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THE EFFECT OF ASCORBIC ACID ON THE INHIBITION OF HEME IRON ABSORPTION IN CACO-2 CELLS

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ABSTRACT

Dietary polyphenolic compounds, such as those found in grape seed and green tea extracts, have a wide range of effects in vivo and vitro including chelation of metals such as iron. Because iron deficiency is the most common nutritional problem found throughout the world, it is wise to explore the effects of those compounds on the intestinal absorption of iron and to try to discover ways to reverse those effects. Previous research has shown that epigallocatechin-3-gallate (EGCG) and grape seed extract (GSE) both have an inhibitory effect on the absorption and transport of heme and non-heme iron across a cellular membrane. The objective of this study was to examine whether ascorbic acid (AA) counteracts the inhibitory effects of EGCG and GSE on heme iron absorption in Caco-2 cells. The fully differentiated intestinal Caco-2 cells grown on mircoporous membranes were incubated for 7 h with heme ^{55}Fe in uptake buffer that contained EGCG, GSE, or a combination of each of the previous and AA in the apical compartment. GSE and EGCG significantly decreased ($p < 0.05$) heme iron transport at 5 and 7 h. AA increased ($p < 0.05$) apical heme iron uptake and reversed EGCG-mediated inhibition of heme iron uptake. AA also increased ($p < 0.05$) the transepithelial heme iron transport in the presence of both EGCG and GSE. This study shows that EGCG and GSE significantly decrease the transport of heme iron. GSE does so by working at the level of the basolateral, not the apical, membrane. EGCG works at the basolateral and apical membranes. In addition, AA reversed the inhibition of cellular iron uptake when added to EGCG but not when added to GSE.

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Chapter 1

Introduction

I. Iron as a Vital Micronutrient

Iron is a vital micronutrient for humans and is utilized in numerous biological processes including binding and transporting oxygen as an essential component of hemoglobin, synthesizing DNA as a part of ribonucleotide reductase, and electron donators and accepters in cytochromes. The ability of iron to participate in so many reactions comes from the fact that it is a transition metal that can act as either a reductant or oxidant by donating or accepting electrons, respectively. Despite the fact that iron is essential for proper function in the human body, iron deficiency is the number one diet-related problem on earth. More than two billion people worldwide have such severe iron deficiency that it has led to the point of anemia [1]. As a result of this iron deficiency, hemoglobin production is greatly impaired thus damaging the ability of cells to transport oxygen. This leads to fatigue, weakness, palpations and light-headedness. Iron deficiency can also lead to growth retardation and intellectual impairment, especially in young children [2].

However, individuals can also suffer from iron toxicity. Iron forms free radicals from peroxides in the body such as lipid peroxides or hydrogen peroxide. Those free radicals can cause oxidative stress throughout the body by damaging membranes, proteins and nucleic acids. Because both iron deficiency and iron toxicity can cause death, iron levels in the body must be maintained. Unlike other nutrients, there is no excretory mechanism for iron so iron homeostasis is regulated by tightly controlling intestinal iron absorption. The mechanism of intestinal iron absorption and dietary factors influencing iron absorption will be reviewed in this section [3].

a. Two dietary sources

There are two forms of iron consumed by humans: non-heme and heme iron. Heme iron is iron that is derived from animal sources in which the iron is bound to a porphyrin ring. It is found in the form of myoglobin and hemoglobin. Non-heme iron comes from all other sources [2]. Even though heme iron only makes up one third of ingested iron, it constitutes two thirds of a human's iron stores, because it is much more bioavailable in the lumen of the small intestines than non-heme iron. Thus, understanding dietary heme, how it is absorbed and how it is processed in the body is very important for balancing iron in the diet and the body [1].

II. Intestinal Iron Absorption

There are three distinct steps in absorbing dietary iron across the intestinal enterocyte: apical iron uptake, intracellular distribution and basolateral iron release. Heme iron absorption and non-heme iron absorption have discrete mechanisms.

a. Heme iron absorption

The process by which heme iron is absorbed and transported is not well researched and thus fairly unknown. However, several theories have been proposed and tested. Before it can be used by the body, heme iron must be released from dietary myoglobin and/or hemoglobin by proteolytic enzymes in the stomach and small intestines. It is then taken up as an intact metalloporphyrin. In other studies, it has been shown that similar substances do not get absorbed by enterocytes due to the absence of specific apical membrane carriers. This evidence suggests that heme iron absorption is a facilitated process; heme iron cannot cross the apical membrane by simple diffusion [1, 4-5].

Several possible theories exist to explain heme iron facilitated diffusion across the apical membrane into the intestinal cells. The heme iron can be taken up by receptor mediated endocytosis. This absorption involves a process by which the intestinal cells internalize the heme iron by the inward budding of plasma membrane vesicles. These vesicles enter the cell with the heme iron inside. The heme is then degraded by heme oxygenases releasing ferrous ion and biliverdin. Divalent metal transporter 1 (DMT-1) transports the non-heme iron from the gastrointestinal lumen (GI) into the enterocyte cytoplasm [1, 4-5].

It has also been theorized that apical carrier proteins absorb the entire, intact heme molecule into the cytoplasm. Heme carrier protein 1 (HCP1) is one such protein [6]. Once in the enterocytes, the heme molecule is degraded by heme oxygenases. Inorganic iron and biliverdin are released from each degradation. The inorganic iron can then continue to follow the path that non-heme iron follows. It is also theorized that intact heme iron (absorbed into the enterocyte by HCP-1) can be transported straight across the basolateral membrane by the feline leukemia virus subgroup C receptor (FLVCR) into the bloodstream where it binds to hemopexin and circulates throughout the body. However, the exact method by which heme iron is taken up by the enterocyte and then transports into the blood is not definitely known. At this point, only theories exist [1, 4-5].

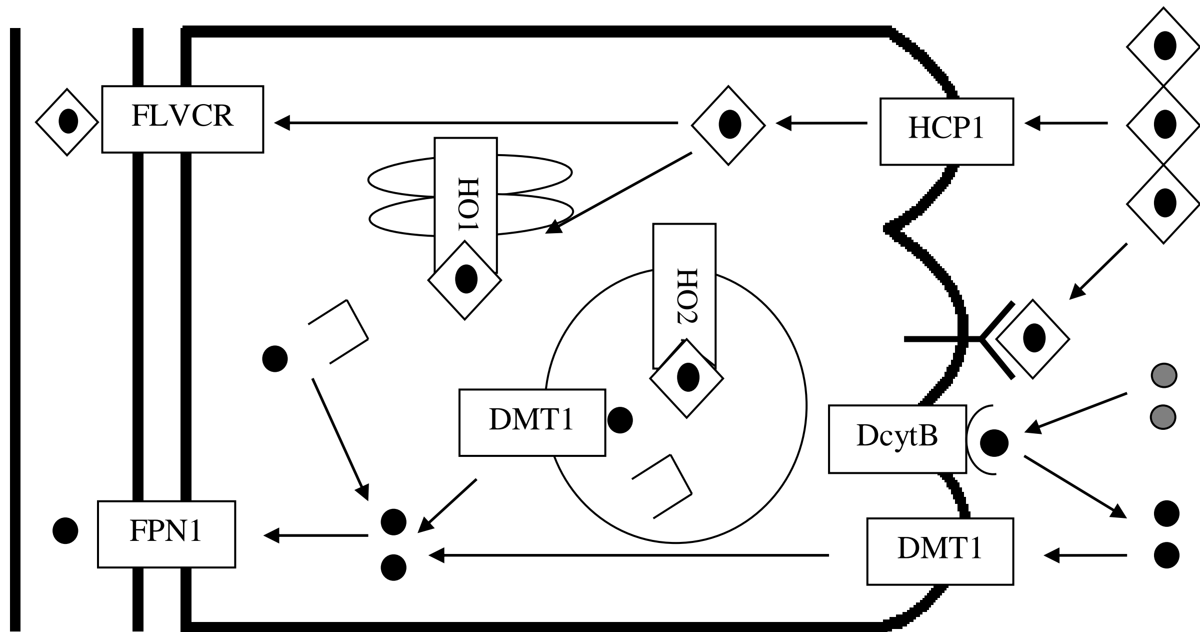
Any and all iron released from ingested heme, regardless of the way it was absorbed, joins the cytoplasmic labile iron pool. With the exception of the iron transported into the bloodstream by the FLVCR transporter, the cytoplasmic iron is transferred across the basolateral membrane into the bloodstream by ferroportin (FPN-1) [1, 4-5].

b. Non-heme iron absorption

Non-heme iron crosses the apical membrane into the enterocyte cytoplasm by the DMT-1 which is located on the microvillus membrane. Once iron enters the intestinal cells, the non-heme iron joins the labile iron pool with the inorganic iron, which is released from the absorbed heme. Ferrous iron is taken up by DMT1 right away whereas ferric iron must first be reduced to ferrous iron by duodenal cytochrome b (Dcytb) before being transported into the cell. Similarly to the iron released from heme, the ferrous iron in the cytoplasmic iron pool is then transported across the basolateral membrane into blood circulation by FPN-1. The ferrous iron is then oxidized by hephaestin prior to release into the blood circulation. The ferric iron binds to apo transferrin and is taken to areas of the body where it is needed [3].

Blood stream

GI lumen



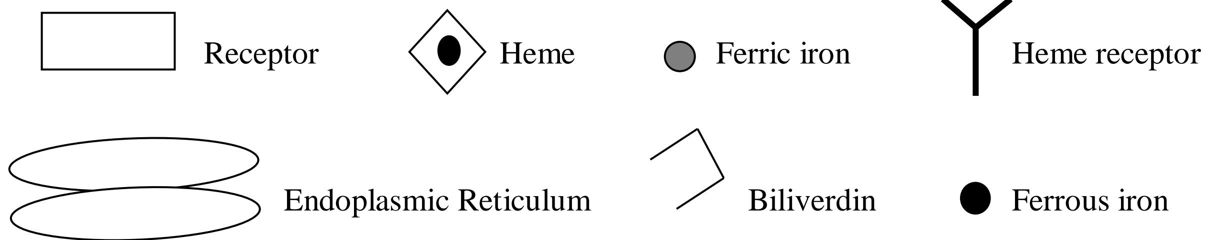


Figure 1: Mechanism of Iron Absorption

This figure illustrates the mechanism of heme and non-heme iron absorption from the GI lumen, through intestinal cells and into the bloodstream.

III. Effects of Nutrients on Iron Absorption

a. Polyphenols and iron absorption

Polyphenols, especially those in grapes and grape seed extract, are well-known and well-advertised for their positive effects on human health. The various phenolic compounds found in grape seed extract are gallic acid (GC), catechin ©, epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EC) and proanthocyanidins. Grape products have been shown to prevent dental caries and function as anti cancer, antifungal, antiviral, antibacterial neuroprotective and anti-inflammatory agents [7, 8]. However, while grape products have been shown to have many positive effects on health, they have been shown to have some negative effects as well. One of the most well-researched effects of grape seed extract is the inhibition of non-heme iron in the intestinal lumen. During digestion they are liberated from foods and are able to complex with the non-heme iron in the lumen, thus making it impossible for the iron to be absorbed. Habitual consumption of polyphenol containing compounds could lead to impaired iron absorption and thus iron deficiency [9]. However, most of the studies done on the effects of polyphenols on iron absorption were done with non-heme iron. Recently, it was shown that grape seed extract significantly decreases non-heme iron absorption across Caco-2 cells. Grape seed extract also inhibits heme iron absorption [10].

1. Green tea

Green tea is a drink made with the leaves of *Camellia sinensis*. It originated in China but has become popular all over the world. Green tea contains compounds such as carotenoids, tocopherols, Vitamin C and numerous minerals (chromium, manganese, selenium, zinc). The

most healthful components are the polyphenols, especially catechins. The six catechins in green tea are EGCG, EGC, ECG, EC, GC and C, the most abundant of which is EGCG.

Because of its polyphenols, especially EGCG, it is also widely advertised as being able to improve health. Due to the phenolic compounds, extracts from green tea have been added to foods and beverages to prevent oxidation, and thus spoilage, by scavenging free radicals and chelating metals [11, 12]. It follows that the same chelating properties that help preserve food and beverages could cause iron chelation in the small intestines and prevent proper iron transport. EGCG, the major polyphenol compound in green tea, has been shown to decrease transepithelial non-heme iron transport in Caco-2 cells and thus may impair the utilization of dietary iron [10, 11].

2. EGCG

EGCG is the ester of epigallocatechain and gallic acid. It is a type of catechin, also known as flavan-3-ols or polyphenols, and the most abundant catechin in green tea (but not black). The leaf bud and first leaves are richest in EGCG and provide about 8 to 12 percent of the total polyphenols in one cup of tea. EGCG (especially that found in green tea) has been showed to help prevent atherosclerosis through its antioxidant and anti-inflammatory effects. It has also been useful in cancer treatment and prevention; EGCG suppresses tumor promotion by inhibiting the release of tumor necrosis factors. The compound has proven helpful in many other medical conditions including chronically dry skin, joint diseases, inflammatory bowel disease, liver disease, diabetes and weight loss. Because EGCG is consistently being advertised as a promoter of health in a world with such an iron deficiency problem, the inhibitory effects of EGCG on iron absorption must be researched [10, 13].

3. Grape seed extracts

Grape seed extracts are industrial derivatives from whole grape seeds. They have high concentrations of Vitamin E, flavonoids and linoleic acid. Grape seed extracts have been shown to treat heart diseases (like high blood pressure and high cholesterol) by inhibiting lipid oxidation, platelet aggregation and inflammation. The extracts may also help with wound healing, tooth decay, osteoporosis, skin cancer and even HIV. However, the main reason for extraction of grape seed is to remove the OPCs (oilomeric proanthocyanidins), flavanols commonly found in grapes and wine. OPCs are widely known to work as antioxidants. Antioxidants are substances that are able to inhibit cellular oxidation, a process that forms an abundance of damaging molecules called free radicals. Although having some free radicals in the body is okay, too many can cause numerous health issues including, but not limited to brain injury and heart disease [7-8, 10, 14-15].

b. Ascorbic acid and iron absorption

Ascorbic acid is a sugar acid essential for humans. It acts as an anti-oxidant, is a cofactor in several enzymatic reactions (including reactions that promote collagen synthesis and wound healing), promotes good immune function and acts as a natural antihistamine. Ascorbic acid is found in many common fruits and vegetables such as lemons, bananas, broccoli, green peppers, corn and much more. It is also found in garlic and watercress. In order to prevent scurvy (a disease resulting from Vitamin C deficiency), ascorbic acid has also been added as a supplement to many foods. Some animals can produce ascorbic acid from glucose but human cannot synthesize it.

c. Effects of nutrients on heme iron absorption

The effects of other ingested nutrients, foods and chemicals on iron uptake is also more studied in relation to non-heme than heme iron. Polyphenols are a group of compounds found in plants and are characterized by the presence of a phenol unit. They are very diverse in chemical structure and work in the body in many capacities, including antioxidant and metal-chelating activities. Dietary polyphenols are found in various commonly consumed foods such as tea, fruits, vegetables, wine, soy and various spices and are especially high in green tea and grapes/grape seed extract.

The availability and transport of iron in the small intestines can be decreased by compounds (such as polyphenols in EGCG, green tea extract, grape seed extract and even calcium) but can also be enhanced by some dietary factors that promote iron absorption [16,17]. Thus, the bioavailability is based on the interaction of the iron promoters and inhibitors. Ascorbic acid, commonly known as Vitamin C, is documented as a promoter of non-heme iron transport. In some cases, it has actually been shown to reverse inhibitory effects of polyphenols and phytates (both proven inhibitors of non-heme iron transport) [18-20].

Unlike polyphenols, adding ascorbic acid actually promotes iron absorption. It reduces ferric iron to ferrous iron that is substrate of DMT-1, an apical iron transporter. In fact, ascorbic acid has even been shown to prevent or reverse the inhibitory effects of polyphenols on non-heme iron absorption [20].

IV. Caco-2 Cells

The Caco-2 cell model was instituted as an in vitro investigation to determine how drugs might be transported across human intestinal cells. The cells form polarized monolayers in

cultures and differentiate into cells that are very similar to intestinal epithelial cells. The cells better resemble colonic than small intestinal cells, so there are some limitations to this model. For the most part, however, the Caco-2 cell model is accepted as a means to accurately determine how chemicals and nutrients move across intestinal absorptive cells [21-22].

V. Objective

The effects of interactions between ascorbic acid and grape seed extract and EGCG on heme iron transport has not yet been effectively studied. The remaining question is whether inhibition of heme iron absorption by bioactive polyphenols can be reversed by ascorbic acid, the promoter of iron absorption. Thus, the objective of this study was to examine whether ascorbic acid counteracts the inhibitory effects of EGCG and GSE on heme iron absorption in Caco-2 cells.

Chapter 2

Methods

I. Caco-2 Cell Culture

The human Caco-2 (HTB-37™) cell line was purchased from American Type Culture Collection (Rockville, MD) and maintained at 37°C in complete medium in a humidified atmosphere of 95% air and 5% CO_2. The complete culture medium consisted of Dulbecco's Modified Essential Medium (DMEM) supplemented with 25 mmol/L glucose, 2 mmol/L glutamine, 100 µmol/L non-essential amino acids, 100 U/L penicillin G, 100 mg/L streptomycin and 10% FBS. Stock cultures were seeded at 10,000 cells/cm^2 and split at ~85% confluence by treatment of 0.5 g/L trypsin and 0.5 mmol/L EDTA in HBSS. For experiments, 50,000 cells/cm^3 in a volume of 1.5 mL complete medium were seeded on the 3µm-microporous membrane insert (4.9 cm^2, BD Biosciences, Bedford, MA) coated with collagen (5 µg/cm^2) (BD Biosciences, Bedford, MA) of a 6-well plate. The basolateral chamber contained 2.5 mL complete DMEM. The culture medium was changed every other day and cells after 17-d post-confluence were utilized for heme-iron transport experiments. The Caco-2 cells are fully-differentiated at 17-d post-confluence in normal cell culture conditions (Louvard and others 1992; Han and Wessling-Resnick 2002). The cell monolayer formed tight junctions at 17-d post-confluence as defined by the transepithelial electrical resistance values of >250 Ω/cm^2.

II. MEL Cell Culture

Mouse erythroleukemia (MEL) cells were utilized to produce heme. MEL cells have been shown to closely mimic the regulatory processes normally associated with red-cell maturation.

The heme produced by MEL cells was then used in transport studies to determine the amount of heme iron that moves across both the apical and basolateral membranes of Caco-2 cells.

To obtain radiolabeled heme, the MEL cells were prepared using the following procedure. The DMEM supplemented with 25 mmol/L glucose, 2 mmol/L glutamine, 100 μmol/L MEM non-essential amino acids, 100 U/L penicillin G, 100 mg/L streptomycin, and 10% FBS was warmed by incubating it in a 37°C water bath. The MEL cells were seeded in T75 and T25 flasks containing 12 and 4 mL of prewarmed complete DMEM, respectively, and then incubated in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2 days. After ~5 days, the flasks were removed from the incubator, and the cells were transferred into a 15 mL tube. The tube was centrifuged for 5 minutes at 800 xg to collect cells. The medium was aspirated and cells resuspended in 2 mL (T25) or 5 mL (T75) fresh medium. The cells were counted using a standard hemocytometer chamber and set up at 1 x 10^5 cells/mL density.

The MEL cells were maintained in suspension in complete DMEM. One hundred thousand MEL cells were seeded in T75 flasks with 10 mL complete DMEM on the first day. On the second and fifth days, the cells were treated with 2 μM /L Transferrin (Tf)-$^{55}Fe_2$ and 2% dimethyl sulfoxide (DMSO). The DMSO induced erythroid differentiation and Hb synthesis. Radiolabeled Hb was produced when the Tf-$^{55}Fe_2$ was added in the presence of DMSO. The cells were harvested after 6 days of treatment. They were washed with phosphate-buffered saline 3 times and collected by centrifugation for 5 minutes at 800 x g and 4°C.

III. Quantification of Hemoglobin by Spectrophotometric Assay

Cells were washed 3 times with 10 mL PBS, resuspended in 0.5 mL distilled water and lysed by several cycles of freezing and thawing (each d1 for 3 minutes). Dry ice and ethanol

were used for the freezing and a 37°C water bath for the thawing. The cell lysate was centrifuged at 14,000 rpm in a microcentrifuge tube for 15 minutes at 4°C and 300 µL of the supernatant was transferred to a new tube. The benzidine assay mixture was prepared by adding the reagents in the order: 10 µL supernatant, 990 µL distilled water, 100 µL benzidine-HCl (10 mg/mL in 0.5% acetic acid) and 40 µL 30% H_2O_2. After exactly 90 seconds, the absorbance at 604 nanometers was measured in a spectrophotometer. Hb concentration was determined by comparing the measurements to a standard curve made using pure bovine Hb solutions at 0, 100, 300 and 1000 µg /mL.

IV. Hemoglobin Digestion

Hb-^{55}Fe solution obtained from MEL cell lysis was digested with 0.1% pepsin at pH 2.0 for 4 hours at 37°C. The digestion rate was estimated by measuring the remaining Hb content in the supernatant. The solution was centrifuged for 5 minutes at 14,000 rpm at 4°C. The supernatant was removed and only heme pellets remained. Pellets of heme were dissolved in 10 mmol/L of NaOH and further diluted in iron uptake buffer (final pH 7.0). The ^{55}Fe-heme specific activity was between 0.4 -0.45 Ci/mole heme.

V. Tf-^{55}Fe Labeling

Apotransferrin (Apo-Tf) was dissolved in 500 µL elution buffer (150 µM /L NaCl, 5 µM /L $NaHCO_3$ and 10 µM /L HEPES, pH 7.2). The ^{55}Fe-nitrilotriacetic acid (NTA) complex was prepared by adding 20 µL 40 µmol/L NTA to 25 µL acidic solution containing 200 nmol of ^{55}Fe Cl_3. NaOH was used to neutralize the acidic ^{55}Fe-NTA solution. The Apo-Tf and the solution were then combined and incubated for 30 minutes at room temperature. Next, the solution was

passed over a Bio-Rad P6 column which had been equilibrated with the elution buffer. Void volume was analyzed spectrophotometrically. The ratio of A465/A280 was 0.046 for these preparations, indicating complete saturation of the two iron binding sites of Tf.

VI. Heme-^{55}Fe Transport and Uptake

The cell monolayer was washed three times with Ca^{2+} and Mg^{2+} free Hanks' balanced salts solution at 37°C. At the same temperature, the cells were incubated with 1.5 mL of 0.5 μmol/L heme ^{55}Fe in iron-uptake buffer containing 46 mg/L of the indicated bioactive compounds in the presence and absence of 100 μmol/L ascorbic acid in the apical compartment and 2.5 mL DMEM in the basolateral compartment. At the indicated time points (1, 3, 5 and 7 hours), 200 μL was removed from the basolateral chamber. It was replaced with 200 μL prewarmed DMEM (time course data were corrected to account for this replacement). During the 7 h incubation, the rate of radiolabeled heme iron transport across the cell monolayer was increased. Transport rates (pmol/h/well) were calculated by linear regression analysis ($r^2 >$ 0.999). Cell monolayers were washed 3 times with ice-cold wash buffer containing 150 μmol/L NaCl, 10 μmol/L HEPES, pH 7.0 and 1 mmol/L EDTA to remove any nonspecifically bound radioisotope before measuring the cellular levels of ^{55}Fe. It was effective in removing all surface bound iron. Cells were homogenized in PBS containing 1 mmol/L EDTA and 0.3% Triton X-100. Liquid scintillation counting in glass vials was used to quantify the amount of ^{55}Fe, and protein levels were determined using a Bio-Rad protein assay kit. Cells were also incubated with various heme ^{55}Fe concentrations (0.5-100 μmol/L) for 1 hour to determine the dose-dependent effect on heme iron apical uptake. During the 7 h incubation, EGCG and GSE did not change cellular protein levels. A cup of green tea contains about 20-200 mg EGCG and most GSE

supplements contain 100-500 mg GSE/capsule. Thus, the 46 mg/L GSE used for transport studies were within physiological levels. The EGCG (46 mg/L = 100 µmol/L) was also within physiological limits. The level of heme iron (1 µmol/L) is similar to the amount one would receive from 10 g of cooked beef per meal.

VII. Measurement of Heme-associated Iron Transport

The Caco-2 cell monolayers were incubated with heme ^{55}Fe (0.5 – 25 mmol/L) for 7 h in the uptake buffer. The medium in the basolateral chamber was then collected and heme-associated iron was determined. The medium was first acidified and centrifuged for 5 minutes at 20,000 x g at 4°C. The supernatant was removed and pellets were dissolved in the basic solution. Liquid scintillation counting in glass vials was used to quantify the amount of ^{55}Fe. During the 7 h assay, the negligible heme from the basolateral chamber was found. Control experiments confirmed that >98% of heme was recovered with this heme acidification method.

VIII. Statistical Analysis

Values were expressed as means $\pm$ SEM, n = 4 - 6. Data were analyzed using 1-way or 2-way (treatment x time) ANOVA followed by Bonferroni's post-hoc multiple comparison tests using Prism 5.0 software (GraphPad). Differences were considered significant at $p < 0.05$.

Chapter 3

Results

I. Transepithelial transport of iron from heme-iron by Caco-2 cell monolayer.

The quantity of heme-derived ^{55}Fe transferred from the apical to the basolateral compartment of Caco-2 cell monolayer was linearly increased between 1 and 7 h of incubation (Figure 2). Caco-2 cells mainly transfer heme-free iron across the basolateral membrane during 7 h incubation time. More than 98% of ^{55}Fe transported from the apical to the basolateral compartment across the cell monolayer was heme free iron.

II. Effects of EGCG and GSE on transepithelial heme iron transport.

During 7 h of incubation, both EGCG and GSE decreased the transepithelial iron transfer across the cell monolayer by 80.2 +/- 0.03% and 77.7 +/- 0.5%, respectively (Figure 3). The addition of bioactive dietary polyphenols did not alter TEER values, thereby confirming the integrity of the monolayer for EGCG or GSE-added cells. Addition of EGCG and GSE modulated apical uptake of heme iron across the brush-border membrane of the enterocyte. The cellular assimilation of heme ^{55}Fe was decreased by 20.5 +/- 2.3% by EGCG (Figure 4). The control cells accumulated 41.4 +/- 0.8 pmol heme ^{55}Fe/well during 7 h incubation. The protein level was almost same for each well and not changed by addition of EGCG or GSE.

III. Effect of ascorbic acid on inhibitory action of EGCG and GSE on heme iron transport across the intestinal cell monolayer

The question remained as to whether this inhibitory effect could be reversed by dietary ascorbic acid. The effect of 100 µmol/L ascorbic acid on the EGCG- and GSE-mediated inhibition of heme iron absorption was investigated. The transepithelial transport of heme-derived ^{55}Fe was decreased by 87.0 +/- 0.05% and 90.5 +/- 0.07% in the cells treated with EGCG and GSE in the presence of ascorbic acid, respectively, compared with the control (Figure 3). The inhibited heme iron transport was similar to that for cells treated with EGCG and GSE alone. However, ascorbic acid significantly increased heme iron transport across the intestinal cell monolayer in the absence of EGCG or GSE during the 7 h transport study (Fig 2). The transepithelial heme iron transport was enhanced by ascorbic acid at 5 and 7 h incubation (Fig 2).

IV. Effects of bioactive dietary polyphenols and ascorbic acid on the apical uptake of heme iron

The apical heme ^{55}Fe uptake was significantly decreased by EGCG but was counteracted by 100 µmol/L ascorbic acid (Fig 4). The addition of ascorbic acid to EGCG increased the apical heme iron uptake by 52.9 +/- 2.3% when compared to EGCG alone. However, the addition of ascorbic acid did not change the apical heme iron uptake in the presence GSE (Fig 4). Similarly, the apical heme iron uptake was not changed by ascorbic acid in the absence of any polyphenols.

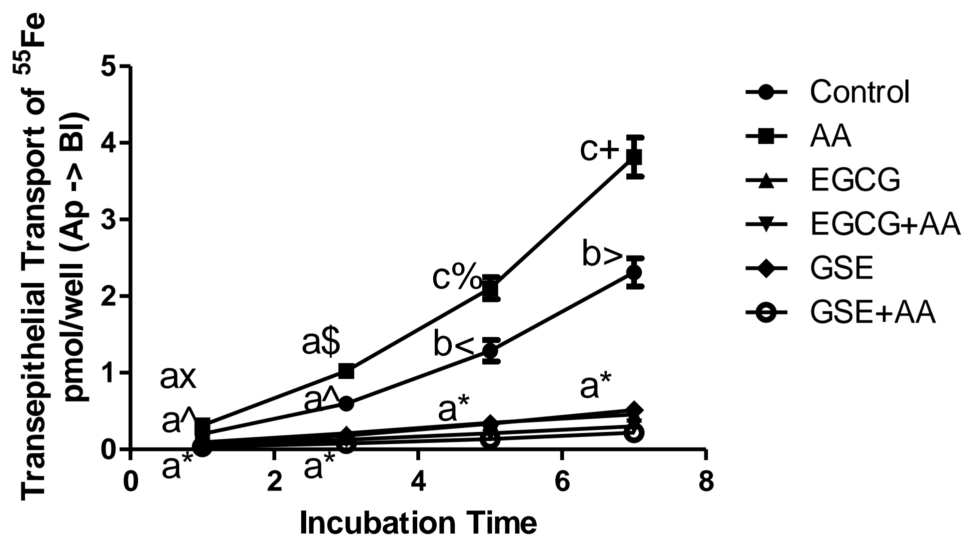


Figure 2: Transepithelial Transport of ^{55}Fe pmol/well (Ap -> Bl) versus Incubation Time

The amount of ^{55}Fe derived from heme that was transferred from the Caco-2 cell apical to basolateral compartment significantly increased between hours 1 and 7 of incubation for the control cells. Ascorbic acid showed a marked increase in the transport of heme ^{55}Fe from the apical to the basolateral compartment. There was no significant decrease in transport for the compartments containing EGCG, grape seed extract, or EGCG or grape seed extract with ascorbic acid. Means at an incubation time point without a common symbol differ at $p < 0.05$. Within a treatment, means without a common letter differ at $p < 0.05$. AP, apical; BL, basolateral.

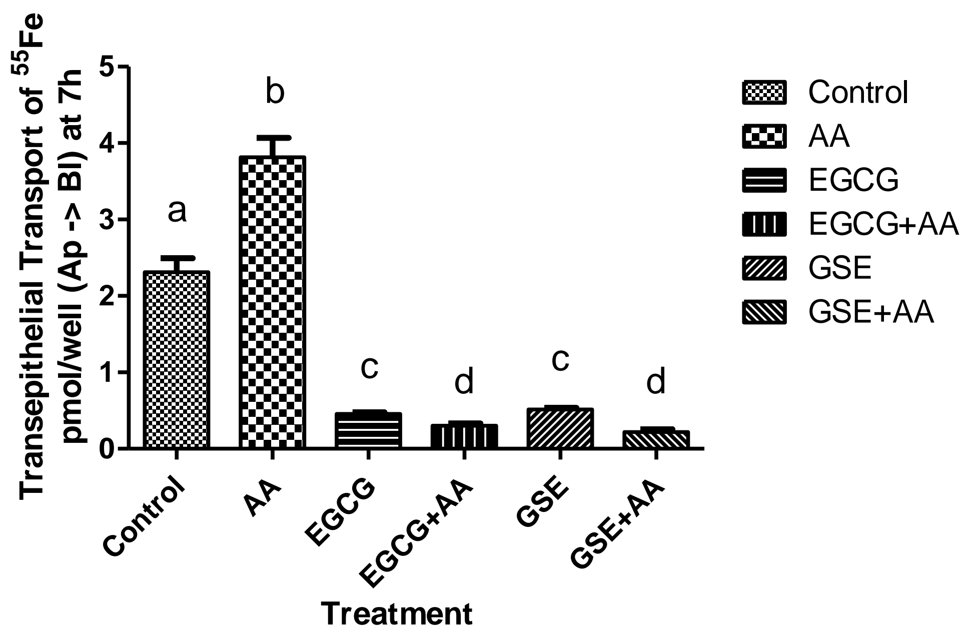


Figure 3: Transepithelial Transport of ^{55}Fe pmol/well (Ap -> Bl) at 7h versus Treatment

At 7h incubation time, EGCG and grape seed extract caused a significant ($p < 0.05$) decrease in ^{55}Fe transepithelial transport compared to the control. Addition of ascorbic acid further decreased ($p < 0.05$) the transepithelial transport in the presence of EGCG and grape seed extract. The well containing ascorbic acid alone showed a significant ($p < 0.05$) increase in ^{55}Fe transepithelial transport from control. Incubations include 0.5 µmol/L heme ^{55}Fe and 46 mg/L EGCG or 46 mg/L GSE in the absence and presence of 100 µmol/L ascorbic acid.

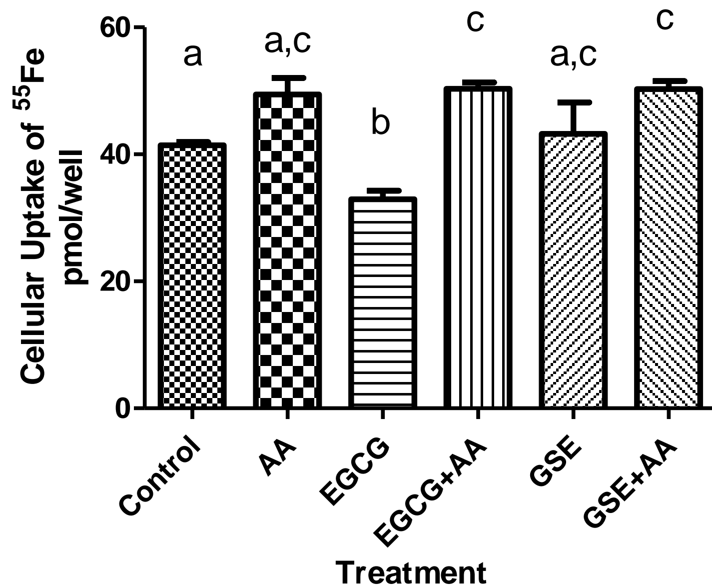


Figure 4: Cellular Uptake of ^{55}Fe pmol/well versus Treatment

Cellular uptake of ^{55}Fe (in pmol/well) was measured after the 7 hours of incubation. When ascorbic acid or grape seed extract were added to the wells, there was no significant change ($P > 0.05$) from the control wells. Addition of ascorbic acid significantly ($p < 0.05$) increased apical heme iron uptake and reversed EGCG-mediated inhibition of heme iron uptake. Ascorbic acid did not significantly ($P > 0.05$) enhance apical heme iron uptake in the presence of grape seed extract. Incubations include 0.5 µmol/L heme ^{55}Fe and 46 mg/L EGCG or 46 mg/L GSE in the absence and presence of 100 µmol/L ascorbic acid.

Chapter 4

Discussion and Conclusions

As expected, we established that EGCG and GSE significantly inhibited the transport of heme iron across the Caco-2 cell monolayer when compared to the control wells and the ascorbic acid wells at incubation times of 5 and 7 hours.

GSE did not inhibit cellular uptake of heme iron at 7 hours. Thus, the mechanism by which GSE inhibits heme iron absorption must be at the level of the basolateral, not the apical membrane. GSE is not preventing the iron from crossing the apical membrane but is preventing it from crossing the basolateral. EGCG inhibited both the transepithelial transport and cellular uptake of heme iron. This suggests that, unlike GSE which must work only at the basolateral membrane, EGCG works at both the apical and the basolateral membranes to inhibit iron absorption.

One possible reason for the inhibitory actions of the GSE and EGCG at the basolateral membrane is that they might decrease the iron exporter FPN protein level. In order to determine if this is the actual mechanism by which GSE inhibits absorption of heme iron, we would have to do a Western blot test to determine whether or not the level of FPN protein changed with the addition of GSE.

In iron deficiency, the body upregulates HO-1 which consequently increases the degradation heme, thus increasing the amount of iron available for absorption. It has been suggested that HO-1 activity can increase by 10 to 100 times normal in certain conditions. That increase in activity may be the reason why more heme iron is absorbed and exported [10]. Thus,

polyphenols (such as those in GSE and EGCG) might downregulate the HO-1 activity. This would cause a decrease in the degradation of iron and decrease the amount of heme iron available for absorption. In order to determine if this is the reason for the decrease in basolateral transport, another Western blot test would have to be performed to determine if the activity and/or expression of the heme oxygenase change with the addition of GSE or EGCG.

It is possible that EGCG decreases the transepithelial transport of heme iron by forming heme-polyphenol complexes. Those complexes would be insoluble in the intestinal lumen and thus would not be able to be taken up by the HCP-1 transporters or enter the cells by way of receptor mediated endocytosis. If EGCG were to inhibit the processes by which heme iron must be transported across the apical membrane, then the iron would not be able to enter the intestinal cells. This would not only explain the decrease in apical membrane transport but could also explain the decrease in basolateral transport. If there is less iron available in the cells, there is less iron available to transport from the cell into the blood (ie across the basolateral membrane).

We also established that ascorbic acid significantly enhanced the transport of heme iron across the Caco-2 cell monolayer when compared to the control wells at incubation times of 5 and 7 hours. In addition, we showed that ascorbic acid significantly increases the cellular uptake of iron at 7 hours. From this data, we cannot make a conclusion about how ascorbic acid promotes iron transport. It is not absolutely clear if the ascorbic acid works at the level of the apical, basolateral or both membranes. Previous research has shown that ascorbic acid does reduce ferric iron to ferrous iron, so it can be assumed that some of the mechanism of action is at the apical level [19]. However, action at the basolateral membrane cannot be ruled out until more research is done on the effects of ascorbic acid within the intestinal cell.

In addition to researching the effects of ascorbic acid within the intestinal cell and the activity/expression of the transporter proteins, it would also be helpful to do more research on the effects of GSE, EGCG, green tea extract and ascorbic acid on heme iron. In this study, the same quantity of heme iron and the same quantity of the added compounds were added to each cell compartment. However, previous studies have shown that heme iron uptake by Caco-2 cells is temperature sensitive and modulated by extracellular compounds [22]. In addition, it has been shown that ascorbic acid can prevent the inhibitory effects of polyphenols on nonheme iron absorption in a dose-dependent manner [20]. Thus, it is possible that the effects of EGCG, GSE and green tea extract on heme iron absorption could be affected by temperature or pH. The effects could also be dose dependent in terms of both the heme and the added compounds.

In conclusion, our study shows that EGCG and GSE significantly decrease the transport of heme iron across Caco-2 cell membranes at incubation times of 5 and 7 hours. GSE does so by working at the level of the basolateral, not the apical, membrane. However, EGCG works at both the basolateral and apical membranes to decrease heme iron absorption. In addition, our study showed that ascorbic acid significantly enhanced iron absorption across the monolayer at 5 and 7 hours and increased the cellular uptake at 7 hours. Ascorbic acid also decreased transepithelial transport of heme iron when from the control and when added to EGCG and GSE compared to the two compounds alone. Ascorbic acid did reverse the inhibition of cellular iron uptake when added to EGCG but not when added to GSE. The exact mechanisms by which these effects happen have yet to be properly researched and explicated.

Annotated Bibliography

1. West, Adrian R, and Phillip S Oates. *Mechanisms of heme iron absorption: Current questions and controversies*. World Journal of Gastroenterology, 2008; 26: 4101-4110. A review describing how heme and non-heme iron is absorbed in the intestines and throughout the cells of the body. Heme receptor mediated endocytosis, heme transporters, heme catabolism and heme oxygenase are all discussed in detail according to the recent advances in iron research.

2. Beard, J. and Okhee Han. *Systemic iron status*, Biochim. Biophys. Acta (2008), doi: 10.1016/j.bbagen.2 0 08.09.005. A review written about systemic iron, its dietary forms, regulation of its absorption in both its heme and non-heme forms and iron deficiency. As stated in the review, iron is essential for growth and development. However, iron can be toxic in large amounts. Thus, iron regulation is crucial and is done at both the level of dietary iron intake and at the level of iron transporters in the intestines.

3. Hentze, Matthias W, Martina U Muckenthaler, and Nancy C Andrews. *Balancing Acts: Molecular Control of Mammalian Iron Metabolism*. N.p.: Cell 2004; 117, 285-297. A review written about iron metabolism at the molecular level in mammals. Cellular iron uptake, storage and export are all discussed. The article also talks about iron distribution in cells and how systemic iron is regulated and kept at a constant level. The authors conclude that not enough is known about iron homeostasis and how tissues communicate about iron needs. Research must be done to know how to treat iron deficiency and toxicity.

4. Latunde-Dada, Gladys O., Robert J Simpson, and Andrew T McKie. *Recent advances in mammalian haem transport*. Trends in Biochemical Sciences 2006; 31: 182-188. A review written about the latest research and findings about mammalian haem transport. The haem-carrier protein 1 has been found to be an important transporter in the apical duodenum that takes haem into the gut cells. The FLCVR and ABC transporters also export haem, usually when a person is under stress. Although more research is needed, cells need a way to regulate haem, which is both essential and toxic. It is thought that this is done by regulating the transporters described in this review.

5. Rouault, Tracey A. *The Intestinal Heme Transporter Revealed*. Cell 2005: n.p. It is known that animals can derive iron from heme, thus indicating that there is an intestinal transporter that transports heme from the gut into the intestinal epithelial cells. This paper describes the heme carrier protein 1 (HCP1) which does that transportation of heme-bound iron. This paper also describes how different conditions such as hypoxia and iron deficiency or overload affect HCP1 activity. Heme uptake is increased during hypoxia and/or iron deficiency. However, the HCP1 transporters retreat to internal membrane compartments when iron-deficient animals get a bolus of iron all at once. Other details of the heme carrier protein are also described.

6. Shayeghi, Majid, et al. *Identification of an Intestinal Heme Transporter*. Cell, 2005; 122, 789-801. A study done to determine specific properties of the intestinal heme transporter (HCP1) and to determine how it is regulated. HCP1 was isolated from mouse duodenum and expressed in oocytes. The results indicate that HCP1 is highly expressed in the duodenum but not in the ileum and was localized in the apical membrane when iron levels were deficient, thus suggesting that iron stores regulate heme transport by

regulating the HCP1 protein. Also, HCP1-mediated heme uptake seems to be temperature dependent and saturable.

7. Kaur, Manjinder, Chapla Agarwal and Rajesh Agarwal. *Anticancer and Cancer Chemopreventive Potential of Grape Seed Extract and Other Grape-Based Products*. The Journal of Nutrition, 2009; 139: 1806S-1812S. A review written about grape seed extract and grape-based products and their value in decreasing the overall incidence of cancer. There has been a lot of research done on chemoprevention, prevention of cancer by dietary constituents. This technique could decrease the incidence of cancer without putting as much economic burden on the population as a whole. Overall, the review concluded that the research showed that grapes and products derived from grapes are sources of anticancer agents. The general population should be encouraged to consume such products.

8. Wu, Christine. *Grape Products and Oral Health*. The Journal of Nutrition, 2009; 139, 1818S-1823S. A study conducted to determine if grape products, such as grapes and grape-containing bran cereal, contain antimicrobial phytochemicals capable of suppressing oral pathogens thus increasing oral health. Compounds associated with pathogen growth inhibition were aleanolic acid, aleanolic aldehyde, linoleic acid and linolenic acid, among others. Oleanolic acid suppressed adherence of *Streptococcus mutans*. Compared to raisins alone, raisin bran cereal caused a lower plaque pH drop. Grape seed extract was also tested and was found to positively affect the mineralization of root caries. The researchers concluded that raisins could be used as a healthy alternative to sugary snacks.

9. Hurrell, Richard, Manju Reddy, and James D Cook. *Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages*. N.p.: British Journal of Nutrition 1999; 81, 289-295. Adult human subjects were fed a bread meal in this study in order to determine the effects of several phenol-containing beverages on non-heme iron absorption. Black tea and peppermint tea inhibited the absorption the most. Herb teas and cocoa were not far behind. Adding milk to the beverages has very little, if any, effect on the amount of absorption. It was determined that the beverages with the highest concentrations of phenolic compounds inhibited iron absorption the most because of their binding properties. However, there are certain polyphenols, such as those found in black tea, that are more inhibitory than other polyphenols due to their chemical make-up. The researchers determined that these results may not be able to be used in regard to complex meals because only bread was used in this study and other foods may change the results.

10. Ma, Qianyi, Eun-Young Kim and Okhee Han. *Bioactive Dietary Polyphenols Decrease Heme Iron Absorption by Decreasing Basolateral Iron Release in Human Intestinal Caco-2 Cells*. The Journal of Nutrition, 2010; 140: 1117-1121. A research study done to test whether iron utilization could be affected by regular ingestion of products containing dietary bioactive polyphenols. It was investigated whether or not EGCG and GSE affect heme iron absorption in intestinal Caco-2 cells when incubated for 7 hours. Both compounds decreased transepithial transport of heme iron. Apical uptake was increased by GSE, but the basolateral transport was impaired by it. Neither compound changed the expression of heme oxygenase, ferroportin and hephaestin proteins. The apical iron uptake was temperature dependent and saturable in the Caco-2 cells. The researchers concluded that polyphenols inhibit heme iron absorption by decreasing basolateral iron

transport rather than decreasing apical uptake.

11. Samman, Samir, et al. *Green tea or rosemary extract added to foods reduces nonheme-iron absorption*. Am. J. Clin. Nutr. 2001;73:607-12. Clinical study involving the administration of test meals containing meat enriched with phenolic-rich extracts from green tea or rosemary to young women aged 19-39 years to determine the effects of those extracts on nonheme-iron absorption. Compared with the control meal, both the meals containing the green tea extract and the meals containing the rosemary extract significantly decreased nonheme-iron absorption. The authors concluded that the chelation of iron may be one of the mechanisms by which the extracts act as antioxidants in vivo and that the effects of phenolic-rich extracts should be further researched for their positive and negative effects in vivo.

12. Disler, P.B., et al. *The effect of tea on iron absorption*. N.p.: Gut 1975; 16, 193-200. A study done with Indian housewives with the goal of determining if tea affects iron absorption in the intestines. They were each given bread, rice with potato and onion soup or minced lamb labeled with ^{55}Fe or ^{59}Fe. Some subjects received ascorbic acid as well. Tea was administered to some. Blood samples were taken at 2 week intervals to determine iron levels. Results indicated that iron absorption was inhibited whether ascorbic acid was present or not. However, tea did not affect absorption if the hemoglobin/lamb was cooked. Iron significantly reduced absorption of non-heme iron.

13. Ryan, Paul, and Michael J Hynes. *The kinetics and mechanisms of the complex formation and antioxidant behaviour of the polyphenols EGCg and ECG with iron(III)*. N.p.: Journal of Inorganic Biochemistry 101 2007;585-593. A study conducted to determine

how EGCG and other related polyphenols form complexes with iron. The mechanism by which this happens and the iron assisted decomposition of complex were also studied. The rate constants in both situations were also determined. The researchers showed that one molecule of EGCG or EGC can reduce a maximum of four iron species.

14. Afsana, Kaosar, et al. *Reducing Effect of Ingesting Tannic Acid on the Absorption of Iron, but Not of Zinc, Copper, and Manganese by Rats*. Biosci. Biotechnol. Biochem. 2004;68 (3), 584-592. Two experiments done with four week old male Sprague-Dawley rats to determine the effects of different levels of iron and different amounts of tannic acid on the absorption of trace minerals, such as Fe, Zn, Cu and Mn. Iron absorption decreased as Fe consumption levels increased, but tannic acid had no absorption effects on any of the trace minerals, except iron which it decreased. The authors concluded that tannic acid significantly reduced iron absorption but not absorption of the other trace minerals tested; the overall effects of tannic acid are not known, so more research needs to be done before recommending a level of tannic acid consumption.

15. Boato, Francesca, et al. *Red Grape Juice Inhibits Availability: Application of an in Vitro Digestion/Caco-2 Cell Model*. N.p.: J. Agric. Food Chem. 2002;50, 6935-6938. A clinical study using seven different fruit juices combined with iron chloride or iron fortified infant cereal then subjected to simulated gastric and intestinal digestion in Caco-2 cells. The pear, apple, grapefruit, orange and white grape juices increased iron bioavailability. The prune and red grape juices inhibited the bioavailability. The researchers concluded that the red grape and prune juices had that effect due to their high concentrations of polyphenolic compounds and those who need to increase their iron uptake should avoid those fruit juices.

16. Lemos, Clara, et al. *Modulation of folate uptake in cultured human colon adenocarcinoma Caco-2 cells by dietary compounds*. N.p.: Eur. J. Nutr. 2007;46: 329-336. A clinical study performed with Caco-2 cells to determine if beverages or their specific components affect the intestinal uptake of ^{3}H-folic acid or ^{3}H-methotrexate. Wine, beer, tea and the components ethanol and phenolic compounds were used in the uptake studies. The amount of phenolic compounds in each beverage was determined. It was found that all of the beverages inhibited uptake of ^{3}H-folic acid and all did the same for ^{3}H-methotrexate (except for wine, which wasn't tested). The researchers determined that the decreased uptake of those compounds can be explained in part by their phenolic compounds.

17. Hallberg, Leif, et al. *Inhibition of haem-iron absorption in man by calcium*. N.p.: British Journal of Nutrition 1992;69, 533-540. A study involving 28 subjects separated into two food consumption studies, one with and one without meat. Each meal was served twice, once with added Ca and once without Ca. Studies done to determine whether Ca works to inhibit iron absorption by directly interfering with the enhancing effect of meat or by directly influencing the absorption of haem-iron. Haem-iron absorption was significantly and similarly reduced for all participants. The authors concluded that since haem-iron was inhibited in all meals, whether meat was included or not, Ca has a direct effect on the inhibition of haem-iron. It does not simply counteract the enhanced haem-iron absorption caused by meat consumption.

18. Mennen, Louise I, et al. *Risks and safety of polyphenol consumption*. N.p.: Am. J. Clin. Nutr. 2005;81(suppl):326S-9S. An overview written about the hazards and potential benefits of polyphenol consumption as discussed at the First International Conference on Polyphenols and Health in Vichy, France in November 2008. Polyphenols have

carcinogenic effects or interfere with thyroid hormone biosynthesis. They inhibit nonheme iron absorption and could lead to depletion of iron stores. Polyphenols may also enhance the effects of pharmaceutical agents. It was concluded that polyphenol-fortified foods should not be ingested unless a person is part of the population for which those foods were made.

19. Reddy, Manju B, Richard F Hurrell, and James D. Cook. *Estimation of nonheme -iron bioavailability from meal composition*. N.p.: Am. J. Clin. Nutr. 2000;71:937-43. A study done to determine whether or not nonheme iron absorption from meals with several food components could be estimated by separately analyzing the determinants of that food before consumption. During nine studies, 25 different meals with multiple ingredients were administered to a total of 86 subjects. According to regression analysis, the most significant variation in iron absorption came from consumption of animal tissue, phytic acid and ascorbic acid. Polyphenols, calcium and nonheme iron were not significant predictors of iron absorption. The authors concluded that dietary factors and food component analysis made only a small contribution to predicting iron absorption in an individual. Other unknown factor or factors contribute the most to iron absorption variation.

20. Siegenberg, David, et al. *Ascorbic acid prevents the dose-dependent inhibitory effects of polyphenols and phytates on nonheme-iron absorption*. N.p.: Am. J. Clin. Nutr. 1991;53:537-41. A study done to determine the extent of the inhibitory effects of several phytates and polyphenols and to document the amount of ascorbic acid needed to overcome the effects of each inhibitor. Eighty grams wheat bread with various quantities of added phytates, polyphenols and ascorbic acid was given to each of 199 Indian

housewives. The researchers found that when phytate P or tannic acid was increased, iron absorption was decreased. Conversely, as ascorbic acid was increased, iron absorption also increased. The authors concluded that the solubility properties of phytate and polymerization by polypheonls have inhibitory effects on iron bioavailability. Both can be reversed by the addition of ascorbic acid.

21. Qiu, Andong, et al. *Identification of an Intestinal Folate Transporter and the Molecular Basis for Hereditary Folate Malabsorption*: Cell 2006;127, 917-928. A study done to identify a proton-coupled, high-affinity folate transporter in the intestines and determine the importance of that transporter. The researchers identified G21, previously known as HCP1 as that transporter and changed the name to PCFT/HCP1. It was determined that that transporter is required for normal folate absorption because a loss-of-function mutation is found in those with hereditary folate malabsorption.

22. Arredondo, Miguel, et al. *Heme Iron Uptake by Caco-2 Cells is a Saturable, Temperature Sensitive and Modulated by Extracellular pH and Potassium*. Santiago, Chile: Biol Trace Elem Res 2008, n.p. A study done to learn more about the mechanism of iron absorption by determining how several variables affect apical iron uptake in Caco-2 cells. After analyzing Caco-2 cells cultured with a radioactive-labeled digest of hemoglobin, it was determined that intracellular iron levels are higher in cells exposed to inorganic iron as opposed to heme iron solutions. It was confirmed that iron uptake is a saturable and temperature-dependant process that could occur through both a receptor and endocytic pathway. Iron absorption is also affected by intracellular iron levels but not by external non-heme iron.

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