THE EFFECT OF THE ANTIOXIDANT, COENZYME Q, ON CACO-2 COLON CANCER CELLS TREATED WITH IRON TO INDUCE LIPID PEROXIDATION

by

Viva M. Johanknecht

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The Graduate School
University of Wisconsin-Stout
Menomonie, WI 54751

ABSTRACT

(Writer)	(Last Name)	(First Name)	(Initial)
	Johanknecht	Viva	M

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The objective of this study was to determine if Coenzyme Q_{10} (also known as ubiquinone) acting as an antioxidant, would protect against free radical damage to cell membranes that can cause cancer. Caco-2 cells were fed experimental media with and without different concentrations of iron (200, 400 or 800 uM) and with and without CoQ_{10} (400 uM). The presence of malondialdehyde (MDA) and 4-hydroxynonenals (4-HNE or HNE) were assayed as an indicator of lipid peroxidation. The results were standardized for the amount of protein in the cell culture well. Iron was not a significant cause of lipid peroxidation in Caco-2 cells. CoQ_{10} appeared to significantly reduce the amount of MDA and 4-HNE in the media and cells combined regardless of the presence of iron, but the analysis did not include the vehicle, mineral oil, for CoQ_{10} due to a limited **n**. Therefore, the mineral oil with CoQ_{10} may be considered protective from free radical damage in the colon.

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CHAPTER ONE

Introduction to the Study

Introduction

Nutrition has important effects on the health and well-being of humans. Optimal nutritional practices can not only enhance an individual's health but also protect against different types of cancers (American Cancer Society 2002). Nutritional status has an influence on how cancers develop and progress (American Cancer Society 2002). Ever since the first population studies linked diets rich in vegetables, fruits and grains to low rates of cancer, scientists have been trying to find out how these foods provide their protective effects (Bright-See 1988; AICR 1998).

Colon cancer is influenced by nutrition (Thun et al. 1992; American Cancer Society 2000; 2002). Possible risk factors for colon cancer include physical inactivity, a high fat and/or a low-fiber diet, as well as inadequate intake of fruits and vegetables (Thun et al. 1992; American Cancer Society 2000; 2002). Other nutrients, such as iron, increase the risk of colon cancer by damaging cell membranes (Bird et al. 1996). High iron consumption has been proposed to increase the risk of colon cancer (Younes et al. 1990; Bird et al. 1996; Lund et al. 2001; Nelson and Davis 1994). Scientific research on prevention of colon cancer should include research on those nutrients that are protective against colon cancer.

Predictions published by the American Cancer Society suggest that about 1,284,900 new cancer cases are expected to be diagnosed in 2002. This year about 555,500 Americans are expected to die of cancer, more than 1,500 people a day. About one-third of these expected

cancer deaths would be related to nutrition, physical inactivity, obesity, and other lifestyle factors that could be prevented (American Cancer Society 2002). This shows that it is important to understand the direct connection between specific nutrients and cancer and take appropriate actions based upon that knowledge. In the past, genetics (nature) and nutrition (nurture) were considered two competing forces in the development of the individual (Simopoulos 1999). Today we understand that it is the interaction of genetics and the environment, including diet and lifestyle that provides the foundation for health and disease (Simopoulos 1999 and 1995).

Humans today live in a nutritional environment that differs from that upon which our genetic constitution was selected. Archaeological findings and historical research indicate that a Paleolithic Cave Person's diet was rich in antioxidants and minerals that influence our evolution and genetic profile (Lane 1999). Rapid changes in our diet, particularly over the last 150 years, have altered both the type and amount of fatty acids that we consume and the antioxidant content of our diet. These dietary changes promote the development of chronic diseases such as arteriosclerosis, essential hypertension, obesity, diabetes, and many cancers (Simopoulos 1999).

Colon cancer in particular can develop as a result of dietary changes or nutritional changes in the human body (Bird et al. 1996). High dietary intakes of iron may enhance the risk of colon cancer, due to the ability of iron to generate free radicals *in vivo* (Bird et al. 1996). Excess levels of dietary iron have serious consequences such as enhanced lipid peroxidation, subsequent cellular damage and carcinogenesis resultant from an increase of hydroxyl radical production (Younes et al. 1990; Nelson 1992; Sobotka et al. 1996).

A direct relationship between the amount of iron ingested and the frequency of colon cancer has been observed in animal studies (Porres 1999). Researchers have examined the susceptibility of pigs to the oxidative stress caused by a moderately high dietary iron intake (Porres 1999). One goal of their experiment was to test the hypothesis that ingestion of moderately high amounts of iron could produce oxidative stress in the colon of the pig. Elevated amounts of dietary iron fed to these animals was associated with a significant increase in colon lipid peroxidation (Porres 1999) and that oxidative stress was related to increased risk of colon cancer (Porres 1999; Stone and Papas 1997). Lipid peroxidation is recognized as a mechanism of cellular injury or oxidative stress (Blache et al. 1999). This stress process leads to the destruction of membrane lipids and the production of lipid peroxides and their by-products such as aldehydes (Blache et al. 1999).

Oxidative stress is becoming an important hypothesis to explain the genesis of several pathologies, including cancer, atherosclerosis and aging (Blache et al. 1999). An imbalance between nutrients, in particular those involved in antioxidant status, could explain the onset of an enhanced production of free radicals (Blache et al. 1999). By definition, a free radical is a molecule containing an odd number of electrons. If two radicals react, both are eliminated; if a radical reacts with a nonradical, another larger, but different, free radical is produced. The latter event may become a chain reaction, playing a role in tissue injury. Once free radicals are formed they attack molecules in the immediate vicinity. In living cells this means taking electrons from cell constituents. Radicals may give rise to more radicals, therefore; causing progressively more damage. Radical-induced changes may result in cancer.

The body has developed methods of defending itself against the harmful effects of free radicals. Superoxide dismutases, enzymes in mitochondria, and antioxidants are effective in counteracting the harmful effects of free radicals (Davis 1997). The primary mechanism by which the body gets rid of radicals is through donation of electrons between oxygen species and antioxidants. Coenzyme Q (CoQ) is a nutrient that acts as an antioxidant with reduction potential to eliminate a free radical (Groff and Gropper 2000).

CoQ is a fat-soluble compound that serves as an antioxidant. It provides hydrogens to terminate lipid peroxyl radicals and complements the antioxidant activity of Vitamin E in low-density lipoprotein (LDL) radical oxidation (Groff and Gropper 2000; Thomas, Neuzil and Stocker 1997). CoQ is efficient against lipid peroxidation in solution and in liposomal membranes, therefore; CoQ plays an important protective role against oxidative stress (Niki 1997).

The protective role of CoQ against oxidative stress prevents this type of cellular injury in tissues. With this knowledge it is critically important to study whether or not CoQ's antioxidant properties can prevent cellular injury in colon tissue. Colon cancer was responsible for 47,700 deaths in the United States in 2000 and there will be an estimated 148,300 new cases of cancers of the colon and rectum this year and an estimated 56,600 deaths (American Cancer Society 2000 and 2002). Therefore, it is imperative to determine whether or not CoQ can play a protective role against colon cancer in humans.

Statement of the Problem

We know that high iron consumption is linked to an increased risk of colon cancer. One mechanism by which this occurs is through the process of lipid peroxidation or oxidative stress on the tissue. This process creates free radicals that attack and cause damage to colon cell membranes. This damage can ultimately contribute to the onset of colon cancer. CoQ is an antioxidant that can act to sequester the free radicals rendering them harmless to the colon cell membrane.

An imbalance between nutrients, in particular those involved in antioxidant status, could explain the onset of an enhanced production of free radicals (Blache et al. 1999). A decrease in the cellular levels of the antioxidant CoQ has been shown to be associated with breast and liver cancer (Lockwood et al. 1995; Portakal et al. 2000; Yamamoto et al. 1998). However, it is not known whether CoQ is similarly associated with colon cancer. Therefore, this research study seeks to demonstrate whether or not CoQ protects colon cells from iron-induced lipid peroxidation. This research complements what is currently known about colon cancer and enhances our understanding of the disease.

Research Objectives

One objective of this cell culture study is to determine whether or not CoQ, acting as an antioxidant, will protect colon cell membranes from iron-induced lipid peroxidation. If so, this would suggest that CoQ can protect against free radical damage to the cell membranes of the colon that is associated with the development of cancer. A second objective is to incorporate results from this research into what is known about preventing colon cancer by promoting the

ingestion of proper amounts and types of nutrients and incorporation of healthy lifestyle habits. Many new cases and deaths from colorectal cancer are preventable by improvements in nutrition and physical activity (Frazier et al. 2000).

Significance of the Study

The significance of this study is that the research results may contribute to the discipline of human nutrition. Prior to this study, iron-induced lipid peroxidation in colon cells (Caco-2 cells) has been researched, but the effects of CoQ on colon cells have not been studied. The results of this study will provide information to further understand the relationship between lipid peroxidation and colon cancer. It will also provide information on whether or not CoQ is protective for colon cells. The results of this study may contribute information for further research to be conducted on the relationship between CoQ and colon cancer.

Limitations of the Study

This research is on selected chemical changes of lipids from human colon cancer cells (Caco-2) grown in cell culture. The research was conducted with a very small budget for supplies and equipment in a cell culture laboratory started from scratch just prior to the initiation of this investigation. With the stringent budgetary constraints minimal replications of data were obtained. In fact, I was actively involved in the grant writing to obtain adequate funding to purchase proper equipment and supplies before beginning the research. The study was limited by the facilities and equipment available at the University of Wisconsin-Stout Biology and Chemistry Department Laboratories. Another limitation is the applicability of information obtained from the use of colon cancer cells grown under culture conditions as a model of the

behavior of human colon tissue *in vivo*. Although the cell culture conditions made it possible to control many variables and focus on the effects of CoQ on iron-induced lipid peroxidation, caution must be exercised when extrapolating the data to human colon tissue.

Assumptions

The assumptions made during the research process include the ability to replicate iron-induced lipid peroxidation accomplished in numerous studies using cell cultures. Therefore, this research study was conducted on the basis of being able to replicate what other laboratories have shown, the ability of iron to initiate lipid peroxidation (Bachowski et al. 1988; Brasitus et al. 1985; Jourd'heuil et al. 1993; Courtois et al. 2000). A second assumption made was that Caco-2 cell culture would be an acceptable model for the study of iron-induced lipid peroxidation in human colon tissue *in vivo*.

Methodology

The methodology of this research includes the maintenance of the Caco-2 cells, subculturing the Caco-2 cells, the experimental design, the cell lysis and freeze thaw cycles, the lipid peroxidation colorimetric assay, the Lowry protein assay, and the statistical analysis of the data using the Statistical Package for the Social Sciences (SPSS) software version 9.0.

CHAPTER TWO

Literature Review

This chapter reviews the literature on the proliferation and prevention of colon cancer both *in vitro* and *in vivo*. The ability of iron to induce lipid peroxidation and the attendant damage to colon cells precedes the initiation and propagation of colon cancer (Younes et al. 1990). Such damage can occur when iron is present at increased levels in the body (Younes et al. 1990). As a result of lipid peroxidation in cells, two products of that process are formed in measurable amounts in cells, malondialdehyde (MDA) and 4-hydroxynonenals (4-HNE or HNE) (Moore and Roberts 1998). Coenzyme Q (CoQ) is an antioxidant that has increasing support for having protective affects against cancer. Studying ways to prevent colon cancer is important to human life since cancers as a whole are one of the leading causes of death in the United States.

Rapid advances in cancer prevention challenge health care workers to look not only at the treatment of the disease but also at its prevention. Understanding cancer and what causes cancer to develop is important in order to determine strategies to prevent the occurrence of cancer. Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. All cancers involve the malfunction of genes that control cell growth and division. Only about 5% to 10% of cancers are clearly hereditary, in that an inherited faulty gene predisposes the person to a very high risk of particular cancers (American Cancer Society 2002). The remainder of cancers lacks a clear hereditary connection, but result from damage to genes (mutations) that occurs throughout a person's lifetime, either due to external or internal factors (American Cancer Society 2002). External

factors include tobacco, chemicals, radiation, nutrients and infectious organisms, while internal factors include inherited mutations, hormones, immune conditions and mutations that occur from metabolism (American Cancer Society 2002). The individual can control many of the external factors.

Scientific evidence suggests that about one-third of the cancer deaths that occur in the US each year are due to the adult diet, including its effect on obesity. Another third is due to cigarette smoking. Therefore, for the majority of Americans who do not use tobacco, dietary choices and physical activity become the most important modifiable determinants of cancer risk (American Cancer Society 2000). The evidence also indicates that although inherited genes do influence cancer risk, heredity alone explains only a fraction of all cancer. Behavioral factors such as tobacco use, dietary choices, and physical activity modify the risk of cancer at all stages of its development (American Cancer Society 2000).

Cancer is a burden on U.S. health care costs. The National Institute of Health estimate overall costs for cancer in 2001 at $156.7 billion: $56.4 billion for direct medical costs (total of all health expenditures): $15.6 billion for indirect morbidity costs (cost of lost productivity due to illness): and $84.7 billion for indirect mortality costs (cost of lost productivity due to premature death) (American Cancer Society 2002). Colon cancer has a significant impact on the overall costs for cancer treatment since the colon and rectum (colorectal) comprise the third most common site of new cases and deaths in both men and women (American Cancer Society 2002).

In the United States, colon cancer is the second most common cancer in adults (after lung cancer) and is also the second most common cause of death due to cancer. Worldwide, it is the third most common malignant neoplasm and the second leading cause of cancer deaths (Mahan and Escott-Stump 2000). Since colon cancer is responsible for such a high number of cancer deaths, finding preventive mechanisms for colon cancer is vital to the health of humans.

Factors that increase the risk of colon cancer include family history, occurrence of inflammatory bowel disease (both Crohn's disease and ulcerative colitis), familial polyposis polyps, adenomatous polyps, and several dietary components (Steele 1995; Eastwood 1995; Potter et al. 1993). Understanding how dietary choices modify the risk of cancer at various stages of its development is the backbone for the prevention of cancers. Dietary factors that may impact the incidence of colon cancer include high meat or fat intake, high intake of fat and iron, and low intakes of vegetables, high-fiber grains, carotenoids, vitamins D, E, and folate, and the minerals calcium, zinc, and selenium (van Poppel and van-den Bergh 1997; Singh et al. 1997; Sawa et al. 1998). Iron in excess can be toxic because it can promote the formation of harmful oxygen radicals, which ultimately cause peroxidative changes to vital cell structures (Britton et al. 1994; Bird et al. 1996; Younes et al. 1990; Nelson 1992; Sobotka et al. 1996).

Iron-Induced Lipid Peroxidation

Iron is necessary for all cells since it is a component of heme-containing proteins (hemoglobin, myoglobin, cytochromes) and numerous nonheme iron-containing proteins (Richardson and Ponka 1997; Cullen et al. 1999). Iron exists in several oxidation states varying from Fe^{6+} to Fe^{2+}, depending on its chemical environment. The only states that are stable in the

aqueous environment of the human body and in food are the ferric (Fe^{3+}) and the ferrous (Fe^{2+}) forms. Dietary iron is found in one of two forms in foods, heme and nonheme. Heme iron is found in meat, fish, and poultry. Nonheme iron is found primarily in plant foods such as nuts, fruits, vegetables, grains, and tofu, and in dairy products such as, milk, cheese, and eggs (Groff and Gropper 2000). Iron can also be found in supplements in the form of ferrous iron, which is provided as nonheme iron (Groff and Gropper 2000).

Iron is absorbed into the body through the intestinal cells (enterocyte) in the jejunum, the second portion of the small intestine extending from the duodenum to the ileum. The mechanisms of heme iron and nonheme iron absorption differ. Heme iron is absorbed intact into the enterocyte and then is hydrolyzed within the intestinal cell to yield ferrous iron. Nonheme iron is typically present in the stomach in the ferric state. Absorption of iron is improved if the iron is present as ferrous iron (Groff and Gropper 2000). Therefore, heme iron found in meat, fish, and poultry is absorbed more readily into the enterocyte than iron found in plant foods. Ascorbate (vitamin C) increases the intestinal absorption of nonheme iron (Groff and Gropper 2000). Once iron is absorbed into the enterocyte, it must either be transported through the enterocyte into the blood for use by the body tissues, be stored in the intestinal cell for future use or be eliminated via the kidneys (Groff and Gropper 2000). Because the absorption of iron in the small intestine is regulated, not all ingested iron is absorbed. Consequently, a high intake of iron may cause higher concentrations of iron to reach the colon. If a high fiber diet is not present to move the iron quickly through the colon, then higher amounts of iron are available for extended periods to initiate cell damage in the colon.

Storage of iron occurs in the body when iron is absorbed in excess of the body's need. Iron stored in cells in the form of ferritin can increase the amount of iron available to the body since ferritin is not a stable compound; it is constantly being degraded and resynthesized to maintain an available intracellular iron pool (Groff and Gropper 2000). Ferritin is synthesized in a variety of tissues, including the liver, spleen, bone marrow, and intestine (Groff and Gropper 2000). The superoxide radical (O_2-) can initiate iron release from ferritin in vitro (Groff and Gropper 2000).

Iron is an essential mineral in the health and maintenance of the human body. The ferrous iron in the center of the heme molecule allows the transport of oxygen to tissues (hemoglobin); the transitional storage of oxygen in tissues, particularly muscle tissues (myoglobin); and the transport of electrons through the respiratory chain (cytochromes) (Groff and Gropper 2000).

Oxygen (O_2) plays a double role in the cell: it is essential for aerobic respiration, but it can also act as a free radical since it contains two unpaired electrons. When an oxygen molecule captures one electron, it becomes a superoxide radical (O_2-). This highly reactive radical is normally produced by macrophages in order to destroy bacteria after phagocytosis. However, this species can also be generated during oxidative phosphorylation by the respiratory chain in mitochondria. It can also be generated in a dismutase reaction by the action of superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2) (Gate et al. 1999). *(Figure 1).*

Iron acts as a proxidant when free ferrous iron (Fe^{2+}) catalyzes the nonenzymatic *Fenton reaction (Figure 1)* in which ferrous iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) to generate ferric iron (Fe^{3+}) and hydroxyl radicals (OH·). The hydroxyl radical, OH·, is an oxygen-centered radical that is a very highly reactive oxidant that can oxidize DNA, lipids, and proteins (Kehler 1989; Groff and Gropper 2000). In a reaction known as the *Haber-Weiss reaction (Figure 1)*, the superoxide radical O_2- may react with another hydrogen peroxide molecule to generate molecular oxygen (O_2) and free hydroxyl radicals (OH·).

Figure 1: Chemical reactions which lead to the generation of reactive oxygen species (Gate et al. 1999).

$$2\ O_2\cdot + 2\ H\text{-} \xrightarrow{SOD} H_2O_2 + O_2\text{-}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH\text{-} \qquad \text{Fenton reaction}$$

$$H_2O_2 + O_2\text{-} \xrightarrow{(Fe^{2+}/Fe^{3+})} OH\cdot + OH\text{-} + O_2 \qquad \text{Haber-Weiss reaction}$$

The hydroxyl radical OH· is a severe threat to living systems (Diplock 1991). It is thought to be one of the most potent reactive radicals and to attack all types of molecules in the body (Buettner 1993; Groff and Gropper 2000). Free hydroxyl radicals rapidly take electrons from the surroundings. The hydroxyl radical is thought to be a major initiator of lipid peroxide

(LOOH) reactions. Thus, removal of free hydroxyl radicals is important to prevent destruction to cell components.

Lipid peroxidation occurs in polyunsaturated fatty acids (Gate et al. 1999). As shown in F*igure 2*, the process is initiated by a hydroxyl radical (OH·) that captures a hydrogen atom from a polyunsaturated fatty acid (LH) in the phospholipids of membranes and produces lipid carbon-centered radical (L·). Lipid carbon-centered radicals (L·) rapidly react with molecular oxygen (O_2) to generate a lipid peroxyl radical (LOO·) *(Figure 2).* The peroxyl radical product is similarly highly reactive and can combine with other peroxyl radicals to alter membrane proteins. The lipid peroxyl radicals (LOO·) can also capture a hydrogen atom from the adjacent fatty acids (LH) to generate a chain reaction that propagates additional lipid carbon-centered radicals (L·) as well as generating lipid peroxides (LOOH) (Gate et al. 1999; Groff and Gropper 2000). If lipid peroxides (LOOH) come in contact with free iron, lipid alkoxyl (LO·) and peroxyl (LOO·) radicals can also be generated. During lipid peroxidation, malondialdehyde (MDA), a highly reactive dialdehyde, can also be generated (Gate et al. 1999).

Figure 2: Mechanism of lipid peroxidation (Gate et al. 1999; Groff and Gropper 2000).

Initiation:

$$LH + OH\cdot \rightarrow L\cdot + H_2O$$

Propagation:

$$L\cdot + O_2 \rightarrow LOO\cdot$$

$$LOO\cdot + LH \rightarrow LOOH$$

In contact with iron:

$$LOOH + Fe^{2+} \rightarrow LO\cdot + OH^- + Fe^{3+}$$

$$LOOH + Fe^{3+} \rightarrow LOO\cdot + H^+ + Fe^{2+}$$

MDA formation:

$$LOOH \rightarrow LO\cdot \rightarrow MDA$$

Lipid peroxide radicals can be generated in the colon. Sawa et al. (1998) found that lipid peroxides and heme components generate peroxyl radical species that damaged DNA. They suggested that the lipid peroxyl radicals generated had originated from common dietary components such as fat and red meat, which usually contains a large amount of heme-iron. The large amounts of heme-iron in red meats may contribute to the high incidence of colon cancer. There is increasing evidence from other research studies that excess quantities of dietary iron have serious consequences in living cells and organisms including lipid peroxidation, cellular damage, and carcinogenesis in response to hydroxyl radical production (Younes et al. 1990; Nelson 1992; Sobotka et al. 1996). Very high dietary iron levels, 2000 mg iron/kg body weight in rats, resulted in a 1.7-fold increase in lipid peroxidation within the colon mucosa compared to

controls (Rimbach et al. 1997). This oxidative stress in the colon was attributed to the formation of hydroxyl radicals. Rats fed either 500 or 2000 mg iron/kg body weight produced 68% and 88% more OH · than controls, respectively. The presence of elevated concentrations of iron in the colon may have catalyzed the formation of hydroxyl radicals via the *Fenton reaction* (Rimbach et al. 1997). Lund et al. (2001) argue that past animal studies used unrealistically high concentrations of iron. Results of their experiments with rats fed a lower dosage of iron for a longer time indicated that dietary iron intake of 29 and 102 mg/kg for 6 months was associated with an increased free radical generating capacity and increased lipid peroxidation in the colon of the experimental animals (Lund et al. 2001). Thus, moderately high dietary iron for a prolonged period may be a risk factor for colon cancer. In cell culture studies, Courtois et al. (2000) showed that Fe^{2+}-ascorbate mediates lipid peroxidation in Caco-2 cells as indicated by the increased formation of MDA with increased Fe^{2+}-ascorbate in a dose-dependent manner.

MDA and 4-HNE as Indicators of Lipid Peroxidation

Lipid peroxidation is a form of oxidative tissue damage that occurs in polyunsaturated fatty acids. The process is initiated when a hydroxyl radical captures a hydrogen atom from a methylene carbon of the fatty acid *(Figure 2)*. The process of lipid peroxidation can also occur upon exposure to hydrogen peroxide and superoxide radicals. Resulting structural alterations the cellular membranes bring about the release of cell and organelle contents, the loss of essential fatty acids, and the formation of cytosolic aldehyde and peroxide products (Comporti 1985; Burton et al. 1990).

Malonaldehyde (MDA) and 4-hydroxynonenals (4-HNE) are good indicators of lipid peroxidation since they are generated in the process of oxidation of polyunsaturated fatty acids (Yoshida et al. 2000). MDA, a highly reactive dialdehyde, is a major end product of free radical reactions with membrane fatty acids (Thamilselvan et al. 2000). Schauenstein and Esterbauer (1977) were among the first to recognize the importance of reactive aldehydes, such as MDA and 4-HNE. These compounds have been used in many studies to indicate the extent of lipid peroxidation in cells and tissues (Thamilselvan et al. 2000; Moore and Roberts 1998; Courtois et al. 2000; Yoshida et al. 2000).

Antioxidants and Their Role in Cancer

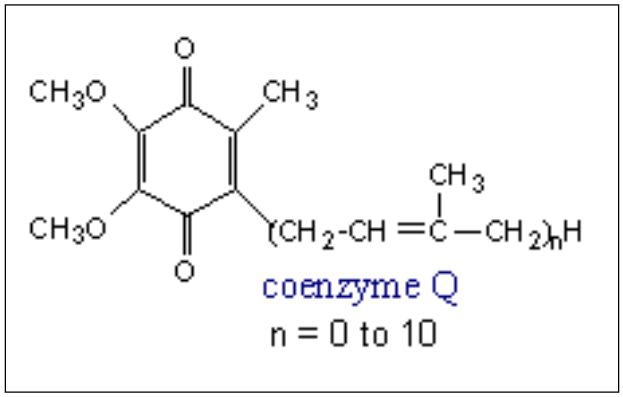
Antioxidants act to sequester free radicals and render them harmless. An antioxidant is an agent that prevents or inhibits oxidation. They are naturally occurring or synthetic substances that help protect cells from the damaging effects of oxygen free radicals (Davis 1997). Antioxidants donate electrons to free radicals to convert them into harmless atoms and molecules. Several nutrients have antioxidant properties. These include vitamin E, manganese, glutathione, CoQ and vitamin C (Groff and Gropper 2000). These antioxidants all appear to be involved in the elimination of carbon-centered radicals and peroxyl radicals (Groff and Gropper 2000).

CoQ as an Antioxidant Involved in Cancer

CoQ has been acclaimed as one of the most exciting nutrient discoveries of our time (Nutrition News 1994). It was originally isolated in 1957 by Dr. Fred Crane of the University of Wisconsin (Crane et al. 1957). CoQ, a ubiquinone, is actually a group of substituted 1,4-

benzoquinone derivatives with an isopentyl side chain of variable length *(Figure 3)* (Mahan and Escott-Stump 2000). CoQ_{10} is that form of ubiquinone with 10 isopentyl groups in its side chain. CoQ_{10} is a naturally occurring compound that our cells use in the electron transfer process in mitochondria during ATP synthesis (Webb 1997; Groff and Gropper 2000). CoQ is essential for normal cell respiration and function; any deficiency in its availability or biosynthesis could disrupt normal cellular function that could lead to abnormal patterns of cell division that might induce cancer (Folkers 1974). It is present in a wide variety of foods including nuts, fish, meat, egg yolks and whole grains (Scheer 1999). Certain factors decrease CoQ_{10} in the body, including illness, physical, mental, and emotional activity, stress, and aging (Scheer 1999).

Figure 3: The structure of CoQ



The antioxidant action of CoQ in the body is similar to that of alpha-tocopherol (vitamin E) where it aids in circulation, stimulates the immune system, increases tissue oxygenation, and counteracts the aging process (Scheer 1999). CoQ provides hydrogens to terminate lipid peroxyl radicals (Thomas et al. 1995; Mohr et al. 1992). The mechanism by which this occurs is described in *Figure 4*. The CoQH· formed may be regenerated into $CoQH_2$ through the action of the electron transport chain in the mitochondria (Groff and Gropper 2000). CoQ also functions

as a supportive antioxidant that restores and regenerates other antioxidants (Scheer 1999), including Vitamin E (Thomas, Neuzil and Stocker 1997). The mechanism by which this occurs is demonstrated in *Figure 5*.

Figure 4: The mechanism by which CoQ provides hydrogens to terminate lipid peroxyl radicals.

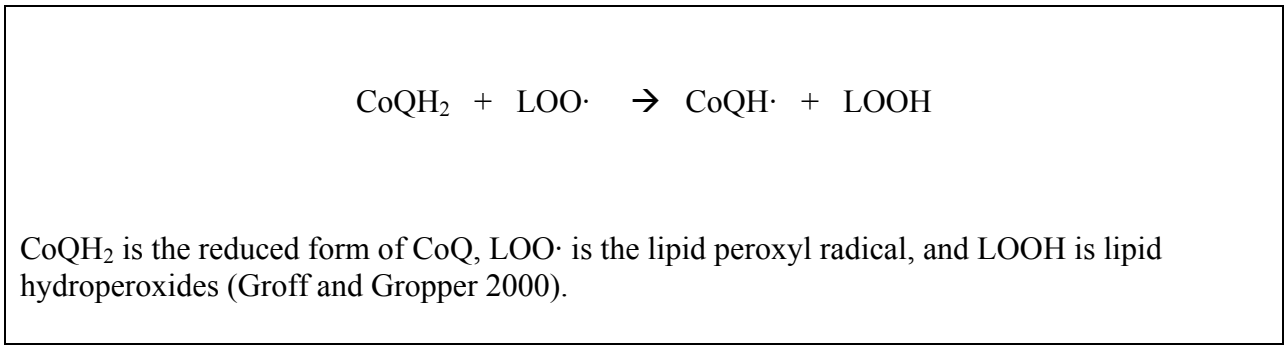
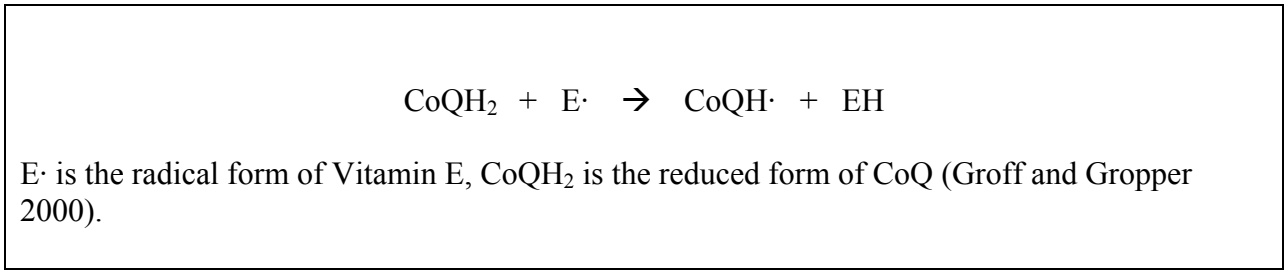


Figure 5: The mechanism by which CoQ helps regenerate Vitamin E.



Diseases that are associated with decreased amounts of CoQ in tissues of the body include heart disease and cancer (Webb 1997). Portakal et al. (2000) and Jolliet et al. (1999) found that the CoQ concentration in tumor breast tissue was significantly decreased as compared to surrounding normal breast tissue. The results of this study imply that administration of CoQ may induce the protective effect against cancer development in breast tissue. The investigators

concluded that since free radicals may promote tumor development, CoQ supplementation for breast cancer patients could be clinically helpful (Jolliet et al 1998). The findings from the previous two studies involving CoQ and breast cancer coordinate with the findings of another study involving patients with breast, lung and pancreas cancer. Folkers et al. (1997) found that the CoQ levels in patients with cancer of the breast, lung and pancreas were lower than normal (Folkers et al. 1997).

CoQ is beneficial to functions of the human body (Scheer 1999; Webb 1997; Portakal et al. 2000). Supplementation with CoQ may have many health benefits including protection against cancers and heart disease (Webb 1997; Folkers et al. 1997; Jolliet et al. 1998). Deficiencies in CoQ require supplementation with higher amounts of CoQ than are available in the diet (Crane 2001). Webb (1997) reported that Peter Langsjoen, M. D., a cardiologist in Tyler, Texas is so convinced of CoQ's effectiveness, that he recommends 120 mg twice daily for his patients diagnosed with cardiomyopathy.

Based on significant clinical benefits of CoQ supplementation for prevention of breast, lung and pancreas cancers (Folkers et al. 1997), and heart disease (Webb 1997), it is important to search for similar benefits of CoQ supplementation for the treatment of colon cancer. Currently, there is no published research concerning the effects of CoQ on colon cancer. Since iron-induced lipid peroxidation is a factor in the development of colon cancer (Courtois et al. 2000) and since CoQ is a significant contributor to the repair of free radical damage caused by iron-induced lipid peroxidation, it is vital to determine whether or not CoQ can help protect against iron-induced lipid peroxidation in human colon cancer cells (Caco-2 cells).

CHAPTER THREE

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) (Cat. # 12100-038) prepared with $NaHCO_3$ (Cat. # 11810-025), penicillin-streptomycin-glutamine (Cat. # 10378-016), Fetal Bovine Serum certified origin: United States (Cat. # 16000-036), and DMEM non-essential amino acids solution (Cat. #11140-050) were all purchased from Gibco (Grand Island, NY). Coenzyme Q_{10} (Cat. # 195108) purchased from ICN Biomedicals, Inc. (Auraro, OH), was made soluble with mineral oil. Ascorbic Acid-Fe (Cat. # A-0207) was purchased from Sigma (St. Louis, MO). PBS-EDTA-Trypsin was prepared with 0.1 M NaCl, 0.01 M NaH_2PO_4-H_2O, 0.02% EDTA (Cat. # E-675), and 0.05% Trypsin (Cat. # T-0303) was purchased from Sigma (St. Louis, MO). Lipid Peroxidation Colorimetric Assay Kits (product # FR 12) purchased from Oxford Biomedical Research (Oxford, MI). Butylated hydroxytoluene (BHT) (Cat. # 101162) purchased from ICN Biomedicals, Inc. (Auraro, OH) was used in the Colorimetric Assay. Caco-2 cells were provided by Dr. Richard Wood (Tufts University, Boston, MA). Other materials included: T-75 cell culture flasks (Nunclon, ™ by Nuno), 6-well cell culture plates (Product # 3516, Corning Inc., Corning, NY), CO_2, water-jacketed cell culture incubator (Model # 2310 manufactured by Sheldon Manufacturing Inc. Cornelius, OR and purchased through VWR Scientific Products), laminar flow hood, and -80º Celsius freezer. Cell count was conducted using a hemacytometer under a brightfield microscope. Colorimetric assay of MDA and 4-HNE was performed using a UV-Visible Spectrophotometer (Varian, Walnut Creek, CA). The following chemicals were used to prepare Lowry A and Lowry B solutions: Na_2CO_3, NaOH, Na-

K-tartrate, $CuSO_4 \cdot 5H_2O$ and Folin & Ciocalteu's Reagent (Cat. # F-9252) are all chemical grade purchased from Sigma (St. Louis, MO).

Methodology

Maintenance of Caco-2 Cells

Caco-2 (human colon cancer cells) cells were grown at 37ºC with 5% CO_2 with 100% humidity in DMEM containing 1% penicillin-streptomycin-glutamine and 1% DMEM nonessential amino acids (this mixture is referred to as DMEM) and supplemented with 20% Fetal Bovine Serum (20% FBS DMEM) for two days then supplemented with 10% Fetal Bovine Serum (10% FBS DMEM). Caco-2 cells were maintained in T-75 cell culture flasks (75 cm^2 growing surface). Cultures were split when they reached 75-90% confluence, using PBS-EDTA-Trypsin. The medium was refreshed every two days with 10% FBS DMEM. Cells were cultured for 4 to 6 days prior to using or subculturing.

Subculturing Caco-2 Cells

After old medium was discarded, 5 ml of PBS-EDTA-Trypsin was added to the cell culture flask to detach the cells. The cell culture flask was placed in the incubator for approximately 10 minutes. The flask was then checked under a microscope to see if the cells had detached from the surface of the flask. The cells were transferred into a 15 ml centrifuge tube containing 5 ml of DMEM with 10% FBS, and centrifuged for 2 minutes at 1700 rpm in a fixed angle rotor with a radius of 12 cm. The supernatant was removed by aspiration. Cells were resuspended into 5 ml of 10% FBS DMEM. A cell count was taken using a hemacytometer.

The suspension of cells was split into T-75 cell culture flasks at a cell density of 2 x 10^6 cells/well and the volume increased to 10 ml using 20% FBS DMEM. For experiments, cells were seeded into 6-well plates at 2 x 10^6 cells/well in a volume of 1.5 ml of 20% FBS DMEM.

Experimental Design

The treatment of the Caco-2 cells in the 6-well plates included the control (nothing added to the cells), Fe^{2+}-ascorbate and the vehicle (mineral oil), and iron plus CoQ_{10} (400 uM) in mineral oil. Mineral oil and CoQ_{10} alone were added to DMEM to control for any interactions with the DMEM. Iron was used at concentrations of 200, 400 and 800 uM. The old media was removed by aspiration from each well. The treatment was added to each well followed by 20% FBS DMEM which brought the final volume to 1.5 ml per well. The cells were then incubated for 24 hours before harvesting the cells and media to be assayed with the lipid peroxidation colorimetric assay.

Cell Lysis and Freeze Thaw

Cells were denatured (lysed) via a freeze/thaw method prior to running the lipid peroxidation colorimetric assay and protein assay. DMEM was removed by aspiration from each well. 500 ul distilled water was added to each well. The 6-well plates were then placed into the -80º Celsius freezer for 5 minutes and then placed into an incubator for 5 minutes to thaw. This freeze/thaw cycle was repeated 4-5 times. Samples were placed on ice and immediately assayed for MDA and 4-HNE or alternatively samples were stored at -80ºC for no more than 2 weeks. Stored samples were thawed in the refrigerator prior to assaying.

Lipid Peroxidation Colorimetric Assay

The lipid peroxidation colorimetric assay was conducted according to the directions provided with the MDA/4-HNE assay kit by the supplier. Standard curve and sample tubes were prepared with 650 ul diluted N-methyl-2-phenylindole, 150 ul methanesulfonic acid, 0-200 ul 1,1,3,3-tetramethoxypropane in 20 mM Tris-HCL buffer for the MDA assay or 0-200 ul 4-hydroxynonenal in 0.5 M BHT for the 4-HNE assay for a final volume of 0-20 uM. Sample tubes were vortexed, incubated at 45ºC for 40 minutes, cooled on ice and then the absorbance measured in a spectrophotometer set at a wavelength of 586 nm.

Protein Assay

The protein content in a sample from each well of the 6-well plate was determined according to the method described by Lowry et al. (1951). Lowry solutions A, B, and C were prepared as needed in the laboratory. Lowry A solution contained: 0.19 M Na_2CO_3, 0.1 M NaOH and 0.7 M Na-K-tartrate. Lowry B solution contained: 20 mM $CuSO_4 \cdot 5H_2O$. Lowry C solution contained a mixture of 50 parts Lowry A and 1 part Lowry B. Commercially prepared Folin reagent was diluted with distilled water to 1 M. Bovine serum albumin (BSA) was diluted with distilled water to make a stock solution (1mg/ml) that was stored at -80ºC until used. The BSA stock solution was further diluted with distilled water to prepare working protein standards containing 0, 5, 10, 15, and 25 ug protein/ul. The samples were diluted 1:10. One ml of Lowry C was added to each tube and vortexed for 2-3 seconds. After 10 minutes, 100 microliters of 1M Folins was added to each tube and the tube vortexed for 2-3 seconds. After 30 minutes, the absorbance was measured in a spectrophotometer set at a wavelength of 750 nm.

Statistical Analysis

Data were analyzed with SPSS version 9.0 using one-way analysis and two-way analysis of variance (ANOVA) with appropriate post-hoc tests. P-values less than 0.05 were considered significant. Each **n** represents an average of 2 or more wells from the same experiment.

CHAPTER FOUR

Results and Discussion

Results

MDA and HNE generation after Fe $^{2+}$ -ascorbate exposure. The effectiveness of Fe $^{2+}$ -ascorbate as an initiator of lipid peroxidation was tested after incubation with Caco-2 cells. Following a 24-hour exposure to Fe^{2+} -ascorbate, the degree of lipid peroxidation was determined by measuring MDA and HNE in the cellular fraction. *Figures 6 and 7* depict the amounts of MDA and HNE formed in the cellular component at iron concentrations of 200 uM, 400 uM, and 800 uM compared to controls.

Figure 6: MDA formation in the cellular component

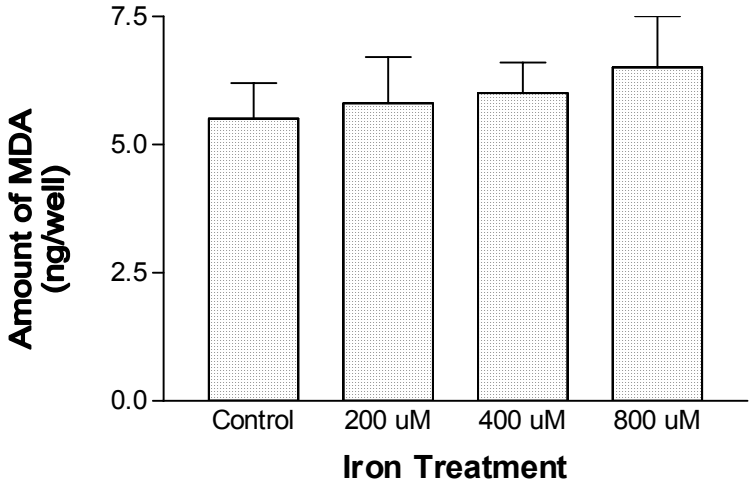
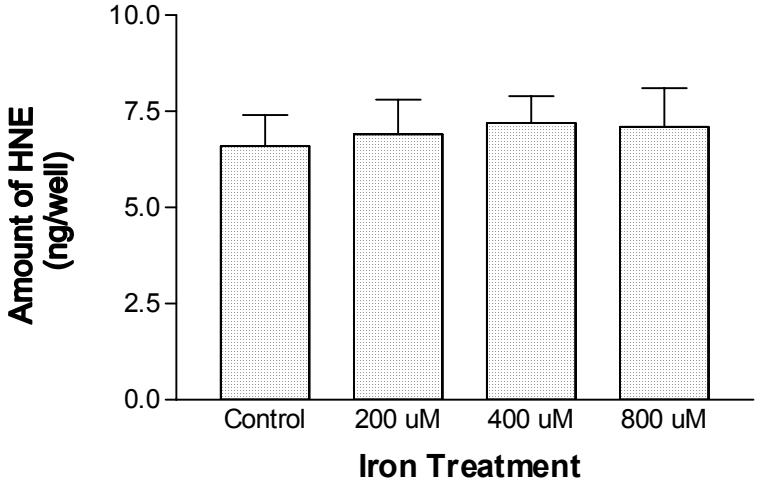


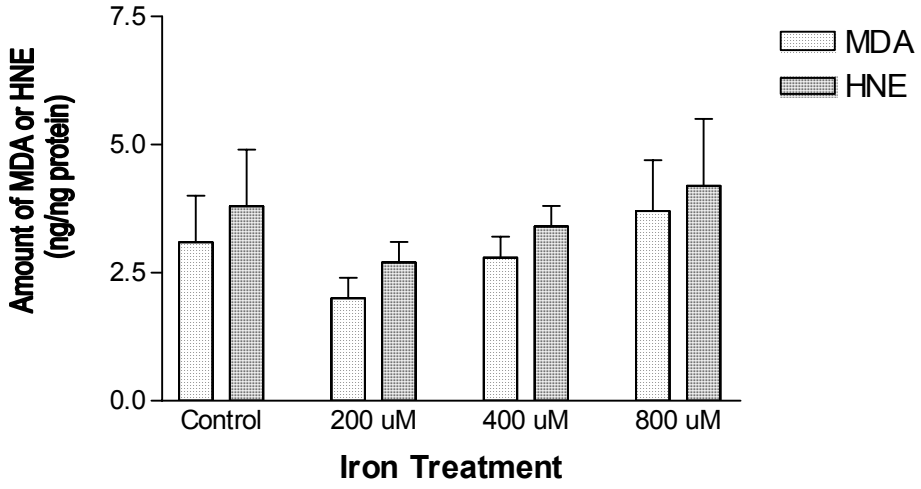
Figure 7: HNE formation in the cellular component



The total MDA *(Figure 6)* or HNE *(Figure 7)* formation is depicted in the cellular fraction of cultured Caco-2 cells grown with 200, 400 or 800 uM Fe^{2+}-ascorbate. Values are mean +/- standard error from 26 experiments done in duplicate. No statistically significant ($p>0.05$) difference in the total amount of MDA or HNE formed during a 24-hour exposure of Caco-2 cells to iron concentrations of 200, 400 and 800 uM for 24 hours was observed using a one-way ANOVA test at each Fe concentration. The levels of MDA and HNE in the cellular component were not consistent with what Courtois et al. (2000) found in their study using Caco-2 cells. Courtois et al. (2000) observed a significant concentration-dependent increase in MDA formation between 50 and 400 uM of Fe^{2+}-ascorbate in cells and in medium, with MDA formation peaking at 400 uM.

Since we were not able to duplicate the results of Courtois et al. (2000), we expressed the data per ng protein in each corresponding well. *Figure 8* illustrates the amount of MDA and HNE in the cells based on the amount of protein in the well, where n=26.

Figure 8: The amount of MDA and HNE in the cellular component corrected for protein



The amount of MDA or HNE (ng protein per well) following treatment with 200, 400 or 800 uM Fe^{2+}-ascorbate for 24 hours is presented in *Figure 8*. Values represent the mean +/- standard error from 26 experiments done in duplicate. No difference was seen using a one-way ANOVA test at each Fe concentration (200, 400 and 800 uM). The data is consistent between MDA and HNE formation.

A significant increase in MDA or HNE formation was not detected in samples obtained from Caco-2 cell cultures exposed to iron concentrations from 200 to 800 uM for 24 hours. A single experiment involving a 48 hour exposure of cultures of Caco-2 cells to 400 and 800 uM Fe^{2+}-ascorbate also failed to demonstrate increased formation of MDA and HNE *(Table 1)*.

Table 1: The amount of MDA and HNE per well with 24 hours or 48 hours of iron treatment, where n=1 or 2.

	MDA		HNE	
Iron concentration	48 hour	24 hour	48 hour	24 hour
Control	2.9 ng, 4.8 ng	0.8 ng, 3.1 ng	3.5 ng, 5.6 ng	1.3 ng, 3.8 ng
400 uM	3.3 ng	5.2 ng	4.0 ng	6.0 ng
800 uM	4.2 ng, 8.4 ng	3.0 ng	5.1 ng, 9.5 ng	3.7 ng

Protective effects of CoQ_{10}. CoQ_{10} was introduced into the cell culture to determine whether or not it affected the formation of MDA and/or HNE. The amount of MDA and HNE was not significantly different in the cellular component by itself but significant differences were apparent when the amount of MDA and HNE in the media and cellular components were analyzed together *(Figures 9 and 10)*. The data is standardized to ng protein per well.

Figure 9: The amount of MDA in the media and cellular component corrected for protein

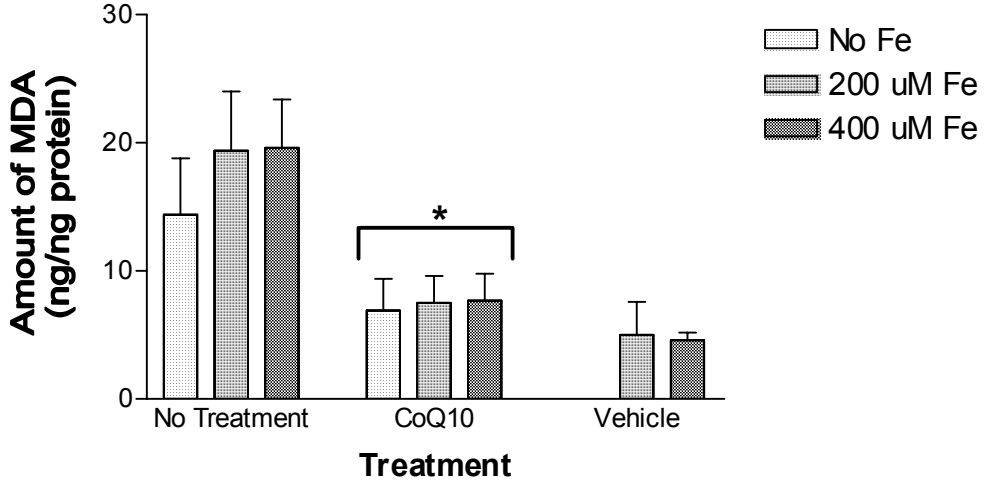
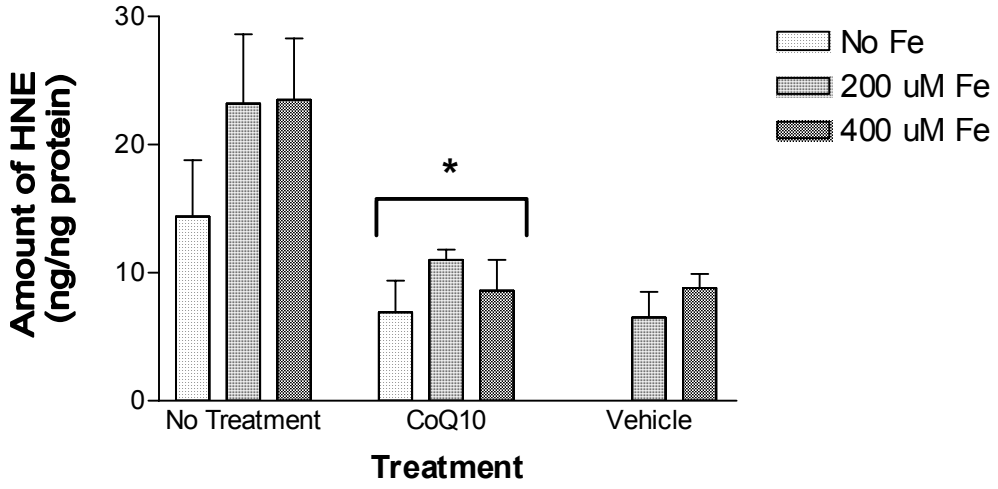


Figure 10: The amount of HNE in the media and cellular component corrected for protein

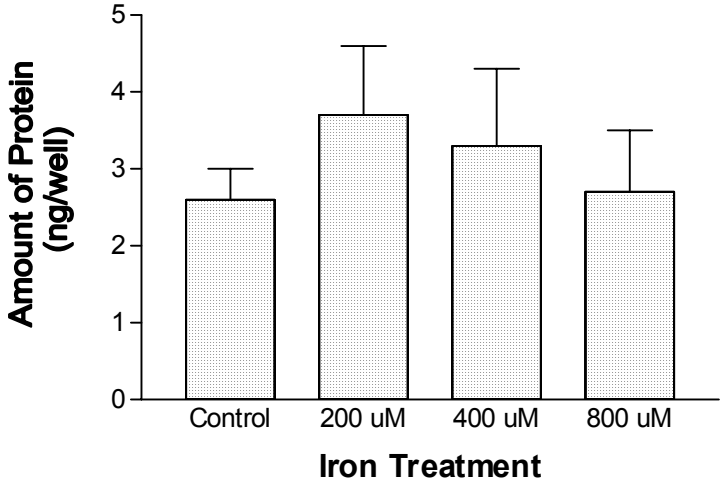


The amount of MDA *(Figure 9)* or HNE *(Figure 10)* in the sample (sum of values obtained from cellular fraction and media fraction) obtained from cultured Caco-2 cells exposed to 200 or 400 uM Fe^{2+}-ascorbate for 24 hours are expressed per ng protein per well. Values are mean +/- standard error from 2-7 experiments done in duplicate. No difference was seen using a one-way ANOVA with the Student-Newman-Keuls Multiple Range test between all three treatments at each Fe concentration (200 and 400 uM). When the data were analyzed with a two-way ANOVA, however, significance was observed. When comparing data on the effects of CoQ_{10} on cells treated or not treated with iron, a two-way ANOVA indicated that CoQ_{10} was protective against lipid peroxidation at all iron concentrations tested (* indicates $p< 0.05$, $p=0.011$ for MDA and $p=0.011$ for HNE). In the two-way analysis the impact of the vehicle carrying CoQ_{10} was not included because the **n** for the vehicle was too small for the analysis to be conducted. The data presented in *Figures 9 and 10* suggest that the vehicle was having an

effect on CoQ_{10}. Therefore, the combination of CoQ_{10} in its vehicle was significantly different based on the two-way analysis.

Protein generation after iron treatment. The amount of protein in the cell cultures after iron treatment was quantitatively evaluated as an indicator of cell growth. This information was also used to standardize the amount of MDA and HNE formed per ng protein. *Figure 11* illustrates the effect of various amounts of iron on cell growth expressed as the amount of protein per well.

Figure 11: The amount of protein based on iron concentration



The amount of protein obtained from cell cultures grown for 24 hours in media supplemented with 200, 400 and 800 uM Fe^{2+}-ascorbate is depicted in *Figure 11*. Values are mean +/- standard error from 26 experiments done in duplicate. No statistically significant difference was observed by a one-way ANOVA at each Fe concentration (200, 400 and 800 uM).

The effect of iron, CoQ_{10} and the vehicle on the assay of MDA and HNE. The possible interactions of iron, CoQ_{10}, and the vehicle with the lipid peroxidation assay were examined to discover any effects of MDA and HNE. No significant difference was detected by the colorimetric assay between control samples (one-way ANOVA), and test samples containing iron, CoQ_{10} or mineral oil. Since the media, DMEM, without cells, was not tested, it remains a possibility that MDA and HNE may have been forming in the media regardless of the treatment.

Discussion

MDA and HNE generation after Fe^{2+} -ascorbate exposure. The Caco-2 cell line has been used to examine a variety of intestinal functions, including nutrient absorption (Levy et al. 1995). Courtois et al. (2000) showed that Fe^{2+}-ascorbate promoted the production of peroxides as evidenced by MDA formation above baseline values. A concentration-dependent equivalent increase in MDA formation was observed between 50 uM and 400 uM of Fe^{2+}-ascorbate in cells and in the medium (Courtois et al. 2000). The results of our experiment are not consistent with what is reported in the literature (Courtois et al. 2000). A concentration-dependent increase in MDA or HNE formation was not observed between 200 uM and 800 uM of Fe^{2+}-ascorbate in cells and in media in our experiments (*Figures 6, 7 and 8).* This data is not consistent with the data reported by Courtois et al. (2000). This may be due to not controlling for substances in the media, such as iron. DMEM has a phenol red color, absorbing visible light maximally at 559 nm whereas the wavelength of absorbance used for the MDA and HNE colorimetric assay was 586 nm. Therefore, it is possible that components of the DMEM may have interfered with the colorimetric assay due to the close proximity of the wavelengths of these absorbance maxima. A

second possibility for the inconsistencies with published data in the literature is the difference between facilities and the methods of detecting MDA and HNE formation. Courtois et al. (2000) used High Performance Liquid Chromatography (HPLC) to quantitatively detect MDA formation. According to Liu et al. (1997) assays of aldehydes from lipid peroxidation also include gas chromatography- mass spectrometry and a thiobarbituric acid (TBA) assay that measures TBA reactive substances (TBARS). Liu et al. (1997) argued that the TBA test overestimates lipid peroxidation due to its nonspecificity and that the bulk of the TBARS material is not MDA.

Protective effects of CoQ_{10}. Statistical analysis for the experimental data suggests that CoQ_{10} is significantly protective against lipid peroxidation in Caco-2 cells, but the vehicle, mineral oil, was not incorporated into the analysis due to a limited **n**. Mineral oil, the vehicle for carrying CoQ_{10} in these experiments, may itself have a protective effect against lipid peroxidation in Caco-2 cells. However, the one-way ANOVA analysis of the limited data from those experiments that indicated included mineral oil, indicated that no significance was observed. *Figures 9 and 10* show that the amount of MDA and HNE formation in Caco-2 cells treated with the vehicle is closer to the amount of MDA and HNE formation in wells treated with CoQ_{10}. In order to conclude that the vehicle did not have an effect on MDA and HNE formation in Caco-2 cells, the amount of MDA and HNE formation in Caco-2 cells treated with the vehicle should be the same as the levels of MDA and HNE formation in the control Caco-2 cells. Additional experiments need to be run to increase the **n** to provide more reliable evidence that mineral oil itself has a protective effect against lipid peroxidation in Caco-2 cells.

Additional investigations of the effects of CoQ_{10} and mineral oil on Caco-2 cells treated with iron appear warranted. Studies have not been done as yet to evaluate the possible protective effect of mineral oil against colon cancer. The results of this study appear to suggest that mineral oil may have a protective action in Caco-2 cells and therefore should be studied further.

In conclusion, we were unable to replicate the reported iron-induced lipid peroxidation in Caco-2 cells (Courtois et al. 2000). However, we did find some interesting preliminary results suggesting that CoQ_{10} and mineral oil inhibit lipid peroxidation in the Caco-2 cells. Mineral oil has the ability to interfere with the absorption of fat-soluble nutrients, and CoQ_{10} is a lipid-soluble quinone present in virtually all cells (Davis 1997). It is possible that mineral oil may have interfered with the ability of the Caco-2 lipid membranes to absorb the CoQ_{10}. If mineral oil has acted in this manner, it cannot be concluded that CoQ_{10} lacks a protective effect against lipid peroxidation in Caco-2 cells treated with iron, but rather that CoQ_{10} may actually have a protective effect against lipid peroxidation if the vehicle used to introduce CoQ_{10} to the cells did not interfere with CoQ_{10}'s action or absorption.

Also, since mineral oil can readily dissolve into lipid membranes, it may dilute the effects of lipid peroxidation or interfere with *in vivo* lipid peroxidation. *Figures 9 and 10* depict mineral oil having a protective effect on iron-induced lipid peroxidation in Caco-2 cells. The action by which this occurs is not yet understood, but it may be because of the ability of mineral oil to dissolve into lipid membranes in the Caco-2 cells and therefore, inhibiting or interfering with iron-induced lipid peroxidation. Additional investigations on the effect of mineral oil on Caco-2 cells should be conducted since it appears that mineral may have a protective effect in the colon.

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