=10 rats for corn oil and fish oil diets, n=8 rats/ Corn oil + Quercetin, n=9 rats/fish oil + Quercetin.

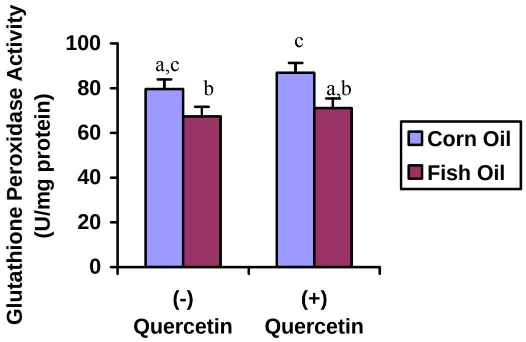

FIGURE 5 Rats fed a corn oil diet (n=20) had higher levels of GPx activity as compared to rats fed a fish oil diet (n=20, p=0.0025). Although there were numerical differences between the corn oil and fish oil fed rats with and without quercetin supplementation, the greatest difference in GPx activity was observed between corn oil and fish oil fed rats with quercetin supplementation (n=10 rats/diet, p=0.0136). Data are means ± SEM for n=10 rats/diet.

Glutathione. To have a representative indicator for the redox environment of the rat colonocyte, the major cellular redox buffer glutathione (GSH/GSSG) was measured. There was no effect of diet or quercetin supplementation on total glutathione or GSH concentrations (**Table 2**). However, the level of GSSG was increased in corn oil fed rats consuming quercetin as compared to fish oil fed rats also consuming quercetin (p=0.0554). The ratio of GSH/GSSG was lower in corn oil rats as compared to fish oil rats (p= 0.0314) with the greatest difference among the corn oil and fish oil rats occurring with quercetin supplementation, suggesting a more oxidized environment among corn oil rats especially with quercetin supplementation (**Fig. 6**).

TABLE 2

Total, reduced, and oxidized glutathione. Concentrations in mucosa of AOM injected rats fed corn oil or fish oil lipid based diets with or without quercetin supplementation for a total of 7wk after AOM injection[1]

Diet	Non Quercetin		Quercetin		
	Corn Oil	Fish Oil	Corn Oil	Fish Oil	P-values
	(nmol/mg protein)		*(nmol/mg protein)*		
Total Glutathione	12.41 ± 2.04	13.34 ± 2.04	10.73 ± 2.15*	14.67 ± 2.04	NS
GSH	11.60 ± 1.99	12.76 ± 1.99	9.61 ± 2.10*	14.19 ± 1.99	NS
GSSG	0.28 ± 0.10^{*,ab}	0.27 ± 0.09^{ab}	0.57 ± 0.20^{b}	0.26 ± 0.09^{a}	0.0554[2]

[1] Values are mean ± SEM, n=10 rats/diet

[2] p-value is for corn vs. fish with quercetin supplementation

[*] outlier removed n= 9 rats

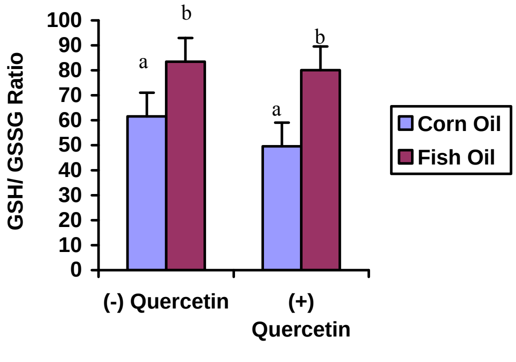

FIGURE 6 AOM injected rats fed a corn oil diet (n=20) had a lower GSH/GSSG ratio in colonic mucosal scrapings suggesting a more oxidized environment in corn oil fed rats as compared to fish fed rats. Quercetin supplementation had little effect on GSH/GSSG. Data are means ± SEM for n=10 rats/diet. Bars not sharing a letter differ, p=0.0314.

Oxidative DNA damage. Because damage to DNA is one consequence of excessive oxidative stress, and several oxidative adducts including 8-oxodG have been implicated in the tumorigenic process, 8-OHdG adduct formation in the distal colon was measured *in situ*. There was no difference in overall levels of oxidative DNA damage as determined by 8-OHdG adduct quantification in fish oil vs corn oil fed rats. However, upon the addition of quercetin to the diet, a decrease in 8-OHdG adduct formation was observed in corn oil fed rats (**Fig. 7**).

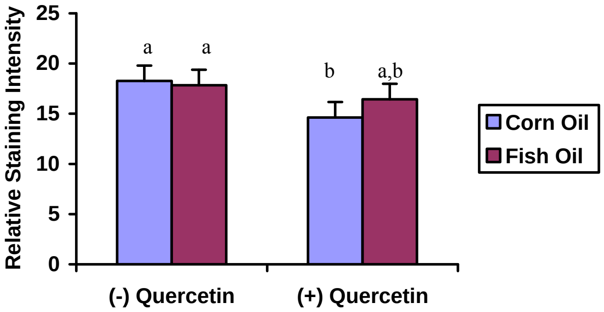

FIGURE 7 Oxidative DNA damage was measured by quantification of 8-OHdG adduct stain intensity in AOM injected rats fed a corn oil or fish oil based diet with and without quercetin supplementation. Quercetin supplementation had more of a protective effect when added with corn oil; when added to fish oil there was no decrease in DNA damage. Data are means ± SEM from 20 crypts for n=10 rats/diet for no quercetin supplementation and n=9 rats/diet for quercetin supplementation. Bars not sharing a letter differ (p=0.0428).

Aberrant crypt formation. All rats were injected twice with the colon specific carcinogen AOM and consumed experimental diets for 7 wk after AOM injection. To measure the protective effect of each experimental diet against colon carcinogenesis, aberrant crypt formations were quantified. Aberrant crypt formation was significantly suppressed in fish oil rats as compared to corn oil animals (**Fig. 8**). Quercetin however, had no effect on aberrant crypt formation.

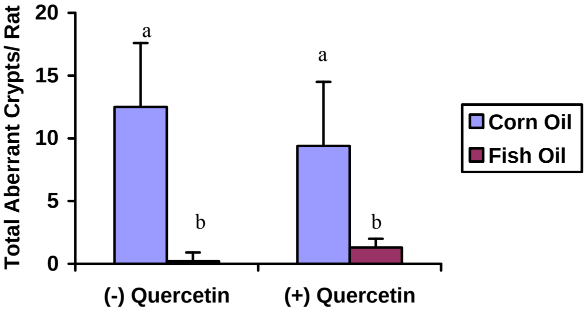

FIGURE 8 AOM injected rats fed a corn oil diet had significantly higher aberrant crypt numbers as compared to fish oil fed rats. Quercetin supplementation had no effect on aberrant crypt formation. Data are means ± SEM for n=10 rats/diet. Bars not sharing a letter differ (p<0.0001).

Discussion

One of the most critical control processes in cancer prevention and treatment has been shown to be apoptosis induction (95). If ROS generation exceeds cellular antioxidant capacity oxidative stress will result. Elevated ROS levels can then initiate and/or modulate signaling cascades, one of which is apoptosis induction. We have previously shown that the dietary combination of fish oil and pectin has the ability to alter the oxidative status of rat colonocytes via ROS generation and modulation of antioxidant enzyme activity, thus creating an environment permissive for apoptosis (9,16). Addition of antioxidants to this pro-oxidant environment may alter cellular redox balance and compromise the ROS-mediated mechanism whereby fish oil and pectin diets initiate apoptosis. Therefore, the intent of this study was to evaluate whether an antioxidant, quercetin, could alter the cellular redox balance of rat colonocytes and compromise the mechanism whereby the dietary combination of fish oil and pectin initiates apoptosis. We hypothesized that the addition of quercetin to a fish oil and pectin enriched diet would decrease apoptosis through its effects on endogenous antioxidant enzyme activities (SOD, CAT, and GPx), which would change redox balance (GSH/GSSG), and increase the level of oxidative DNA damage. The modulation of these aforementioned cellular systems should therefore negate the chemoprotective effect of fish oil and pectin enriched diets leading to an increase in AC numbers during the promotion stage of colon carcinogenesis.

Data from this experiment showed a significant increase in apoptosis in both corn oil and fish oil fed rats (pectin as the fiber source) with the addition of quercetin to the diet. We have also demonstrated an increase in apoptosis in rats consuming diets containing corn oil and quercetin when cellulose was the fiber source (96). Quercetin has been shown in vitro to enhance TNF-related apoptosis-inducing ligand (TRAIL) apoptosis through Akt dephosphorylation in human prostate cancer cell lines (97). In the in vivo study conducted by Warren et al., no change in total Akt or PI3 kinase levels were observed (96). Another mechanism of apoptosis induction is through ROS-mediated pathways involving the mitochondria. A recent study observed that the level of ROS and malondialdehyde was increased in quercetin treated hepatoma cells (23). These results correlated well with quercetin induced cytotoxic effects in isolated rat liver nuclei (98) and comparable results in human leukemic HL-60 cells (99). These studies strongly suggest that quercetin has pro-oxidant activity in vitro and thus, may have contributed to apoptosis induction through an ROS-mediated pathway in the colonocytes from rats consuming quercetin.

We previously showed in normal rat colonocytes that as the levels of ROS rose, the apoptotic index rose exponentially ($p=0.005$) (9). This previous study involved analyzing enzyme activity levels in normal rat colonocytes fed fish oil or corn oil and used pectin or cellulose as the fiber source for 4 wk. Fish oil and pectin fed rats had lower antioxidant enzyme activity levels of SOD and CAT and comparable levels of GPx activity as compared to corn oil and cellulose fed rats (9). Similar experiments in rat colonocytes observed also that dietary fish oil lowers antioxidant enzyme activities of SOD, CAT, and GPx (100). SOD, CAT, and GPx enzymes are critical determinants of cellular antioxidant capacity and ROS elimination. These enzymes act sequentially to quench ROS, therefore the balance of the activities of these enzymes are just as important as the activity of each enzyme alone. SOD coverts superoxide anion ($O_2^{\cdot-}$) into H_2O_2, which is then converted to H_2O and O_2 by GPx or CAT. Although GPx and CAT both decompose H_2O_2, the amount of H_2O_2 produced and whether H_2O_2 is found in the cytosol or mitochondria, dictates the relative contribution of these two enzymes toward H_2O_2 removal (43,101). The current experiment used AOM injected rats, and we saw consistent numerical reduction in SOD activity in the fish oil fed rats, which when the main effect of lipid was analyzed fish oil fed rats had a significantly lower SOD activity level than corn oil fed rats. Quercetin did not diminish this effect. Fish oil fed rats exhibited a greater CAT activity as compared to corn oil fed rats, however upon addition of quercetin, this difference was eliminated. Because a decrease in CAT activity has been found in a variety of animal tumors (102), the significant reduction in CAT activity for the fish oil fed rats with quercetin may negatively affect the chemprotective effects of this diet if the study were continued to the tumor stage, which is possible as suggested by the tendency (N. S.) for AC to increase in fish oil fed rats with quercetin in the diet. The SOD/CAT ratio was higher for corn oil fed rats as compared to fish oil fed rats. In fact we also observed a tendency for AC to decrease in corn oil fed rats supplemented with quercetin (**Fig. 8**). Corn oil fed rats with and without quercetin supplementation exhibited a significantly higher level of GPx activity as compared to fish oil fed rats. The differences in antioxidant enzyme activity levels observed in this experiment compared to previous studies may be due to the fact that the current experiment measured antioxidant enzyme activity of rat colonocytes excised 7 wk post 2nd AOM exposure whereas the previous experiments were in normal rats (9) and an ulcerative colitis rat model (100), and the ages of the rats where also different than those in the current study.

Dietary fish oil and pectin did protect against AC formation in this study. We still observed that protection with the addition of quercetin to the diet. The ability of the fish oil and pectin enriched diet to enhance apoptosis appears to be due in part to the modulation of the redox environment (9,16). This study examined the redox environment using GSH/GSSG, a frequently used indicator of oxidative status. Several studies suggest that a shift in the cellular environment towards a more oxidative state is the major initiator for apoptosis rather than the cellular oxidative status at a particular timepoint (68-71). In this investigation we demonstrate that the combination of dietary fish oil and pectin tends to promote a more reduced cellular environment (elevated GSH/GSSG) as compared to the combination of dietary corn oil and pectin. This reduced environment produced by fish oil and pectin suggests that upon exposure to a chemical carcinogen, fish oil and pectin enriched colonocytes are better able to endure an oxidative shift sufficient to trigger apoptosis, but not so severe as to enhance oxidative DNA damage (9,18). The elevated GSSG levels for corn oil fed rats supplemented with quercetin further suggests that quercetin may indeed affect recycling of GSSG back to GSH. This correlates with a previous study which observed that quercetin can change the activity of glutathione reductase, the enzyme responsible for reducing GSSG back to GSH (103).

Although dietary fish oil and pectin alter the antioxidant enzyme activities of SOD, CAT, and GPx as compared to corn oil and pectin, and produced a more reduced environment as reflected in the GSH/GSSG ratios, there was not a reduction in 8-OHdG DNA adducts. However, upon addition of quercetin to the diet, corn oil fed rats did have a significant decrease in 8-OHdG adduct formation. Despite the significant reduction in oxidative DNA damage to the corn oil fed rats supplemented with quercetin and dramatic increase in apoptosis, fish oil fed rats with and without quercetin supplementation displayed a significantly lower level of AC. However, there was a tendency for AC to be reduced in the corn oil fed rats with quercetin supplementation as compared to those without supplementation. These finding suggest that fish oil and quercetin are utilizing distinctly different mechanism to induce apoptosis, and the mechanism utilized by fish oil seems to more chemoprotective than the mechanism utilized by quercetin.

In summary the combination of dietary fish oil and pectin have been shown to enhance colonocyte apoptosis by modulation of the cellular redox environment. In this study dietary fish oil and pectin decreased the enzyme activities of SOD and GPx, while increasing CAT activity and GSH/GSSG in the rat colonocyte of AOM injected rats. Although fish oil and pectin did not reduce oxidative DNA damage as compared to corn oil and pectin, fish oil did significantly reduce AC. Upon the addition of quercetin to the fish oil pectin diet antioxidant enzyme activities of SOD and GPx were comparable to the non-quercetin enzyme activities. Although CAT activity was significantly higher in fish oil fed rats than corn oil fed rats without quercetin in the diet, the addition of quercetin to the diets diminished the effect of higher CAT observed in fish oil fed rats. Quercetin also had a tendency to create a shift to a more oxidized environment in corn oil fed rats as analyzed by GSH/GSSG, yet we observed a significant increase in apoptosis and a slight tendency to lower AC with this dietary combination. This study also demonstrates that the mechanisms used by chemopreventive agents are critical to whether combinations of chemopreventatives are synergistic or antagonistic. Further investigations should evaluate the exact in vivo mechanism of dietary fish oil and pectin as well as quercetin, and whether quercetin is more beneficial to an n-6 as compared to an n-3 enriched diet in the initiation, promotion, and progression stages of colon cancer.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

The oxidative environment of the colonocyte plays a critical role in the susceptibility of the colon to rid itself of malignant cells via apoptosis. The cellular redox environment is determined by the balance of ROS generation and antioxidant defenses. Therefore modulation of this environment through dietary measures is indeed a beneficial strategy in colon cancer chemoprevention.

Colon cancer is 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. Studies have shown that pectin in combination with fish oil has a synergistic protective effect on multiple stages of colon cancer. This effect is largely due to the up-regulation of apoptosis (7,15). Evidence suggests that this up-regulation of apoptosis by these dietary components is through modulation of the redox cellular environment resulting in a pro-oxidant environment such that colonocytes are more receptive to pro-apoptotic signals, especially those generated by ROS.

Other studies have shown that the proportion of the population that has the lowest intake of fruits and vegetables has twice the incidence of cancer of epithelial origin (3,20,21). More studies show that people whose diets were rich in fruits and vegetables had a lower incidence of diabetes, stroke, heart disease, dementia, and certain types of cancer. These diseases are all associated with free radical damage (87). Since fruits and vegetables are rich sources of antioxidants and thus have the power to quench free radicals by donating electrons, scientists assumed that taking antioxidants as supplements or fortifying foods with antioxidants should decrease oxidative damage and diminish disease. Randomized clinical trials using antioxidants to prevent several diseases have shown that antioxidant supplementation can increase cancer incidence and mortality (84,85,89). Dietary quercetin is postulated to contribute to the chemoprotective activities of fruits and vegetables through its antioxidant properties (22,23,25,26,28-31,90). The purpose of our study was to determine if the addition of quercetin to an experimental diet of fish oil and pectin would decrease apoptosis because of modifications in cellular redox status, thus increasing aberrant crypt formation.

In this investigation, we show that apoptosis is enhanced by dietary quercetin in AOM exposed rat colonocytes. This increase in apoptosis was seen in both the corn oil and fish oil fed rats. The greatest differences in enzyme activities were seen in rats without quercetin in the diet. With addition of quercetin to the diet the differences observed stayed the same for SOD and diminished for CAT. In the case of GPx we observed a numerical increase in activity for corn oil fed rats, causing a significant difference in activity between corn oil and fish oil fed rats with quercetin supplementation. GSH was not affected by diet; yet, GSSG was elevated in corn oil fed rats compared to fish oil fed rats with quercetin. Quercetin also had a tendency to create a more oxidized environment in corn oil fed rats as analyzed by GSH/GSSG, yet we observed a significant increase in apoptosis and a tendency to lower AC with this dietary combination. There was no difference in overall levels of 8-OHdG adducts in fish oil vs corn oil fed rats. However, upon the addition of Q, a decrease in 8-OHdG adduct levels was observed in corn oil rats.

Conclusions

Despite increasing apoptosis, and lowering 8-OHdG adduct levels in corn oil fed rats, quercetin had little effect on AC formation. These data suggest that quercetin may have created a greater oxidant load than corn oil alone, which shifted redox balance to a more oxidized state as compared to corn oil only animals. The small decrease in AC caused by quercetin in the corn oil fed rats may have resulted from the accumulation of GSSG making the colonocyte more susceptible to apoptosis induction and thus lowering AC formation. The small increase in AC caused by quercetin in the fish oil fed rats may have resulted from the lower CAT activity caused by this dietary combination. The distinct effects of the diets suggest that the corn oil and quercetin and fish oil and quercetin combinations are functioning through different mechanisms, yet the mechanism used by fish oil appears to be more protective in the promotion stage of colon carcinogenesis as observed in this study and in agreement with Chang et al. (7). This may be in part due to intrinsic vs extrinsic apoptosis induction. Kolar et al. (8) demonstrated that the combination of fish oil and pectin exhibit an enhanced ability to induce apoptosis and protect against colorectal cancer in part by recruiting a Ca^{2+}-mediated intrinsic mitochondrial pathway in addition to a nonmitochondrial, Fas-mediated, extrinsic pathway. This study and the current study demonstrate that an understanding of the mechanisms used by putative chemopreventive agents is critical in determining whether combinations of chemopreventatives will be synergistic or antagonistic.

Because quercetin had a tendency to lower AC and 8-OHdG adducts in corn oil fed rats and increase AC in fish oil fed rats, the long-term consequences of supplementing antioxidants to a diet thought to exert its anticancer effect through a pro-oxidant mechanism are unknown and deserve further study. Further investigations should also evaluate the exact in vivo mechanism of dietary fish oil and pectin as well as quercetin, and whether quercetin is more beneficial to an n-6 as compared to an n-3 enriched diet in the initiation, promotion, and progression stages of colon cancer.

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APPENDIX A

EXPERIMENTAL PROTOCOLS

Kill Day Set-up & Checklist

General

- Tape bench papers on lab benches and line with extra bench papers; replace bench papers as necessary.
- Put up instruction/reminder sheets and Rat Kill List at every station.
- Put out gloves of all sizes, boxes of Kim wipes, sufficient napkins, and sharpies at each station.
- Make sure liver Rnase-free utensils and supplies are properly "Zapped" the day before kill.
- Label timers, ACF tub covers, homogenization tubes and pestles
- Tape 2 - 3 biohazard bags on benches where needed
- Make solutions, ensure plenty of reserves; chill those that require chilling.
    - RNase-free & regular 1X PBS
    - 4% PFA
    - 50% & 70% EtOH
    - Heparin Soln.
- Complete rat's 48-hr diet intakes
- Move homogenization buffer from freezer to fridge
- Put up rat kill lists and instruction sheets
- Inform Aleta to prepare cages, covers and a cart for rats to be moved up to 2nd floor
- Tapes & Dispenser
- Assemble rat transport cages

Kill Station

- Check CO_2 tank, chamber, weights, cover cloth
- Black trash bags for rat bodies
- PBS in squirt bottle-RNAse Free
- Surgical tools
    1. Straight scissors (black handle)
    2. Bent large scissors
    3. Forceps-(1) bent, (1) straight
    4. Small blunt tip scissors
- Extra bench papers
- Weigh dishes, 1 big, 1 medium, 2 small labeled with rat #
- Extra weigh dishes all sizes
- Rat packs
- Gauze – lots
- Ice bucket for PFA cubes
- 1 ml pipette with tips

- Instruction sheet
- Liquid Nitrogen Container
- Sharps Container
- Sharpie
- Gloves

ACF Station

- Big weigh dishes labeled with rat # (for holding colon)
- PBS in squirt bottle (1)
- 600mL glass beaker (1) covered w/foil
- Ice bucket (to hold PBS beaker) (1)
- 10cc syringe attached with RNase-free round-tip needle (4)
- Scapels & blades (4)
- Forceps (1 straight, 1 bent)
- Small blunt-tip scissors (1)
- Medium weigh boats for colon
- Labeled Whatman filter papers (in ziplock bag)
- ACF tupperwares and glass plates (2)
- Boat with 70% ethanol
- 70% EtOH
- “ART XLP 200”, P200 pipette (1)
- Biohazard bag
- Record sheet for any observations

Cassetting Station

- Ice tubs for holding small specimen cups (1)
- Labeled timers with rat # (8)
- Labeled big specimen cup with lid for holding cassettes fixed in 70% EtOH (1)
- Labeled cassettes with sponges (2/rat)
- Forceps (1 straight, 1 bent)
- Small scissors (1)
- 1X PBS in squirt bottle
- Labeled rat #, date and fixation small specimen cups with lids (wrap ones) for holding cassettes fixed in 4% PFA (8)
- Large weigh boats labeled with rat #
- Cold 4% PFA
- 70% EtOH
- Igloo coolers for holding ice (2)
- Record sheet for PFA fixation EtOH changes

Mucosal Scrapping and Homogenization Stations

- Ice buckets for storing homogenization buffer and eppetubes
- Homogenization buffer
- Denaturation Buffer
- 2ml RNAse free eppitube /rat
- Ice box for holding glass
- Piggyback racks for holding eppitubes
- Square ice box for holding square glass plate
- Microscope slides
- Curved forcep (1)
- Water in squirt bottle (from bottled sterile water)
- 1X PBS in squirt bottle
- “ART XLP 200”, “ART 1000E”, and “10 Reach” pipette tips
- P1000 pipette (2), P200 pipette (1), and P10 pipette (1)
- Small specimen cups for rinsing (2)
- 2-mL homogenization tubes and pestles (labeled and in ziplock bags) 2/rat
- Pre-weighed tubes for GSH/GSSH
- Homogenization instruction sheet
- Balance
- Data sheet

Protein Isolation Station

- Centrifuge machine
- Timer labeled (4)
- 1cc Luer-Lok syringe with 27G 1¼ needle (8+)
- Sharps container
- Labeled 0.65mL
- 2 mL eppetubes for protein (2) (1 white to centrifuge, 1 yellow to transfer supernatant)
- Ice chest to hold eppitubes (2)
- Piggyback rack
- P200 and P1000 pipettes and pipette tips
- Calculator
- Instruction sheet
- Remarks record sheet and sharpie
- Gloves

Liver Station

- 3 ice buckets
- Zapped glass plate (4)
- 1.8 ml tubes (2/rat)

- Labeled RNA later bottle – 5 ml (1/rat)
- Chopping tools (4)
- Zapped scissors
- NaCl and heparin solution (300 ml/ rat)
- Beaker 250 ml (covered w/foil)
- 10 ml syringe(2) and needles (1/rat)
- Cryobags for extra liver (1/rat)
- Kimwipes
- Gloves
- Catch Tub for liver perfusion
- Tub of Soapy Water
- Large liquid nitrogen container
- Spatulas –Spoon shaped (2), Straight (2)
- Pen, Sharpie
- Ziplock bag
- Instruction Sheet

Blood Station

- 1 ice bucket next to centrifuge
- 0.6 and 0.3 ml heparin tube
- 1 ml cryotube
- Container for liquid N_2
- Rotator and ice in cold room
- Centrifuge at 4 °C
- Balance
- Syringe 2 ml/needle
- Pipette heparinated
- 3 Marma tubes
- 1 marma – Ac tube
- Balancing tubes for centrifuge
- Recording Sheet
- Sharpie Pen

Morning of Kill

1. Take protease inhibitor and protein buffer (previously aliquoted) out of freezer to thaw.
2. Scoop lots of ice and fill necessary tubs, boxes, and mug; store the remaining in ice igloos.
3. Take out heparin solution keep on ice
4. Remove denaturation solution from fridge; keep in ice.
5. Turn on both centrifuges at appropriate temp.
6. Add protease inhibitor to buffer and keep in ice.
7. Prepare and move rats from basement to kill station; keep food in cages.
8. Assemble specimen cups for cassetting – put in tub filled with ice, fill with ice-cold 4% PFA, store in ice chest.
9. Fill ACF tubs and EtOH fixation specimen cups with 70% EtOH.

After Kill

1. Store samples in appropriate place
2. Clean up
3. Autoclave biohazard trash and bring to dumpster
4. Bring rat bodies to freezer in basement diet mixing room
5. Wash tools and glassware; put to dry in oven
6. Solution changes

ABERRANT CRYPT FOCI ENUMERATION PROTOCOL

(Cindy Warren, 2001. Modifications by Kim Paulhill, 8/2006)

Supplies:

Methelyne blue in clear specimen cup
PBS in clear specimen cup
70% Ethanol in clear specimen cup
Rubber gloves
Long stem cotton swabs
Labeled tissue samples in clear specimen cups
Clear grid (with ½ cm squares)
ACF score sheets
Flash Drive
Paper towels
Clip board

Procedure:

1. Place all supplies in small cooler with waffle lab bench paper in the bottom of cooler in case of spillage.
2. In the microscope room, turn on the four switches and prepare to use the microscope (computer, scope, TV screen, grey box).
3. Stain the tissue by dipping in the methylene blue for 10-45 seconds. If too dark dip it back into 70% ethanol.
4. Place on plastic grid taped onto microscope stage, beneath lens, 10 X magnifications. Take care not to touch the lens.
5. Note rat ID number, length of colon, date, etc. on the score sheet.
6. Starting on the distal end (this end doesn't have tissue architecture with ridges). Go slowly through each box, examining the tissue.
7. Note any ACF, both position and multiplicity.
    - Normal crypts are oval or round and a lighter blue stain.
    - ACF are darker and have swirl-like appearance.
    - Many of the ACF have increased size, thicker epithelial lining, and darker staining luminal openings.
    - Compare the ACF to the surrounding tissue.
    - Hyperproliferative crypts are not as distorted as the ACF.
    - ACF have a larger white, distorted zone in the luminal opening.
    - Peyer's patches are lymphatic tissue; look like big cloudy spots

8. Make sure that tissue does not dry out—using cotton swap, moisten tissue periodically with PBS. It should remain shiny.
9. **This procedure is totally subjective.** Determine individual standards for scoring ACF and remain consistent with each tissue.

Date **Sample**

_______ ____________________
____

Apoptosis – ApopTag

Modified by Lisa Sanders for NSBRI initiation study
Used by Kim Paulhill for Quercetin Project
Original Protocol: ApopTag Kit with modifications by Wen-Chi Chang & April Carney
02/2006

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

***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_2O, 2x, 2 min

___4. Quench Endogenous Peroxidase: 0.3% H_2O_2 in 100% Methanol:
    3.0 ml 30% H_2O_2 in 297 ml 100% Methanol or 2.0 ml in 198 ml(add fresh H_2O_2 immediately before quenching). 30 min, RT

___5. Wash in dH_2O, 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 1/10 (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 dH_2O). 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 ($\leq$ 1 min).

___18. Wash in dH_2O, 3X, 1 min
Leave in 4th for 5 min

___19. Counterstain w/ Methyl Green (reusable):
Dip quickly into Methyl green

Rinse in dH_2O 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

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

Tissue: **Name:**

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BUFFER SELECTION – PROTEIN HOMOGENIZATION

3 rats were killed to practice antioxidant assay in colon mucosa. 2 different kinds of buffers were used to homogenize the protein. Particulate buffer and Dr. Wu's modified buffer.
The buffer was modified by Jairam, Laurie and Dr. Turner, so the samples can be used for Western blot assays. Dr. Wu's modified buffer seemed to offer better results for antioxidant enzyme quantification than the particulate homogenization buffer. Apparently the protein recovery is also higher with Dr. Wu's modified buffer. However, this buffer was used for the actual experiment. 10 mL of buffer were prepared for each killing day (8 rats).

DR. WU'S HOMOGENIZATION BUFFER

1. Preparation of Stock solutions

Stock 1. 50mM K_2HPO_4: Dissolve 4.35g of K_2HPO_4 in 500ml of deionized H_2O

Stock 2. 50mM KH_2PO_4: Dissolve 3.4g of KH_2PO_4 in 500ml of deionized H_2O (use 1L bottle)

Stock 3. Prepare 50mM Potassium Phosphate buffer (pH 7.2)
Add 50mM K_2HPO_4 (stock 1) to 50mM KH_2PO_4 (stock 2) until pH is 7.2

Stock 4. Prepare 250mM Sucrose/1mM EDTA solution by dissolving 42.8g of sucrose and 186mg of disodium EDTA in 500ml of potassium phosphate buffer (stock 3). pH again to 7.2 (use KOH or HCl)

2. Preparation of homogenization buffer (adapted from Dr. Wu's assay for enzyme extraction). 250mM sucrose/1mM EDTA/1mM DTT in 50mM potassium phosphate buffer:

5ml	250mM sucrose/1mM EDTA solution (stock 4)
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

DR. WU'S MODIFIED HOMOGENIZATION BUFFER

(Modification suggested by Jairam, Laurie and Dr. Turner)

250 mM sucrose/1mM EDTA/1mM DTT in 50 mM potassium phosphate buffer. Modification consists of final concentration of 0.1% triton rather than 0.0001% and use of sodium orthovanadate.

PREPARATION OF DR. WU'S MODIFIED HOMOGENIZATION BUFFER

1. Preparation of Stock solutions

Stock 1. 50mM K_2HPO_4: Dissolve 4.35g of K_2HPO_4 in 500ml of deionized H_2O

Stock 2. 50mM KH_2PO_4: Dissolve 3.4g of KH_2PO_4 in 500ml of deionized H_2O (use 1L bottle)

Stock 3. Prepare 50mM Potassium Phosphate buffer (pH 7.2)
Add 50mM K_2HPO_4 (stock 1) to 50mM KH_2PO_4 (stock 2) until pH is 7.2

Stock 4. Prepare 250mM Sucrose/1mM EDTA solution by dissolving 42.8g of sucrose and 186mg of disodium EDTA in 500ml of potassium phosphate buffer (stock 3). pH again to 7.2 (use KOH or HCl)

2. Preparation of homogenization buffer

Stock Solutions	**10 mL Buffer**	**50 mL Buffer**	**Company cat. #**	**Storage**
250 mM Sucrose/1mM EDTA solution (stock 4)	9.4 mL	47 mL	K_2HPO_4 & KH_2PO_4 (Sigma) Sucrose (Sigma S7903) EDTA (disodium) Sigma ED4SS	Frozen
Triton X-100 10%	100 µl	500 µl	Peroxidase free Calbiochem 648464	Frozen
1M DTT	10 µl	50 µl	Sigma D9779	Made fresh
10 mM Sodium orthovanadate	100 µl	500 µl	Sigma S6508	Frozen
Protease inhibitor	400 µl	2 mL	Sigma P8340	Add just before use

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_2O = 1µg/µl
   125µl of 2µg/µl + 1000µl ddH_2O = 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.

Catalase Assay Kit
Catalogue # 219265
www.calbiochem.com

Materials Provided:
10X Assay Buffer
10X Sample Buffer
Formaldehyde Standard Kit
Catalase Control
Potassium Hydroxide
Methanol
Hydrogen Peroxide
Purpald
Potassium Periodate
96 Well Plate and Plate Sealer

Materials Required but not Provided:
Plate Reader (540 nm filter)
Adjustable Pipettor
Repeat Pipettor
Distilled or HPLC-grade water

Reagent Preparation

1. 10X Assay Buffer
    a. Dilute 2ml 10X Assay Buffer with 18ml of HPLC-grade water
    b. This is 1X Assay Buffer to be used in Assay
    c. Store at 4°C-Stable for at least 2 months
2. 10X Sample Buffer
    a. Dilute 5 ml of 10X Sample Buffer with 45 ml HPLC-grade water
    b. This is 1X Sample Buffer should be used to dilute standards, controls, samples prior to assaying
    c. Store at 4°C-Stable for at least 2 months
3. Catalase (Control)
    a. Add 2 ml of 1X Sample Buffer and Vortex well
    b. Take 100µl of reconstituted enzyme and dilute with 1.9ml of 1X Sample Buffer
    c. Diluted enzyme is stable for 30 min
    d. Reconstituted CAT is stable for one month at -20°C
4. Potassium Hydroxide
    a. Place vial on ice, add 4ml cold HPLC-grade water and vortex
    b. Store at 4°C stable for at least 3 months
5. Hydrogen peroxide
    a. Dilute 40µl Hydrogen Peroxide with 9.96 ml HPLC-grade water
    b. Dilute Hydrogen Peroxide solution is stable for 2h.

Detailed Protocol

1) Prepare Formaldehyde Standards

   a) Dilute 10µl of Formaldehyde Standard with 9.99 ml 1X Sample Buffer

   b) Add the amount of Formaldehyde Standard and 1X Sample Buffer to each tube as laid out below

Tube	Formaldehyde Stock (µl)	Sample Buffer (µl)	Final Concentration (µM formaldehyde)
A	0	1000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

2) Formaldehyde Standard Wells

   a) Add 100µl 1X Assay Buffer

   b) 30µl Methanol

   c) 20µl Formaldehyde Standards (tubes A-G)

3) Positive Control

   a) Add 100µl 1X Assay Buffer

   b) Add 30µl Methanol

   c) Add 20µl diluted Catalase Control

4) Sample Wells

   a) Add 100µl 1X Assay Buffer
   b) Add 30µl Methanol
   c) Add 20µl sample

5) Initiate the reactions by adding 20 µl dilute Hydrogen Peroxide to all wells
   a) Note time Started

6) Cover Plate with plate sealer and incubate on shaker for 20 min at room temp.

7) Add 30µl Potassium Hydroxide to each well to terminate reaction

8) Add 30µl of Purpald to each well

9) Cover plate with plate sealer and incubate for 10 min at room temp. on shaker

10) Add 10µl Potassium Periodate to each well. Cover with plate sealer and incubate at room temp. on shaker for 5 min.

11) Read absorbance at 540nm

Calculating Results of Catalase Assay

1) Calculate avg. absorbance for each standard or sample

2) Subtract avg. absorbance of standard A from itself and all other standards and samples.

3) Plot corrected absorbance of standards as a function of final formaldehyde concentration

4) Calculate the formaldehyde concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample
   a. Formaldehyde (µM)=(Sample absorbance - y-intercept/ slope) x(0.17 ml/0.02 ml)

5) Calculate Catalase Activity of the Sample using the following equation
   a. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol formaldehyde per min at 25°C
   b. CAT Activity = (µM of sample/20 min)x Sample dilution = nmol/min/ml

Superoxide Dismutase Assay

Caymen Chemicals
Catalog #706002
Range of Kit is 0.025-0.25 units/ml SOD

Contents of Kit

1. Assay Buffer (10X)
2. Sample Buffer (10X)
3. Radical Detector
4. SOD (standard)
5. Xanthine Oxidase
6. 96 Well Plate
7. Plate Cover

Additional Items Required

1. Potassium Cyanide
2. Plate Reader with 450nm filter
3. Adjustable pipettors and a repeat pipettor
4. Glass distilled water or HPLC-grade water

Pre-Assay Preparation

1. Assay Buffer-(10X)-(Vial #1)
    a. Dilute 3ml of Assay Buffer with 27ml of HPLC-grade water

       Assay buffer is used to dilute Radical Detector

       Store at 4°C-Stable for 2 Months
2. Sample Buffer-(10X)-(Vial #2)
    a. Dilute 2ml of Sample Buffer concentrate with 18 ml of HPLC-grade water
        i. Final Sample Buffer should be used to prepare the SOD standards and Dilute xanthine oxidase and SOD samples prior to assaying

       Store at 4°C-Stable for 2 Months
3. Radical Detector-(Vial #3)

   a. Transfer 50:l of tetrazolium salt to another vial and dilute with 19.95 ml of *diluted* Assay Buffer
   b. Cover with Foil
   c. Stable for 2 hours

4. SOD standard-(Vial #4)
   a. Bovine Erythrocyte SOD(Cu/Zn)
      i. Ready to use as supplied
      ii. Store thawed enzyme on ice
5. Xanthine Oxidase (Vial #5)
   a. Thaw one vial
   b. Transfer 50:l of enzyme to another vial
   c. Dilute with 1.95 ml of *diluted* Sample Buffer
   d. Store on ice-stable for 1 hour
      i. Do not refreeze thawed enzyme

Performing the Assay

The Assay Temp is 25°C and Absorbance at 450 nm

All reagents except samples and xanthine oxidase must be equilibrated to room temperature before beginning the assay.

1. Preparation of SOD standards

    a. Dilute 20:l of SOD standard (Vial #4) with 1.98 ml of *diluted* Sample Buffer to each tube as described below:

Table 1

Tube	**SOD stock(:l)**	**Sample Buffer (:l)**	**Final SOD Activity (U/ml)**
A	**0**	**1,000**	**0**
B	**20**	**980**	**0.025**
C	**40**	**960**	**0.05**
D	**80**	**920**	**0.1**
E	**120**	**880**	**0.15**
F	**160**	**840**	**0.2**
G	**200**	**800**	**0.25**

2. **SOD Standard Wells**-Add 200:l of diluted Radical Detector and 10:l of Standard(tubes A-G) in designated wells on plate

3. **Sample Wells**- Add 200:l of diluted Radical Detector and 10:l of Sample or :

    a. 190:l of diluted Radical Detector

    b. 10:l of inhibitor (potassium cyanide)

    c. 10:l of sample

4. Initiate reaction by adding 20:l of diluted xanthine oxidase to all wells

   a. ***Note Precise time started***
   b. ***Add as quickly as possible***

5. Shake Plate for 5 sec. to mix and cover with plate cover
6. Incubate on a shaker for **20 min** at room temp
7. Read absorbance at ***450nm*** using a plate reader

Calculating Results

1. Calculate average absorbance of each standard and sample
2. Divide Standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR)
   a. (*i.e. LR for Std A= Abs Std A/ Abs Std. A; LR for Std B=Abs Std A/ Abs Std B)*
3. Plot the linearized SOD standard rate (LR) as a function of final SOD Activity (U/ml) from Table 1
4. Calculate the **SOD activity** of the samples using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample.
   a. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical.
   b. SOD(U/ml)= [(Sample LR-y-intercept/slope)x (0.23ml/0.01ml)] x sample dilution

Glutathione Peroxidase Assay Kit
Catalog No. 703102

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage.

$$\text{R-O-O-H} + 2\text{GSH} \longrightarrow \text{R-O-H} + \text{GSSG} + H_2O$$

$$\text{GSSG} + \text{NADPH} \longrightarrow 2\text{GSH} + \text{NADP}^+$$

Pre-Assay Preparation

Preparation of reagents:

1. Assay Buffer (10X)-(vial #1)
   a. Dilute 2 ml of Assay Buffer concentrate with 18 ml of HPLC-grade water
   b. Stare at 4ºC – stable for at least 2 months
2. Sample Buffer (10X)- (vial#2)
   a. Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water
   b. Store at 4ºC – stable for at least 1 months
3. Glutathione Peroxidase (Control)-(vial #3)
   a. Transfer 10 µl of enzyme and dilute with 490 µl of *diluted* Sample Buffer and keep on ice.
   b. Stable for 4 hours on ice.
4. Co-Substrate Mixture- (vial # 4)
   a. Add 2ml of HPLC-grade water to each vial needed (each vial is enough for 40 wells)
   b. Reconstituted Reagent should be kept at 25°C while assaying and then stored at 4°C
   c. Stable for 2 days at 4°C
5. Cumene Hydroperoxide-(vial #5)
   a. Reagent is ready to use as supplied
   b. Store at -20°C

Performing the Assay

- The final Volume of the assay is 190µl in all the wells
- The assay temperature is 25°C
- Use the Assay Buffer dilute in the assay
- Monitor the decrease in absorbance at 340 nm using a plate reader

1. Background or Non-enzymatic Wells- Add
   a. 120µl of Assay Buffer
   b. 50µl of co-substrate mixture to three wells
2. Positive Control Wells (bovine erythrocyte GPx)- Add
   a. 100µl of Assay buffer,
   b. 50µl of co-substrate mixture,
   c. 20µl of diluted GPx (control) to three wells.
3. Sample Wells- Add
   a. 100 µl of Assay Buffer,
   b. 50µl of co-substrate mixture,
   c. 20µl of sample to each well
4. Initiate reaction by adding 20µl of cumene hydroperoxide
5. Shake plate for a few seconds to mix
6. Read absorbance once every minute at 340 nm using plate reader to obtain at least 5 time points.

Calculating the Results

Determination of the Reaction Rate:

1. Determine the change in absorbance per minute by:
   a. Plotting the values as a function of time to obtain the slope (rate) of the linear portion of the curve -OR-
   b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:
      i. ΔA_{340}/min=A_{340}(Time 2) – A_{340}(Time 1)/ (Time 2(min.)- Time 1 (min.))
2. Determine the rate of ΔA_{340}/min. for the background or non-enzymatic wells and subtract this rate from that of the sample wells.
3. Use the following formula to calculate the GPx activity.
   a. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25ºC.

GPx Activity= ((ΔA_{340}/min)/ 0.00373µM^{-1})x (0.19ml/0.02ml)x sample dilution = nmol/mim/ml

Immunohistochemistry of 8-hydroxy-2’-deoxyguanosine

Note: For 70%EtOH fixed tissue.

Day 1

___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_2O_2** (Sigma #H-1009) **in methanol for 30 min** to quench endogenous peroxidase.
(Add 1ml H_2O_2 for every 10 ml of methanol. Add H_2O_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_2O and place in ddH_2O. Finish one slide before moving to next slide.
1) 5 ml dH_2O.
2) 2 drops of buffer, mix.
3) 4 drops of DAB, mix.
4) 2 drops of H_2O_2, mix.

___**20. Wash in ddH_2O 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 8-oxodG

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$_2$O

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$_2$O

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$_2$O

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$_2$O

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_2O – 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_2O_2 in methanol
30% H_2O_2 (Sigma H-1009)
100% Methanol

Add 1ml of 30% H_2O_2 to every 10ml of MeOH for 3% H_2O_2.

HPLC Analysis of GSH, GSSG, Cysteine and Cystine

Modified By Kim Paulhill 7/2007

The principle: the reduced form of glutathione (GSH) an the oxidized form of glutathione (GSSG), as well as cystine and cysteine, are derivatized with dansyl chloride in the presence of iodoacetic acid and KOH/teraborate (pH 9.0) to yield fluorescence derivatives. The derivatives are separated by HPLC and quantified using fluorescence detection.

1. **Chemicals:**
    a. Sodium Heparin
    b. Bathophenanthroline Disulfonate Sodium Salt (BPDS)
    c. Iodoacetic Acid
    d. Dansyl Chloride
    e. L-serine
    f. GSH
    g. GSSG
    h. Cystine
    i. Cysteine
    j. Sodium Acetate Trihydrate
    k. Boric Acid
    l. Sodium Tetra Borate

2. **Preparation of Solvents and Reagents:**
    a. 100 mM Boric Acid: Dissolve 3.1g boric acid in 500ml HPLC H_20
    b. 100 mM Sodium Tetraborate: Dissolve 19.1g sodium tetraborate in 500ml HPLC H_20

        i. **Solution A (Presevation Solution):**
            1. Dissolve 1.05g L-Serine
            2. 50 mg sodium Heparin
            3. 100 mg BPDS
            4. 200 mg iodoacetic acid
            5. in 80 ml of 100mM boric acid
            6. 20 ml of 100mM sodium tetraborate
            7. Solution is stable at -80°C for 6 months
            8. *(Note: Heparin to inhibit coagulation, serine·borate to inhibit degradation of GSH by (-glutamyltranspeptidase, bathophenanthroline disulfonate to inhibit GSH oxidation, and iodoacetic acid to alkylate GSH).*

        ii. **Solution B (10% perchloric acid, w/v; 0.2 M boric acid):**
            1. Dissolve 6.2 g boric acid in 300 ml HPLC water
            2. Add 71 ml of 70% perchloric acid
            3. quiesce to 500ml with HPLC water

- **iii. Derivatization solutions:**
  - **1. KOH/tetrahydroborate solution (pH 9.0):**
    - a. Add 5.6 g KOH
    - b. to a plastic bottle containing 50g $K_2B_4O_7 \cdot 4H_20$ and 100ml HPLC water
    - c. Mix- (*Stable indefinitely at 25°C*)
  - **2. Iodoacetic Acid:**
    - a. Dissolve 14.8 mg iodoacetic acid in 2ml of HPLC water
    - *b. Made fresh the day of derivatization*
  - **3. Dansyl chloride:**
    - a. Dissolve 200mg dansyl chloride in 10ml of HPLC acetone
    - *b. Made fresh the day of derivatization*

3. **GSH, GSSG, Cystine, and Cysteine standards:**
   - **a. 2 mM GSH, GSSG, Cys, and Cystine:**
     - i. Dissolve 6.16 mg GSH
     - ii. 12.2 mg GSSG
     - iii. 2.44 mg cysteine
     - iv. 4.8 mg cystine
     - v. In 5ml of Sol A + 5ml of Solution B
   - **b. 500 nmol/ml GSH, GSSG, Cys, and Cystine:**
     - i. Mix 50µl of 2 mM standards
     - ii. With 75µl of Sol A+ 75µl of Sol B
   - **c. 50 nmol/ml GSH, GSSG, Cys, and Cystine:**
     - i. Mix 50µl of 2 mM standards
     - ii. With 975µl of Sol A+ 975µl of Sol B
   - **d. Blank (0 nmol/ml):**
     - i. Mix 200µl of Sol A+ 200µl of Sol B

4. **Extraction of Glutathione from Tissue**
   - a. Tissue Homgenization for small sample size;(~ 5 to 10 mg tissue)
     - i. Homogenize the tissue in 0.1 ml Sol A+ 0.1 ml Sol B
     - ii. Rinse Homogenizer with 0.1 ml Sol A + 0.1 ml Sol B
     - iii. Combine Homogenates
     - iv. Centrifuge all tubes at 10,000 g for 1 min.
     - v. Use supernatant for derivatization
5. **Derivatization**
   - a. Add to 1.5 ml microcentrifuge tube (use amber tubes):
     - i. 150µl of standards (0, 50, 500 nmol/ml) or samples
     - ii. 30µl of Iodoacetic acid solution
     - iii. 100µl of KOH/tetraborate sol. (pH ~9)
     - iv. Vortex and wait 20 min
   - b. Add 150µl of dansyl chloride solution to each tube
     - i. Vortex

        - ii. Keep all tubes in dark at room temp. for 16 h.
    - c. Add 250µl of HPLC-grade chloroform to each tube(to extract the free dansyl-Cl)
        - i. Vortex and centrifuge at 10,000 g for 1 min
        - ii. (*The dansyl derivatives are stable in the dark at 0-4°C for 12 months*)
        - iii. Use upper aqueous layer for HPLC analysis
    - d. Add 100µl of sample (or standard) derivatives to a micro-insert tube in a brown vial
        - i. Vortex
        - ii. Injection volume: 25µl

6. **HPLC Analysis**
    - a. HPLC solvents:
        - i. Solvent A (Acetic Acid Buffer, pH 4.6)
            1. 640 ml Methanol + 200 ml Acetate stock solution +125 ml Glacial acetic acid + 50 ml HPLC water
            2. (*Acetate Stock solution: Dissolve 272 g sodium acetate trihydrate in 122 ml HPLC water and 378 ml glacial acetic acid*)
        - ii. Solvent B (80% Methanol; v/v):
            1. 800 ml HPLC Methanol + 200 ml HPLC water
    - b. HPLC Column: 3-Aminopropyl column (5 um; 4.6 x 250 mm)
        - i. Custom LC, Houston, TX: Tel 800-537-9339)
    - c. HPLC solvent gradient:

Solvent	Time(min)					
	0	10	30	33	33.1	38
Solvent A (%)	20	20	80	80	20	20
Solvent B (%)	80	80	20	20	80	80

Flow rate: 1.0 ml/min

Fluorescence detection (Waters 2475 Multi λ Fluorescence Detector):

0.0 to 7.5 min: 590 nm excitation; 610 nm emission
7.5 to 38 min: 335 nm excitation; 610 nm emission

Gain: 10 (0 to 32.2 min): 100 (32.2 to 38 min).
(For liver samples)
Gain: 100 (0 to 32.2 min): 1000 (32.2 to 38 min).
(For other samples)

Attenuation: 1

Retention time: Cys-16.7 min, Cysteine-20.1 min, GSH- 29.4 min,
GSSG- 3.8 Min

d. References:(104)

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
Cystiene Standard	Sigma C-7352
Cystine Standard	Sigma C122009
Sodium acetate trihydrate	Sigma 236500
Boric acid	Sigma B0394
Sodium tetraborate	Sigma 221732
70% Perchloric acid	Fisher A469
HPLC grade water	Fisher W7
Potassium hydroxide (KOH)	Sigma P5958
Potassium tetraborate tetrahydrate	Sigma P5754
Acetone	Fisher A949
Chloroform	Sigma 650498
Glacial acetic acid	Bio/Bio
Methanol (HPLC grade)	Fisher A452
3-aminopropyl column	CEL Associates #132-204 Ph# 800-537-9339 Pearland, TX

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 insert tubes	Waters Wat72030

HPLC Recording Sheet

Sample Description	Cryo/Sample Weight (mg)	Cryo-Only Weight (mg)	Sample Weight (mg)	Sol A/Sol B Added Vol (µl)	Total Vol. (µl)	20 min Incub. (In Dark)	Start Time 16 hr	End Time 16 hr	Storage Date

APPENDIX B

TABLES OF RESULTS

TABLE 3

Average Food Intake (g)[1]

Diet	Non-Quercetin		Quercetin		
	Corn Oil	Fish Oil	Corn Oil	Fish Oil	p-value
Day 18-20	16.84 ± 0.33^{a,b}	16.60 ± 0.33^{a}	17.19 ± 0.33^{a}	17.66 ± 0.33^{b}	0.0385
Day 56-58	20.02 ± 0.60	19.72 ± 0.60	19.39 ± 0.60	19.49 ± 0.60	NS
Day 67-69	19.94 ± 0.38	20.04 ± 0.38	20.12 ± 0.38	18.90 ± 0.38	NS

[1] Values represent LS Means ± S.E.M. of average food intake for n= 10 rats/ diet, means without a common letter differ. NS=Not Significant

TABLE 4

Average Weight Gain Day (g)[1]

Diet	Non-Quercetin		Quercetin		
	Corn Oil	Fish Oil	Corn Oil	Fish Oil	p-value
Day 18-20	6.66 ± 0.46	6.62 ± 0.46	6.30 ± 0.46	6.39 ± 0.46	NS
Day 56-58	2.84 ± 0.57	3.01 ± 0.57	3.32 ± 0.57	3.07 ± 0.57	NS
Day 67-69	1.99 ± 0.45	1.89 ± 0.45	2.23 ± 0.45	2.46 ± 0.45	NS

[1] Values represent LS Means ± S.E.M. of average weight gain for n= 10 rats/ diet. NS= Not significant.

TABLE 5

High Multiplicity ACF (4+) [1]

Diet	Corn Oil	Fish Oil
(-) Quercetin	0.60 ± 1.26^{a}	0.00 ± 0.00^{b}
(+) Quercetin	0.20 ± 0.42^{a}	0.00 ± 0.00^{b}

[1] Values represent Means ± Std D of average high multiplicity ACF with 4 or more aberrant crypts in foci for n= 10 rats/ diet, means without a common letter differ, p= 0.0184

TABLE 6

Average Mitochondrial SOD (Mn-SOD) Enzyme Activity (U/mg protein)[1]

Diet	Corn Oil	Fish Oil
(-) Quercetin	8.14 ± 1.17	7.88 ± 1.17
(+) Quercetin	9.28± 1.17	8.29 ± 1.17

[1] Values represent LS Means ± S.E.M. of average Mn-SOD enzyme activity for n= 10 rats/ diet.

VITA

Kimberly Jones Paulhill

Permanent Address

215 Kleberg MS 2253
College Station, TX, 77844-2253

Education

Institution	*Degree*	*Discipline*	*Graduation Date*
Texas A&M Univ.	M.S.	Nutrition	Aug. 2008
Texas A&M Univ.	B.S.	Nutrition	Dec. 2002
Blinn College	A.S.	Mathematics	Dec. 2000

Honors&Awards

Scholarships- Louis Stokes Alliance for Minority Participation (2007), IFN (2007)
Fellowship- Bridge to the Doctorate Fellow Cohort II (2004-2006)
Travel Awards- OGS Student Research (2007), IFN(2007,2008)
Finalist- Procter and Gamble Student Abstract Competition (2008), Coca-Cola Student Poster Competition (2007, 2008)
Recipient-Excellence in Science Award (IFN, 2007)

Publications

KJ Paulhill, S.S. Taddeo, G. Wu, R.J. Carroll, R.S. Chapkin, J.R. Lupton, and N.D. Turner. 2008. Endogenous antioxidant enzyme activities and colonocyte redox balance are altered by dietary lipids and quercetin. FASEB J.

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KJ Paulhill, SS Taddeo, RJ Carroll, RS Chapkin, JR Lupton, and ND Turner. 2007. Quercetin does not significantly affect the protection of a fish oil diet in early colon carcinogenesis. FASEB J. 21: 112.3

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