THESIS

INGESTION OF WHEAT GERM IN HEALTHY SUBJECTS DOES NOT ACUTELY ELEVATE PLASMA WHEAT GERM AGGLUTININ CONCENTRATIONS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JESSICA KUZMA ENTITLED INGESTION OF WHEAT GERM IN HEALTHY SUBJECTS DOES NOT ACUTELY ELEVATE PLASMA WHEAT GERM AGGLUTININ CONCENTRATIONS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

INGESTION OF WHEAT GERM IN HEALTHY SUBJECTS DOES NOT ACUTELY ELEVATE PLASMA WHEAT GERM AGGLUTININ CONCENTRATIONS

Because the fiber content (15 g/day) of the typical U.S. diet is considerably lower than recommended values (25-30 g) the public is encouraged by USDA Dietary Guidelines to increase consumption of high fiber foods such as whole grains, fruits, and vegetables. Because whole grains are also a rich source of the lectin wheat germ agglutinin (WGA), those abiding by current recommendations would consequently increase their lectin intake. The WGA lectin has been poorly studied in humans, and it is unclear if it can breach the gut barrier, enter circulation, and influence physiology. Apart from animal studies, characterization of lectin behavior in humans is limited to tomato lectin (TL) and peanut lectin (PNA) feeding studies; both of which were able to detect lectin in systemic circulation within hours of ingestion of tomato juice and peanuts, respectively. Because lectin consumption has elicited adverse health effects in laboratory animals, a prudent first step in humans will be to determine if common dietary lectins such as WGA can enter circulation in quantities capable of influencing normal cell function.

The purpose of this study was to determine if WGA enters the circulation of healthy subjects following ingestion of wheat germ and to determine the potential time

course and concentration of this lectin in venous plasma. Fourteen normal males and females between 17 and 40 yrs completed preliminary screening and reported to the lab after an overnight fast. Following baseline blood measurements, subjects consumed 50 g wheat germ within a 60 min interval. Blood samples were obtained at 30, 60, 90, 120, 240 min, and at 24 hrs post ingestion. Venous plasma concentrations of WGA were determined via WGA-specific Enzyme-Linked Immunosorbent Assay (ELISA) at the six time intervals, including baseline.

According to the protocol utilized, WGA was not detected in venous plasma samples from any of the subjects following consumption of 50 g of wheat germ, thereby indicating that the available WGA in wheat germ either did not breach the gut barrier, or preferentially bound tissues other than plasma proteins. These data represent an important first step in determining the safety of WGA found in common whole-wheat food products. Further research will be required to determine if WGA enters the bloodstream and binds formed elements such as erythrocytes, platelets, and leukocytes in addition to other tissues and organs.

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CHAPTER I

INTRODUCTION

The United States Department of Agriculture (USDA) is urging the public to increase dietary fiber by eating more whole grains. Updated 2005 dietary guidelines advise those following a 2,000 kcal diet to consume six to eight servings of grains each day, preferably in the form of whole grains, in order to achieve 14 grams of fiber per 1,000 kilocalories consumed (United States Department of Health and Human Services, United States Department of Agriculture, & Committee, 2005).

Whole wheat grain products contain several non-to-poorly digestible naturally occurring secondary compounds. Specifically, wheat germ agglutinin (WGA) belongs to the lectin family; proteins identified by their ability to bind common carbohydrate residues found on integral membrane proteins of eukaryotic cells (Lis & Sharon, 1998). Consequently, if intact biologically active lectins enter circulation, they have the potential to interact with virtually all cells in the body.

Our current understanding of the cell-lectin interaction has come from numerous in vivo and in vitro animal studies (Pusztai, 1991; Pusztai, et al., 1993; Vasconcelos & Oliveira, 2004) and two studies in humans

(Kilpatrick, Pusztai, Grant, Graham, & Ewen, 1985; Wang, Yu, Campbell, Milton, & Rhodes, 1998). A wide range of adverse health effects, including disruption of normal gut function (Vasconcelos & Oliveira, 2004), persistent immune system stimulation (Yarden & Sliwkowski, 2001), and altered hormonal state (Pusztai, 1993) have been documented. However, the human studies have not been replicated, nor has the in vivo human interaction with WGA been described.

Because of the potential for adverse health effects associated with proteins in wheat, a common staple food item, the need exists to uncover any mechanisms of action in humans. Hence, a necessary first step must be to determine whether WGA does indeed enter systemic circulation in an intact and biologically active form.

Statement of the Problem

The purpose of this study was to determine whether upon consumption of 50 g wheat germ, biologically active WGA could be detected in the plasma of normal, healthy human subjects.

Hypothesis

There will be a significant ($p < 0.05$) increase in plasma concentrations (ng/ml) of WGA following consumption of 50 g wheat germ.

Delimitations, Limitations, and Assumptions

The study was delimited to 14 healthy males and females aged 17 to 40 years from Colorado State University. Subjects had no known allergies to wheat or other diagnosed GI disease. A medical questionnaire and a three-day dietary intake record were required in order to determine health status and habitual wheat intake. Following an overnight fast and baseline blood draw, subjects consumed 50 g (dry weight) raw wheat germ within one hour. Subjects remained seated for an additional four hours prior to release and were asked to abstain from strenuous physical activity for 24 hours.

It was assumed that food intake was accurately documented, subjects were fasted prior to the study, and physical activity was avoided for the prescribed time period. Additionally, it was assumed that WGA occurred in wheat germ at concentrations similar to those previously reported (Vincenzi, et al., 2002).

CHAPTER II

LITERATURE REVIEW

Originally discovered in 1888 (Stillmark, 1888), lectins were distinguished from common protein molecules because of their unusual ability to agglutinate, or clump, erythrocytes and to cause coagulation of plasma (Freed, 1985). The field of lectinology officially emerged with characterization and description of lectins beginning in 1948. Boyd and Reguera chose the Latin verb legere (to select) to name this family of naturally occurring plant proteins with the ability to agglutinate red blood cells of specific blood type within the ABO blood group system (Boyd & Reguera, 1949). It was later determined that the specificity of different lectins for erythrocytes of differing types was dependent on the sugars present on the surface of the cell (Watkins & Morgan, 1952). Accordingly, lectins have since been defined as proteins (or glycoproteins) of non-immune nature capable of specific recognition of, and reversible binding to, carbohydrate moieties of complex glycoconjugates (Pusztai, 1991). All plant lectins exhibit strong binding affinity for one of five common monosaccharide configurations expressed on the surface of eukaryotic cells (Lis & Sharon, 1998). Hence, lectins are divided into five families based on preferential binding to: D-mannose/D-glucose, D-galactose/N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-fucose, or N-acetylneuramine

(Goldstein, Winter, & Poretz, 1997). Wheat germ agglutinin is a member of both the N-acetylneuramine (sialic acid) and the N-acetyl-D-glucosamine (NAG) families, along with lectins found in potatoes, tomatoes, and other cereals (Pusztai, 1991).

Consumption of plant lectins is frequent as their presence in the food system is widespread. In a laboratory survey of common foods, 30 percent of those tested showed significant haemagglutinating activity, while a review of the literature revealed an additional 53 edible plants (including carrots, cantaloupe, raspberries, cereals, nuts, and spices) with documented lectin activity (Nachbar & Oppenheim, 1980). The majority of which however do not appear to interact with human gut cells. One explanation for the pervasive abundance throughout the plant kingdom is lectins' role as chemical defense proteins. Unlike animals with developed immune systems, plants have evolved other means to discourage predation. In theory, barrier methods, such as thorns or thick cell walls, work in combination with endogenously synthesized chemicals to provide the plant with the best chance of survival in a hostile environment (Peumans & Van Damme, 1995). In support of this theory, lectins tend to be concentrated in the edible, calorically dense, valuable seed parts of plants. This is especially true of plants in the *Gramineae* (grass) family; WGA has a high affinity for chitin, a polymer of N-acetyl-glucosamine and a component of the cell wall of fungi, insects, arthropods, and other small predators. Additionally, WGA binds N-acetyl-neuraminic acid (sialic acid), a monosaccharide that is widely abundant in the animal kingdom, but virtually non-existent in plants (Zeleny, Kolarich, Strasser, & Altmann, 2006). Co-localization of antinutritive components along with valuable genetic material appears to confer a selective advantage by enhancing the odds for survival and proliferation of the species (Pusztai, 1991).

Because lectins appear to exert low-level toxic effects in nature, it is prudent to ask what health consequences might arise in humans as a result of chronic dietary intake. To date, no human studies have been conducted to evaluate the acute or long-term effects of WGA ingestion from consumption of wheat products.

Composition of a Wheat Seed

Because wheat has become a staple source of protein and calories for both animals and humans, the chemistry of the wheat plant has been known since the early 1900s (Osborne & Harris, 1906). The wheat kernel, or seed, is structured to provide the maximum amount of nutrition and protection to the embryo (germ). In order to pass along genetic material, germination must occur and the wheat plant must ensure the seed is equipped with sufficient nutrition to support growth. The food reserves for the embryo are located in the endosperm and consist primarily of starch (20-30% amylase and 70-80% amylopectin) and proteins (12%) (Srivastava, 2002), whereas the embryo itself makes up approximately 3% of the entire grain and consists of concentrated nutrient sources with approximate ratios of 28% protein, 12% lipid, 30% starch, and 24% fiber (Hernot, Boileau, Bauer, Swanson, & Fahey, 2008; Sjovall, Virtalaine, Lapvetelainen, & Kallio, 2000).

Since the agricultural revolution, humans have come to rely on the concentrated food reserves stored in seed endosperms. Today, wheat, rice, and maize seeds supply approximately 50 % of all food calories consumed by humans ("Biodiversity

International: Cereals," 2008). To enhance the calories available for exploitation, seed sizes have been dramatically increased beyond their wild-type size through selective breeding techniques (Srivastava, 2002). Because the proteins in seeds are synthesized for use specifically by the developing embryo, the composition is unique from those proteins found in the vegetative parts of the plant (Srivastava, 2002). Seed proteins are contained in membrane-bound protein storage vacuoles and are classified, based on their solubility, into four groups:

1. Albumins: soluble in water or dilute buffers at neutral pH
2. Globulins (legumins and vicilins): soluble in salt solutions
3. Prolamins: soluble in alcohols (70-90% solution)
4. Glutelins: soluble in dilute acids or alkalis (Srivastava, 2002)

As early as 1906, scientists had characterized the proteins of the wheat kernel and diagramed their location. Of significance, gliadin and glutenin, a prolamin and a glutelin, respectively, were identified as the primary proteins present in the endosperm, whereas globulin and albumin proteins appeared to be contained solely within the embryo (Osborne & Harris, 1906). In the production of white flour, the starches and proteins of the endosperm are separated from the germ and bran and pulverized into a fine powder. Gliadin comprises the majority of protein in the endosperm and due to its dense interchain disulfide bonds, has the unique ability to form gluten upon the addition of water—a coherent, elastic mass that remains after the starch and water soluble proteins have been eliminated (Srivastava, 2002).

A staple for the baking industry, the composition of flours are also important for health reasons. Increasingly, gluten-free products are requested in an attempt to avoid the gliadin fraction, the protein responsible for symptoms associated with celiac disease

(Drago, et al., 2006). Implicit in this request is the assumption that gliadin can be completely eliminated by removing the endosperm components of wheat and flour products. However, gluten proteins are difficult to separate into discrete fractions and globulin proteins can remain trapped in the gluten matrix allowing several proteins to exist in more than one fraction (MacFarlane, et al., 2003). Further, the conventional milling process eliminates the majority of non-endosperm seed parts in the production of white flour (approximately 1-2% remain), yet it is difficult to quantify the ratio of different grain tissues in each fraction due to lack of an efficient detection method (Hemery, et al., 2009). On average, the protein composition of typical flours appears to be 10%:40%:48% albumin/globulin:gliadin:glutenin (DuPont, Chan, Lopez, & Vensel, 2005). Additionally, the soil characteristics affect the final nutrient composition of the wheat prior to milling. The availability of nitrogen and sulfur during seed filling for instance, might favor starch or oil deposition over proteins (Srivastava, 2002), affecting the final nutrient profile of the wheat seed.

As previously mentioned, the wheat seed is the most valuable, and vulnerable, component of the plant because the genetic material, offspring, and energetically expensive food resources are contained here. In light of this, the seed is protected by the plant and is packaged with defense proteins aimed at warding off predation of these vital plant components. Several secondary compounds, or defense proteins, have been identified in seeds of plants with differing functions. In addition to lectins, alpha-amylase inhibitors, protease inhibitors, chitinases, and ribosome-inactivating proteins are all found packaged along with seeds to deter predation (Srivastava, 2002). The lectin WGA is specifically associated with wheat seeds and appears to be constrained solely to

the embryo with an approximate concentration of 1 μg WGA per grain (Mishkind, Raikhel, Palevitz, & Keegstra, 1982). Further, the lectin is condensed in external surfaces encasing tissues that will develop into the vital organs of the plant. For instance, the internal organs appear devoid of WGA while dense localization occurs in tissues surrounding the organs that will become the leaves, first adventitious roots, radicle, and all tissues that will be in direct contact with the soil during germination and growth. This pattern of distribution fits with WGA's role as a defense protein; microbes in the soil preying on vulnerable seedlings receive dense dosages of lectin in germination until the plant can grow and acquire stronger immunity. Indeed, newly emerged adventitious roots contain approximately 20-fold greater lectin density as compared to older parts of the root system (Mishkind, et al., 1982).

Dietary Exposure to WGA

Upon germination, the WGA concentration diminishes gradually with maturation. After one month of growth, WGA levels are approximately one-third to one-half of those in ungerminated embryos (Mishkind, et al., 1982). Therefore, it seems probable that the WGA concentration in the wheat kernel could also vary based on timing of harvest, soil properties, and other regional variations between cultivars. On average, however, it appears that the WGA concentration in raw wheat germ, composed solely of extracted embryos, is approximately 0.30 mg/g (Vincenzi, et al., 2002) or 0.03 %.

Due to the aforementioned inconsistencies associated with wheat milling, and dependent upon the dominate wheat products consumed in the diet, exposure to WGA is variable. White flour, the type utilized in the majority of processed baked goods or in all purpose flour, aims to selectively retain endosperm starches and proteins only. The consumer could expect 1-2 % non-endosperm tissues included in these refined flours (Hemery, et al., 2009) and an anticipated WGA concentration of around 4.4 μg/g (Matucci, et al., 2004). Whole grain or wholemeal flours, utilized in whole grain products, retains all three portions of the grain—bran, endosperm, and germ—and averages 50 μg/g WGA (Matucci, et al., 2004). If one were to follow USDA recommendations and consume primarily whole-grain products, WGA intake could range from 10 to 20 mg per day.

Processing appeared to alleviate the bioactivity of WGA in one study that showed that the lectin was no longer detectable in wholemeal pastas after cooking at 65º C for 30 minutes (Matucci, et al., 2004). However, a study of the agglutinating activity of common foods revealed that Wheaties, Product 19, All Bran, Shredded Wheat, Special K, Raisin Bran, and Total cereals, in addition to roasted wheat germs, were able to agglutinate human erythrocytes (Nachbar & Oppenheim, 1980), suggesting these foods retain bioactive WGA throughout processing. Further, the detection of biologically intact WGA in the fecal matter of humans after consumption of wheat germ confirms the lectins' high resistance to proteolysis and stabilization throughout harsh conditions (Brady, Vannier, & Banwell, 1978).

Resistance to Proteolysis

Plant proteins, in general, are more resistant to proteolysis than proteins of animal origin (Friedman, 1996) due to high dietary fiber, lectin, phytate, and tannin content (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997). Plant lectins in particular are resistant to gut enzymes in vitro, and even more so in vivo (Vasconcelos & Oliveira, 2004). The basis for this resistance is inherent in their structure: through extensive analysis of kidney bean lectin (PHA), it is apparent that three amino acids (aspartic acid, asparagine, and an aromatic amino acid: phenylalanine, tryptophan, or tyrosine, depending on the lectin) make up the monosaccharide binding site. A strong hydrophobic surface is created when the complimentary sugar ring stacks above the side chain of the aromatic residue (Sharon & Lis, 2002). A fourth key amino acid, glycine, participates in hydrogen bonding with the monosaccharide, and two metal ions, calcium and magnesium, are also required for sugar binding. The combination of these features results in lectins' high affinity for their complimentary monosaccharide, thereby lowering the energy state of the complex upon binding. It appears stabilization and resistance to proteolytic degradation is greater in vivo, where there is an abundance of free mono- and oligosaccharides (Vasconcelos & Oliveira, 2004).

The presence of lectins in the gut can also inhibit the absorption of other dietary macronutrients; upon ingestion, normal digestive processes attempt to degrade the proteins into amino acids and small peptides in preparation for transport across the gut epithelium. In addition to their resistance to proteolysis, lectins inhibit digestive enzymes and bind saturable amino acid transporters on the brush border which in turn, interferes

with protein absorption and utilization (Vasconcelos & Oliveira, 2004). Disruption to absorptive brush border cells leads to shedding and shortening of microvilli, which further decreases the surface area available for macronutrient absorption (Pusztai, 1991). In rat models, after 10 days on a mixed WGA diet (7g/kg body weight), digestibility and utilization of dietary macronutrients was depressed as evidenced by significantly lower total body growth with a concomitant increase in fecal nitrogen content, indicative of poor protein absorption (Pusztai, et al., 1993). In general, experimental animals fed high lectin diets exhibit signs of loss of appetite and decreased body weight eventually leading to death (Vasconcelos & Oliveira, 2004).

Interaction with the Gut

Because the gut is essentially open to the outside environment, it must offer a surface for nutrient absorption while also providing a critical physical barrier for protection against pathogenic materials to which humans are constantly exposed. Currently, three pathways have been proposed by which lectins may bypass innate immunity barriers, access lymph, and theoretically enter systemic circulation. The process begins in the intestinal lumen where lectins must first penetrate a mucus layer lining the gut composed of mucins (glycoproteins with a molecular weight greater than 2 x 10^6 Da) with a mean thickness of 192 μm (Gabor, Bogner, Weissenboeck, & Wirth, 2004). These intestinal mucus glycoproteins are primarily carbohydrate (75.5%) with a ratio of 1.0:0.6:0.7:0.3:0.5 N-acetyl-galactosamine:N-acetyl-glucosamine:galactose:fucose:sialic acid (Gabor, et al., 2004). As WGA binds both N-

acetyl-glucosamine and sialic acid with high affinity (Goldstein & Poretz, 1986), WGA is a strongly mucoadhesive lectin. In Caco-2 monolayer studies, WGA-combining sites of mucins were saturable (Gabor, et al., 2004); thereby eliminating exposed terminal residues available to bind other antigens, and potentially compromising one aspect of the mucosal innate immunity. Furthermore, the intestinal mucus layer appears to exist in an inconsistent layer with an overall permeability greater than that of gastric mucosa (Gabor, et al., 2004).

Secondly, the glycocalyx, a dense filamentous polysaccharide web, extends from the brush border of enterocytes and offers a second line of defense against potential pathogens. The glycocalyx is composed of a uniform 400 kDA mucin-like glycoprotein terminating in O-acetylated sialic acid (Massey-Harroche, 2000). Due to WGA's affinity for sialic acid, this filamentous structure does not prevent lectin binding. Because of the identical oligosaccharide composition of the mucins and glycocalyx, in combination with complete reversibility of lectin binding, WGA could conceivably find its way to the cell surface membrane undeterred (Gabor, et al., 2004).

Mechanisms of Absorption: Transcellular

The first mechanism of absorption was proposed upon numerous observations in tissue (Gabor, et al., 2004; Kramer & Canellakis, 1979) and animal (Pusztai, et al., 1993) models of the 'cytoinvasive' properties of lectins and the rapid uptake from the gut via endocytosis. Specifically, the WGA lectin binds a wide variety of human and animal

cells including stomach, brush border, skin, platelets, and myelin. In a recent review of food lectins, WGA appears to stand out among all others in its ability to bind 'almost everything in the human body' (Freed, 2002). Indeed, binding to the epithelial cell membrane via surface glycosides appears to be stimulus enough to trigger vesiculation and endocytosis (Gabor, et al., 2004; Kramer & Canellakis, 1979). Theoretically, they are then released via exocytosis into the intracellular space and subsequently transported throughout the body (Vasconcelos & Oliveira, 2004). This event was implied in the aforementioned rat model in which orally-administered WGA was taken up from the gut, transported into circulation, and deposited, biologically intact, in the walls of blood and lymphatic vessels (Pusztai, et al., 1993). It should be noted however that the amount of purified WGA consumed, 7 g/kg, was considerably higher than that which can be achieved by eating wheat food products.

In addition to spontaneous phagocytosis upon apical cell membrane binding, receptor-mediated endocytosis offers a likely mechanism by which WGA gains access to the cytoplasm. Perhaps the most well-studied example is the binding and uptake of WGA via exploitation of the epidermal growth factor receptor (EGF-R), a unique lumenally-facing receptor localized on the apical membrane of human (Hormi & Lehy, 1994) and rat (Montaner & Perez-Tomas, 1999) enterocytes. Similar to other hormone receptors, EGF-R binds its ligand via hydrophobic interactions between glycosyl side chains (Playford & Wright, 1996). The EGF-R, associated with epithelial cells throughout the body, normally binds EGF—a mitogenic hormone that initiates a signal cascade via phosphorylation of tyrosine kinase resulting in increased glycolysis, protein synthesis, gene expression, upregulation of additional EGF-R, and cell proliferation

(Fallon, et al., 1984). It has been speculated that because EGF is synthesized in salivary glands and secreted continuously into the gut lumen (at low concentrations), it acts as a surveillance peptide to initiate healing and stimulate repair at sites of injury (Playford & Wright, 1996). When EGF binds its receptor in the gut, invagination of the plasma membrane occurs and the entire receptor-ligand complex is endocytosed into the cell as second messenger cascades are initiated (Bardocz, Ewen, Grant, & Pusztai, 1995; Yarden & Sliwkowski, 2001).

To study this phenomenon, the immortalized Caco-2 cell line, despite a lack of mucus-producing capability, is widely used to model binding, uptake, and transport of molecules in the gut because of its similarity to in situ human enteroctyes (Gabor, et al., 2004). From these models, the EGF-R has been implicated as a primary mode of entry for WGA into epithelial cells (Lochner, Pittner, Wirth, & Gabor, 2003) and systemic circulation due to the presence of N-acetyl-glucosamine at the binding site. In fact, the binding affinity of WGA for EGF-R is high enough to inhibit binding of EGF, its natural ligand (Zeng, et al., 1995). Caco-2 studies showed rapid uptake and accumulation of fluorescent-labeled WGA—at least 50% of internalized lectin localized to lysosomes within 1 hr (Gabor, et al., 2004). Once taken up by the cell, the intracellular trafficking of the lectin is unknown; conceivably, because of its inherent resistance to enzymatic degradation, WGA reaches venous circulation via lysosomal transport and exocytosis to the lymph. Evidence of a direct transcytosis shuttle route to the basolateral compartment also exists, and it is speculated that approximately 3% of bound lectin enters this transport pathway (Gabor, et al., 2004).

The implications for WGA affinity for EGF-R extend beyond the gut. Upon binding by either EGF or a mimetic, the same cascade of events is initiated (Bardocz, et al., 1995), resulting in epithelial cell proliferation and increased EGF-R gene expression. Because the presence of WGA in the intestine causes damage to epithelial cells (Pusztai, 1991), healing is initiated by upregulation of EGF-R. The combination of these events could theoretically perpetuate a continuous positive feedback mechanism sustaining the entrance of WGA into enterocytes (Bardocz, et al., 1995) and, potentially, systemic circulation. Since EGF-R are associated with virtually every epithelial cell in the body at a density of 2×10^4 to 2×10^5 per cell (Todderud & Carpenter, 1989), there is the potential for circulating ligands to bind to remote tissues and alter metabolic and immune function, or induce abnormal cell proliferation and angiogenesis.

Mechanisms of Absorption: M cells

The second potential mechanism of entry, via M-cell transcytosis, exploits the immuno-protective mechanisms in place in the gut. Indeed, the largest immune organ is located in the gut where the mucosal immune system interacts with host and intestinal microbes and maintains homeostasis via innate and acquired immunity along the epithelial surface (Moreto & Perez-Bosque, 2009). The largest component of the mucosal immune system are gut-associated lymphoid tissues (GALT) including Peyer's Patches, isolated follicles, mesenteric lymph nodes, and lymphocytes dispersed throughout the epithelium. The GALT functions in both innate and acquired immunity; the tissues offer both a physical barrier to foreign substances as well as an inducible 'seek

and destroy' response when stimulated by antigens or pathogens (Moreto & Perez-Bosque, 2009). Ingested lectins have the potential to interact with the GALT and elicit an immune response by recognition as an antigen when coming into contact with lymphocytes. Specifically, microfold (M) cells are located within follicle-associated epithelium of Peyer's Patches, sections of lymphoid tissue localized to the lower small intestine. The protective role of this segment of the GALT is to continuously sample the contents of the intestine and present potential antigens to cells of the mucosal immune system, such as T and B lymphocytes. To aid in this function, M cells lack dense villi but contain instead broad microfolds. Additionally, the glycocalyx is thin or completely lacking in this area, allowing for direct contact between intestinal contents and the cell membrane (Neutra, Phillips, Mayer, & Fishkind, 1987). The potential for exploitation of this pathway and entrance into the body by WGA was proposed upon evidence of other pathogens utilizing this pathway, such as the polio virus (Ouzilou, et al., 2002). It has been shown that WGA avidly binds M cells (Freed, 2002) and in a rabbit model of M cell transport, was taken up rapidly and transported in vesicles to the basolateral membrane (Neutra, et al., 1987). Secondary to direct entry of the lectin, WGA's interaction with GALT provides a mechanism of sensitization to WGA and immune activation potentially resulting in an immune response upon each ingestion of wheat or products containing WGA.

Mechanisms of Absorption: Paracellular

Lastly, transport of WGA into circulation could occur via the paracellular transport pathway through tight junctions between enterocytes. Under normal physiological conditions, tight junctions are strictly regulated and passage of macromolecules is virtually nonexistent (Drago, et al., 2006) as the paracellular diameter is approximately 10 to 15 Å, which excludes any molecule larger than approximately 3.5 kDa (Fasano, 2008). Inflammation, both localized and systemic, is associated with an increase in gut permeability which, it appears, occurs exclusively via paracellular means rather than as a result of enterocyte damage (Hietbrink, et al., 2009). It could be argued, therefore, that anything that induces an injury response and/or inflammation in the gut would loosen tight junctions and increase permeability. Based on animal studies documenting the physical degradation to the gut mucosa induced by WGA (Pusztai, 1991), and the role of EGF-R binding as a gut-repair signal (Playford & Wright, 1996), it is likely that WGA could induce leaky gut via its pro-inflammatory tendencies. Furthermore in vitro, WGA binds human dendritic cells (El Sherbini, et al., 2000), induces inflammatory cytokine differentiation (Muraille, Pajak, Urbain, & Leo, 1999), and binds N-actyl-glucosamine-rich cell wall components of Gram-negative bacteria (lipopolysaccharides) (Heath, 1971), all of which initiate an immune response. It is conceivable that chronic, low level exposure to WGA and/or wheat products could sustain a background low-grade inflammatory state in otherwise healthy individuals with normal functioning immunity.

Early evidence in vivo established that application of 1 mg WGA to the distal small intestine of rats induced morphological changes to the microvilli including shortening, irregularity of the brush boarder, and infiltration of the villi with inflammatory cells (Sjolander, Magnusson, & Latkovic, 1984). As a result, permeability of the intestine to large molecular weight molecules increased significantly over controls, which was attributed to entry via the paracellular route, as evidenced by fluorescent-labeled dextran molecules (MW 3,000) (Sjolander, et al., 1984). Further, in Caco-2 studies measuring transepithelial electric resistance (TEER) as an indicator of tight junction permeability, WGA treatment decreased the TEER value and induced greater transport of isoflavones to the basolateral membrane (Ohno, Naganuma, Ogawa, & Muramoto, 2004).

Significant strides have been made recently in elucidating the complex tight junction network regulating paracellular transport and communication between enterocytes and underlying GALT. Perhaps most intriguing is the recent discovery that zonulin, an endogenously secreted growth-hormone like peptide, is the primary protein responsible for tight junction regulation (Fasano, et al., 2000). The protein is most likely part of the innate immunity of the gut and when released, intracellular actin filaments, which are directly connected to tight junction complex proteins, polymerize (Drago, et al., 2006) thereby increasing the diameter of the tight junction. Since the zonulin system operates in the small intestine only, and the small intestine is essentially sterile, it has been speculated that the zonulin system has been conserved as a means of protection from bacterial colonization. In mammalian small intestines, the presence of non-specific bacteria induces a mucosal response resulting in zonulin-induced opening of tight

junctions, secretion of water into the lumen, and flushing of bacteria from the small intestine (Fasano, 2008).

It was recently discovered that this mechanism of increased tight junction permeability is also stimulated upon exposure to alpha-gliadin, the wheat protein responsible for intestinal damage in celiac disease sufferers. Using intestinal biopsies from celiac and non-celiac patients, gliadin-induced zonulin release in both groups resulted in an increase in intestinal permeability to macromolecules (Drago, et al., 2006). It has since been determined that gliadin causes zonulin release by binding to the chemokine receptor CXCR3, another lumenally expressed receptor, involved in lymphocyte migration into inflamed tissues (Lammers, et al., 2008). CXCR3s are predominately expressed on the apical membrane of enterocytes and are upregulated during the active phase of celiac disease, suggesting a role in the immune response. The receptor is also expressed on the surface of immune cells, such as T lymphocytes, T helper cells, and natural killer cells, and acts as a mechanism for these cells to migrate to their target tissues and bind their intended ligands—chemokines (Lammers, et al., 2008). It is relevant that gliadin binds this receptor and induces zonulin release, and this function appears to be unique to gliadin as chemokine-binding exerts different effects in regards to zonulin and tight junction permeability (Lammers, et al., 2008).

These data raise important implications for the consumption of wheat and mechanisms of WGA entry into the body. Gliadin, a wheat endosperm protein, is exposed upon milling and occurs in combination with WGA in whole-wheat flour products. The potential for these two proteins to work in tandem in the gut presents a

compelling case for the role of gliadin in increasing gut permeability and essentially shuttling WGA through tight junctions.

Further complicating the task of determining the effects of WGA in vivo is gliadin's 'lectin-like' ability to agglutinate cells on its own, irrespective of the presence of WGA. Purified alpha-gliadin agglutinates undifferentiated human K 562(S) cells and is inhibited by both mannan and N-acetyl-glucosamine (Auricchio, et al., 1990), suggesting a potential morphological effect on the gut even in non-celiac cases. Moreover, gliadin interacts with the EGF-R and elicits the same initial response as EGF-binding in terms of cell proliferation and initiation of DNA synthesis, but also delays the inactivation of the pathway by interfering with endocytosis of the EGF-R complex (Barone, et al., 2007). Gliadin does not share sequence similarity with any growth factors and does not directly bind the EGF-R, rather it appears to enhance the effect of the ligand, such as EGF and potentially others, by prolonging receptor activation (Barone, et al., 2007). Zonulin itself belongs to a family of serine proteases that have structural similarity to several growth hormones, including EGF (Fasano, 2008); it is conceivable that gliadin could also interact with zonulin's target by amplifying and sustaining its effect on tight junction permeability.

All, or none, of the previously described mechanisms, transcellular, M cell, or paracellular, could play a role in the internalization of WGA from dietary sources. Currently, no human studies have unequivocally demonstrated the presence of biologically active WGA in systemic circulation following the ingestion of wheat germ or wheat products. Human-dietary lectin interaction studies are limited in general; there

exists two published accounts of orally administered lectin recovered from human circulation. The first (Kilpatrick, et al., 1985) attempted to determine whether the fate of tomato lectin was the same in humans as had been established in rats. Using ^{125}I-labelled tomato lectin in fresh tomato juice, one male subject drank a solution after an overnight fast and blood was drawn immediately before and repeatedly after consumption. By measuring circulating radioactivity and based on crude calculations, the authors concluded that at the plateau level (30 min), approximately 2.3 % of ingested labeled lectin could be detected in circulation (Kilpatrick, et al., 1985). Contrary to WGA, however, tomato lectin does not disrupt, flatten, or otherwise damage the intestinal villus (Kilpatrick, et al., 1985) potentially highlighting a key difference between the mechanisms by which these two different lectins gain access to circulation.

The second human study (Wang, et al., 1998) attempted to establish whether intact peanut agglutinin (PNA) enters the circulation following consumption of peanuts. Blood samples were collected immediately before, and up to four hours after, seven subjects ate 200 g of peanuts with a PNA concentration of approximately 0.11 mg/g (Vincenzi, et al., 2002). Within one hour of consumption, up to 5.0 μg/mL intact PNA was detected in serum samples (Wang, et al., 1998). Because of the differing preference for sugar specificities between WGA and PNA, these results offer no indication of the amount of WGA that might be recovered from plasma. Furthermore, by measuring only the plasma fraction, other binding sites were overlooked perhaps underestimating the amount of lectin that gained access to the circulation. However, these studies pioneered the field of human-lectin research and offer valuable guidance for future studies.

Systemic Effects

Short of experimental evidence of WGA entrance into systemic circulation of humans, one can only extrapolate from tissue and in vivo animal studies to speculate what adverse health effects can be expected from ungoverned circulation of lectins throughout the human body. Recent research into the exploitation of WGA as a potential drug delivery mechanism (Gabor, et al., 2004) and oral insulin carrier (Kim, Jeong, Park, & Kim, 2005; Zhang, Ping, Huang, & Xu, 2005) suggests that the scientific community is aware of WGA's unique characteristics and likely entrance into the system intact—a disturbing thought given WGA's reputation as the lectin that 'binds to almost everything in the human body' (Freed, 2002).

As a result of its strong affinity for the EGF-R, WGA has the potential to interact with, and gain entry into, any cell expressing the EGF-R or other receptor presenting N-acetyl-glucosamine at its binding site. Organs with especially dense localization of EGF-R are those with an epithelial component, such as the skin, lungs, pancreas, and GI tract (Yarden & Sliwkowski, 2001); overexpression of the EGF-R is a common theme in human malignancies of these organs, which could be linked to the aforementioned positive feedback mechanism between continuous binding of the EGF-R and increased gene expression (Bardocz, et al., 1995).

Additionally, in vitro studies have shown that WGA completely inhibits the passage of nucleoplasmin, a nuclear transport protein, through the nuclear pore (Finlay, Newmeyer, Price, & Forbes, 1987). Nuclear pores traverse the nuclear envelope and route mRNA and ribosomes from the nucleus to the cytosol for protein synthesis.

Blockage of the pore by WGA impairs vitamin D metabolism by interfering with nuclear transport of vitamin D receptors (Barsony, Pike, DeLuca, & Marx, 1990) which are principally located in the nuclei of target cells (Haussler, et al., 1998). Upon binding with its ligand, 1,25 hydroxyvitamin D3, the activated receptor-ligand complex modulates a variety of maintenance functions including blood calcium homeostasis, immune system support, and bone remodeling (Holick, 2004). Impaired vitamin D metabolism has been associated with osteomalacia, cardiovascular disease, multiple sclerosis, and Type 1 Diabetes, among others (Holick, 2004).

Recently, WGA has been identified as an inhibitor of the human leptin receptor complex. In an in vitro tissue study, leptin binding was reduced by up to 70 percent in the presence of WGA (Kamikubo, Dellas, Loskutoff, Quigley, & Ruggeri, 2008). Twenty potential N-glycosylation sites expressed on the surface of the receptor seem to regulate receptor function and permit ample opportunity for WGA interaction. Leptin, an adipocyte-secreted hormone, regulates food intake and energy expenditure through constant communication with the brain (Woods, 2005), and plays a role in the association between obesity and cardiovascular disease.

In light of the myriad subtle, yet significant, health maladies suspected to occur in connection with dietary WGA, and the ever-increasing worldwide consumption of wheat, further research into human-WGA interactions is warranted. The first in a series of experiments must be the detection and quantification of biologically active WGA in systemic circulation of humans.

CHAPTER III

METHODS AND PROCEDURES

Prior to recruitment of subjects, Institutional Review Board (IRB) approval at Colorado State University (CSU) was obtained (Appendix A). Subjects were screened for eligibility; exclusionary criteria included pregnant females, individuals with wheat allergy or other known GI disease, and those under age 17 or above age 40. The study purpose, protocol, procedure, risks, and benefits were explained during screening, each volunteer signed an informed consent (Appendix B). It should be noted that this protocol was originally part of a larger study entitled 'Bioavailability of Dietary Lectins' which sought to characterize the entry of both WGA and peanut lectin (PNA) into the circulation of separate populations of healthy individuals. The methodology and results of the wheat germ portion of the study are presented here.

Subjects were recruited from the CSU community via email announcements and fliers. After completion of a health history questionnaire (Appendix C), volunteers were asked to keep a food record (Appendix D) for three days prior to the testing day. Subjects arrived at the Human Performance and Clinical Research Laboratory (HPCRL) at CSU following an eight-hour fast. An indwelling catheter was place in the antecubital vein and 5 ml of blood was drawn for baseline characterization. A 500 ml saline

reservoir was connected to the catheter with a flow rate of 22 ml/min to prevent clotting. After which, subjects consumed 50 g raw wheat germ (Bob's Red Mill) within 1 hr. Portion sizes for wheat germ were determined based on intakes that can be expected for individuals following USDA dietary guidelines of 6-8 servings of (whole) grains per day for a 2,000 kcal diet (United States Department of Health and Human Services, et al., 2005).

Repeated blood draws were collected at 30, 60, 90, 120, and 240 minutes following complete consumption of the wheat germ portion. Proper techniques were followed to prevent contamination of blood samples. Subjects remained seated for the duration of the study and were released with emergency contact information (Appendix E) and were asked to return to the lab 20 hours later for a final blood draw.

Blood samples were refrigerated at 2ºC immediately after collection and plasma was separated via centrifuge (Thermo Corp., Waltham, MA) at 2800 rpm for 15 min at 0ºC within 1 hr. The plasma portion was alloquoted into two micro-centrifuge tubes and stored at -80°C until thawed for analysis.

Biochemical Analysis

Previously unthawed plasma samples were shipped at -80°C to laboratories at the University of Vienna, Austria and analyzed for WGA content according to methods previously published (Gull, Wirth, & Gabor, 2007). Briefly, a sandwich ELISA was created utilizing porcine gastric mucin (PGM) as the glycoprotein bound to the

polystyrene wells with lectin-binding sites exposed, and polyclonal rabbit anti-goat IgG to detect PGM-bound WGA. The ELISA allows for quantitative determination of WGA over the range from 10 to 1000 ng/ml WGA with a regression coefficient of 0.1438 and coefficient of determination of 0.9991 (Figure 1) (Gull, et al., 2007).

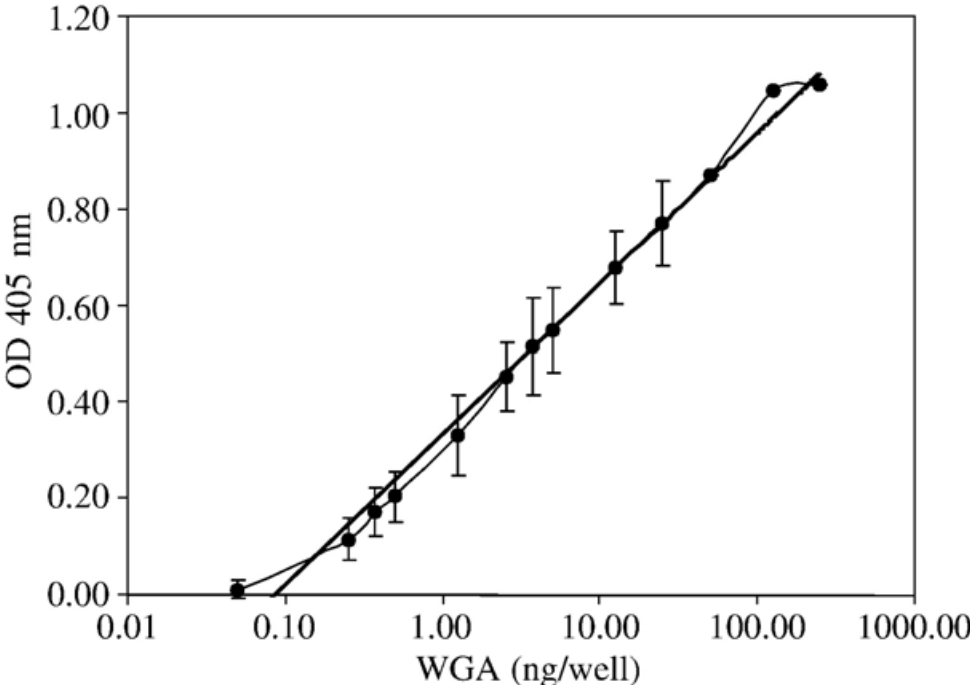

Figure 1. Calibration curve for quantification of WGA by ELISA-protocol (from Gull, et. al., 2007).

Statistical Analysis

Because no baseline differences in WGA concentration were detected among all samples at a minimum 1 ng/ml resolution, the need for further statistical analysis was eliminated. Subject characteristic means were calculated using SPSS (version 17.0).

CHAPTER IV

RESULTS

Fourteen subjects (8 male, 6 female, mean age 23 yrs) took part in the study; their characteristics are presented in Table 1. One subject (WG-01) was only able to finish 45 g of the 50 g wheat germ portion. Otherwise, all subjects complied with the protocol and arrived at the lab following an 8-hr fast. A total of seven blood samples were collected for each subject at t = 0(baseline), 30, 60, 90, 120, 240, and 1440 min; to standardize the procedure, time was tracked beginning 15 min after subjects began to eat the wheat germ. All plasma samples remained frozen at -80ºC for approximately one year before WGA analysis was completed.

Table 1. Subject characteristics

Subject	Height (cm)	Weight (kg)	BMI	Age	Gender
WG-01	162.6	56.4	21.3	21	F
WG-02	177.8	75	23.7	20	M
WG-03	177.8	63.6	20.1	20	F
WG-04	172.7	55.9	18.7	23	F
WG-05	177.8	74.1	23.4	23	M
WG-06	185.4	89.1	25.9	22	M
WG-07	167.6	72.7	25.9	28	F
WG-08	175.3	79.5	25.9	23	M
WG-09	177.8	68.2	21.6	24	M
WG-10	177.8	61.4	19.4	28	F
WG-11	185.4	74.5	21.7	24	M
WG-12	170.2	62.3	21.5	19	F
WG-13	180.3	78.6	24.2	25	M
WG-14	170.2	79.5	27.4	22	M
mean	**175.6**	**70.8**	**22.9**	**23**	

Figure 2 illustrates a typical calibration curve created for each plate using WGA diluted in phosphate buffered saline (PBS), a method that correlates well to spiked WGA concentrations in rabbit serum (Gull, et al., 2007). Utilizing this method, WGA was not detected in any of the plasma samples sent from the CSU laboratory to Dr. Gabor's laboratory in Vienna. Samples were measured in triplicate; raw data are included in Appendix F.

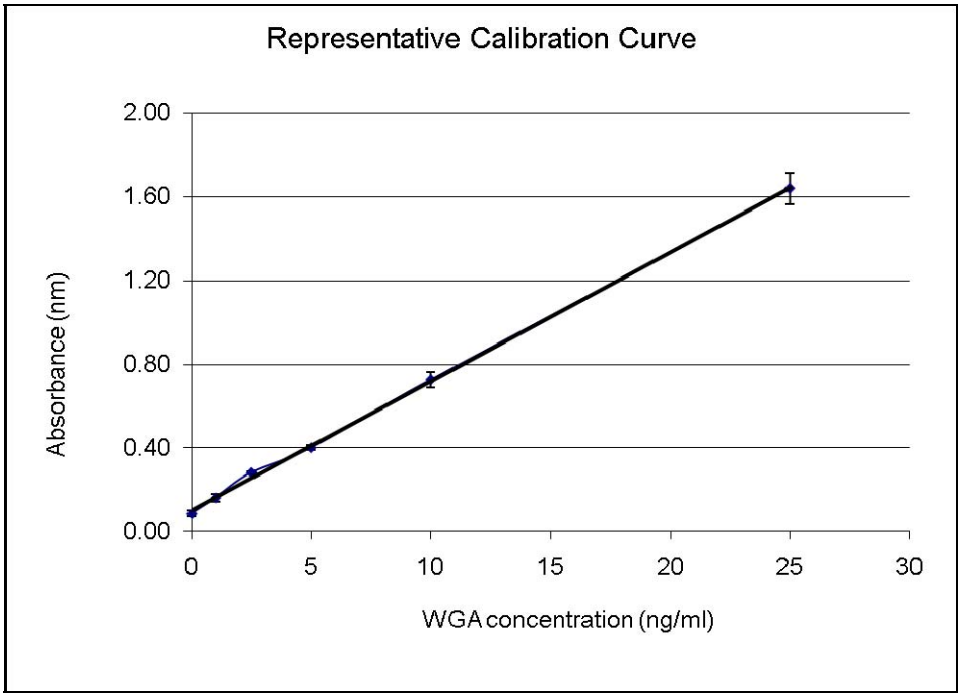

Figure 2. Representative calibration curve. Concentrations of WGA in Phosphate Buffered Saline (PBS) for each plate were: 0 (blank), 1, 2.5, 5, 10, and 25 ng/ml. Each sample was measured in triplicate; concentration was determined as mean minus the blank. Regression coefficient was 0.0616 and coefficient of determination (R^2) was 0.9993.

In order to determine whether the ELISA could detect WGA in human plasma samples, the baseline sample from subject WG-05 was spiked with known concentrations of WGA. Ten percent of plasma was replaced by PBS only (blank), or 10, 25, and 50 ng/ml WGA in PBS; the resulting curve is presented in Figure 3. In general, the absorbance values of all samples occurred at approximately the same value as the blank.

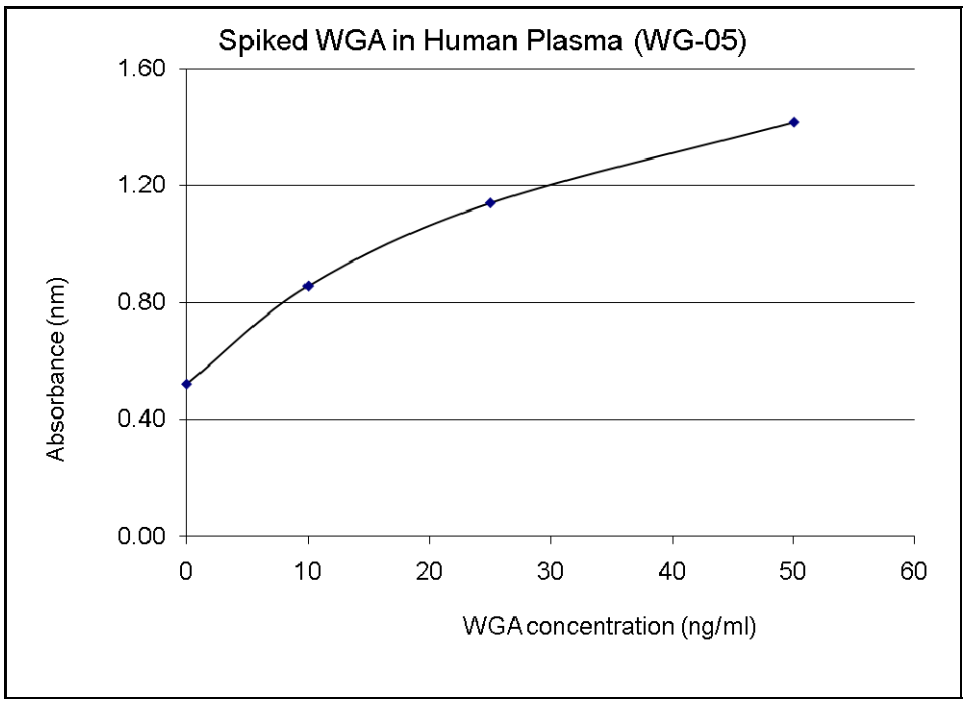

Figure 3. Baseline sample (subject WGA-05) spiked with known concentrations of WGA to show theoretical detection of WGA in human plasma.

CHAPTER V

DISCUSSION & RECOMMENDATIONS

With the exception of one subject, each person consumed 50 g of wheat germ which equates to an effective dose of 15 mg WGA, assuming 0.30 mg WGA per g raw wheat germ (Vincenzi, et al., 2002). Rather than scale dosage to body weight, all subjects were given the same portion (6.6 g fiber) because recommendations for dietary fiber are not based on caloric intake, but rather a combined total of 28 g of fiber per day is encouraged (United States Department of Health and Human Services, et al., 2005). Still well below the recommended daily intake for fiber, the dose was intended to represent typical exposure to WGA one might receive when consuming a Western diet, while remaining realistic about what subjects could eat in one sitting.

According to the ELISA protocol utilized in this study (Gull, et al., 2007), WGA was not detected in any of the plasma samples from the 14 subjects fed wheat germ. Explanation of these results includes the possibility that WGA does not bypass the gut barrier intact and therefore was not identified in plasma samples. Conversely, if WGA did in fact enter circulation, non-detection could be a consequence of one of several methodological details. First and foremost, lectins were originally defined upon their ability to agglutinate red blood cells and hence have a high affinity for the carbohydrate

moieties expressed on cell surfaces. Because only the plasma fraction was retained in this study, there is no way to determine whether WGA, if present in circulation, was bound and discarded with the erythrocyte portion. Additionally, WGA binds platelets (Freed, 2002) and white blood cells, all of which are separated from plasma upon centrifugation.

Caco 2 studies have shown that direct application of WGA to the apical membrane resulted in 0.1 % transport of intact lectin across the epithelial layer (Dalla Pellegrina, et al., 2005); therefore if even half of the ingested WGA bound enterocytes in vivo, 7.5 µg WGA could potentially have been transported into circulation. Furthermore, using the kinetics of the human PNA study as a rough guide, 22 mg PNA was ingested and a maximum 5.0 µg/ml PNA was detected in serum samples after 1 hr (Wang, et al., 1998), or 63 % of that consumed. If WGA is absorbed similarly, one might expect to find a maximum of 3.4 µg/ml WGA in plasma samples following consumption of 50 g wheat germ (15 mg WGA). However because PNA and WGA possess differing sugar-binding preferences, and because WGA binds a wider variety of cells in the body, it is quite possible that the dose did not saturate all potential sites and therefore was not detected in plasma samples. Indeed in previous studies (Pusztai, et al., 1993), detrimental effects to the growth and health of rats were observed after daily consumption of 7 g/kg purified WGA for 10 days. To reach these concentrations of WGA by consuming wheat germ, a 70 kg human would have to eat over 1600 kg each day.

Obviously, the present study introduced WGA to the system at much lower doses; however, because WGA is most likely treated as an oral antigen, the way in which it is

processed by the immune system depends largely on this variable. By analyzing plasma only, the immune response evoked by WGA due to the ingestion of wheat germ is unclear and likely varied among individuals. One of two potential responses could have occurred: control of the antigen at the gut level through innate immunity (IgA secretion) or engaging peripheral organs in the acquired immune pathway (helper-T cells). In response to oral antigen at any dose, it appears dendritic cells (DCs) are the key antigen-presenting cells (APCs) in the gut and the pathway by which antigen is processed and presented to naïve T cells determines the action the immune system will take when dealing with the antigen (Alpan, 2001).

In the typical Western diet, WGA is introduced to the immune system chronically and at low doses. Dendritic cells in the gut-associated lymphoid tissue (GALT) are unique from those found elsewhere in the body and are one of the first immune cells to come into contact with oral antigens. These cells are strategically located within Peyer's patches, interepithelial areas, and mesenteric lymph nodes (Alpan, 2001), and densely throughout the lamina propria (the sub-epithelial compartment), making them ideally positioned to bind any material transported through or between epithelial cells (Mowat, 2003). In addition to M cells, it appears DCs themselves play an important role in sampling the contents of the luminal environment. Indeed, DCs located in the villus mucosa have been observed to migrate to the intact epithelium to sample contents of the gut directly via extension of cellular processes into the lumen, then promptly retracting to the lamina propria with the bound antigen (Mowat, 2003; Strober, 2006).

Immature DCs are highly mobile and upon loading with antigen, they travel to the mesenteric lymph nodes (MLN), which drain the GALT (Nagler-Anderson, 2000). Herein lies the crucial step dividing innate from acquired immunity: the MLN is the crossroads between the peripheral and mucosal recirculation, and although the details of the mechanism have yet to be completely elucidated, it appears that APC presentation to naïve T-cells occurs here and the severity of the immune response is subsequently determined (Mowat, 2003). Within the MLN, T-cells can mature into one of two types depending on antigen presentation by DCs. Partially mature DCs stimulate gut-homing, IgA-promoting T-cells; those that restore homeostasis by binding antigen locally and dealing with antigen at the level of the GALT. It is believed that antigen-binding to Toll-like receptors (TLRs) on the surface of macrophages and epithelial cells results in local inflammation and stimulus for DCs to undergo full maturation (Mowat, 2003). Mature DCs provoke a full-blown immune response by inducing a subset of helper-T cells to produce IFN-γ and perpetuate inflammation. These helper-T cells migrate from the MLN to distal lymphoid organs through the thoracic duct—the largest lymph vessel in the body—which subsequently drains to systemic circulation at the left subclavian vein (Mowat, 2003).

Because WGA does not bind TLRs, which recognize conserved molecular patterns that uniquely characterize microbes (Nagler-Anderson, 2000), an acute inflammation co-stimulus did not exist to prompt full maturation of DCs. Therefore, in the present study, any WGA in the GALT or epithelial cells may have been dealt with at the local level via IgA—the major mucosal immunoglobulin that disposes of antigen without stimulating the complement cascade (Wittig & Zeitz, 2003). Conversely, chronic

low-dose WGA in healthy subjects could have provided ample stimulus to cause WGA-specific T-cell deletion (anergy), upon which T cells no longer respond to antigen and oral tolerance is achieved (Alpan, 2001). Without characterization of inflammatory factors in circulation, it is difficult to speculate which mechanism likely occurred.

As a result of its central position within the gut immune system, perhaps the MLN is the gateway for dietary antigen as well; it is conceivable that WGA, transferred through the GALT to the MLN, could migrate with MLN effluent into systemic circulation. Additionally, WGA might gain direct access to the bloodstream from the gut thereby draining to peripheral lymph tissues or to the liver through the portal vein. Or, even more ambiguous, intestinal epithelial cell-derived lipid vesicles called 'tolerosomes' have been detected in the bloodstream of antigen-fed rats and are believed to play a role in dissemination of immunological information (Mowat, 2003).

Assuming WGA entered circulation through one, or several, of the proposed pathways, it is likely that the WGA was bound to other cells with exposed N-acetyl-glucosamine binding sites. Of the plasma samples retained, albumin comprises approximately 60 % of the protein and is not glycosylated; hence if WGA were detected, it would have been bound to alpha and beta-globulins, coagulation proteins, and immunoglobulins, which occur in varying concentrations depending on the individual. Table 2 presents the ten most abundant plasma proteins and their concentration in a typical healthy human.

Table 2. Human plasma proteins and their carbohydrate content. Ranked in descending order according to average concentration. (From Putnam, 1984).

Protein	Amount in Normal Plasma (mg/100 ml)	Carbohydrate Content (%)	WGA binding
Albumin	3,500 - 5,000	0	None*
Immunoglobulin G (IgG)	800 - 1,800	3	N/A
Haptoglobins	380 - 780	16.4	N/A
Fibrinogen	200 - 450	4	N/A
a-Globulin a_1-Antitrypsin	200 - 400	13	N/A
a-Globulin a_2-Macroglobulin	150 - 420	9.4	N/A
Immunoglobulin A (IgA)	90 - 450	8	N/A
B-Globulin Transferrin	200 - 320	5.9	N/A
Immunoglobulin M (IgM)	60 - 250	10	N/A
a-Globulin a_1-Acid glycoprotein	55 - 140	42	N/A

*(Ketis, Girdlestone, & Grant, 1980)

Paralleling this concept, and irrespective of immune system stimulation, naturally occurring highly specific, anti-WGA antibodies have been isolated from human sera (Tchernychev & Wilchek, 1996). Perhaps, any WGA in circulation was rapidly bound by these antibodies, preventing WGA-binding to any other proteins. The antibodies, however, did not appear to interfere with the agglutination properties of the lectin (Tchernychev & Wilchek, 1996) suggesting the formation of lectin-antibody complexes while lectin binding sites remain exposed and retain their ability to bind erythrocytes. It is possible to envision a scenario in which anti-WGA antibodies quickly bound any free lectin, either preventing widespread circulation or allowing agglutination of blood cells, and the resulting complexes were subsequently discarded along with the erythrocyte portion of the sample.

Based upon the variables previously mentioned, it is not altogether surprising that WGA was not detected in the plasma samples at concentrations within the range of ELISA sensitivity. The method was created to detect WGA in the 10-1000 ng/ml concentration range; it is possible that any WGA, bound or free, circulated at concentrations below 10 ng and was interpreted as 'noise'. Indeed, the lab technician reported 'considerable signal' even without added WGA when creating the spiked WGA curve (Figure 3) (Ratzinger, 2009). The ELISA itself utilizes glycans from porcine gastric mucin (PGM) with a carbohydrate composition different from that of humans. However, because the method was able to detect WGA in a spiked baseline sample from a human subject, one can conclude that WGA, if present in significant concentrations, would have been detected. Assuming an average 5.0-liter human blood volume (2.8 L plasma) only 0.028 mg, or 0.18 % of the 15 mg WGA ingested, would have been required to enter circulation in order to reach the lower detection limit of the ELISA's sensitivity.

Strengths and Limitations

The greatest strength of the present study was its novel nature. The research group chose to undertake a previously un-attempted protocol in order to characterize the behavior of dietary WGA in human subjects. Additionally, biochemical analysis was completed by unbiased members outside the research group and outcome variables were objective in nature. Approved laboratory techniques were utilized at all times to ensure accurate and consistent collection of blood samples.

The project was limited by a small subject set and by details of the study design. In hindsight, it would have been preferable to determine the presence of WGA in whole-blood samples. However, a reliable method to detect WGA in whole blood does not currently exist; therefore the group chose to initiate research in this area by utilizing an established method of detection in plasma.

Summary and Recommendations for Future Studies

This study represents the first of its kind aimed at determining whether WGA breeches the gut barrier intact and can be recovered from systemic circulation in humans. According to the protocol utilized, ingestion of a 50 g dose of raw wheat germ did not result in detection of WGA in plasma samples. However, these data are an important first step and provide a template for future studies.

Primarily, the preservation and analysis of whole blood samples should be a goal of subsequent research on this topic. Additionally, a larger dose of wheat germ could be administered because 50 g was easily tolerated by most subjects and perhaps did not provide enough WGA stimulus to saturate all potential binding sites leading to a 'spill over' into the plasma portion, as seemed to be the case in animal models (Pusztai, et al., 1993). Additionally, a larger dose might have provoked increases in IgA or helper T-cells; the measurement of which could help elucidate the immune system's recognition and response to WGA. Tracking anti-WGA antibodies and probing for antibody-WGA complexes would help clarify mechanisms as well.

In light of recent research connecting gliadin and the zonulin system to increased gut permeability (Drago, et al., 2006), perhaps introducing the combination of gliadin and WGA into the gut at the same time would result in detection of the lectin in circulation. It would be interesting to repeat the study using germ flour, which consists of the milled endosperm and germ components of the wheat kernel.

Overall, there has been a recent resurgence of interest in the field of lectinology, specifically in terms of application of WGA to biotechnology. Significant understanding of the kinetics and binding behavior of WGA has been gleaned from in vitro studies, however it remains crucial to describe the complex, immune-system mediated human-lectin interaction in vivo before WGA-modified tools can be utilized in a safe and dependable manner.

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APPENDIX A

IRB NOTICE OF APPROVAL FOR HUMAN RESEARCH

Colorado State University

Research Integrity & Compliance Review Office
Office of Vice President for Research
Fort Collins, CO 80523-2011
(970) 491-1553
FAX: (970) 491-2293

Notice of Approval for Human Research

Principal Investigator: Loren Cordain, HES, 1582
Co-Principal Investigator: Matt Hickey, HES, 1582
Title: Bioavailability of Dietary Lectins
Protocol #: 06-031H **Funding Source:** n/a

Number of Participants/Records: Remaining 25 participants
Board Action: **Approval Date:** April 19, 2008 **Expires:** April 18, 2009

IRB Administrator: Janell Barker Janell L Barker

Consent Process:
The above-referenced project was approved by the Institutional Review Board with the condition that the attached consent form is signed by the subjects and each subject is given a copy of the form. *NO changes may be made to this document without first obtaining the approval of the IRB.*

Investigator Responsibilities:

- It is the PI's responsibility to obtain this consent form from all subjects.
- It is the responsibility of the PI to immediately inform the IRB of any serious complications, unexpected risks, or injuries resulting from this research.
- It is also the PI's responsibility to notify the IRB of any changes in experimental design, participant population, consent procedures or documents. This can be done with a memo describing the changes and submitting any altered documents.
- Students serving as Co-Principal Investigators must obtain PI approval for any changes prior to submitting the proposed changes to the IRB for review and approval.
- The PI is ultimately responsible for the conduct of the project.
- A status report of this project will be required within a 12-month period from the date of review. Renewal is the PI's responsibility, but as a courtesy, a reminder will be sent approximately two months before the protocol expires. The PI will be asked to report on the numbers of subjects who have participated this year and project-to-date, problems encountered, and provide a verifying copy of the consent form or cover letter used. The necessary continuation form (H-101) is available from the RICRO web page http://ricro.research.colostate.edu.
- Upon completion of the project, an H-101 should be submitted as a close-out report.
- If approval did not accompany a proposal when it was submitted to a sponsor, it is the PI's responsibility to provide the sponsor with the approval notice.
- **Should the protocol not be renewed before expiration, all activities must cease until the protocol has been re-reviewed.**

This approval is issued under Colorado State University's OHRP Federal Wide Assurance 00000647.

Please direct any questions about the IRB's action on this project to me for routing to the IRB.

Attachment Date of Correspondence: 4/29/08

APPENDIX B

INFORMED CONSENT

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY: Bioavailability of dietary lectins

PRINCIPAL INVESTIGATOR: Loren Cordain, Ph.D. 491-7436

CO-PRINCIPAL INVESTIGATOR: Matt Hickey, Ph.D. 491-5727
Dave Sampson, Ph.D. 491-5234

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH? You are a healthy adult who has no history of gastrointestinal disease or any current food allergies (particularly to wheat).

WHO IS DOING THE STUDY? This study is being conducted by Professors Cordain, Hickey, and Sampson. These faculty members have appointments in Health & Exercise Science and in Food Science and Human Nutrition. They will be assisted by a team of graduate students.

WHAT IS THE PURPOSE OF THIS STUDY? The study is meant to find out if a part of your diet can be absorbed from your gut without being broken down. In general, what we eat is broken down in our gut, and we absorb the fragments of that breakdown (for instance, starch is broken down into individual sugars, table sugar is broken down into it's 2 component sugars, dietary proteins are broken down into the individual parts (called amino acids), and dietary fat is broken down into it's parts (called fatty acids). The focus of this study is dietary lectins. Lectins are large proteins that are found in a number of plant-based foods (for instance, wheat germ, wheat, peas, soybeans, & kidney beans all have lectins). These lectins have the ability to bind to cells in your mouth, stomach, intestines, and colon. Because most of the world consumes a variety of lectins on a daily basis, it is important to determine if lectins readily pass through the gut to the blood. Future recommendations for intake of lectin-containing foods may be developed on the basis of studies like this one.

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST? The study will be conducted at the Human Performance Clinical Research Laboratory (HPCRL) on the CSU campus. Your total time commitment will be ~ 16-20 hours.

WHAT WILL I BE ASKED TO DO? You will be asked to come to the HPCRL as follows:

Visit 1: Preliminary screening: you will complete a health history questionnaire. This will take ~ 15-20 minutes.

Visit 2 – 5: On study days, you will be asked to report to the HPCRL after an overnight fast. On each of the 4 study days, a catheter will be placed in a vein in your forearm. You will be asked to consume either no wheat germ (a "control" day), or one of three different amounts of wheat germ (ranging from ~ 1-3 tablespoons). Blood samples will be obtained prior to the start of the test, and at 30, 60, 90, 120, 180, and 240 minutes, and at 24 hours after eating the wheat germ.

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?
If you have a wheat allergy, a history or diagnosis of a gastrointestinal disease (such as celiac or gluten enteropathy), or are unable to commit to staying in the lab for 4 hour blocks of time, you should not participate in this study.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

- Catheter placement and blood sampling: There is a small risk of infection and/or bruising at the site of catheter placement, and some people do have a reaction in which they get dizzy or even faint. The catheters will be placed by trained study staff using sterile techniques. In addition, you will be asked to lie down while the catheter is being placed, and will be seated in a comfortable chair or bed for the duration of the test.
- There is a small chance that the ingestion of the wheat germ may cause some stomach discomfort. However, the amount of wheat germ you will be asked to eat is small, and, unless you have a wheat allergy, the likelihood of a problem is small.
- Dietary lectins have been reported to be toxic when administered to rodents (at doses far higher (~10x times higher) than used in this study). We are proposing to study small amounts of wheat germ intake (the largest amount is only ~ 3 tablespoons). There is no evidence that a small amount of wheat germ will be toxic in humans. In fact, studies in humans have been conducted with doses of ~3 tablespoons every day for an entire year with no reports of side effects.
- It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

WILL I BENEFIT FROM TAKING PART IN THIS STUDY? There is no direct benefit to you for participating in this study. We hope to obtain information about the degree to which specific components of a normal diet may enter your blood without being digested. This information may be useful in modifying dietary recommendations.

DO I HAVE TO TAKE PART IN THE STUDY? You do NOT have to participate in this study. Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.

WHAT WILL IT COST ME TO PARTICIPATE? There is no cost to your for participation in this study, beyond the time-commitment stated above.

WHO WILL SEE THE INFORMATION THAT I GIVE?
We will keep private all research records that identify you, to the extent allowed by law. Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep you name and other identifying information private. We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key.

You should know, however, that there are some circumstances in which we may have to show your information to other people. For example, the Human research Committee at CSU has the right to audit research records.

CAN MY TAKING PART IN THE STUDY END EARLY? Yes, if you miss more than 2 scheduled appointments, the research staff reserves the right to remove you from the study. In addition, if your reaction to either the catheter or the wheat germ suggests that it is in your best interests NOT to continue, the research staff will explain this to you, and you will not continue with the study.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? No, there is no compensation for this study.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

RETENTION OF BLOOD SAMPLES

You should understand that we would like to keep any extra blood samples that are not used in the analysis for this study. In other words, if we have any "extra" blood we will keep it in a freezer in our lab. It is very possible that we will use all of the blood obtained in this study and will have none left, but in the event that we do, we would like your permission to keep the samples in the event that they can be used for further research. We will use these samples in the future solely for additional research on dietary lectins; specifically, all future research will simply be an extension of what we hope to accomplish with the current study. We may simply analyze your blood for the presence of other hormones or metabolites. We have NO plans to store or analyze DNA in this study. Your stored samples will be coded in such a way that your confidentiality will be maintained. Only the Principal Investigator (Dr. Cordain) will have access to the coding system for your samples. There is a possibility that your samples may be shipped to other departments on the CSU campus, or to colleagues at other Universities for assistance with analysis. Under such circumstances, the same coding system will be used, so researchers in other labs will not be able to identify you. We do not anticipate ANY commercial product development from your samples; they will be used solely for research purposes. You should be advised that we do NOT have plans to recontact you in the future regarding any additional analyses, but will seek full approval of the CSU Regulatory Compliance Office prior to initiating any further research on your samples. By checking "Yes" below and signing on the accompanying line, you are agreeing to allow the investigators retain any blood samples obtained during this study. If you do not wish the investigators to retain any samples, please check the box marked "No" and also sign on the accompanying line.

The investigators may keep any blood samples obtained during the course of this study for future research on obesity and metabolism YES ☐ NO ☐

_____

Signature Date

WHAT IF I HAVE QUESTIONS?
Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the investigator, Loren Cordain at 491-7426. If you have any questions about your rights as a volunteer in this research, contact Janell Meldrem, Human Research Administrator at 970-491-1655. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 4 pages.

_____ _____
Signature of person agreeing to take part in the study Date

_____
Printed name of person agreeing to take part in the study

_____ _____
Name of person providing information to participant Date

_____
Signature of Research Staff

PARENTAL SIGNATURE FOR MINOR

As parent or guardian I authorize _____ (print name) to become a participant for the described research. The nature and general purpose of the project have been satisfactorily explained to me by _____ and I am satisfied that proper precautions will be observed.

_____
Minor's date of birth

_____
Parent/Guardian name (printed)

_____ _____
Parent/Guardian signature Date

APPENDIX C

HEALTH HISTORY QUESTIONNAIRE

HUMAN PERFORMANCE CLINICAL/RESEARCH LABORATORY
COLORADO STATE UNIVERSITY
CONFIDENTIAL HEALTH HISTORY QUESTIONNAIRE

STUDY_____ **DATE**_____ **SUBJECT ID #**_____

Reviewed by (must be PI):_____

PLEASE PRINT

GENERAL MEDICAL HISTORY

Do you have any current medical conditions? YES ☐ NO ☐
If Yes, please explain:

Have you had any major illnesses in the past? YES ☐ NO ☐
If Yes, please explain:

Have you ever been hospitalized or had surgery? YES ☐ NO ☐
If Yes, please explain: (include date and type of surgery, if possible)

Have you ever had an EKG? YES ☐ NO ☐
If Yes, please explain:

Have you been diagnosed with diabetes? YES ☐ NO ☐
If Yes, please explain:

Age at diagnosis_____

Are you currently taking any medications, including aspirin, hormone replacement therapy, or other over-the-counter medications?
YES ☐ NO ☐ If Yes, please explain:

Medication	**Reason**	**Times taken per Day**	**Taken for how long?**

SPECIFIC MEDICATIONS

	YES	NO
Aspirin (chronically)	☐	☐
Ibuprofen	☐	☐
Acetominophen	☐	☐
Steroids (costisone, etc.)	☐	☐
Other anti-inflammatories	☐	☐

FAMILY HISTORY

	Age (if alive)	Age of Death	Cause of Death
Father	_____	_____	_____
Mother	_____	_____	_____
Brothers/Sisters	_____	_____	_____
	_____	_____	_____
	_____	_____	_____
	_____	_____	_____

Do you have a family history of any of the following: (Blood relatives only, please give age at diagnosis if possible)

	YES	NO	Relation	Age at Diagnosis
a. High blood pressure	☐	☐	_____	
b. Heart Attack	☐	☐	_____	
c. Coronary bypass surgery	☐	☐	_____	
d. Stroke	☐	☐	_____	
e. Diabetes	☐	☐	_____	
f. Obesity	☐	☐	_____	

TOBACCO HISTORY (check one)

None ☐
Quit ☐ (when)_____
Cigarette ☐

Cigar ☐
Pipe ☐
Chew Tobacco ☐

Snuff ☐

CURRENT TOBACCO USE
(if applicable)

	# per day
Cigarette	_____
Cigar	_____
Pipe	_____
Chew Tobacco	_____
Snuff	_____

Total years of tobacco use_____

CARDIORESPIRATORY HISTORY

If you checked YES to any of these, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

	YES	NO
Are you presently diagnosed with heart disease?	☐	☐
Do you have any history of heart disease?	☐	☐
Do you have a heart murmur?	☐	☐
Occasional chest pain or pressure?	☐	☐
Chest pain or pressure on exertion?	☐	☐
Episodes of fainting?	☐	☐
Daily coughing?	☐	☐
High blood pressure?	☐	☐
Shortness of breath?		
At rest?	☐	☐
lying down?	☐	☐
After 2 flights of stairs?	☐	☐
Do you have asthma?	☐	☐
Do you have a history of bleeding disorders?	☐	☐
Do you have a history of problems with blood clotting?	☐	☐

MUSCULOSKELETAL HISTORY	**YES**	**NO**
Any current muscle injury or illness?	☐	☐
Any muscle injuries in the past?	☐	☐
Do you experience muscle pain at rest?	☐	☐
Do you experience muscle pain on exertion?	☐	☐
Any current bone or joint (including spinal) injuries?	☐	☐
Any previous bone or joint (including spinal) injuries?	☐	☐
Do you ever experience painful joints?	☐	☐
Do you ever experience swollen joints?	☐	☐
Do you ever experience edema (fluid build up)?	☐	☐

Do you have pain in your legs when you walk? ☐ ☐

GASTROINTESTINAL SURVEY

Do you have any know food allergies? YES ☐ NO ☐
If Yes, please explain:

Do you have any current gastrointestinal disease? YES ☐
NO ☐ If Yes, please explain:

NUTRITIONAL SURVEY

How many times do you usually eat per day? _____

What time of day do you eat your largest meal? _____

How many times per week do you eat out ? _____

Are you taking any diet supplements? **YES** ☐ **NO** ☐. **IF YES, PLEASE PROVIDE DETAILS:**

How many times per week do you normally eat the following:

Ground beef_ Sausage_____ Bacon_____ Beef_____

Pork_____ Cheese_____ Fish_____ Poultry_____

Shellfish___ Fried Foods_ Breads_____
Cereals_____

Fruits_____ Vegetables__ Eggs_____ Desserts_____

Other_____ (describe)

How many servings per week of the following do normally consume:

Whole milk__ 2% Milk_____ Skim milk___ Buttermilk__

Coffee_____ Tea_____ Soft-Drinks_ Beer_____

Wine_____ Liquor_____ Water_____

Have you ever dieted? YES ☐ NO ☐

If YES, have you dieted within the past 12 months or are you currently on a diet?

YES ☐ NO ☐

If YES, please describe the diet:

a). Name (if applicable):_____

b). Prescribed by a Physician/nutritionist?YES ☐ NO ☐

c). Have you lost weight? YES ☐ NO ☐

d). Duration of diet_____

What was your weight 12 months ago? _____

What is your current weight?_____

Have you dieted other than in the past 12 months?YES ☐ NO ☐

If YES, please answer the following:

a). How many times have you dieted?

b). How old were you?

c). Weight loss (amount)?

You may be asked to complete a more detailed diet survey if you are volunteering for a research study.

PHYSICAL ACTIVITY SURVEY

Compared to a year ago, how much regular physical activity do you get? (Check one)

Much less ☐
Somewhat less ☐
about the same ☐
somewhat more ☐
much more ☐

Have you been exercising regularly for the past three months?

YES ☐ NO ☐

If YES, what type of exercise do you regularly participate in? (check those that apply)

		Days per week	Minutes per session	**Intensity** (1=easy, 10=very hard)
Walking	☐	_____	_____	_____
Running	☐	_____	_____	_____
Cycling	☐	_____	_____	_____
Swimming	☐	_____	_____	_____
Aerobics	☐	_____	_____	_____
Weight Training	☐	_____	_____	_____
Martial Arts	☐	_____	_____	_____
Other (describe)		_____	_____	_____

APPENDIX D

THREE-DAY NUTRITIONAL ANALYSIS FORM

Bioavailability of Dietary Lectins
Human Performance Clinical/Research Laboratory

Three-Day Nutritional Analysis

Name:_____Physician:_____

DOB:_____ Age:_____ Gender:_____

Company/Clinic:_____

Address:_____

Phone: (h)_____ (w)_____ Fax:_____

Email:_____

Height:_____ Weight:_____

Health Concerns/Comments:_____

_____

_____

Food Allergies: _____
_____
_____
_____

See Following Page for Instructions

Instructions

1. Record food and beverage intake for three consecutive days. If possible, include two weekdays and one weekend day (i.e.- Thurs./Fri./Sat. or Sun./Mon./Tues.).

2. Please record *everything* you eat and drink *immediately* after meal. (Do not try to recall everything at the end of the day)

3. The more details you provide, the better able we are to assess your risk of cardiovascular disease.

    - Record portion size using units such as 1 cup, ½ cup, 1 oz., 3 tsp., 2 Tbsp.
    - Specify cooking preparations when appropriate (i.e.-baked, broiled, fried, fast).
    - Include nutritional label when appropriate (i.e. – international foods, "unique" foods).
    - It is OK to use name brands of fast food meals. (i.e. – McDonalds Big N' Tasty)
    - Do not forget added condiments (i.e. salt, sugar, pepper)

4. An example recording is below.

Example Day

Time	*Food Item*	*Portion Size*
6:30 AM	Frosted Mini Wheats (Medium size) Milk (Fat Free Lucerne) Orange Juice (pulpless, MinuteMaid)	1.5 cups 1 cup 1 small cup
10:00 AM	Poptart (untoasted, Blueberry) Grapes (red, seedless) Water bottle 12 oz.	1 package (=2) 1 cup 2 full
12:00 AM	Pepsi (diet) Sandwich – bread (Pepperdine Farm, french, Enriched) - cheese (Feta cheese) - mustard (brown) - ham (5% fat roasted) - lettuce (romaine) Apple Chocolate Chip Cookies (Tollhouse, chewy)	1 can 2 slices 2 ounces 1 Tbsp. 4 ounces 2 leaves 1 item 4 items
3:00 PM	Lays Potato Chips (Baked) Picante sauce (Mild) Water bottle 12 oz.	1 bag 2 ounces 3 full
6:30 PM	**Casserole – specific brand, include recipe** Milk (2% Lucerne)	2 cups *2 2 cups (large)

Day 1

Time	*Food Item*	*Portion Size*

Day 2

Time	*Food Item*	*Portion Size*

Day 3

Time	*Food Item*	*Portion Size*

APPENDIX E

CONTACT INFORMATION

Bioavailability of Dietary Lectins

Contact Information

The following contact information is provided in the unlikely event that you have any problems that develop after eating the wheat germ. You will be observed in the lab for 4 hours after eating, and we do have a follow-up visit the following day. Nonetheless, if any problems or concerns arise, we provide the contact information below. As noted in the consent form, we do not have the resources to pay for any medical services. However, we remind you that what you are being asked to eat are commonly consumed items – we do not anticipate any problems but believe we must provide this information "just in case".

1. Dr. Cordain: Office 491-7436
2. Dr. Hickey: Office 491-5727 Cell 217-3628
3. Hartshorn Health Services: 491-7121
4. Poudre Valley Hospital: 495-7100
5. Emergency: 911

APPENDIX F

RAW DATA

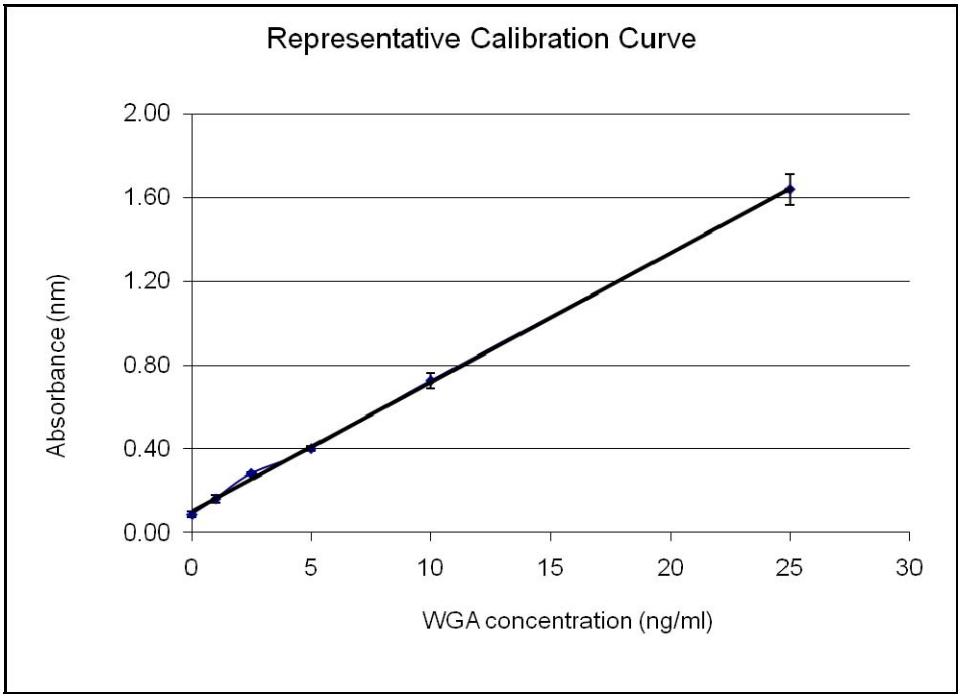
test person	t (min)	V1	V2	V3	mean	standard deviation	blank	mean-blank
1	0	0.1614	0.1511	0.1721	0.1615	0.0105		0.0169
	30	0.1483	0.1644	0.1479	0.1535	0.0094		0.0089
	60	0.1372	0.1482	0.1693	0.1516	0.0163		0.0070
	90	0.141	0.1342	0.148	0.1411	0.0069		-0.0035
	120		0.1308	0.1402	0.1355	0.0066		-0.0091
	240		0.1405	0.1382	0.1394	0.0016		-0.0053
	1440		0.1295	0.1168	0.1232	0.0090		-0.0215
2	0	0.1198	0.1314		0.1256	0.0082		-0.0190
	30	0.1325	0.1178	0.1178	0.1227	0.0085		-0.0219
	60	0.1294	0.1429	0.1271	0.1331	0.0085		-0.0115
	90	0.0994	0.1314		0.1154	0.0226		-0.0292
	120	0.1048	0.139	0.0993	0.1144	0.0215		-0.0302
	240	0.1158		0.1147	0.1153	0.0008		-0.0294
	1440	0.093		0.1098	0.1014	0.0119		-0.0432
3	0	0.1465	0.1802		0.1634	0.0238		0.0188
	30	0.1331	0.1124	0.099	0.1148	0.0172		-0.0298
	60	0.1466	0.1176	0.1526	0.1389	0.0187		-0.0057
	90	0.1085		0.0887	0.0986	0.0140		-0.0460
	120	0.1238	0.1011	0.0881	0.1043	0.0181		-0.0403
	240	0.1477	0.1213	0.1777	0.1489	0.0282		0.0043
	1440	0.1476	0.1414	0.0999	0.1296	0.0259	0.1446	-0.0150
4	0	0.1594	0.105	0.1019	0.1221	0.0323		0.0362
	30	0.0904	0.1013	0.0977	0.0965	0.0056		0.0106
	60	0.094	0.1047	0.1631	0.1206	0.0372		0.0347
	90	0.0887	0.1018	0.0973	0.0959	0.0067		0.0100
	120	0.1049	0.1033	0.0997	0.1026	0.0027		0.0167
	240	0.1049	0.1379	0.1278	0.1235	0.0169		0.0376
	1440	0.0963	0.1697	0.1477	0.1379	0.0377		0.0520
5	0	0.0649	0.0593	0.0626	0.0623	0.0028		-0.0236
	30	0.0485	0.0726	0.0659	0.0623	0.0124		-0.0236
	60	0.0581	0.105	0.0837	0.0823	0.0235		-0.0036
	90	0.0598	0.0531	0.0645	0.0591	0.0057		-0.0268
	120	0.0771	0.0982	0.0653	0.0802	0.0167		-0.0057
	240	0.1378	0.0778	0.0707	0.0954	0.0369		0.0095
	1440	0.1076	0.0813	0.0625	0.0838	0.0227		-0.0021
6	0	0.0822	0.0765	0.1198	0.0928	0.0235		0.0069
	30	0.0628	0.0564	0.0647	0.0613	0.0043		-0.0246
	60	0.1184	0.0576	0.1524	0.1095	0.0480		0.0236
	90	0.0643	0.0674	0.0684	0.0667	0.0021		-0.0192
	120	0.0648	0.054		0.0594	0.0076		-0.0265
	240	0.0554	0.0568	0.0988	0.0703	0.0247		-0.0156
	1440	0.0573	0.0562		0.0568	0.0008	0.0859	-0.0292

test person	t (min)	V1	V2	V3	mean	standard deviation	blank	mean-blank
7	0		0.2974	0.2446	0.2710	0.0373		0.1867
	30	0.1136	0.0904	0.0983	0.1008	0.0118		0.0165
	60	0.2252	0.1431	0.2142	0.1942	0.0446		0.1099
	90	0.1118	0.093	0.0926	0.0991	0.0110		0.0148
	120	0.1341	0.0762	0.0927	0.1010	0.0298		0.0167
	240		0.123	0.126	0.1245	0.0021		0.0402
	1440	0.1311	0.168	0.1128	0.1373	0.0281		0.0530
8	0	0.1506	0.1515	0.133	0.1450	0.0104		0.0607
	30	0.1182	0.1176	0.0845	0.1068	0.0193		0.0225
	60	0.1129	0.1188	0.0871	0.1063	0.0169		0.0220
	90	0.0698	0.1042	0.122	0.0987	0.0265		0.0144
	120	0.0869	0.0841	0.0824	0.0845	0.0023		0.0002
	240	0.0954	0.0591	0.0902	0.0816	0.0196		-0.0027
	1440		0.0736	0.0861	0.0799	0.0088		-0.0045
9	0	0.2804		0.1715	0.2260	0.0770		0.1417
	30		0.1276	0.0988	0.1132	0.0204		0.0289
	60	0.1896	0.1182	0.1018	0.1365	0.0467		0.0522
	90	0.0659	0.1077	0.1066	0.0934	0.0238		0.0091
	120	0.0725	0.0761	0.0935	0.0807	0.0112		-0.0036
	240	0.1189	0.1573	0.1228	0.1330	0.0211		0.0487
	1440	0.1332	0.1363	0.2177	0.1624	0.0479	0.0843	0.0781
10	0	0.1141	0.1364		0.1253	0.0158		0.0159
	30	0.1637	0.1115	0.1038	0.1263	0.0326		0.0169
	60	0.1122	0.113		0.1126	0.0006		0.0032
	90	0.0983	0.0917		0.0950	0.0047		-0.0144
	120	0.0898	0.0942	0.074	0.0860	0.0106		-0.0234
	240	0.1451		0.2123	0.1787	0.0475		0.0693
	1440	0.2848	0.3429		0.3139	0.0411		0.2045
11	0		0.1823	0.1925	0.1874	0.0072		0.0780
	30	0.1362	0.1244		0.1303	0.0083		0.0209
	60		0.0919	0.1297	0.1108	0.0267		0.0014
	90	0.0952	0.1004	0.0878	0.0945	0.0063		-0.0149
	120	0.0765	0.1132	0.0869	0.0922	0.0189		-0.0172
	240		0.2531	0.2366	0.2449	0.0117		0.1355
	1440	0.1489	0.2125		0.1807	0.0450		0.0713
12	0	0.1599	0.2383	0.164	0.1874	0.0441		0.0780
	30	0.0758	0.0829	0.1461	0.1016	0.0387		-0.0078
	60	0.1178	0.0876		0.1027	0.0214		-0.0067
	90	0.0826	0.1086	0.1161	0.1024	0.0176		-0.0070
	120	0.0872	0.107	0.1528	0.1157	0.0336		0.0063
	240		0.3728	0.3206	0.3467	0.0369		0.2373
	1440	0.1795		0.1396	0.1596	0.0282	0.1094	0.0502

test person	t (min)	V1	V2	V3	mean	standard deviation	blank	mean-blank
13	0	0.1095	0.1083	0.1015	0.1064	0.0043		0.0291
	30	0.0474	0.0572	0.0519	0.0522	0.0049		-0.0251
	60	0.0631	0.1021	0.093	0.0861	0.0204		0.0088
	90	0.0363	0.0429	0.0595	0.0462	0.0120		-0.0311
	120	0.0446	0.0573	0.043	0.0483	0.0078		-0.0290
	240		0.0717	0.0699	0.0708	0.0013		-0.0065
	1440	0.0394	0.0755	0.0492	0.0547	0.0187		-0.0226
14	0	0.1028	0.0807	0.0865	0.0900	0.0115		0.0127
	30	0.0586	0.0699	0.0664	0.0650	0.0058		-0.0123
	60	0.0969	0.0756	0.0652	0.0792	0.0162		0.0019
	90	0.0575	0.0696	0.0658	0.0643	0.0062		-0.0130
	120	0.0583	0.0655	0.0643	0.0627	0.0039		-0.0146
	240	0.0836		0.074	0.0788	0.0068		0.0015
	1440	0.0842	0.0838	0.139	0.1023	0.0318	0.0773	0.0250

A typical WGA/PBS calibration curve (determined for each plate)

ng/ml	V1	V2	V3	mean	standard deviation	mean-blank
25	1.5511	1.6905	1.6753	1.6390	0.0765	1.5531
10	0.721	0.7652	0.6924	0.7262	0.0367	0.6403
5	0.4041	0.3872	0.412	0.4011	0.0127	0.3152
2.5	0.2809	0.2785	0.2872	0.2822	0.0045	0.1963
1	0.1814	0.1484	0.157	0.1623	0.0171	0.0764
0	0.0898	0.0974	0.0705	0.0859	0.0139	0.0000



Plasma samples spiked with WGA

Test person 5, t=0:

10% of the plasma was replaced by PBS or WGA/PBS:

							Calculated WGA conc (ng/ml)	
Spike-WGA (ng/ml)	V1	V2	mean	mean-blank	standard deviation	standard deviation (%)	MW-LW	minus Spike-WGA
0	0.529	0.5127	0.5209	0.4296	0.0115	2.6832	32.9820	32.9820
10	0.9227	0.7885	0.8556	0.7643	0.0949	12.4158	60.6039	50.6039
25	1.1027	1.1749	1.1388	1.0475	0.0511	4.8738	83.9722	58.9722
50	1.4133	1.418	1.4157	1.3244	0.0033	0.2509	106.8164	56.8164