WHEAT BIOACTIVES AND ANTIOXIDANT ACTIVITY

By

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To my mother, Kathleen Johnson, and my grandparents, Patricia and Robert Johnson

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
AA	Ascorbic acid
BMI	Body mass index
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenedidaminetetraacetic acid
FITC	Fluorescein isothiocyanate
g	Gram
H_2O_2	Hydrogen peroxide
LPS	Lipopolysaccharide
mL	Milliliter
mM	Millimolar
NaCl	Sodium chloride
NSAIDS	Non-steroidal anti-inflammatory drugs
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI	Propridium iodide
RNase	Ribonuclease
TE	Trolox equivalents
TdT	Terminal deoxynucleotidyl transferase

TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
USDA	United States Department of Agriculture

Abstract of Thesis Presented to the Graduate School
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The objectives of this pilot study were (1) to show that consumption of wheat bran cereal by normal, healthy human subjects will provide antioxidant protection as measured by DNA strand breaks and (2) to determine if there is a dose response in cereal consumption. Following a baseline blood draw, 44 volunteers were randomized to consume either one serving (50g) or two servings of wheat bran cereal for 21 days at which time a second blood draw was performed. The antioxidant protection of the phenolic compounds was determined in peripheral blood mononuclear cells by measuring DNA strand breaks using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. If the antioxidants in the cereal were bioavailable, then the DNA will show fewer strand breaks in the form of a smaller percentage of apoptotic cells when the cells are cultured with H_2O_2. The results showed that there was no apparent protection of lymphocyte DNA from oxidative stress. Reasons for this may be poor bioavailability, however, the study was not designed to observe lack of bioavailability. Phenolic acids are bound to large amounts of fiber found in the cereal product. This binding could prevent absorption of the phenolic acids, keeping them from exerting their

antioxidant effects in the body. Other reasons may include variability in DNA strand breaks among subjects.

CHAPTER 1
INTRODUCTION

Background

Until recently, the phenolic compounds providing antioxidant activity in fruits and vegetables have received more attention than the phenolics in grains. This may be due to the underestimation of the amounts of phenolic compounds contained in grains. Most of the phenolic compounds found in grains are bound to the cell wall. The refining process of wheat removes the wheat bran, therefore removing the phenolic compounds found there. The emphasis on eating whole grains has now been increased in the USDA's Dietary Guidelines but Americans continue to over-consume refined grains and fall short on whole grain intake [1].

Specific Aims

Our proposed aim is to show that wheat bran cereal has important bioactive compounds that confer antioxidant protection to DNA in the form of reduced strand breaks. We will study two serving sizes of the cereal to determine if there is a dose response in the protection of DNA of the PMBCs.

Specifically,

- Our objective is to show that consumption of the wheat bioactives in the cereal in normal, healthy human subjects will provide that antioxidant protection to their DNA. The antioxidant protection will be determined by measuring DNA strand breaks using the TUNEL assay. If the antioxidants in the cereal are bioavailable, then the DNA will show fewer strand breaks when cells are cultured with H_2O_2.
- Another objective is to determine if a greater amount of cereal consumption will have a greater impact on protecting against DNA stand breaks if the cereal does indeed provide antioxidant protection.

Research on the bioavailability of the compounds in this cereal is limited. Positive results from this study could benefit consumers by knowing that their cereal is a source of antioxidants that can easily be consumed on a regular basis.

Hypothesis

Wheat bran phenolics have been shown to have positive effects on health. If there are bioavailable phenolic compounds in the wheat bran cereal, then they may provide antioxidant protection in the form of protection against oxidatively-induced DNA strand breaks.

CHAPTER 2
LITERATURE REVIEW

Whole Grains

The American Association of Cereal Chemists have defined a whole-grain as "the intact, ground, cracked, or flaked caryopsis, whose principal anatomical components, the starchy endosperm, germ, and bran, are present in substantially the same relative proportions as they exist in the intact caryopsis" [2]. This means that for products such as flour to be considered whole grain, the three major components of bran, germ, and endosperm must be present in the same amounts that occur in the grain's native state. The 2010 Dietary Guidelines for Americans have established that at least half of grain foods consumed daily should be whole grain [3]. Reasons for this include that the bran and germ fractions provide a majority of the biologically active compounds found in a grain, some of which are unique to grain products and are not found in other plant foods [4].

Disease Prevention

Epidemiological studies have shown associations between higher intakes of whole grain products and lower risk of chronic diseases such as heart disease [5, 6], diabetes [7-9], and cancer [5, 10-13]. Intakes of refined grains have not shown these associations. One cause of this may be that refined grains do not contain the bran and germ layers that are seen in whole grain products which are known to contain fiber, vitamins, minerals, and antioxidants [14]. One study [15] looked at the antioxidant content of grain products, fruits, and vegetables purchased at a local grocery store. Antioxidant content was determined using the DPPH reaction. They found that whole grain bread was 2000 Trolox equivalents (TE), compared to white bread at 1200 TE,

indicating the contribution of bran and germ to antioxidant activity. The researchers believe that whole grain products contain biologically active antioxidants that could act independently or synergistically and/or additively with the fiber found in the whole grain provide health benefits beyond basic nutrition to reduce the risk of diseases.

Bound Phytochemicals

Foods commonly known to have antioxidant activity are fruits and vegetables, with little attention given to grains. This same study [15] found that the average antioxidant activity of cereal products is equal to or exceeds most vegetables or fruits. A possible reason for these findings is that this study took into account the bound phytochemicals producing antioxidant activity found in grains as well as the free phytochemicals producing antioxidant activity. Studies before this did not measure bound phytochemicals and therefore it was reported that grains had lower antioxidant activity than fruits and vegetables. It has been found that in wheat, 90% of the antioxidants are bound [16]. It is believed that bound wheat phenolics associated with the cell walls may be resistant to upper gastrointestinal tract digestion and finally reach the colon, where colonic digestion by intestinal microflora may release the bulk of the bound phenolics [17]. These results show that while fruits and vegetables have been found to be an important source of antioxidants, whole grains should be given more attention for their potential antioxidant activity.

Antioxidants

Antioxidants are molecules that react with and destroy free radicals, preventing them from inflicting oxidative damage or mutation to vital components such as DNA or cell membranes [14, 18]. Free radical compounds result from normal metabolic activity as well as from the diet and environment [14]. Families of endogenous antioxidant

enzymes have evolved including superoxide dismutases for the elimination of the superoxide radical, and catalases and glutathione peroxidases for the elimination of hydrogen peroxide and organic peroxides [18]. One example of an endogenous antioxidant is glutathione. The most important antioxidant function of glutathione is to remove hydrogen peroxide and organic peroxides catalyzed by the selenium-dependent enzyme glutathione peroxidase, forming water or alcohol, respectively [19]. Endogenous antioxidant defenses can be supported by dietary antioxidants for maintaining health.

Disease Prevention

Several epidemiologic studies have shown that dietary antioxidants reduce the risk of many diseases and conditions including Parkinson's disease [20], Alzheimer's disease [21], myocardial infarctions [22], and coronary heart disease [23]. There are many individual compounds that contribute to the overall antioxidant activity of grains. They fall under the term phytochemical which is defined as a plant-based substance that may have a beneficial effect in the body but is non-essential. Phytochemicals can be classified as nitrogen-containing compounds, polyphenolics, terpenes, or organosulfur compounds as seen in Figure 1-1. Numerous studies [24-33] have found that ferulic acid (Figure 1-2) is the dominant phenolic acid in wheat accounting for 46-70% of the total phenolic acids in wheat on a per weight basis [24]. As mentioned before, most of the antioxidants in wheat are in the bound form and ferulic acid is no exception. One study [17] found that bound ferulic acid contributed more than 97% of total ferulic acid for all wheat varieties tested. This was in agreement with a later study [34] that found 95.8% of ferulic acid was found in the bound form and 4.2% in the free form in wheat bran.

Other phenolic acids found in wheat are syringic, caffeic, vanillic and *p*-coumaric acids [35]. It is thought that there are other antioxidants in wheat that may act synergistically and/or additively with the phenolic acids to produce a stronger antioxidant activity of the wheat. These antioxidants are known to be carotenoids such as lutein [17, 24, 25] and tocopherols [24].

Antioxidant Location in Wheat

Additional research [36] was done to further determine whether it was the bran, the germ, or the whole grain of wheat together that provided the most antioxidant activity. It was found that the polyphenolics in the bran/germ fraction was 15- to 18-fold higher when compared to the corresponding endosperm samples. They also looked at ferulic acid content and found that it was 50- to 70-fold higher in bran/germ fractions when compared to the endosperm fractions. This further shows that the processing of wheat is likely to be important when determining its antioxidant activity. Other research [25, 28, 35, 37, 38] found that the bran in particular had the most antioxidant activity than other products from the same species. They hypothesize this is due to the localization of the phenolic acids in the grain with the outer layers of the grain containing the greatest concentrations of total phenolic acids.

Fiber and Disease Prevention

Studies have shown that consuming foods such as whole grains that are high in fiber helps to reduce the risk of oral, pharyngeal, and esophageal cancers [39] and coronary heart disease [40]. Fiber is believed to play a significant role in the utilization of the phenolic acids found in wheat bran as most of the antioxidants are associated with dietary fiber constituting a “dietary fiber-phenolic acid” complex [37,41]. It is thought that the antioxidants in wheat bran can act independently or synergistically and/or additively

with fiber to reduce disease [15] but it is difficult to separate the potential effect of fiber from that of the other components of the wheat bran. This could explain data showing that it is much better for the health of the human body to consume the dietary fiber as part of whole fiber-rich foods with respect to the intake of only purified fiber, tablets, pills, and other medical preparations [37].

It is because of the high amounts of fiber in bran that scientists question the bioavailability of the phenolics bound to this fiber. As stated before, it is possible that there is a synergistic and/or additive relationship with bound antioxidants and fiber. Antioxidants that are covalently bound to cell wall fiber can be transported to the large intestine to be released by fermentation in the presence of microbiota [42]. In fact, one in vitro study [43] using a model gut system to examine the release of ferulic acid from wheat bran shows that, over 95% of the total release of ferulic acid groups takes place during fermentation in the colon. Enzymatic hydrolysis in the colon can free phenolics such as ferulic acid to release the free acid for absorption into the epithelium [42, 43]. This allows whole grain foods to provide antioxidant protection over a long time period through the entire digestive tract which provides unique protection that is not possible by any single component.

Numerous researchers [29, 37, 44] believe that it is the complex mixture of phytochemicals in foods that provides better protective health benefits than individual isolated components through the combination of additive and/or synergistic effects. Previous studies [45, 46] have even shown a detrimental effect on disease when using only the concentrated antioxidant to prevent disease. This further shows why for some

antioxidants, it may be more beneficial to consume the dietary antioxidant in the food it is naturally found in so it can exert the positive effects.

Food Processing and Antioxidant Activity

Although foods have been shown to have antioxidant activity, it is thought that the processing they go through to create foods such as breakfast cereals will reduce their antioxidant activity. According to one study [42], the reverse is true. Antioxidant activity increased gradually during cooking steps of breakfast cereal processing with the biggest increase coming from toasting. It appears that there may be no loss of natural antioxidants while there is a formation of new antioxidant activity, most likely Maillard reaction products. This is demonstrated by the comparison of crust antioxidant activity to that of crust-free white bread. The crust was about double in antioxidant activity compared to the crust-free part showing little change in antioxidants in the interior of the bread loaf during baking. Maillard reaction products may explain the increase in crust antioxidant activity.

Studies Measuring Bioavailability

Few studies have examined the influence of the food matrix on the bioavailability of phenolic acids such as ferulic acid. One study by Adam [47] investigated the bioavailability of ferulic acid in rats in a complex wheat bran cereal matrix. The rats were fed the experimental diet for 21 days. They found that the recovery of ferulic acid in urine was quite limited and they hypothesize that the cereal matrix appears to severely limit ferulic acid bioavailability.

Another study [34] on the influence of the cell wall linkage on the bioavailability of ferulic acid of an oral short-term intake of wheat bran mixed with a standard diet in rats was evaluated. There were three groups, the first received standard food, the second

received standard food and pure ferulic acid, and the third received standard food and bran. There was a fast appearance of ferulic acid in plasma after intake of pure ferulic acid. This could be explained by a fast absorbance of the compound in the jejunum or maybe in the stomach which was shown for other antioxidants found in grains. There was also an early appearance of ferulic acid in the bran group but only a small amount compared to the free ferulic acid group. This could be explained by the fact that only 4.2% of ferulic acid in bran is in the free form, the rest is bound. Over 24 hours, the amount of ferulic acid in the plasma remained constant in the bran group whereas it decreased to zero after only 4 hours in the free ferulic acid diet. The antioxidant activity was better after consumption of the bran than the pure ferulic acid. This result emphasizes that ferulic acid alone cannot fully explain the antioxidant activity of plasma. This further indicates how the components of wheat bran may act synergistically and/or additively to produce antioxidant activity and supplementation with wheat bran seems more efficient than a supplementation with pure ferulic acid.

Kern [48] investigated the bioavailability of ferulic acid from high-bran wheat and determine if some of the covalently bound forms of this phenolic were absorbed. The study used six human volunteers that underwent a two-day low polyphenol diet. Volunteers were asked to consume 100 grams of a commercial breakfast cereal and their blood was collected 1, 3, 6, 9, and 12 hours after the test meal. Volunteers were asked to collect urine for 24 hours on the day prior to the study day and throughout the study day. The results show a maximum absorption of ferulic acid between 1 and 3 hours after the test meal. This suggests that absorption of ferulic acid from the high-bran breakfast cereal occurs primarily in the small intestine. In this study, the low levels

of ferulic acid found in the plasma 6 hours after consumption of the cereal are indicative of little or no absorption from the large intestine. If ferulic acid from cereal is released into the colon, it is more likely to be further metabolized by the microflora or excreted via feces indicating that bound ferulic acid is either not absorbed or absorbed only in very small amounts.

A recent human study [49] looked at the effect of bioprocessing the bran in whole wheat bread on the bioavailability of phenolic acids, the postprandial plasma antioxidant capacity, and ex vivo anti-inflammatory properties. After consumption of a low phenolic diet for three days and an overnight fasting, eight healthy men consumed 300 grams of whole wheat bread containing native bran, the control, or bioprocessed bran, the treatment. The bran was bioprocessed by fermenting it with baker's yeast and enzymatically treating it with cell-wall degrading enzymes. This was a randomized, blind cross-over study. Their results showed the bioavailability of ferulic acid from the bioprocessed bread was about 3-fold higher than that of the control bread. They also found that the absorption of ferulic acid from the bioprocessed bread mainly took place in the small intestine although a large proportion of ferulic acid is known reach the colon bound to fiber. The researchers then looked to see if there were any immunomodulatory effects as a result of consuming the bioprocessed wheat bran. Blood samples were taken before bread ingestion and then at 1.25, 6, and 12 hours after bread ingestion. The blood was incubated with LPS from *Escherichia coli* in a final concentration of 1 µg/L for 24 hours at 37°C and 5% CO_2 to stimulate an inflammatory response. When looking at the pro-/anti-inflammatory cytokine ratios (IL-6:IL-10 and IL-1β:IL-10), the researchers found that compared with the control bread, the bioprocessed bread led to

a lower ratio in the ex vivo LPS-stimulated blood. Looking at these results, it can be concluded that the bioprocessing of bran increases the bioavailability of ferulic acid from whole wheat bran and it may have immunomodulatory effects. This type of study results could prove useful to optimize a food such as bread or cereal for the prevention of diet-related diseases as well as other chronic diseases that could be prevented by increased phenolic acid absorption.

To our knowledge, no study has been done to assess the effect of wheat bran cereal on oxidative stress in the human body. Wheat bran has high antioxidant activity in vitro so it is plausible that consuming wheat bran in the form of a ready-to-eat breakfast cereal may provide antioxidant protection for the cells of the body. Therefore, the purpose of the proposed study is to demonstrate whether wheat bran cereal supplemented in the diet will lead to increased antioxidant protection in healthy human subjects.

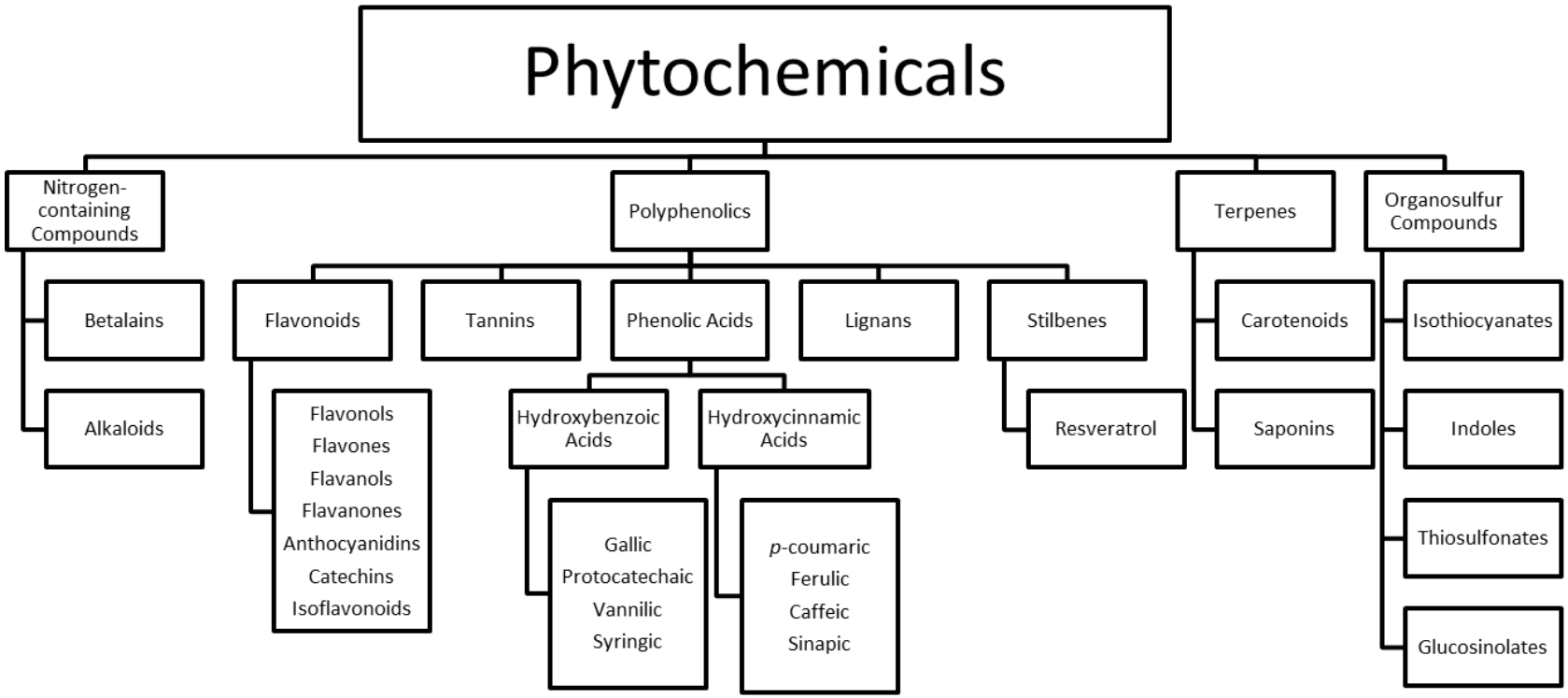

Figure 1-1. Breakdown of phytochemicals.

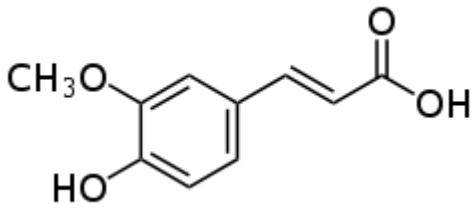

Figure 1-2. Chemical structure of ferulic acid.

CHAPTER 3
MATERIALS AND METHODS

Composition of Study Materials

Wheat bran cereal (Kellogg's All Bran, Battle Creek, MI) was the supplemental cereal used in this study. The Nutrition Label and ingredients can be found in Appendix A.

DPPH Assay

A stock solution of 2,2-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma, St. Louis, MO) was prepared in methanol (0.0049 g DPPH in 50 mL methanol at 0.25mM). A stock solution of ascorbic acid (Sigma, St. Louis, MO) was prepared in Milli-Q water (0.011 g ascorbic acid in 50 mL Milli-Q water). Tubes were labeled 1 through 6. Milli-Q water and ascorbic acid were added to corresponding tubes in the amounts shown in Table 3-1. Fresh-squeezed orange juice from Valencia oranges was centrifuged. The supernatant of the orange juice was collected and diluted in Milli-Q water (30 µL orange juice, 70 µL Milli-Q water) and placed in a separate tube. The wheat bran cereal was ground with a mortar and pestle. The ground cereal was weighed and 0.1 g was collected in a tube. Added to the cereal tube was 2 mL of methanol. The cereal tube was vortexed, sonicated, and centrifuged (1600 rpm, 3 minutes). The supernatant was removed and place in a separate tube. From each tube (tubes labeled 1-6, the orange juice, and the wheat tubes) 10 µL was transferred to a 96-well plate in triplicate. Next, 40 µL of DPPH was added to the wells and mixed with the same pipette tip (50 µL total volume). The plate was incubated for 20 minutes. Absorbance was read at 517 nm using a SPECTRAmax (Molecular Devices, Sunnyvale, CA) spectrophotometer.

Subject Description

Healthy adult males and females between the ages of 18 and 50 years were recruited to participate in a 21 day pilot study. Subjects were recruited via flyer (Appendix B), word of mouth, and a listserv announcement from the University of Florida campus, and the Gainesville, Florida community during June of 2010. The study was approved by the University of Florida's Institutional Review Board. Screening for the study occurred by telephone and/or personal interviews. Exclusion criteria (Appendix C) included the use of dietary supplements (not including multivitamins), strict vegetarian or vegan diet, use of antibiotics, chronic use of NSAIDS, ongoing illness or infection, females that are pregnant or planning to become pregnant, and BMI greater than 35. A study email (Appendix D) was sent to those who were eligible with the written informed consent (Appendix E) attached so they may read it and discuss it with family and physicians if needed. Written consent was obtained from each subject. Subjects were randomly placed into one of two treatment groups. Group A subjects were instructed to consume one bag (50 g) of cereal per day and group B subjects were instructed to consume two bags (100 g) of cereal per day. Subjects were told to return any uneaten bags of cereal at the end of the study.

Study Design

On day 1, the subjects were asked to come in to sign the informed consent and give a fasting baseline blood draw. They were then given their cereal and instructed to consume it corresponding with their group. Since the cereal is very fibrous, to prevent too much gastrointestinal distress, the subjects were told to eat the cereal throughout the day until their bodies adjusted.

On day 21, when the study participants arrived for their final blood draw, a questionnaire (Appendix F) was used to evaluate compliance. Compliance was also assessed by the returned cereal bag count. The exit questionnaire also included questions to determine if the subjects experience any side effects, changes in weight or changes in appetite that would be attributed to the cereal. The study design can be seen in Figure 3-1 and includes the start and end dates.

Blood Collection

Fasting blood was collected into a serum tube and a PBMC tube on day 1 (baseline) and day 21. Blood was collected into one 10 mL sodium heparin tube for PBMC separation, and one 10 mL SST^{TM} tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) for serum. Tubes for PMBC were maintained at room temperature and the serum tubes were placed at 4ºC. Both tubes were processed within one or two hours after collection under sterile conditions.

Serum Collection

Serum was removed from SST^{TM} tubes after centrifugation (2000 g, 10 minutes, 4ºC) and 400 µL was added to 3.6 mL RPMI-1640 (Cellgro, Mediatech, Herndon, VA) complete (100 U/mL Penicillin; 100 µg/mL Streptomycin; 0.25 µg/mL Fungizone; 50 µg/mL Gentamycin; 2 mM 1-glutamine; 25 mM HEPES) to prepare culture medium with 10% autologous serum. This was stored at 4ºC until use.

Blood Cell Separation

Whole blood was diluted and placed on a gradient to separate PBMC. The blood was diluted 1:1 with 0.9% NaCl. Diluted blood (6.5 mL) was layered over 3 mL of Lympholyte-H cell separation medium (Cedarlane Diagnostics, Burlington, Ontario), and

centrifuged (800 g, 20 minutes, 20^0C). Using a fine-tip sterile transfer pipette, the PBMC band was removed from the gradient tube, washed twice with RPMI-1640 complete by centrifugation (400 g, 10 minutes, 4^0C). Individual cell pellets were resuspended in 2 mL RPMI-1640 complete without serum and counted on a Z1-S Particle Counter (Beckman Coulter, Brea, CA) at 1:1000 dilution (10 µL cells + 10 mL Isoton). An example of the PBMC band can be seen in Figure 3-2.

Culture of PBMC for DNA Strand Breaks

On day 1, 2.0 x 10^6 cells/mL in RPMI with 10% autologous serum were plated into a 12-well non-treated plate. Added to each well was 5.5 µL of 30% hydrogen peroxide to achieve a final concentration of 25 mmol/L. The plate was incubated in a humidified 5% CO_2 atmosphere at 37ºC for 2 hours. After incubation, the cells were transferred to a 15 mL conical tube. Each well was washed with 5 mL PBS twice, with the cells being transferred to the 15 mL tube. A last wash with 1 mL PBS was done and added to the 15 mL conical tube. This tube was centrifuged (800 g, 12 minutes, 4ºC) and then aspirated being careful to avoid the cell pellet. The pellet was resuspended in 5 mL cold PBS and vortexed to ensure washing. The tube was again centrifuged (800 g, 12 minutes, 4ºC), aspirated, resuspended in 5 mL cold PBS, and vortexed. Another round of centrifuging (800 g, 12 minutes, 4ºC) and aspirating occurred followed by resuspending the pellet in 1mL cold PBS and vortexing. The cells were then fixed by adding 1 mL cold 2% paraformaldehyde to the 1 mL suspension. The tubes were incubated at 4ºC for 20 minutes. After the incubation, the tubes were centrifuged (800 g, 12 minutes, 4ºC), aspirated, resuspended in 5 mL cold PBS, and vortexed. This process was repeated two more times, with the second time using 1.5 mL cold PBS. After being

vortexed, the cell membranes were permeabilized by adding 3.5 mL ice-cold neat ethanol dropwise while vortexing each tube. The cell suspensions were stored at -20ºC.

The cells were processed using the ApoAlert™ DNA Fragmentation Assay Kit (BD Biosciences Clonetech, Palo Alto, CA) for flow cytometric analysis. This assay is based on the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay. Terminal deoxynucleotidyl transferase catalyzes the incorporation of fluorescein-dUTP at the free 3'-hydroxyl ends of fragmented DNA. The tubes were removed from the freezer and centrifuged (800 g, 12 minutes, 4ºC). The supernatant was aspirated, the cells were resuspended in 5 mL cold PBS, and then vortexed. The tubes were again centrifuged (800 g, 12 minutes, 4ºC), aspirated, resuspended in 1 mL cold PBS, and vortexed again. These cell suspensions were transferred to a 5 mL round-bottom polystyrene "snap cap" tube. The original 15 mL conical tube was washed with 1 mL PBS again and transferred to the snap cap tube. From this point forward the cells were protected from light. The cells were centrifuged (800 g, 12 minutes, 4ºC) aspirated, resuspended in 50 µL equilibration buffer, and vortexed. The cells were equilibrated at room temperature for 5 minutes. Next, the cells were centrifuged (800 g, 12 minutes, 4ºC), aspirated, resuspended with 26 µL TdT incubation buffer, and vortexed. The cells were incubated in a closed 37ºC water bath for 60 minutes with gentle tapping every 15 minutes to mix. After incubation, 0.5 mL of 20 mM EDTA was added to terminate the reaction and then vortexed. The cells were centrifuged (800 g, 12 minutes, 4ºC), aspirated, resuspended with 1 mL of 0.1% Triton X-100/BSA/PBS (5 mL PBS; 0.2% Triton X-100; 50 mg BSA), and vortexed. This was repeated first with the 1 mL of 0.1% Triton X-100/BSA/PBS, then with 0.25 mL propidium iodide/RNase/PBS

(2.5 µg/mL PI; 0.5 µg/mL DNase-free RNase). The tubes were incubated at room temperature for 30 minutes and then store in the dark at 4ºC for same day flow cytometry analysis.

Flow Cytometry

The fluorescein-labeled DNA was quantified by flow cytometry. This was done within 24 hours using the FACSort instrument located in the Flow Cytometry laboratory at the Interdisciplinary Center for Biotechnical Research. The populations were gated to omit debris and data was analyzed as a percent of apoptotic cells using FlowJo version 7.6.1 software. Apoptotic cells were determined by looking at the PI negative, fluorescein isothiocyanate positive (FITC+) quadrant.

Statistical Analysis

All statistics were performed using SigmaStat, version 11.0 Systat Software. A t-test was performed on the data to look at the difference in the percent of apoptotic cells and serving size. Noting no statistical difference in response between the two groups, all subjects were pooled together for further statistical analysis. An unpaired t-test was run to look at the difference in the percent of apoptotic cells from day 0 to day 21. When variances were unequal, the data was analyzed using a Mann-Whitney Rank Sum test.

Table 3-1. Volume of ascorbic acid, Milli-Q water, and DPPH added to each tube and final ascorbic acid concentration in mM.

Tube	Ascorbic Acid (AA)	Milli-Q Water	DPPH	AA mM Concentration
1	0 µL	100 µL	40 µL	0.000
2	10 µL	90 µL	40 µL	0.089
3	20 µL	80 µL	40 µL	0.178
4	30 µL	70 µL	40 µL	0.268
5	40 µL	60 µL	40 µL	0.357
6	50 µL	50 µL	40 µL	0.446

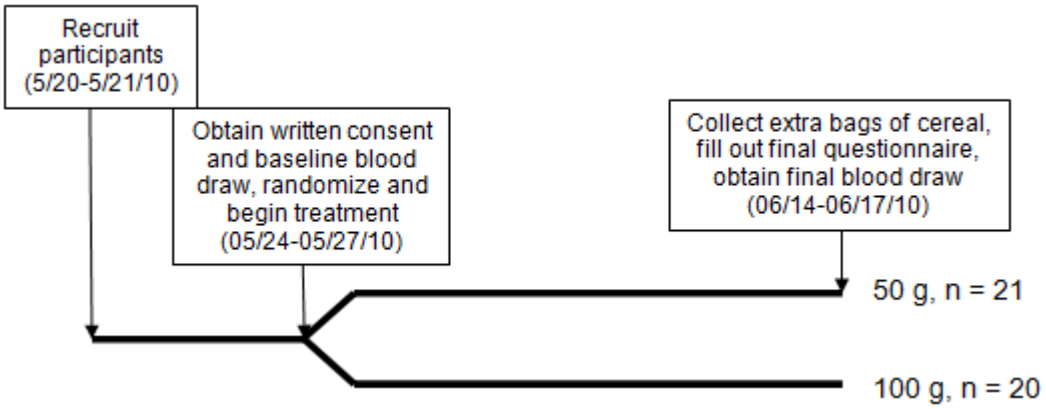

Figure 3-1. Study design.

CHAPTER 4
RESULTS

The DPPH radical scavenging activity of the fresh squeezed orange juice was calculated to be 113.66 mM ascorbic acid equivalents (AAE). The DPPH radical scavenging activity of the ground wheat bran was calculated to be 0.366 mM AAE.

Fifty subjects were recruited and assessed for eligibility. Six participants were excluded for not meeting the age and illness inclusion criteria. Of those 44 subjects (18 males, 26 females), 43 showed up for the first blood draw, their demographics are listed in Table 4-1. Forty-one of those 43 subjects came back for the final blood draw. One withdrew because of mild adverse effects such as gastrointestinal distress. One did not show up to the final blood draw and was withdrawn. This is shown in Figure 4-1.

The leftover bags were collected, counted, and compared with the total number of days missed eating the cereal as noted on the final questionnaire. A total of 41 participants consumed the wheat bran cereal 810 out of the 861 total days. This yields 94% compliance among the study participants.

When looking at the responses on the final questionnaire, the participants listed five different side effects they experienced that they attributed to eating the cereal. They were increased bowel movements, bloating, gas, stomach discomfort, and increased thirst. Increased bowel movements were listed by 19.5% of the subjects (4 people in group A, 4 people in group B). Bloating was listed by 12% of the subjects (4 people in group A and 1 person in group B). Gas was listed by 7% of the subjects (3 people in group A, none in group B). Stomach discomfort was listed 10% of the subjects (3 people in group A and 1 person in group B). Increased thirst was listed by 7% of the subjects. (3 people in group A, none in group B). When asked if they noticed any changes in their

satiety after consuming the cereal, 66% of the subjects (14 people in group A and 13 people in group B) said they felt full and ate less.

There was no significant difference observed when comparing the percent of apoptotic cells between group A consuming one serving of cereal and group B consuming two servings of cereal which is shown in Figure 4-2. After making this observation, both groups were pooled together to compare the percent of apoptotic cells in the blood draws for day 0 and day 21. Figure 4-3 shows that the percent of apoptotic cells seen in the baseline and day 21 blood draws were not significantly different ($p = 0.837$).

Table 4-1. Subject demographics.

	Total	1 Serving	2 Servings
Mean Age (years)	26.4	23	26.3
Age Range (years)	18-43	21-43	18-41
Males	18	9	9
Females	26	13	13

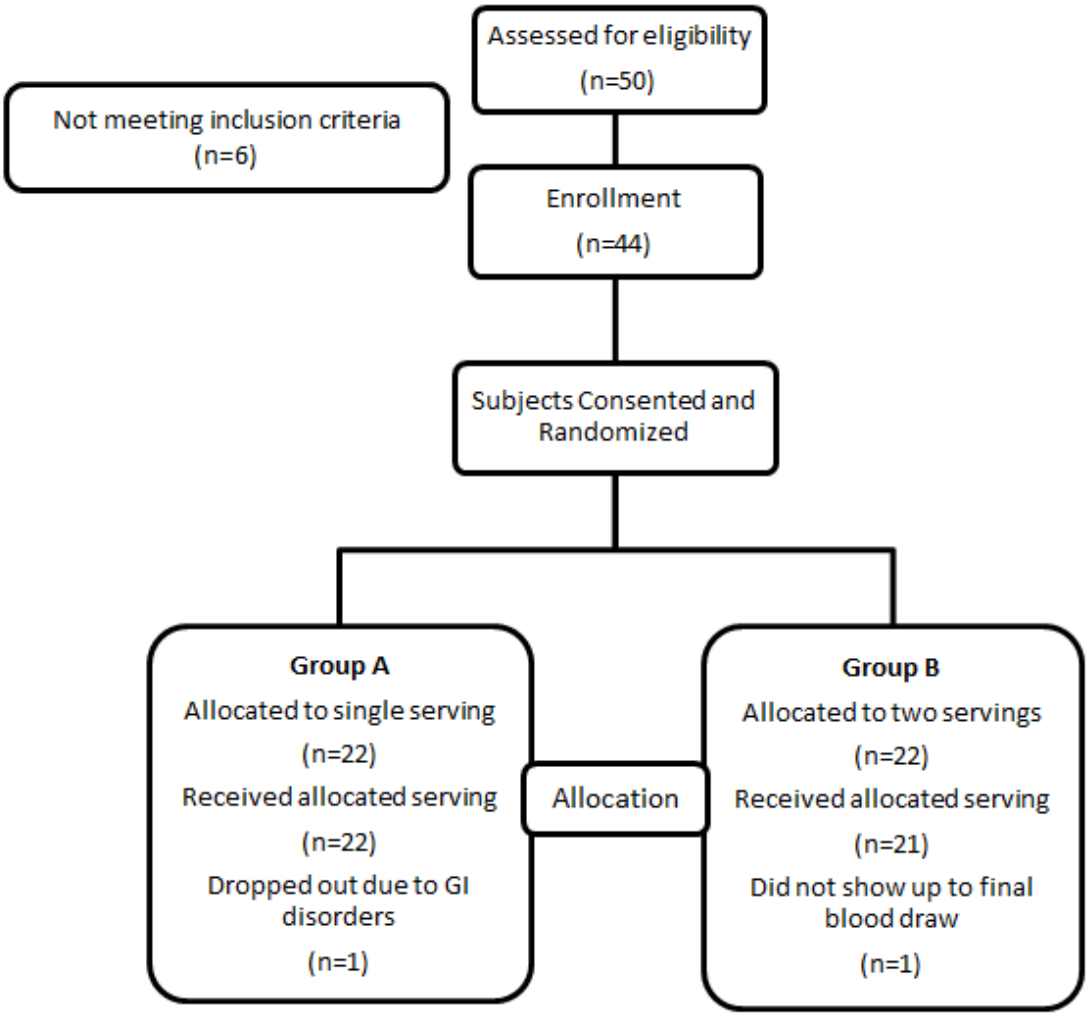

Figure 4-1. Flow chart presenting the number of subjects at each stage of the study.

Table 4-2. Side effects and effects on satiety seen in participants after consuming wheat bran cereal for 21 days.

Subject	Side Effects	Effect on Satiety	Subject	Side Effects	Effect on Satiety
1	none	No effect	23	Bloating	Ate less
2	Increased bowel movements	No effect	24	none	Full
3	Bloating	Ate less	25	none	No effect
4	none	No effect	26	Increased bowel movements	Ate less
5	Bloating Thirsty	Full	27	none	No effect
6	Increased bowel movements	No effect	28	Increased bowel movements	Full
7	none	Full	29	none	Full
8	increased bowel movements	Ate less	30	Increased bowel movements	Ate less
9	Gas Thirsty	Full	31	none	Full
10	none	Ate less	32	none	No effect
11	Bloating Stomach Discomfort	Ate less	33	none	Full
12	none	Full	34	Stomach discomfort	Ate less
13	none	Ate less	35	none	Ate less
14	Stomach Discomfort Gas	Ate less	36	Increased bowel movements	No effect
15	Thirsty	Ate less	37	WITHDRAWN	
16	Bloating Gas	Ate less	38	none	Ate less
17	none	No effect	39	none	No effect
18	none	No effect	40	none	Ate less
19	none	No effect	41	none	Ate less
20	none	No effect	42	none	Full
21	WITHDRAWN		43	Increased bowel movements Stomach discomfort	Full
22	none	No effect	44	WITHDRAWN	

Table 4-3. Number of subjects with side effects in group A, group B, total and percent of the total.

Side Effects	One Serving	Two Servings	Total (Percent)
Increased Bowel Movements	4	4	8 (19.5%)
Bloating	4	1	5 (12%)
Gas	3	0	3 (7%)
Stomach Discomfort	3	1	4 (10%)
Thirsty	3	0	3 (7%)
Full/Ate Less	14	13	27 (66%)

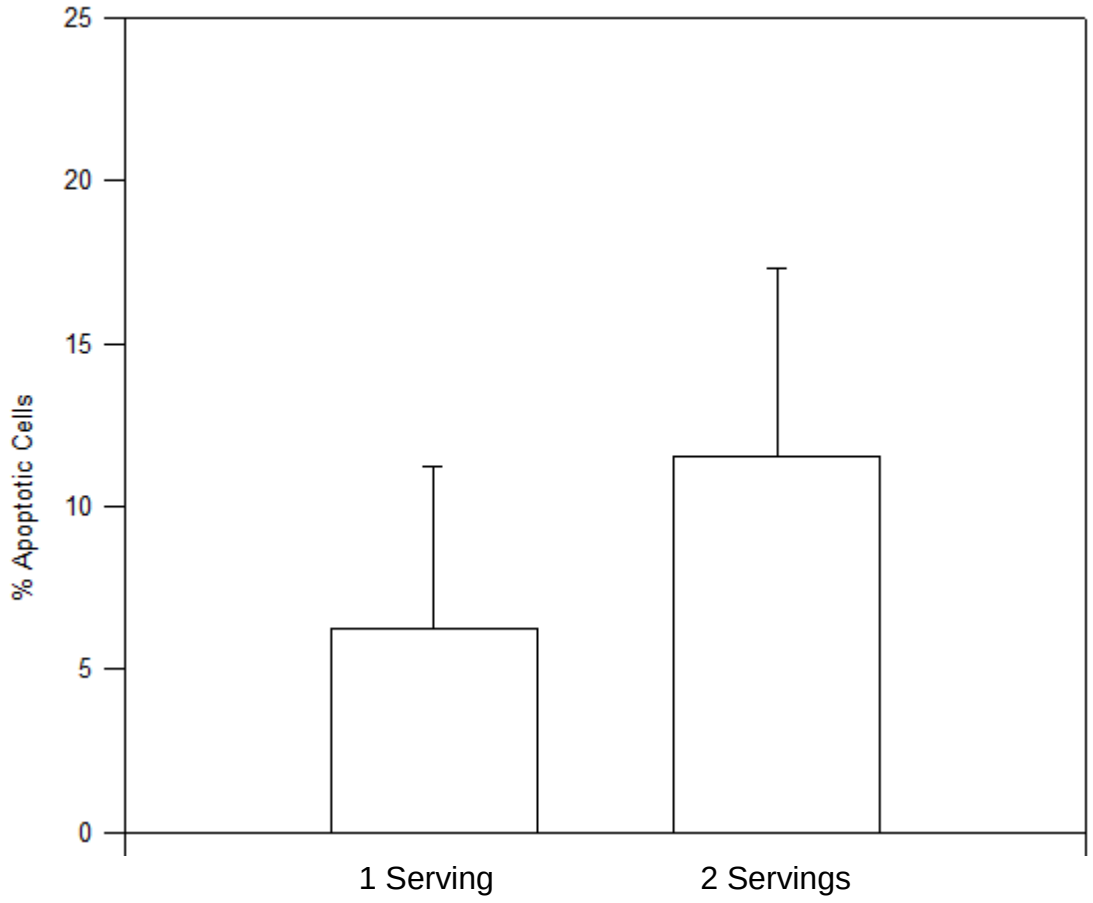

Figure 4-2. Bar chart comparing percent apoptotic cells (mean ± SD) on day 21 seen in the groups consuming one (n = 21) or two (n = 20) servings of cereal. Statistical comparisons were determined using a t-test (p = 0.255).

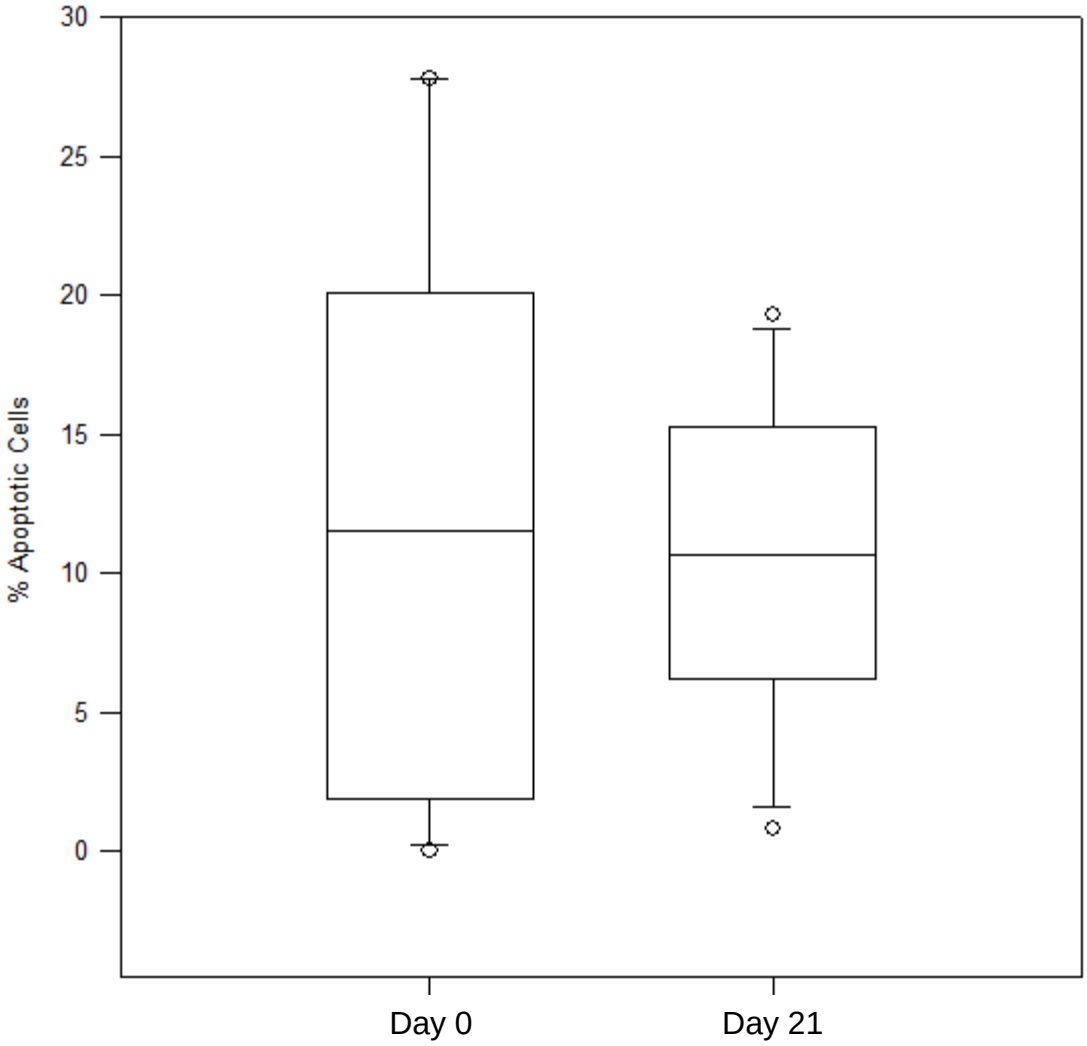

Figure 4-3. Box plot comparing the median values of the percent of apoptotic cells seen in the baseline (n = 43) and day 21 (n = 41) blood draws. Statistical comparisons were determined using a Mann-Whitney Rank Sum test on medians due to unequal variance (p = 0.837).

CHAPTER 5
DISCUSSION

Dietary antioxidants reduce the risk of many chronic diseases. Previous literature suggests that they are mainly found in fruits and vegetables. Emerging studies confirm that wheat bran contains phenolic acids that can offer extensive antioxidant benefits to the consumer. However, their binding to fiber may impact their bioavailability. The results of the DPPH assay show that there is antioxidant activity in wheat bran cereal. When compared to the amount seen in fresh squeezed orange juice it is quite small.

The effect of wheat bran cereal on oxidative stress in vivo has not been investigated. The purpose of this pilot study was to see if the antioxidants in the wheat bran would provide protection to healthy human subjects when consumed as a cereal.

In the present study, forty-four subjects were randomized to one of two groups with different serving sizes of cereal. Their blood was drawn at day 0 and they were asked to consume their cereal each day for three weeks and come back to give a final blood draw. As seen in Figure 4-3, significant differences were not found between the percent of apoptotic cells when consuming one or two servings of cereal. All results were then pooled together to compare the percent of apoptotic cells in the blood draws for day 0 and day 21. Figure 4-3 shows no significant difference between the baseline blood draw and the final blood draw.

While this data does not show increased antioxidant activity after consuming the wheat bran cereal, it cannot be definitively stated that the wheat bran cereal does not contain antioxidants that may provide antioxidant activity. There were some disadvantages to this study. Compliance was measured by having the subjects report how many days they did not eat the cereal as well as having them returned unused

bags of cereal. It is possible that some of the participants were not entirely truthful. They should have had an extra bag of cereal and some did not bring back any bags stating they ate the cereal every day. Also, looking at the reports of fullness after eating the cereal, you would expect to see more reports of being full after the eating the cereal in group B who had two servings of cereal compared to group A. This was not the case which may mean poor compliance of study participants.

Another limitation related to the purpose of this study is the variability in DNA strand breaks among subjects. To correct for this, further studies could include the use of a control cereal containing low fiber and a low amount of antioxidants. A crossover study design would reduce the influence of confounding covariates because each crossover patient would serve as their own control.

APPENDIX A
COMPOSITION OF STUDY MATERIALS

Nutrition Facts

Per 1 box (50 g)

Amount	% Daily Value
Calories 130	
Fat 1.5 g	3 %
Saturated 0.3 g Trans 0 g	2 %
Cholesterol 0 mg	0 %
Sodium 440 mg	18 %
Potassium 580 mg	17 %
Carbohydrate 38 g	13 %
Fibre 17 g	68 %
Sugars 9 g	
Protein 6 g	
Vitamin A	0 %
Vitamin C	0 %
Calcium	4 %
Iron	50 %
Thiamin	80 %
Riboflavin	6 %
Niacin	35 %
Vitamin B_6	15 %
Folate	15 %
Pantothenate	10 %
Magnesium	70 %
Zinc	35 %

Ingredients:
Wheat bran, sugar, malt (corn flour, malted barley), salt, vitamins (thiamin hydrochloride, pyridoxine hydrochloride, folic acid, d-calcium pantothenate), iron.
Contains wheat ingredients.

APPENDIX B
FLYER FOR RECRUITING PARTICIPANTS

RESEARCH PARTICIPANTS WANTED

To take part in a Nutrition Study of the Effect of Wheat Bioactives on the Immune System

If you are between the ages of 21 and 50 and are generally healthy, you may qualify to take part in this study examining the potential health benefits of a wheat bran cereal.

Here is what you will do if you qualify:

- Eat one (50 grams) or two (100 grams) boxes of cereal a day for 3 weeks.
- Make two (2) visits to the University of Florida, Dept. of Food Science and Nutrition. Give a sample of blood on each visit. Have your blood pressure measured at each visit.
- Complete a final questionnaire at the end of 3 weeks.

Total time commitment will be approx. 4 hours.
Compensation up to $100.00.
Interested? Please call (352)392-1991 x255 and ask about Cereal Study Eligibility.

APPENDIX C
INCLUSION/EXCLUSION EVALUATION TO DETERMINE PARTICIPANT ELIGIBILITY

"Wheat Bioactives and Immune Function"

Inclusion/Exclusion Evaluation

P.I. Dr. Percival and Suzanna Bonard

These questions are for evaluating inclusion and exclusion for purposes of enrollment in the study. These answers to these questions are not recorded in association with an individual identification. Names and telephone numbers are kept once their eligibility to enroll is established. The participant is then assigned a number that is used exclusively for identification while their name and phone number (the key to their ID number) remain in a locked file cabinet within a laboratory's interior office.

Introductory Statement:

"[Thank you for calling] (or) [We are returning your call] about a research study we will be doing at the University of Florida in the Department of Food Science & Human Nutrition. The purpose of the study is to evaluate the effects of a wheat bran cereal on human immune function and to determine if bioactives in wheat bran increase antioxidant activity in humans. Participation in this study would last 21 days and will require 2 visits to our lab. You will be required to consume cereal daily for 21 days and come to the lab for blood draws between 8:00a.m.-9:00a.m. after fasting overnight.

To see if you might qualify for this study, I need to ask you some questions about your health history and present condition. [for female participants} If you are pregnant or planning to become pregnant you should not participate. I will now ask you about your age, height and weight, current health and medication.

Questions:

1. Are you between the ages of 18 and 50 years old? Yes/no: yes is acceptable into the study
2. What is your current weight and height? BMI over 35 is not acceptable only because we do not have the equipment to take blood pressure
3. Do you have any ongoing or chronic illness or infection? Yes/no: no is acceptable
4. Are you on any of the following: antihypertensive medication, immunosuppressive drugs, antibiotics, or chronic use of NSAIDS? Yes/no; no is acceptable
5. Do you take any dietary supplements? If yes, are you willing to refrain from taking them during the 21 days of the study? [*Note: Subjects consuming a daily vitamin/mineral supplement with "normal" levels can be recruited into the study].* Yes/no: yes is acceptable
6. During the study, will you consume no more than 2 glasses of alcoholic beverages per day? Yes/no: yes is acceptable

Thank you. You qualify for participation in the study. [an appointment location, time and date are set]

Thank you. I'm sorry, but you do not qualify for this particular study. We appreciate your interest.

APPENDIX D
STUDY EMAIL

Dear ,
If you could be so kind, **please reply** to this email to let me know that you have received it and read it completely.
Thank you again for volunteering to participate in our nutrition study on Wheat Bran Cereal.
You are scheduled for your first visit on . This first visit should take less than an hour. You will need to **fast** after midnight the night before (no food, however, you can and should drink water). We will call you to remind you on
When you come in on morning, first you will have some paperwork to fill out. After that, we will take your blood pressure and your blood will be drawn by our phlebotomist. We will provide a grab-and-go breakfast for you. You will also receive cereal supply along with instructions and additional information regarding the study and subsequent visits. After consuming the cereal for 21 days, you will return to the clinical lab for your second blood draw, complete your final questionnaire and return uneaten bags of cereal.
If are not familiar with our location, I have attached a map. When you get to the **Food Science & Human Nutrition Building**, please come to the North end of the building, enter through the double glass doors and take the elevator to the **2nd floor, Room 227**.
If you have any problems on the morning of the study, please call **352- 392-1991 ext. 287**.

I am also attaching an Informed Consent form for you to read over before you arrive for the study. Reading this in advance should shorten the time you are here during your visit.

Note: Both attachments to this email are pdf documents. If you cannot open these files, please email me back and I will send them to you in a different format.

If you have any questions about any of this information, please call me at **392-1991 x255** or email me at suzanana@ufl.edu.

Finally, if you know of anyone else who might be interested, please have them call us.

With Thanks,

Suzanna Bonard

Graduate Student in Dr. Percival's Lab
University of Florida

449 Food Science & Human Nutrition
Gainesville, FL 32611

APPENDIX E
INFORMED CONSENT

UF | Institutional Review Board
UNIVERSITY of FLORIDA

INFORMED CONSENT FORM
to Participate in Research, and

AUTHORIZATION
to Collect, Use, and Disclose Protected Health Information (PHI)

INTRODUCTION

Name of person seeking your consent:_____

Place of employment & position: _____

This is a research study of how wheat bioactives may benefit immune function in humans.

Could participating in this study offer any direct benefits to you? Yes, as described on page 49.

Could participating cause you any discomforts or are there any risks to you? Yes, as described on page 48.

Please read this form which describes the study in some detail. I or one of my co-workers will also describe this study to you and answer all of your questions. Your participation is entirely voluntary. If you choose to participate you can change your mind at any time and withdraw from the study. You will not be penalized in any way or lose any benefits to which you would otherwise be entitled if you choose not to participate in this study or to withdraw. If you have questions about your rights as a research subject, please call the University of Florida Institutional Review Board (IRB) office at (352) 846-1494. If you decide to take part in this study, please sign this form on page 54.

GENERAL INFORMATION ABOUT THIS STUDY

1. Name of Participant ("Study Subject")

_____

2. What is the Title of this research study?

Wheat Bioactives and Immune Function

3. Who do you call if you have questions about this research study?

Dr. Susan S. Percival

work: 352-392-1991 x217

cell: 352-562-9670

email: percival@ufl.edu

4. Who is paying for this research study?

The sponsor of this study is Kelloggs Corporate Citizen's Fund

5. Why is this research study being done?

The purpose of this research study is to evaluate the effects of wheat bioactives on human immune function and to determine if wheat bioactives provide antioxidant protection to DNA.

You are being asked to be in this research study because you are a healthy individual between the ages of 21 and 50.

WHAT CAN YOU EXPECT IF YOU PARTICIPATE IN THIS STUDY?

6. What will be done as part of your normal clinical care (even if you did not participate in this research study)?

Nothing will be done as part of your normal clinical care because you are a healthy volunteer and therefore do not have any normal clinical care.

Because this study is not related to your normal clinical care, your physician will not be informed that you are taking part in this study.

7. What will be done only because you are in this research study?

This is a 3 week long study.

At the beginning of the study, you will first complete the initial paperwork which includes this informed consent form and the form required to pay you, which should take about 10 minutes.

A trained phlebotomist will obtain a sample of venous blood (four teaspoons total in two tubes) from you for an immune assessment, if you decide to take part in this study. You will be randomly assigned to consume either one serving (50 grams) or two servings (100 grams) of wheat cereal.

You are required to fast a minimum of 6 hours prior to the blood draw and it is preferable if you fast overnight.

1. You will be given a 3 week supply of your assigned cereal serving. For the first 4 days you will consume ½ in the morning and ½ at night. By the 5th day you will consume each serving in its entirety. Water consumption should be maximized as well.

2. After 3 weeks of consuming the cereal, you will be asked to return to have a trained phlebotomist obtain a sample of venous blood (four teaspoons total in two tubes) from you for immune assessment. You are required to fast a minimum of 6 hours prior to the blood draw and it is preferable if you fast overnight.

3. Your blood pressure will be measured at both of your two (2) visits to the lab. If on your first visit, if either one of your blood pressure values are high, defined as 140/90, you will not be allowed to participate.

If you have any questions now or at any time during the study, please contact Dr. Susan S. Percival in question 3 of this form.

8. How long will you be in this research study?

The total time commitment for this research is estimated to be 4 hours, over a 3 week period. Each of the two blood draw sessions (first day and at the end of 3 weeks) is expected to last no more than 1 hour (equivalent to 2 hours total). You will be

required to come to the Food Science and Human Nutrition building for the blood draws. You will consume either one serving (50 grams) or two servings (50) of cereal every day for 3 weeks. The time to eat the cereal daily and to fill in the final questionnaire is expected to total approximately 2 hours.

9. How many people are expected to take part in this research study?

40 people are needed to participate in this research study.

WHAT ARE THE RISKS AND BENEFITS OF THIS STUDY AND WHAT ARE YOUR OPTIONS?

10. What are the possible discomforts and risks from taking part in this research study?

There are no risks associated with consuming one to two servings of cereal a day. The increased fiber may cause some discomfort, at first, in the form of gastrointestinal distress. This may include, but is not limited to bloating, cramps, and gas. To minimize the discomfort of high fiber, we ask the participant to gradually increase their intake of the high fiber cereal over 4 days. We also recommend that participants drink a lot of water to help with the digestion of the fiber.

The risks of drawing blood from a vein include discomfort at the site of puncture; possible bruising and swelling around the puncture site; rarely, an infection; and, uncommonly, faintness from the procedure.

An overnight fast is required and may cause physical discomfort, however we will have a “grab-n-go” breakfast for you after the blood draw.
Other possible risks to you may include:
Researchers will take appropriate steps to protect any information they collect about you. However, there is a slight risk that information about you could be revealed inappropriately or accidentally. Depending on the nature of the information, such a release could upset or embarrass you, or possibly affect your insurability or employability. Questions 17-21 in this form discuss what information about you will be collected, used, protected, and shared.
This study may include risks that are unknown at this time.

Participation in more than one research study or project may further increase the risks to you. If you are already enrolled in another research study, please inform Dr. Susan S. Percival (listed in question 3 of this consent form) or the person reviewing this consent with you before enrolling in this or any other research study or project.

Throughout the study, the researchers will notify you of new information that may become available and might affect your decision to remain in the study.

If you wish to discuss the information above or any discomforts you may experience, please ask questions now or call the PI or contact person listed on the front page of this form.

11a. What are the potential benefits to you for taking part in this research study?

You may or may not personally benefit from participating in this study. You may experience better immune health if the wheat bioactive works as we predict.

11b. How could others possibly benefit from this study?

Consuming the wheat bran cereal may result in benefits such as stronger immunity.

11c. How could the researchers benefit from this study?

In general, presenting research results helps the career of a scientist. Therefore, Dr. Susan S. Percival may benefit if the results of this study are presented at scientific meetings or in scientific journals.

Dr. Percival does not receive any compensation, monetary or otherwise, from the sponsor outside of the funding for this research.

12. What other choices do you have if you do not want to be in this study?

The option to taking part in this study is doing nothing. If you do not want to take part in this study, tell the Principal Investigator or her assistant and do not sign this Informed Consent Form.

You have been invited to participate in this research project because you qualify as a member of the generally healthy population. The investigators associated with this project may or may not teach in your college or be associated with courses for which you are enrolled or might be expected to register in the future. Your participation in this study is voluntary and any decision to take part or not to participate will in no way affect your grade or class standing.

If you believe that your participation in this study or your decision to withdraw from or to not participate in this study has improperly affected your grade(s), you should discuss this with the dean of your college or you may contact the IRB office.

13a. Can you withdraw from this study?

You are free to withdraw your consent and to stop participating in this study at any time. If you do withdraw your consent, you will not be penalized in any way and you will not lose any benefits to which you are entitled. In addition, you have the right to refuse to answer any specific question that you do not want to answer.

If you decide to withdraw your consent to participate in this study for any reason, please contact Dr. Susan S. Percival at 352-392-1991 ext. 217 or study coordinator at 352-293-1991 ext. 255. They will tell you how to stop your participation safely.

If you have any questions regarding your rights as a research subject, please call the Institutional Review Board (IRB) office at (352) 846-1494.

13b. If you withdraw, can information about you still be used and/or collected?

If you withdraw, no new information will be collected about you. However, information that was already collected may still be used and disclosed to others if the researchers have relied on it to complete and protect the validity of the research.

13c. Can the Principal Investigator withdraw you from this study?

You may be withdrawn from the study without your consent for the following reasons:

You are unable to keep appointments, complete a final questionnaire or take the study capsules as directed, or the study is cancelled by the Food and Drug Administration (FDA), the National Institutes of Health (NIH), the company supplying the study treatment, and/or other administrative reasons.
You may also be withdrawn from the study if you have a change in your medical-health status including blood pressure.

WHAT ARE THE FINANCIAL ISSUES IF YOU PARTICIPATE?

14. If you choose to take part in this research study, will it cost you anything?

It will not cost you anything to take part in this study. The grain cereal will be provided at no cost to you while you are participating in this study.

The Sponsor will pay for all activities provided as part of your participation in this study. There will be no cost to you. If you receive a bill related to this study, please contact Dr. Susan S./ Percival at 352-392-1991 x 217 or the study coordinator at 352-392-1991 x 255

15. Will you be paid for taking part in this study?

You will receive compensation for taking part in this study. We will pay you $25 for participation in each of the two blood draws, and $50 for completing the final questionnaire, totaling $100. You will receive payment after the study is completed. Please allow between 4-8 weeks after the completion of the study for payment.

If you are paid for taking part in this study, your name and social security number will be reported to the appropriate University administrative personnel for purposes of making and recording the payment. The information will be used for the purpose of payment and will be kept confidential. You are responsible for paying income taxes on any payments provided by the study. If you are a University of Florida employee, taxes will be deducted from your payment which will be added to your regular paycheck. If the payments total $600 or more, the University must report the amount you received to the Internal Revenue Service (IRS).

16. What if you are injured because of the study?

If you are injured as a direct result of your participation in this study, any resulting medical expenses will be billed to you or your insurance provider. You will be responsible for any deductible, co-insurance, or co-payments. Some insurance companies may not cover costs associated with research studies. Please contact your insurance company for additional information.

No additional compensation is offered. The Principal Investigator and others involved in this study may be University of Florida employees. As employees of the University, they are protected under state law, which limits financial recovery for negligence.

Please contact the Principal Investigator listed in question 3 of this form if you experience an injury or have questions about any discomforts that you experience while participating in this study.

17. How will your health information be collected, used and shared?

If you agree to participate in this study, the Principal Investigator will create, collect, and use private information about you and your health. This information is called protected health information or PHI. In order to do this, the Principal Investigator needs your authorization The following section describes what PHI will be collected, used and shared, how it will be collected, used, and shared, who will collect, use or share it, who will have access to it, how it will be secured, and what your rights are to revoke this authorization.

Your protected health information may be collected, used, and shared with others to determine if you can participate in the study, and then as part of your participation in the study. This information can be gathered from you or your past, current or future health records, from procedures such as physical examinations, x-rays, blood or urine tests or from other procedures or tests. This information will be created by receiving study treatments or participating in study procedures, or from your study visits and telephone calls. More specifically, the following information may be collected, used, and shared with others:

- name
- height
- weight
- blood pressure measurements
- Social Security number for compensation purposes
- address
- phone number
- birth date
- a list of the medications you are taking and the conditions for which they were prescribed
- diaries and questionnaires.

This information will be stored in locked filing cabinets or on computer servers with secure passwords, or encrypted electronic storage devices.

Some of the information collected could be included in a "limited data set" to be used for other research purposes. If so, the limited data set will only include information that does not directly identify you. For example, the limited data set cannot include your name, address, telephone number, social security number, photographs, or other codes that link you to the information in the limited data set. If limited data sets are created and used, agreements between the parties creating and receiving the limited data set are required in order to protect your identity and confidentiality and privacy.

18. For what study-related purposes will your protected health information be collected, used, and shared with others?

Your PHI may be collected, used, and shared with others to make sure you can participate in the research, through your participation in the research, and to evaluate the results of the research study. More specifically, your PHI may be collected, used, and shared with others for the following study-related purpose(s):

To evaluate the effects of wheat bioactives on human immune function and to determine if grain bioactives provide antioxidant protection to DNA.

Once this information is collected, it becomes part of the research record for this study.

19. Who will be allowed to collect, use, and share your protected health information?

Only certain people have the legal right to collect, use and share your research records, and they will protect the privacy and security of these records to the extent the law allows. These people include the:

- the study Principal Investigator, Dr. Susan S. Percival and research staff associated with this project.
- other professionals at the University of Florida or Shands Hospital that provide study-related treatment or procedures
- the University of Florida Institutional Review Board (IRB; an IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research).

20. Once collected or used, who may your protected health information be shared with?

Your PHI may be shared with:

- the study sponsor *Kellogs Corporate Citizen's Fund*
- United States and foreign governmental agencies who are responsible for overseeing research, such as the Food and Drug Administration, the Department of Health and Human Services, and the Office of Human Research Protections
- Government agencies who are responsible for overseeing public health concerns such as the Centers for Disease Control and federal, state and local health departments
- Malcom Randall VA Medical Center (Gainesville)
- Your insurance company for purposes of obtaining payment

Otherwise, your research records will not be released without your permission unless required by law or a court order. It is possible that once this information is shared with authorized persons, it could be shared by the persons or agencies who receive it and it would no longer be protected by the federal medical privacy law.

21. If you agree to take part in this research study, how long will your protected health information be used and shared with others?

Your PHI will be used and shared with others for up to three (3) years after the study ends. If you withdraw your permission for the use and sharing of your protected health information, then your information will be removed from the databse.

You are not required to sign this consent and authorization or allow researchers to collect, use and share your PHI. Your refusal to sign will not affect your treatment, payment, enrollment, or eligibility for any benefits outside this research study. However, you cannot participate in this research unless you allow the collection, use and sharing of your protected health information by signing this consent and authorization.

You have the right to review and copy your protected health information. However, we can make this available only after the study is finished.

You can revoke your authorization at any time before, during, or after your participation in this study. If you revoke it, no new information will be collected about you. However, information that was already collected may still be used and shared with others if the researchers have relied on it to complete the research. You can revoke your authorization by giving a written request with your signature on it to the Principal Investigator.

SIGNATURES

As an investigator or the investigator's representative, I have explained to the participant the purpose, the procedures, the possible benefits, and the risks of this research study; the alternative to being in the study; and how the participant's protected health information will be collected, used, and shared with others:

Signature of Person Obtaining Consent and Authorization	Date

You have been informed about this study's purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your protected health information will be collected, used and shared with others. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask questions at any time.

You voluntarily agree to participate in this study. You hereby authorize the collection, use and sharing of your protected health information as described in sections 17-21 above. By signing this form, you are not waiving any of your legal rights.

Signature of Person Consenting and Authorizing	Date

APPENDIX F
FINAL QUESTIONNAIRE

"Wheat Bioactives and Immune Function"

Subject # ____

***Thank you** once again for participating in our Wheat Study. Please answer the following questions as completely as possible.*

1. During the study, did you experience any side effect(s) that might be attributed to the cereal?
   Yes ___ No ___
   If Yes, please explain: _____
   _____

2. During the study, did you take any dietary supplement(s) other than a vitamin/mineral?
   Yes ___ No ___
   If Yes, what type(s) and how often:_____
   _____

3. During the study, did you notice any changes in your satiety (feeling of fullness) after consuming the cereal?
   Yes ___ No ___
   If Yes, how did this effect your usual eating habits:_____
   _____

4. During the study, did you experience any weight loss or weight gain?
   Yes ___ No ___

5. Did you consume the cereal we provided to you daily, for the entire 3 weeks?
   Yes ___ No ___

6. If you missed eating the cereal, approximately how many days did you miss?
   _____

7. During the study, did you find yourself feeling more hungry than usual?
   Yes ___ No ___

8. Any additional information you would like us to know, or any comments regarding this study?
   _____
   _____
   _____
   _____
   _____
   _____

LIST OF REFERENCES

1. Wells, HF, Buzby JC: Dietary assessment of major trends in U.S. food consumption, 1970-2005. USDA, 27, 2008.

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BIOGRAPHICAL SKETCH

Michelle Patricia Smith was born in Bellevue, Washington. Michelle grew up in Clearwater, Florida, where she attended Countryside High School. After high school, she attended the University of Florida, where she earned a Bachelor of Science in food science and human nutrition, specializing in nutritional sciences in May of 2009. In the summer of 2009, Michelle was accepted as a master's student in the Food Science and Human Nutrition Department at UF and began her classes that fall. She began working with Dr. Susan Percival in March of 2010. After graduation, she intends on working toward becoming a registered dietician.