

**THE EFFECT OF WHEAT CLASS AND PROCESSING ON
MARKERS OF COLON CANCER RISK IN
CARCINOGEN-TREATED RATS**

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Abstract

A previous study in this laboratory found that hard red wheat is more effective than soft white wheat in reducing colon cancer risk, regardless of processing state, based on fewer aberrant crypt foci (ACF), a morphological marker of colon cancer risk. Here we examined the effect of wheat class (red vs. white) and processing (whole vs. refined) on reducing markers of colon cancer risk during the early and late promotion stage of colon cancer development. Rats adapted to a basal diet were treated twice with the colon-specific carcinogen, dimethylhydrazine (DMH).. After the last dose of carcinogen, rats were divided into either the basal diet or the wheat flour-based diet groups. Both hard red and soft white wheat flour significantly reduced morphological markers such as ACF, and sialomucin producing ACF (SiM-ACF), an ACF with greater tumorigenic potential, compared to the basal diet. These reductions occurred equally with whole and refined wheat. Both hard red and soft white wheat diets significantly reduced a biochemical marker of risk, β -catenin accumulated crypts (BCAC), compared to the basal diet, but hard red wheat did so to a greater degree. Only hard red wheat significantly reduced a marker of stem cells mutation, metallothionein positive crypts, compared to soft white wheat. Hard red wheat caused regression of ACF, suggesting it can reduce the risk level of colon cancer. Overall, hard red wheat reduced colon cancer risk more than soft white wheat, regardless of processing state. The differences between wheat flours were greater in the late promotion stage.

Introduction to Thesis Project

Colorectal cancer is a major public health problem in the United States. It is the third most common cancer and second leading cause of cancer deaths in the United States. Of various risk factors, environmental factors have been found to have the greatest influence on the etiology of colon cancer development. Studies of migrant populations show that when people move from an area with low colon cancer incidence to an area with high incidence, colon cancer of the migrant population quickly becomes similar to that of the host country.

It has been estimated that over 70% of colorectal cancer is influenced by diet. There are dietary recommendations to increase the intake of whole grains, fruits and vegetables and to decrease intake of red meat and avoid low fiber diets to protect against colon cancer development. Epidemiological studies have identified an association between the consumption of whole grains and a reduced risk of colon cancer, whereas a lack of whole grains or increased intake of refined grains has been associated with an increased risk of colon cancer.

Of the various types of grains, wheat is the largest cereal grain produced and consumed in the United States. There are four major classes of wheat. Hard red and soft white wheats make up the majority of wheat-containing foods. Hard red wheat makes up 66% of US wheat production and soft white wheat makes up 13% (1); the remaining wheat crop production was composed of soft red (15%), hard white (1%) and durum (5%). Wheat is composed of various types of nutrients, fibers and phytochemicals. The phytochemical composition and antioxidant activity of individual wheat flours vary depending on the growing location as

well as wheat variety (2, 3). Some of these individual components of wheat have been shown to prevent against colon cancer risk.

Epidemiological studies support the preventive effect of whole grains on reducing colon cancer risk. However, few experimental studies have examined the effect of whole grains focusing on wheat and the reduction of colon cancer risk. The purpose of the first experiment of this thesis was to examine the effect of wheat class and processing on reducing colon cancer risk during the early promotion stage of colon cancer development. Rats treated with a colon-specific carcinogen were fed either a basal diet or wheat flour-based diet made from whole and refined hard red, hard white, soft red and soft white wheat. Aberrant crypt foci (ACF), which are pre-cancerous lesions of colon cancer, and sialomucin producing ACF (SiM-ACF), which are ACF thought to have greater tumorigenic potential, were measured as the main end points of colon cancer risk.

The purpose of the second experiment was to examine the effect of hard red and soft white wheat, either as whole flour or refined flour, in reducing colon cancer risk during the late promotion stage of colon cancer development. Rats were fed a basal diet before, during and six weeks after the carcinogen treatment. After six weeks one group of rats were killed and the remaining rats were switched to a wheat flour-based diet for eight more weeks. ACF, SiM-ACF and mucin depleted foci (MDF), which are speculated to be more advanced towards tumor development compared to ACF and SiM-ACF, were measured as major end points of colon cancer risk.

Our first two experimental studies have observed that diets containing red wheat are effective in reducing colon cancer risk in the early and late promotion stages of colon cancer. The effect of a red wheat diet, either whole or refined, on reducing ACF was significantly greater

than that of soft white wheat in the late promotion stage of colon cancer. It is not known why a hard red wheat diet is more effective than the soft white wheat diet. The final study of this thesis investigated the effect of wheat-containing diets on reducing colon cancer risk in both the early and late promotion stages. In addition to studying morphological markers, a biochemical marker and a stem cell mutation marker were also employed. In addition, the ability of the wheat-containing diets to regress animals from a state of high colon cancer risk to a state of lower risk was evaluated.

Chapter-1 surveys the epidemiological and experimental evidence related to whole grains and protection against colon cancer, followed by a discussion on pathways of colon cancer development, and a discussion on biomarkers of colon cancer. In chapter-2, chapter-3 and chapter-4, the experimental designs, results and discussions are presented. The thesis project summary is given in chapter-5.

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Chapter-I
Literature Review

Colorectal Cancer Incidence

Colorectal cancer is one of the most common cancers in the United States. According to the American Cancer Society, it is the third most common type of cancer and the second leading cause of cancer death in the United States. In western countries, including the United States, colon cancer represents 12.6% of all incident cancer in men and 14.1% in women (1, 2). It has been estimated that 148,810 new cases will be diagnosed in 2008, of which 108,070 will be colon cancer cases and 40,740 will be rectal cancer cases. The incidence of colon cancer has increased more rapidly than that of rectal cancer. The incidence of colon cancer is much higher in westernized countries, especially in North America, Northern and Western Europe, and Australia, compared to Asia and Africa (3). Indeed, there is a 20 fold international difference in colorectal cancer rates. Colorectal cancer rates vary by race and ethnicity. The highest rates were observed among Caucasians of northern European origin in both their native countries and in the areas to which they migrated, for example Australia and North America. Lower rates were observed in Caucasians of southern European origin, Asian and African. However, rates tend to increase with migration (4). Studies on incidence rates in migrant populations indicate that when people migrate from a region with low cancer risk to a region with high cancer risk, the development of colon cancer incidence increases in the migrating population to that of the host country (5, 6). For example, Asian immigrants in Australia exhibit a three times greater risk of colorectal cancer compared to people living in Asia and have a risk rate similar to those originating from Australia (7, 8). In US born

Japanese, the colorectal cancer incidence rate is twice as high as rates for foreign born Japanese, and almost 60% higher than those who were originally born in the US (9).

Epidemiological studies, such as migration studies just described, demonstrate that environmental factors show the major influence on the development of colorectal cancer. Among environmental factors, dietary factors in particular have shown to influence colon cancer incidence. It has been suggested that dietary factors might account for approximately 35% of cancer deaths (10). In both men and women high dietary intake of processed and red meat, refined grain, and sugar have been associated with increased risk of colon cancer (11, 12). Reduced risk of colon cancer was associated with those who consumed vegetarian diets, particularly with fruit, vegetable, and grains. Although environmental factors are clearly the most important, 20-30% of colorectal cancer cases may have some sort of genetic link (13). The most common hereditary forms of colorectal cancer are familial adenomatous polyposis (FAP) and hereditary non-adenomatous polyposis coli (HNPCC). However, these hereditary forms account for <5% of colon cancer. The genetic factors in most cases may determine the susceptibility to develop colorectal cancer, while environmental factors mostly determine which of the genetically predisposed individuals will develop colorectal cancer (14). In addition to environmental and genetic factors, the other factors considered to be associated with a greater risk of colorectal cancer include age, gender, smoking, obesity, and family medical history. Perhaps something indicating that, overall, environment plays a much bigger role than genetics, and that diet is mostly likely the most important environmental factor.

Whole Grain and Colon Cancer Risk

Epidemiological Studies

Recently, a number of epidemiological studies have brought attention to whole grain consumption as a possible dietary factor to reduce cancer risk. This association was first reviewed by Jacobs and his colleagues (15), who found that, of fourteen studies, thirteen showed an inverse association between whole grain consumption and the risk of cancers including colon, gastric and endometrial cancer. Although whole grain intake was associated with healthy lifestyle behaviors, the pooled odds ratio remained statistically significant after adjusting for a number of covariates. The protective effect of whole grain was further examined in a meta-analysis of 40 case-control studies involving 20 different types of cancers including colorectal cancer. The subjects with high whole grain intake had a 34% lower risk of overall cancer than those with low whole grain intake (16, 17). In these studies the whole grains intake included whole wheat bread, crispy bread or whole grain hot cereals whereas refined grains intake included white bread, pancakes or waffles. The odds ratio was less than 1 in nine of ten studies involving colorectal cancer and polyps, indicating an association between whole grain intake and decrease in colon cancer risk. In contrast to whole grain, refined grain had a positive association with colon cancer risk. The first eight studies listed in Table 1-1 summarize the details of the colon cancer studies included in the meta-analysis and show that five out of eight studies found a statistically significant association between whole grain intake and colon cancer risk.

Since the meta-analysis by Jacobs et al. (16, 17) several case-control studies on whole and refined grain intake and colorectal cancer have been published. By systematic analysis of a series of case-control studies conducted in Northern Italy between 1983 and 1993 Chatenoud et al. (18) observed an association between intake of refined grain cereals and an increased

risk of cancers of the large intestine, stomach and other organs of the gastrointestinal tract. The odds ratio for the highest tertile of refined grain intake was 1.5 for colon cancer, and 1.3 for rectal cancer. A case-control study by La Vecchia et al. (19) conducted in Italy between 1983 and 1996 showed that whole grain consumption as whole grain bread or pasta was associated with decreased incidence of all types of cancers except thyroid cancers. Based on the analysis of 828 cases of colon cancer and 7990 controls, the odds ratio of the high vs. low whole grain intake group was 0.5 for colon cancer (95% CI, 0.3 to 0.6). Another case-control study by Levi et al. (20) involving 223 cases of colon cancers and 491 controls found that the intake of whole grain was associated with a reduced risk of colon cancer (OR= 0.85), whereas the intake of refined grain showed a positive association (OR= 1.32). Comparing the dietary intake information between 1993 cases of colon cancer and 2410 controls, Slattery and colleagues (21) reported that whole grain consumption was inversely associated with the risk of colon cancer in women (OR = 0.7, 95% CI, 0.5 to 1.0), whereas refined grain intake was positively associated with the risk of colon cancer in men (OR 1.7, 95% CI 1.3 to 2.3). Table 1-1 summarizes several case-control studies which support a statistically significant association between whole grain consumption and reduction of colon cancer risk, except for one study which reported no association between different whole grain foods and colorectal cancer risk in men and women. This study reported an odds ratio for consumption of a variety of whole grain foods of 1.1 for men and 1.3 for women, which was not statistically significant (21).

Of these case-control studies, some analyzed the effect of bread based on whole and refined categories of wheat flour. Tuyns et al. (22) found that the overall protective effect of total grain intake (both whole and refined) was due to bread consumption (OR=0.57). Both white

bread and whole-meal bread consumption were inversely associated with colon cancer risk. Similarly, another case-control study in Southern Italy observed that the consumption of total bread reduced the risk of colon cancer (OR=0.57). However, the odds ratio of whole-meal bread was slightly higher than 1 (OR=1.03). The majority of the studies which examined food type and risk of colorectal cancers found an inverse association between total bread intake and reduced risk of colorectal cancer, while rice, pasta and pastries were associated with increased risk (23, 24). The consumption of whole grain by the participants of these studies was mostly in the form of bread, thus the inverse association between whole grain and colon cancer risk may be considered mostly due to bread consumption.

Results from several prospective studies also support an association between an increased intake of whole grain and reduced risk of colon cancer. A recent prospective American Association of Retired Persons (AARP) diet and health study conducted by the National Institutes of Health reported that whole grain intake was associated with a reduction in risk of colon cancer. The study involved 291,298 men and 197,623 women age 50-71 years, and the subjects were followed for 5 years. After adjusting for age, physical activity, menopausal hormone therapy, total energy intake, red meat consumption, calcium, and folate intake the study observed that the risk of colon cancer was lower in the subject groups with a high intake of whole grain foods (1.3 servings per 1000 kcal per day) compared to the subjects with a low intake of whole grain foods (0.2 servings per 1000 kcal per day). This effect was statistically significant (RR=0.86, 95% CI, 0.75 to 0.99, p=0.03) (25). The association between whole grain consumption and colorectal cancer risk was also investigated by Larsson and colleagues (26) in a prospective study involving a cohort of 61,000 Swedish women followed for 15 years. Dietary information was derived from a food-frequency

questionnaire at baseline (1987-1990). Whole grain foods included hard whole grain rye bread, whole grain soft wheat bread, porridge, and breakfast cereal. Refined grain included soft white bread, pasta, rice, pancakes or waffles, and sweet buns or biscuits. The study found an inverse association between colon cancer risk and whole grain food intake. The multivariate relative risk (RR) was 0.74 (95% CI, 0.55 to 0.98) for women who consumed two or more slices of hard whole grain bread per day compared with those who consumed less than four slices of bread per week. Refined grain consumption was not associated with risk of colon cancer (RR=1.29, 95% CI, 0.92 to 1.82). Contrary to these prospective studies, the Cancer Prevention Study II Nutrition Cohort study examined the relation between whole grain, fruit, vegetables, dietary fiber and colon cancer risk. The study involved 62,609 men and 75, 554 women who completed questionnaires on their medical history, diet and lifestyle behaviors. The study reported no association between whole grain consumption and colon cancer risk in men and women whether analyzed alone or combined with fruits and vegetables (RR=1.17; 95% CI, 0.73 to 1.87) (27). Likewise, in the Nurses' Health Study the analysis of dietary intakes found no association between cereal fibers intake and colorectal cancer risk in women (RR=1.00; 95% CI, 0.79 to 1.27) (28). Furthermore, Pietinen et al. (29) analyzed intake data from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC Study). The study involved 27, 111 male subjects who completed dietary questionnaires at baseline and were followed for 8 years. The result reported a null association between whole grain cereal consumption and colorectal cancer risk.

The results overall of epidemiological studies are generally supportive of a protective effect by whole grain in reducing colon cancer risk, although some studies fail to suggest the association. The lack of statistical significance in the association between whole grain intake

and colon cancer risk might be due to several reasons. In epidemiological studies the food frequency questionnaire (FFQ) is a commonly used method to examine the relationship between diet and cancer. FFQ measurement error is important because it often leads to under estimates of relative risks of a disease, therefore reducing the statistical power of the study to detect their significance. To compensate for this problem, investigators have used more accurate reference instruments, such as food records or a 24-hour dietary recall. Proper classification and identification of whole and refined grain plays a major role in evaluating the effect of grain consumption on colorectal cancer risk. Subjects may identify grain incorrectly if they report whole grain or refined grain bread consumption based on the brown coloring of the food items. Proper classification of foods by researchers is also very important to analyze and to interpret data. If one type of grain or grain food is more strongly associated with reduction of colon cancer risk, the association may be attenuated by the other grain foods included in the whole grain foods category. Epidemiological studies have suggested an association between whole grain consumption and reduced risk of colon cancer and this association may represent a relationship between grain type and colon cancer risk. Further experimental research is required to investigate whether colon cancer prevention is due to a particular type of grain.

Table 1-1. Summary of case-control studies on whole grains and colon cancer risk

Subjects	Methodology	Odds ratio for high to low grains	Adjustments	Reference
Cases-123 Controls-699	Home interview (1986)	Whole grain bread and pasta, OR= 0.7 Pasta and rice, OR= 1.2 All bread, OR= 2.1 Pastries, OR= 1.1	Age, sex, social status	(30)
Cases-119 Controls-119	Home interview (1987-89)	All grain, OR= 0.93, p=0.97 Whole meal bread, OR= 1.03 All bread, OR= 0.58 Pasta, OR= 1.1	Age, sex, social status	(31)
Cases-339 Controls-778	Hospital interview (1985-87)	Whole grain bread/pasta, OR= 0.5 Pasta and rice, OR=2.97 All bread, OR=0.82	Age, sex	(23)
Cases-106 Controls-147	Home interview (1975-84)	Whole grain bread, OR= 0.6 White bread, OR= 1.0	Age, education	(32)

Subjects	Methodology	Odds ratio for high to low grains	Adjustments	Reference
Cases-58 Controls-178	Home interview (1970-77)	All grains, OR= 1.3 Whole grains, OR=0.77	Age, sex, residence	(33)
Cases-1993 Controls-2410	CARDIA diet history interview	Refined grains food, OR _{Men} = 1.5, OR _{Women} =1.1 Whole grains food, OR _{Men} = 0.80, OR _{Women} =0.56	Age, BMI, physical activity, energy intake	(34)
Cases-453 Controls-2851	Dietary history (1978- 82)	All grains, OR= 0.6 All bread, OR=0.57 White bread, OR= 0.86 Pastries, ginger bread, OR=1.05	Age, sex, province	(22)
Cases-488 Controls-488	Frequency questionnaire (1978-82)	All grains, OR= 0.545 Whole grains food, OR=0.65 Refined grains, OR= 0.88	Race, BMI, physical activity, energy intake	(35)

Subjects	Methodology	Odds ratio for high to low grains	Adjustments	Reference
Cases-955 Controls-3526	Hospital interview (1983-93)	Refined grain, OR= 1.5 Breakfast cereal, OR=0.53	Age, sex, BMI, education, smoking, physical activity	(36)
Cases-828 Controls-7990	Hospital interview (1985-89)	Whole grain, OR= 0.5	Age, sex, BMI, education, smoking	(19)
Cases-119 Controls-491	Frequency questionnaire (1992-97)	Whole grains, OR= 0.54 Refined grains, OR= 1.79	Age, sex, education, physical activity, energy intake	(20)
Cases-1993 Controls-2410	CARDIA diet history interview (1991-94)	Whole grains food, , OR _{Men} = 1.1, OR _{Women} =1.3 Refined grains food, , OR _{Men} = 1.7, OR _{Women} =1.1	Age, BMI, energy intake, physical activity	(12, 34)

Subjects	Methodology	Odds ratio for high to low grains	Adjustments	Reference
Cases-106 Controls-309	Home interview (1985-90)	All bread, OR= 0.7 Refined grains, OR=2.0 Pasta and rice, OR= 1.8	Age, sex, BMI, education, smoking, physical activity	(24)
Cases-1225 Controls-4154	Frequency questionnaire (1992-96)	Whole grains bread, OR= 0.90 Refined bread, OR= 1.40	Age, sex, education, physical activity, energy intake	(37)
Cases-424 Controls-410	Frequency questionnaire (1985-89)	Hot and cold breakfast cereals, $OR_{Men} = 0.67$, $OR_{Women} = 0.47$	Age, sex, education, physical activity, energy intake	(38)
Cases-119 Controls-119	Home interview (1987-89)	All grains, OR= 0.93 All bread, OR=0.58 Whole-meal bread, OR= 1.03	Age, sex, education, smoking	(31)

Experimental Studies

There have been many studies investigating the effect of individual components of whole grain on colon carcinogenesis in animal studies, but only a few of observed the effect of whole grain. A variety of individual components of whole wheat such as wheat bran (39), wheat germ extract (40), and wheat aleurone flour (41) have been shown to protect against colon cancer risk in animals. To date, only two animal studies have examined the effect of whole grains, especially wheat flours, on colon cancer risk. The first study was conducted by Maziya-Dixon and colleagues (42) who investigated the effect of wheat diets on tumor incidence in carcinogen (dimethyl hydrazine) treated mice. The wheat flours used in the study came from two varieties of wheat, either hard red or hard white winter. Both of these wheat varieties were processed into whole and refined wheat flours and wheat bran. The mice were fed diets containing whole wheat flours, refined wheat flours or wheat bran through the initiation and progression stage of colon carcinogenesis. After 40 weeks of feeding, mice fed red wheat bran had a lower incidence of colon tumor development compared to the white bran diet. Based on these findings the authors suggested that red wheat had a greater chemo-preventive effect than the white wheat.

Yu and his colleagues (43) observed the effect of only soft white wheat flour (whole or refined) compared with that of a wheat-free control diet during the initiation and post-initiation stages of colon carcinogenesis induced by heterocyclic aromatic amines. There was no statistically significant difference in aberrant crypt foci (ACF), which are pre-cancerous lesions, in all treatment groups. ACF multiplicity, which was calculated as number of AC per ACF, was significantly greater in the refined soft white wheat diet group in the post-initiation

stage compared to the initiation stage of colon cancer development. ACF multiplicity did not increase in the group fed the whole wheat diet during post-initiation stage. The number of small ACF with 2-3 crypts was significantly lower in the whole soft white wheat diet group in the post-initiation stage compared to the initiation stage, suggesting that the whole wheat diet had an inhibitory effect on development of ACF.

A recent study in this laboratory (Iovinelli and Gallaher, unpublished data) examined the effect of wheat class and processing on colon cancer risk in a rat model. Rats treated with a colon carcinogen were fed diets containing whole and refined red wheat or whole and refined white wheat. Rats fed red wheat flour had significantly fewer ACF compared to rats fed white wheat flour regardless of the state of processing. However, colonic mucosal cell proliferation, fecal bile acid concentration, and oxygen radical absorption capacity (ORAC) of cecal contents supernatant were reduced in animals fed the whole wheat flours, compared to the refined flours. Colonic mucosal apoptosis was unaffected by either processed state or wheat class. From this study, it was hypothesized that wheat class but not processing influences colon cancer risk in this animal model.

Several studies have evaluated the possible mechanisms of individual whole wheat components in preventing colon cancer risk (41). Wheat bran has been suggested to reduce colon cancer development by a number of different mechanisms, including decreasing fecal bile acid concentration, increasing fecal bulk, resisting bacterial fermentation, and increasing butyrate concentration in the distal colon (44, 45). Wheat bran was found to enhance apoptosis (44) and decrease colonic mucosal cell proliferation in carcinogen treated rats (46). Wheat bran contains significant amounts of phenolic compounds such as ferulic acid,

flavonoids, lutein, zeaxanthin, and phytic acid. All of these have been shown to reduce colon cancer risk when fed as isolated compounds, as discussed below. These studies suggest that the chemo-preventive effect of wheat bran might be due to the presence of these biologically active compounds in wheat bran (2, 47).

Ferulic acid is the predominant phenolic compound present in wheat bran and mainly functions as an antioxidant. About 98% of ferulic acid is bound to bran polysaccharide and only 2% of ferulic acid is in either a free form or as a soluble conjugate (2, 47). The complete absorption of free ferulic acid occurs in the small intestine; however bound ferulic acid is not absorbed in the small intestine and reaches the colon. There it is hydrolyzed by colonic bacteria and released for absorption (48). Ferulic acids have been shown to increase colonic cell differentiation, induce apoptosis (49), and decrease ACF formation and tumor growth in carcinogen-treated rats (50). The effect of ferulic acid on reducing colon carcinogenesis may be mediated by activation of the phase II enzyme, glutathione-S-transferase, which helps to detoxify carcinogens and mutagens (51, 52).

Carotenoids, xanthophylls, and flavones are the major yellow pigments in wheat flour. Lutein is one of the major carotenoids of red wheat. It has the ability to prevent oxidation by quenching free radicals. A study by Qu and his colleagues (53) observed that in dimethylhydrazine (DMH) treated mice lutein was associated with inhibition of cell proliferation and ACF formation. Low doses of lutein supplementation inhibited ACF formation whereas high doses of lutein increased ACF numbers in azoxymethane (AOM) treated rats (54) indicating that the anticarcinogenic effect of lutein is dose dependent. Consistent with the results of these animal studies, epidemiological studies also support that

the high dietary intake of lutein reduces the risk of colon cancer (55). Anthocyanin, which is the major red pigment in wheat flour, was reported to suppress colon tumor development (56) and growth of cancer cells in culture (48).

Phytic acid (inositol hexaphosphate) is the predominant storage form of phosphorus in cereals, and is concentrated in the bran. By chelating iron and copper it acts as an antioxidant, thereby limiting free radical formation and reducing lipid peroxidation. The negatively charged phosphate groups of phytic acid binds with starches and limits its absorption, thus enhancing bacterial fermentation and increasing fecal bulk. Carcinogen treated rats fed diets supplemented with phytic acid had fewer sialomucin-producing ACF, a category of ACF considered to be more dysplastic and therefore more tumorigenic. In addition, animals fed phytic acid supplemented diets also had a lower rate of colonic cell proliferation and higher rate of apoptosis (49).

Wheat germ is a very good source of polyunsaturated fatty acids, proteins, vitamins and minerals (57). Rats fed a diet supplemented with wheat germ extract before, during and after carcinogen treatment showed fewer numbers of ACF and neoplastic tumors compared to the group fed a wheat germ extract-free diet (40). The authors hypothesized that the wheat germ extract shows an inhibitory effect by inducing apoptosis in the initiation phase of colon cancer.

Epidemiological studies have strongly suggested an association between intake of whole grains and reduced risk of colon cancer. However it is not clear from these associations what is the relationship between colon cancer risk and specific type of grains. Experimental studies

have focused almost exclusively on components of grains. Therefore to further understand the relationship between whole grains and colon cancer, further investigation is required.

Wheat-a Major Whole Grain

Grains provide approximately two-thirds of the energy intake in the developing countries of the world, and one-quarter of the energy intake in the United States (58). Among grains, wheat, rice and corn are the major cereal grains, whereas barley, oat, rye, sorghum, and millet are the minor grains available worldwide. Wheat accounts for one-third of the total grains produced worldwide, while rice accounts for one-fourth. In the United States, wheat is the principal cereal grain of human consumption and export. It is considered to be the fourth leading US field crop and leading export crop.

The Kernel of Wheat

The wheat kernel is the seed from which the wheat plant grows. Wheat kernel contains three distinct parts i.e. bran, germ and endosperm, that are separated during milling.

Bran: Bran is the outer layer of the wheat kernel and makes up about 14.5% of the kernel weight. The bran consists of seven layers and these layers are a concentrated source of dietary fiber. The aleurone layer is the largest portion of the bran, constituting 75% or more of its dry weight. The aleurone layer is a concentrated source of vitamins, minerals and other nutrients. Bran is included in whole wheat flour, but it is removed in the refining process during extraction of white flour. Bran, in addition to fiber, contains proteins and vitamin B complex such as niacin, pyridoxine, pantothenic acid, riboflavin, and thiamin.

Germ: Germ is the embryo part of the wheat kernel from which the wheat plant sprouts, and it accounts for about 2.5% of the kernel. Germ is a concentrated source of unsaturated fats. That is why it is often necessary from the perspective of shelf life to remove the germ to prevent lipid oxidation. Germ is also a very good source of protein, vitamin B-complex, alpha and beta tocopherol, iron, quinine and enzyme inhibitors. Of the nutrients in the whole kernel, the germ contains about 64% of thiamin, 26% of riboflavin, 21% of pyridoxine, 7% of pantothenic acid, 2% of niacin and 8% of protein.

Endosperm: Endosperm is the inner part of the wheat kernel. It is about 83% of the kernel weight. The main nutrients in the endosperm are protein and carbohydrate. The endosperm, excluding the aleurone layer, contains 50 to 75% of starch. It also contains typically 8 to 18% of proteins. Relatively few vitamins, minerals, fiber, or phytochemicals are found in the endosperm. It is composed of tightly packed cells containing starch granules held in a protein matrix. The phytonutrient concentration of endosperm is very low; however it contributes a significant amount of bioactive compounds due to its large size. The endosperm contains about 17% of total wheat phenolic compounds, 21% of total flavonoids and about 50% of the carotenoids, cryptoxanthine and lutein, in the whole wheat kernel (59). Of the nutrients in the whole kernel, the endosperm contains about 43% of pantothenic acid, 32% of riboflavin, 12% of niacin, 6% of pyridoxine, 3% of thiamine and 70 - 75% of protein.

Wheat Classes

In the United States wheat has two distinct growing seasons. Winter wheat is planted in the fall and harvested either in the spring or summer. This type of wheat accounts for 70 to 80 percent of the US production. Spring wheat is planted in the spring and harvested in the late

summer or early fall. There are many varieties of wheat produced in the United States, all of which fall into six major classes of wheat. These classes are determined not only by the time of planting and harvesting but also by hardness, color and shape of the kernels. The six major classes of wheat are discussed below.

Hard Red Winter: This is the predominant class of wheat produced and exported each year. It has medium to high protein content (10-13.5%), and has either a hard or soft endosperm. It has good milling and baking characteristics. It is mostly used for making breads and all purpose flour. This wheat has no subclasses.

Hard White Wheat: This is the newest class of wheat grown in the United States. This wheat is closely related to red wheat except for the absence of color genes. It has a milder and sweeter flavor. It has equal fiber content to red flour and has similar milling and baking qualities. Hard white wheat is used in yeast breads, tortillas, hard rolls and oriental noodles. There are no subclasses of this wheat.

Soft Red Winter: This wheat is high yielding, but has relatively low protein content, (usually about 10%) and a soft endosperm. It is used in making cakes, pastries, flat breads and crackers.

Soft White Wheat: The use of this type of wheat is the same as soft red wheat i.e. mostly used for bakery products and breads. Soft white wheat has a protein content of about 10%. Soft white wheat represents about twenty percent of total U.S. exports. There are three subclasses: soft white, white club and western white.

Hard Red Spring: This wheat contains the highest amount of protein (13-16.5%) of all the wheats. It is excellent for making bread as it has high quality milling and baking characteristics. Subclasses are based upon the color and hardness of the kernel, which include dark northern spring, northern spring and red spring.

Durum: This wheat also has high protein content (12-16%). It is mainly used for pasta products such as macaroni, spaghetti and other noodles. This is the hardest of the wheats. It has the lowest export volume, accounting for less than 5 percent of all U.S exports. The major subclasses are hard amber durum, amber durum and durum.

Pathways of Colon Carcinogenesis

Colon carcinogenesis has been considered a multistep process in which normal cells are transformed into malignant cells through a series of events occurring at the cellular and molecular levels. It has been suggested that this stepwise progression to colon cancer arises from an accumulation of multiple mutations in proto-oncogenes and tumor suppressor genes (60-64). Mutations in the tumor suppressor gene, adenomas polyposis coli gene (APC) occurs at an early stage of colon carcinogenesis. APC gene mutations leads to dysplasia, abnormalities in adult cells or formation of polyps, which form benign growths on the surface of mucous membranes. These polyps can remain dormant for many decades, but when one cell in these polyps develops a second mutation in the proto-oncogene, *K-ras*, it grows at a faster rate resulting in a larger tumor or intermediate adenoma. Subsequent mutations in other genes, *DCC* and *p53*, which are tumor suppressor genes, lead to late adenoma and finally carcinoma (61, 62). The adenoma-carcinoma sequence represents a

process by which most, if not all, colon cancer develop (61, 62). Figure 1-1 shows genetic mutations in adenoma-carcinoma sequence.

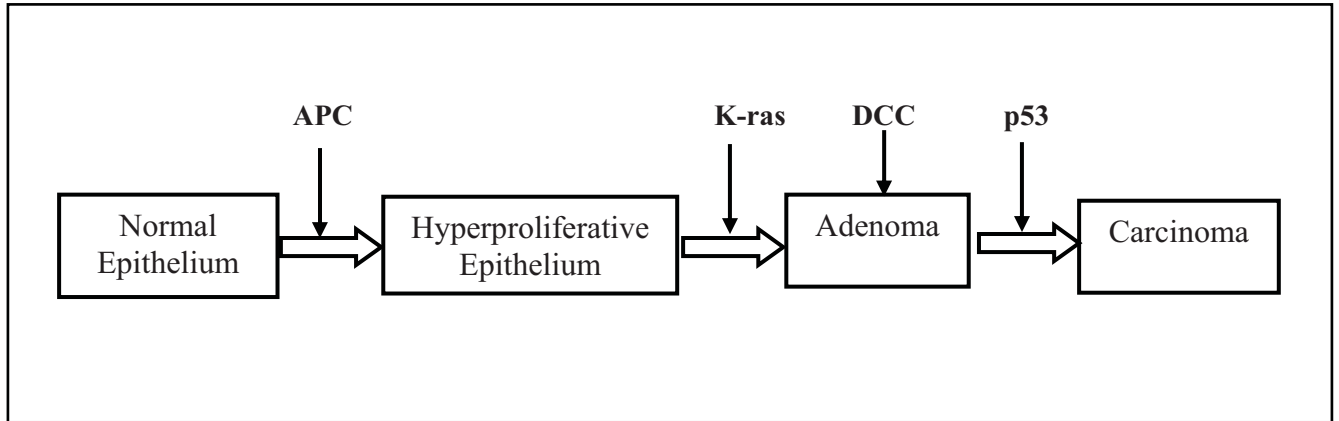


Figure 1-1: Genetic mutations in adenoma-carcinoma sequence (65)

The entire process of carcinogenesis may occur through three major stages: initiation, promotion and progression.

Initiation: Initiation is the first stage of colon carcinogenesis. This stage involves a mutation in the DNA molecule which is either spontaneous or inherited. This mutation leads to genotypic changes of the cell. These genotypic changes may also occur during the interaction of a carcinogen with DNA which may cause DNA damage. The initial damage to the DNA molecule rarely results in cancer because the cell has many mechanisms to repair this damage. However, if the repair system is lost or dysfunctions and the damage to DNA is in the location of a gene that regulates cell growth and proliferation, DNA repair, or a function of the immune system, then the cell may obtain a growth advantage and become more prone to be cancerous. The most common types of initial mutations observed in animals are simple gene transition, transversion, or small deletions (66).

During initiation of colon carcinogenesis, mutation in the *APC* gene is often one of the earliest events. The *APC* gene is a tumor suppressor gene located on chromosome 5q21 (67, 68). The product of the *APC* gene is a large 312 kDa protein consisting of 2843 amino acids. This protein is expressed ubiquitously. One of the major functions of the protein product of the *APC* gene is thought to be the regulation of β -catenin induced signaling. The APC protein is multifunctional and consists of several domains which form a multi-protein complex with β -catenin, axin and glycogen synthase kinase (69, 70). Within this multi-protein complex, glycogen synthase kinase phosphorylates β -catenin. As a result β -catenin is released from the complex and is degraded. This occurs by a pathway known as ubiquitin-dependent proteosomal pathway (71, 72). Due to the mutation of *APC* or *β -catenin* genes, the formation of the multi-protein complex is inhibited, and therefore β -catenin protein does not degrade. Free β -catenin protein accumulates in the cytoplasm of the cells, and ultimately translocates to the nucleus. In the nucleus, β -catenin protein binds with a DNA binding protein called T-cell factor (Tcf) and forms a β -catenin-Tcf complex, which in turn activates transcription of oncogenes *c-myc* and *cyclin D₁*, and thereby promotes colon carcinogenesis (73, 74).

The frequency of *APC* gene mutation is very high in colorectal cancer patients. Germline mutations in the *APC* gene were detected in 80% of familial adenomatous polyps (FAP) patients. Somatic mutation of the *APC* gene has been detected in most colorectal carcinomas, whether these are familial or sporadic origin (68, 75).

Promotion: The promotion stage of colon carcinogenesis is associated with altered morphological and phenotypic changes. During promotion, a mutated cell divides faster and grows continuously. Later these cells undergo clonal expansion and form a benign tumor.

Promotion requires prolonged exposure to promoting agents that activate clonal expansion. It is speculated that the acquisition of a mutant *K-ras* gene is either an early event or depends upon previous APC mutation to exert its oncogenic effect. *K-ras* is a proto-oncogene located in chromosome 12p12 and encodes a small 21 kDa protein. *K-ras* participates in signal transduction of regulatory pathways essential for normal cell proliferation and differentiation (76). It is a GTP binding protein located at the cytoplasmic membrane, which has intrinsic GTPase activity (77). The GTPase activity of the Ras protein is regulated by several other proteins such as epidermal growth factor, platelet-derived growth factor, or cytokines e.g. interleukin-2. The activity of Ras is regulated by the GTP/GDP cycle. The Ras protein is active when bound to GTP, but when GTP is hydrolyzed to GDP this protein become inactive. When mutated, Ras protein becomes constitutively active, and stimulates several transcription factors such as c-Jun, c-Myc and c-Fos, leading to enhanced cell proliferation, and suppressed apoptosis (63, 76). Several studies have shown that 50% of sporadic colorectal cancers have mutations in at least one of 3 codons of the *K-ras* gene (78-81). *K-ras* mutation has also been observed in 35-42% of large adenomas (82-86).

Progression: Progression involves both genotypic and phenotypic changes that ultimately lead to metastasis and malignancy. This stage is more complex, and involves structural changes of the chromosome that activates uncontrolled cell proliferation, cell growth, metastasis, invasiveness, and malignancy. During progression, the genetic material of the cancerous cell is more fragile and prone to additional mutations. These mutations occur in genes that regulate growth and functions of the cell such as oncogenes, tumor suppressor genes, and DNA mismatch-repair genes (87). Mutation in the tumor suppressor gene, *p53*, usually occurs at the progression stage of colon carcinogenesis (88). This gene is located on

chromosome 17p and encodes a sequence specific DNA binding protein which functions as a transcription factor and controls the expression of a large number of genes. The *p53* gene is considered as the “guardian of the genome” because of its ability to block cell proliferation during the presence of DNA damage. In normal cells, p53 protein level is low. DNA damage and other environmental stresses such as ultraviolet light, trigger the increase of p53 protein, which arrest cell cycle, stimulate DNA repair and promote apoptotic cell death. The effect of p53 in cell cycle arrest is mediated via the activation of p21 protein which is an inhibitor of cyclin-dependent kinase (CDK), a key regulator of the cell cycle.

Mutation in the *p53* gene is very common in colorectal cancer. Based on immunohistochemical detection, DNA sequencing and 17p allelic loss study, it was observed that 4-26% of adenomas, 50% of adenomatous polyps and 50-75% of adenocarcinomas have either *p53* gene alteration or allelic loss of 17p (85, 86, 89-94)

Other tumor suppressor genes that have been identified to play a role in colon carcinogenesis are *DCC*, *TGF- β* and *Smad*. Studies have found that about 70% of primary colorectal cancers have deletion of at least one *DCC* allele. A mutation of the *DCC* gene is more common in large, advanced adenomas than small adenomas (75). Mutations in *TGF- β* and *Smad* occur at the later stage of colon carcinogenesis and have been detected in approximately in 10% of colorectal cancer (95, 96). *TGF- β* acts as a tumor suppressor protein that inhibits cell growth and *Smad* protein is an intracellular mediator of *TGF- β* signaling pathway, which exerts a wide range of effect on many cells, including regulation of cell growth, differentiation and apoptosis. Table 1-2 summarizes the common gene changes during colon carcinogenesis.

Table 1-2. Gene changes identified in colon cancers

Gene	Type of change of function	Function	Cancer showing that change (%)
APC	loss	Cell adhesion	>70%
K-ras	gain	Signal transduction	50
DCC	loss	Proliferation, differentiation	>70
p53	loss	DNA repair, apoptosis	>70
TGF, Smad	loss	Growth, differentiation, apoptosis	10
MSH2, MLH1	loss	DNA repair	5-35

Biomarkers of Colon Carcinogenesis

Morphological Markers

Aberrant crypt foci (ACF): Aberrant crypt foci (ACF) are focal lesions of the colonic mucosal cells characterized by either single or multiple enlarged crypts. They were first identified as pre-cancerous lesions on methylene blue stained whole mount colonic mucosa of rodents treated with a colon carcinogen (97). ACF are easily identified under the microscope as slightly elevated from the surrounding normal mucosa with an oval slit-like opening and are characterized by their larger size than adjacent normal crypts, thick epithelium, and altered luminal openings. They are quantified based on their number, size, or multiplicity (the number of AC per ACF). Studies have shown that in rodents ACF develop as early as 2 weeks after a single dose of colon carcinogen administration. ACF are numerically greater in the distal colons of rats and mice (98, 99) where most colon tumors develop (100, 101). The average number of crypts per foci increases over time after a dose of carcinogen and after 34 weeks more than 50% of ACF contains at least four or more crypts. These large ACF have been shown to have higher proliferative activity compared to adjacent normal crypts (102, 103).

Phenotypic and genotypic changes associated with ACF in rodents have been clarified. The most common phenotypic change observed in ACF developed in azoxymethane (AOM) treated rats is reduction of hexosaminidase activity, which is associated with colon cancer development. Altered expression of several growth factors and signaling molecules have been found in carcinogen-treated rodents. Reduced expression of TGF- α and TGF- β and

increased expression of EGFR were also reported in some studies (97, 104, 105). The genotypic changes include increased expression in proto-oncogenes, *c-myc* and *cyclin-D1* in AOM treated mice (106). Mutation in the tumor suppressor genes *K-ras* and *p53* genes have also been found in ACF of carcinogen-treated rats (107).

Although ACF share altered morphological, biochemical and genetic characteristics with tumors, the relationship between ACF and tumor formation is still controversial. A study by Shivapurkar et al. (108) observed that rats fed a wheat bran rich diet had fewer ACF and colon tumors compared to those fed a diet low in wheat bran after 12 weeks of carcinogen treatment. There was a strong correlation between the number of ACF and tumor incidence. Likewise, Pretlow et al. (103) reported an association between large ACF and tumor formation. The study found fewer large ACF at 12 weeks and a lower tumor incidence at 36 weeks compared to the control groups in carcinogen-treated rats. Magnuson et al. (109) reported that crypt multiplicity, not the number of ACF, was an early predictor of tumor incidence. The number of ACF was lower ($P < 0.033$) in animals fed cholic acid, a tumor promoter, measured at all time points until 14 weeks. However, after 8 weeks, average crypt multiplicity of ACF was greater ($P = 0.045$) in animals fed cholic acid compared to animals fed the control diet. Tumor incidence was significantly higher measured at 18 weeks in the cholic acid fed group (63.2%) compared to the control diet group (29.4%). On the other hand, several studies reported either no relation or an inverse relation between ACF formation and tumor incidence. In one study the number of ACF obtained at 7 or 15 weeks did not correlate with the number of tumors obtained at 26 weeks or 8 months in AOM treated rats (110). A study by Harman et al. (111) also failed to show a correlation between the mean number of aberrant crypt foci and the incidence of adenocarcinomas in rats treated

with dimethylhydrazine (DMH) after 24 weeks. It is unclear why there is an inconsistent relationship between development of ACF and tumors in these studies. However, differences in study design, carcinogen dosing, type of carcinogen, and the time point to measure end points may be factors that affect this inconsistent correlation between ACF and tumor incidence.

It is clear that not all ACF develop into colon tumors, as many more ACF are present than the number of tumors that would be expected to develop. It is likely that only certain populations or types of ACF might be associated with development into tumors. Histological evaluation of ACF has shown that ACF can be identified as hyperplastic or dysplastic, depending on the degree of alteration, ranging from mild cellular atypia to dysplasia (112). Dysplastic ACF are characterized by abnormal nuclear stratification and elongation. Dysplastic ACF show higher rates of luminal alteration, higher degrees of dysplasia, and greater cell proliferation (105, 113). On the other hand, hyperplastic ACF show an increase in the number of cells per crypt; however they maintain normal cellular and nuclear differentiation. Table 1-3 shows dysplasia ranking of ACF and their criteria for identification. Molecular analysis of dysplastic ACF has shown that the frequency of mutations in several tumor suppressor genes such as *APC*, *β -catenin* and *ctnmb1* are higher in dysplastic ACF than hyperplastic ACF (114-116). Expression of nitric oxide synthase (iNOS) and sialomucin are much greater in dysplastic ACF compared to hyperplastic ACF (117). Interestingly, dysplastic ACF often lack mutations in the proto-oncogene, *K-ras*, whereas most hyperplastic ACF accumulate *K-ras* mutation (114, 118). In humans, the frequency of dysplastic ACF is much greater in FAP patients (119, 120). Dysplastic ACF have also been detected in patients with or without sporadic colorectal carcinoma suggesting that dysplastic

ACF are the true precancerous lesions of colon cancer and therefore are the more important biological markers to be considered.

The identification and quantification of ACF is usually limited to animal models. Although development of ACF is common in humans, it is difficult to quantify ACF in human colon tissue. It is still under debate whether or not ACF are a reliable marker for colon carcinogenesis in humans. However their ability of rapid proliferation and growth advantage in addition to their dysplastic criteria would suggest that ACF are a useful early marker of colon carcinogenesis.

Markers of Crypt Dysplasia

Sialomucin producing ACF: Mucin is a high molecular weight epithelial glycoprotein. It has a high content of oligosaccharides that are linked to tandem repeat peptides rich in threonine, serine and proline by glycosidic bonds. The primary function of mucin is to protect the intestine from physical and chemical damage. Normal mucosal cells usually produce sulfomucin, which has a higher degree of sulfate in the molecule. But due to an abnormality or a change in mucosal cells, the structure and chemical nature of the large intestinal goblet cell mucin changes to producing sialomucin, which contain mostly sialic acid (121).

ACF can differ in the type of mucin produced, with some producing predominantly sialomucin, and others producing sulfomucin. It has been reported that a decrease in sulfomucin and gradual increase in sialomucin is predictive of ACF that have a higher degree of dysplasia (105, 122). The degree of dysplasia in turn has been found to be closely related

to the degree of alteration in cellular morphology and increase in ACF multiplicity (122). A study by Jenab and colleagues (123) observed that ACF predominantly producing sialomucin or ACF producing a combination of sialo and sulfomucin show a higher degree of alterations including irregularity of crypt structure, changes in nuclei localization, reduction in number of goblet cells, and alterations of mucin structure and glycosylation. Sialomucin producing ACF also have higher cell proliferation at the top regions of the crypts, and enlarged morphological structure compared to that of sulfomucin producing ACF. Moreover, sialomucin producing ACF express more ulex europaeus agglutinin-1 (UAE-1) compared to sulfomucin producing ACF. UAE-1 is predominant found in large ACF. UEA-1 is a lectin which specifically binds with glycoproteins such as mucin. Increased expression of UEA-1 and their binding with glycoproteins is closely associated with a high degree of dysplasia and high proliferation rate of cancerous cells (124). Sialomucin producing ACF are suggested to be more dysplastic and are more advanced towards carcinogenesis, hence a more reliable predictor of colon carcinogenesis in addition to other morphological markers (122).

Mucin depleted ACF (MDF): Mucin depleted ACF (MDF) are characterized by either an absence or limited production of mucin. Histological analysis revealed that MDF are more dysplastic than ACF that express either sialomucin or a combination of sialo and sulpho mucin (125). In addition to a lack of mucin production, MDF are also identified as focal lesions that show distorted crypts compared to surrounding normal crypts. Elevation of the lesions above the surface of the colon and greater multiplicity are also important features of MDF. In MDF, expression and accumulation of β -catenin protein is much more intense than

ACF. Mutation in the *ctnmb1* gene that encodes β -catenin protein was observed in 25% of MDF, where as only 7% of ACF showed *ctnmb1* gene mutation (126).

In carcinogen-treated rats the total number of MDF that develop in the colon somewhat depends on the dose of carcinogen administered. Compare to the total number of ACF, the total number of MDF per colon is much lower. However studies have found a strong association between MDF and tumor incidence in animals (126, 127). Supplementation of the diet with either cholic acid (a strong colon cancer promoter) or beef or sausage increased the number of MDF (126, 127) whereas administration of chemopreventive agents such as piroxicam or synbiotics decreased the incidence of MDF formation and crypt multiplicity in rats (122, 128). The strong correlation of MDF with carcinogen treatment suggests that MDF may be more direct precursors to tumors.

Flat ACF: Flat ACF were first identified in Min/+ mice, which is a model with a germline mutation in APC and therefore is analogous to FAP in humans. Unlike classical ACF detected in AOM treated rats, these ACF in Min/+ mice were detected as flat lesions, not elevated above the surrounding mucosa, and as a compressed crypt under trans-illumination microscopic examination. These are the only ACF detected in Min/+ mice, and have been identified as dysplastic crypts, suggesting they are precancerous lesions (129). When Min/+ mice were treated with AOM, both flat ACF and classical ACF were detected, however only flat ACF showed a significant degree of dysplasia (130). Recently, Paulsen and his colleagues have also detected flat ACF in both F344 and A/J rat models treated with AOM (131, 132). Small flat lesions, designated as flat ACF, were characterized by bright blue staining, compressed crypt openings, and crypts not elevated above the surrounding mucosa

in these animal models. The authors observed that the crypts surrounding large flat ACF enlarge at a later stage of carcinogenic development and acquire a change whereby they are slightly raised the structure above the surface.

Large flat ACF and nascent tumors were found to have similar morphology. Furthermore, large flat ACF and tumors showed a uniform picture of severe dysplasia with presence of paneth cells, compressed crypts, cytoplasmic/nuclear over-expression of β -catenin, and nuclear over-expression of cyclin D1. In contrast, classical elevated ACF with more than 4 or more crypts did not display such changes. These classical elevated ACF showed mainly hyperplasia, either mild or moderate dysplasia but never severe dysplasia. The number of flat ACF and tumors, including microscopic and macroscopic, was virtually constant over time (approximately 2.5 lesions/rat), whereas the number of classical elevated ACF was initially higher (approximately 180 lesions/rat), but the total number decreased at a later stage (approximately 80 lesions/rat). It was also reported that the proliferation rate of flat ACF was significantly higher than classical elevated ACF. The authors suggested that small flat dysplastic ACF may undergo continuous developmental growth to the stage of a tumor, and thus need to be considered as an important end point of colon carcinogenesis (131, 132) .

Table 1-3. ACF dysplasia rankings and criteria for identification

Dysplasia Ranking	Criteria for Identification
Non-dysplastic foci	<ul style="list-style-type: none"> ■ Crypts have increased height with slight dilation ■ Cells exhibit normal nuclei with regular location, orientation, shape, color, number of nuclei ■ No visible nuclear stratification ■ Apical localization of mucus and minor loss of mucin with normal looking goblet cell ■ Crypts are mildly basophilic and hypercellular
Mild to moderate dysplastic foci	<ul style="list-style-type: none"> ■ Crypts have greater increased height with more dilation ■ Cells exhibit moderately enlarged nuclei with irregular location, orientation, shape, darker color ■ Some focal nuclear stratification ■ Loss of cytoplasm and mucin production with abnormal location of mucin ■ Crypts are moderately basophilic and hypercellular
Moderate to severe dysplastic foci	<ul style="list-style-type: none"> ■ Crypts have increased crypt height with severe dilation ■ Cells exhibit enlarged nuclei with irregular location, loss of polarity, abnormal shape and color ■ Extensive nuclear stratification ■ Greater loss of mucin production with irregular mucin pattern ■ Crypts are strongly basophilic and hypercellular

Biochemical Markers

β -Catenin Accumulated Crypt (BCAC): β -Catenin is one of the important components of the β -catenin/Tcf signaling pathway. It is a cadherin binding protein and a key regulator of the cadherin-mediated cell-cell adhesion system. This protein functions as a transcriptional activator of several downstream oncogenes, when complexed with other DNA binding proteins of the Tcf family (116). Mutations in the *APC* or *β -catenin* genes or activation of the Wnt signaling pathway prevent degradation of β -catenin protein (133, 134). As a result β -catenin accumulates in the cytoplasm, translocates into the nucleus and binds with a transcription factor, Tcf-4 (69, 72, 135, 136). In the nucleus it up-regulates several oncogenes such as *c-myc* (135), cyclin-D1 (136), *c-jun* (137) and ultimately activates the Tcf pathway (73, 135, 136). Figure 1-2 shows the Wnt/ β -catenin signaling pathway.

Studies in animals treated with carcinogen have observed that accumulation of β -catenin protein is a consequence of either mutation in the *β -catenin* gene (138) or allelic loss of the *APC* gene (139), and mutations of these genes occur early in the process of carcinogenesis. In ACF with hyperplasia, the accumulation of β -catenin protein is mostly localized at the cell membrane as in normal colon epithelium (140-142), whereas in ACF with dysplasia, β -catenin expression increases in the cytoplasm and nucleus, and decreases in the membrane, depending on the degree of dysplasia. These dysplastic crypts accumulated with β -catenin protein are known as β -catenin accumulated crypts (BCAC) (143-145). Over-expression of membranous and nuclear β -catenin in ACF, adenomas and carcinomas suggest that altered expression of β -catenin plays an important role in the progression of colon carcinogenesis.

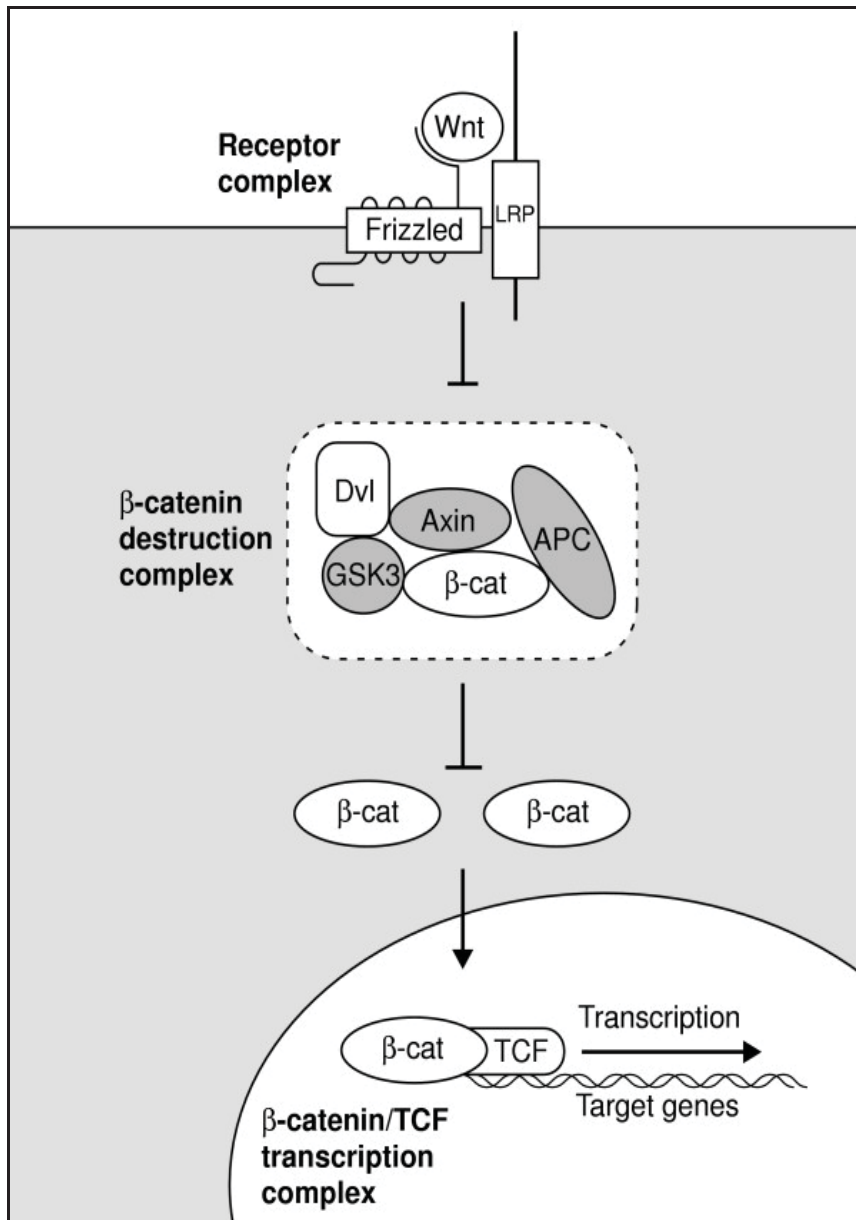


Figure 1-2. Wnt/β-catenin signaling pathway (146)

Components shown shaded in gray have an inhibitory effect on downstream signaling in the nucleus. Abbreviations: APC, the adenomatous polyposis coli protein; GSK-3, glycogen synthase kinase-3

BCAC do not appear as typical ACF in a whole mount of colon. They are identified in the histological sections on *en face* preparation of paraffin embedded colon tissues using a β-

catenin antibody. Typical ACF do not show accumulation of β -catenin protein in the *en face* sections. It is hypothesized that BCAC are preneoplastic lesions independent of ACF and are therefore another early biomarker of colon cancer (145). Histological analysis of BCAC has revealed that BCAC exhibit several pathological properties that are different than that of ACF (147). BCAC proliferate at a higher rate compared to ACF and show dysplasia with disruption of cellular morphology. Studies have shown that the number of BCAC and their histological abnormality significantly increase with time after carcinogen treatment. Some of the BCAC acquire pathological characteristics of adenomatous crypts with extensive branching (143-145, 148). Over-expression of cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) was observed in BCAC, characteristics thought to be associated with colon cancer cell growth (149-151). Reduced hexosaminidase activity was detected both in the cytoplasm and in the nucleus of BCAC (145). Crypts with decreased hexosaminidase activity have been linked with colon neoplasms in humans and in rats (152, 153). A decrease in hexosaminidase activity, suggesting BCAC coincide with hexosaminidase-altered foci and are more likely to be an early precursor of colon cancer.

The existence of mature paneth cells is rare in normal colonic epithelium; however they are often present in colonic tumors. Interestingly, these cells are frequently present in BCAC, which indicates that an association with paneth cells may be an important morphological characteristic for identifying BCAC. The biological function of paneth cells has not been established yet; it is known that paneth cells produce several biological factors including tumor necrosis factor- α , guanylin, epidermal growth factor, and matrilysin (154-157). These factors have important roles in cell growth, regulation of epithelial cell proliferation and differentiation. Of them, particularly, matrilysin has been shown to play a role in intestinal

carcinogenesis, suggesting paneth cells are possibly involved in carcinogenesis (158).

BCAC have been reported to show increased expression of matrilysin at both the mRNA and protein levels, indicating the existence of paneth cells in BCAC and suggesting that they are more prone to tumor development.

Mutational analysis has shown that the mutations of the *β-catenin* gene are frequently identified in BCAC in codons 28, 30, 32, 34, and 41 (144). The frequency of mutation of the *β-catenin* gene was higher in BCAC (46%) compared to ACF (32%). In contrast, greater than 70% of ACF were found to have mutations in the *K-ras* gene in rats treated with AOM (148). Similar to carcinogen-treated animals, a higher frequency of mutation in the *β-catenin* gene has also been reported in human colorectal cancer patients. In human colon, 54% of dysplastic ACF were shown to have excessive expression of β -catenin protein suggesting that dysfunction of β -catenin is an early event of colon carcinogenesis.

Stem Cell Mutation Marker

Stem cells and cancer: Stem cells are the cells upon which the entire cell production process is ultimately dependent. Regardless of the tissue, all stem cells have the ability to divide, renew and regenerate themselves for long periods of time. A stem cell usually remains undifferentiated. During formation of specialized cells or tissues, stem cells divide asymmetrically into two daughter cells. One daughter cell retains the identity of the parental stem cell and the other daughter cell undergoes differentiation to form specialized cells (159, 160).

Current evidence supports that stem cells can become cancerous and that these cancer stem cells lose their normal regulatory controls on cell division, leading to over proliferation and formation of a tumor (161). This is referred to as the cancer stem cell hypothesis of carcinogenesis. A tumor is composed of heterogeneous populations of cells. The cancer stem cell hypothesis suggests that this heterogeneity is due to an ongoing differentiation within a tumor. In this heterogeneous population, it appears that only a small subset of cells with self-renewal ability, the cancer stem cells, give rise to differentiated cells, whereas the majority of the tumor cells undergo differentiation and lose this ability (160, 162). For example, Bonnet and Dick (163) observed that only a small subset of acute myeloid leukemia (AML) cells was capable of producing leukemic progenitors upon transplantation into immunodeficient mice. Similarly, in immunodeficient mice, only the mutated cells that expressed stem cells markers were able to produce AML. These leukemic cells obtained chromosomal translocations only in the stem cell population and subsequent generations acquired additional mutations leading to leukemia, suggesting that stem cells are the target of transformation (164-166). Since the first demonstration of cancer stem cells in leukemia, they have also been identified in breast cancer (167), prostate cancer (168), pancreatic cancer (169), liver cancer (170), and colorectal cancer (171-173). Based on these studies it has been hypothesized that leukemia and other malignancies must be maintained by a small population of cancer stem cells that have the potential for self-renewal and extensive proliferation (174, 175).

Normal human colon consists of millions of crypts, each of which contains several thousand cells. These colonic crypts are maintained by stem cells located at the bottom of each crypt (176-178). These stem cells maintain the homeostasis of colonic epithelial tissues and respond to changes within the crypts to regulate stem cells number and crypt volume (177,

179). During the self-renewal process of these stem cells, a few stem cells have the ability to generate progenitor cells; these are called transit amplifying cells (TA). The stem cells and TA cells reside near the base of the intestinal crypt. In their normal environment, TA cells proliferate and eventually differentiate along a particular cell lineage to form the tissue (180, 181). The TA cells retain the ability to divide for several differentiation stages and are the major contributor to normal tissue renewal. In rapidly proliferating tissues, such as epithelial tissues, the stem cells are normally quiescent and do not divide, and the tissue is renewed by TA cells. Differentiated cells that are originated from the stem cells migrate towards the lumen of the colon and subsequently die (182). Colonic epithelial cell turnover is very rapid; all cells except the crypt stem cells are replaced with a week (183). By self-renewing, stem cells often survive for longer periods of time instead of undergoing terminal differentiation or dying. Therefore, there is a much greater likelihood for mutations to accumulate in individual stem cells than in most mature cells (159, 184).

The Wnt signaling pathway usually maintains the proliferation of colonic crypt stem cells or its progenitors (TA cells), thus, Wnt signaling is essential for homeostasis of the colonic epithelium (185). The role of Wnt/ β -catenin in proliferation and maintenance of epithelial stem cells has been investigated by gain and loss-of-function studies in mice. It has been observed that mice lacking Tcf-4 transcription factor, a part of the Wnt signaling pathway, quickly exhaust the undifferentiated progenitor cells in the crypts of the epithelium during fetal development. The neonatal epithelial crypts of mice were found to be composed entirely of differentiated, non-dividing cells, indicated that Tcf-4 maintains the crypt stem cells of the intestine (186).

In crypts, Wnt signaling drives the formation of Tcf/ β -catenin complexes, which maintains a proliferative phenotype in crypt epithelial cells. Over proliferation of cancer stem cells is likely due to a dysregulation of Wnt/ β -catenin signaling pathway (187-190), and dysregulation of this pathway is often involved in the development of familial and sporadic cancers (191). β -Catenin is one of the important components of the Wnt/ β -catenin pathway. In the absence of a Wnt ligand, β -catenin is sequestered in a multiprotein degradation complex containing the scaffold protein Axin, the tumor suppressor protein APC, and glycogen synthase kinase-3 β (GSK-3 β). In absence of Wnt, β -catenin is phosphorylated and subsequently degraded by proteasomes. As a result, there is no transcription of Wnt target downstream oncogenes. In the presence of Wnt signaling, the extracellular Wnt molecules bind to frizzled (Fz) receptors on the cell membrane of colonic crypt cells. The activated frizzled receptor in turn activates Dishevelled (Dvl) protein which sequesters axin proteins from the complex and removes GSK-3 β from the complex (192, 193). β -catenin is then released from the multiprotein complex, accumulates in the cytoplasm in a stabilized non-phosphorylated form and subsequently translocates into the nucleus where it binds to transcription factor, Tcf-4. The β -catenin/Tcf-4 complex activates the downstream oncogenes *c-myc* and *cyclin D1* (60, 64, 194). The β -catenin/Tcf-4 complex also induces the assembly of cell cycle engines via inactivating p21, an inhibitor of cell cycle kinases.

Putative colon cancer stem cells have been isolated based on the positive expression of the specific cell surface biomarker CD133 (162, 171-173). CD133 is a transmembrane glucoprotein expressed in normal haematopoietic, endothelial and epithelial cells. CD133 expression in normal epithelial cells of colon tissue is very infrequent and barely detectable as compared with colon tumor tissue. The increased number of CD133- positive cells in

cancer tissues may result from their oncogenic transformation. Cancer stem cells show a high density of the CD133 cell surface marker. Studies have observed that these CD133-positive cells readily developed into tumors after transplantation at a low concentration into immunodeficient mice, whereas CD133-negative cells did not induce tumor formation. This suggests that colon tumors are generated and maintained by a small subset of undifferentiated cells, cancer stem cells, which are able to self-renew and differentiate into heterogeneous tumors (171-173). Besides CD133, other cell surface proteins have been reported to identify colon cancer stem cells, such as CD44 and CD166. However, the number of CD133-positive colon cancer stem cells was always greater (171).

Metallothionein (MT) as a Stem Cell Mutation Marker: Recently, over-expression of the protein metallothionein (MT) has been identified as a biomarker of neoplastic progression. MT is a small intracellular protein with a molecular weight of only 6-7 kilodaltons (195). This protein is characterized as a metalloprotein because of its high metal binding capacity and unusual bioinorganic structure. MT has a high cysteine and thiol content and a lack of aromatic amino acids. Due to the high thiol content, MT binds to a number of trace elements. MT plays a homeostatic role in the control of cellular zinc and copper concentration and participates in detoxification of heavy metals such as mercury and cadmium.

MT is also associated with metalloregulatory functions such as cell proliferation, growth and differentiation. In recent years, MT expression has been linked with carcinogenesis and tumor progression (196-198). In human malignant tumors, over-expression of the MT gene has been observed, however why this occurs is not yet known. It has been hypothesized that

somatic mutations in the MT gene might be responsible for over-expression of MT protein in neoplasia. Over-expression of MT occurs either by cis-acting mutations of the MT gene itself or by trans-acting mutation of other genes involved in controlling MT expression (199). Several studies have shown an association between MT over-expression and the grade of the tumor (199-201). MT over-expression has been found in various types of cancers such as breast cancer (197, 202-205), hepatic cancer (206), bladder cancer (207), pancreatic cancer (208), gastric cancer (209), esophageal cancer (210), and colorectal cancer (137, 199).

Recent studies have reported that the presence of MT-positive crypts in the mouse colon can be used as a marker of colon crypt stem cell mutations (199, 211, 212). A study by Jasani et al. (199) reported crypt-restricted immunopositivity for MT in human and mouse colon following administration of a carcinogen, dimethylhydrazine (DMH). The study found that the frequency and time course of the appearance of crypt-restricted MT positivity in mice treated with DMH was similar to that of glucose-6-phosphate dehydrogenase (G6PD). G6PD, an endogenous reporter gene, has been used in several studies as a marker to evaluate somatic mutation in stem cells. Mutation of the G6PD gene leads to a loss of its activity, and as a result, crypt-restricted phenotypic change occurs within the colonic crypt stem cells. Cook et al. (211) examined the dose response and time course of appearance of MT-positive crypts in a large group of Balb/c mice treated with a mutagen, N-ethyl-N-nitrosourea (ENU). The authors also examined the stability of MT-positive crypts 6 months after mutagen administration. The study reported that administration of ENU to mice created scattered crypt-restricted immunopositivity for MT. The frequency of crypt-restricted immunopositivity for MT increased proportionately with the dose of mutagen administered. The frequency of MT-positive crypts was stable for as long as 6 months after the mutagen

treatment. There was a strong correlation between MT and G6PD positivity ($r>0.9$). The authors speculated that the stable, crypt-restricted immunopositivity for MT in the mouse colon may be due to a mutation which affects expression of the MT gene in a colonic stem cell.

Overall a positive correlation between MT-positive crypts and loss of G6PD activity was observed in the studies discussed above. The results of these studies were consistent and show that, as with loss of G6PD activity, the frequency of MT-positive crypts is very low in control animals (mutagen untreated), but it increases proportionately in treated animals with the dose of mutagen administration. These studies show that the MT-positive phenotype develops over time after mutagen administration and stabilizes after a few weeks. As the effect of carcinogen treatment and time course of appearance of MT-positive crypts is similar to that found with G6PD activity, it appears that stable crypt-restricted immunopositivity for MT results from a mutation affecting expression of MT gene in a colonic stem cell.

Another study by Donnelly et al. (212) investigated the correlation of MT-positive crypts with ACF in Balb/c mice. Two mutagens, N-methyl, N-nitrosourea (MNU) and lambda carrageenan (λ CgN), were tested either alone or in combination. The total number of MT-positive crypts and ACF was determined. The study observed that a MT-positive phenotype in the colonic crypts developed by λ CgN alone. However, combined λ CgN/MNU treatment produced a greater number of MT-positive crypts in the mouse colon. The number of ACF and ACF crypt multiplicity was also greater in the combined λ CgN/MNU treatment group compared to the group treated with MNU alone. There was a strong correlation between MT-positive crypts number and multiplicity of ACF. The results of this study suggest that

The over-expression of MT in colonic mucosal cells in mutagen-treated animals indicates that MT immunopositivity in the colonic crypt cells can be used as a marker for crypt stem cell mutation. In addition to other morphological and biochemical markers, MT over-expression may be a useful early biochemical marker to assess the risk of colon cancer development. However, further research is required to understand the underlying mechanism through which MT relates to colon cancer development.

Chapter-2
First Study

**The Effect of Wheat Class and Processing State on Reducing
Colon Cancer Risk in the Early Promotion Stage**

Abstract

In a previous study in this laboratory red wheat flour was found to decrease precancerous lesions (aberrant crypt foci, ACF) relative to white flour. This study aimed to determine the effect of wheat color, hardness, and processed state on reducing ACF and sialomucin (SiM) producing ACF in early promotion stage of colon cancer. Rats adapted to an AIN-93G (basal) diet were administered the colon specific carcinogen, dimethylhydrazine, twice, a week apart. Rats were adapted to the basal diet for a week before and through the carcinogen treatment. Five days after the last dose of carcinogen, rats were divided into 9 groups. One group continued to be fed the basal diet and the rest were fed diets containing 61.5% of hard red, hard white, soft red, and soft white, in either the whole or refined state, for 7 weeks. ACF were significantly lower in whole and refined hard red, whole and refined soft white, and refined soft red groups compared to the basal diet group. There was a significant interaction between wheat hardness and color with hard red wheats having fewer AC and ACF than soft white wheats. SiM-producing ACF, which are suggested to be more advanced towards tumorigenesis, were reduced in all wheat flour diets compared to the basal diet. These results suggest that all wheat flours reduce colon cancer risk, regardless of type or processed state.

Introduction

Colorectal cancer is one of the major public health problems in North America and Western Europe. It is the third most common cancer and the second leading cause of cancer death in the United States, yet the etiology of this disease is unknown. Colorectal cancer risk rates vary by geography approximately 20 fold worldwide (3, 213), and the highest rates are seen largely in the developed countries such as North America, Western Europe and Australia. Populations that have previously been at low risk of colorectal cancer are now, with westernization, experiencing a greater incidence of this disease, with mortality rates rapidly increasing. Lower rates are observed among Caucasians of southern European origin but these tend to rise with migration (214). The studies of migrant populations suggest that the large international difference may be due to the change in dietary and other life-style factors (215).

Several observational studies have suggested that high intakes of whole grains, fruits and vegetables may lower the risk of colon cancer, whereas a high intake of red meat and fat may increase the risk of colorectal cancer (11, 28, 215, 216). Types of whole grains such as wheat, rice, oat and barley have been shown to be associated with a reduced risk of colon cancer (217). In a meta-analysis of whole grains intake and cancer (17), whole grains were shown to protect against colon cancer risk in 46 of 51 studies involving a high intake of whole grains and in 43 of 45 studies after exclusion of 6 studies which reported low whole grains intake or flaws in study design. Evidence from case-control studies suggest that a high intake of whole grains is associated with a lower risk of developing colon cancer (26, 34, 36).

Although there are inconsistencies among epidemiological studies, overall whole grains consumption has been found to be associated with a lower incidence of colon cancer compared to refined grains (17, 20, 21, 36). The reported odds ratios ranged from 0.5 to 1.48 in the studies which measured the intake of whole and refined grain foods. In these epidemiological studies, the intake of whole grains was recorded as whole or refined wheat bread, crispy bread or whole grain hot cereal, whereas intake of refined grains was recorded based on intakes of white bread, waffles, pancakes or spaghetti (16, 36). With regards to these studies, the participants consumed whole grains mainly in the form of bread. The studies which measured exclusively intake of whole-grain breads or whole-grain breads and pasta found that odds ratios were less than one. As whole grain bread is consumed in greater quantity than pasta, the protective effect is likely due to the intake of whole-grain bread.

When the independent effect of bread was analyzed it was observed that total bread intake (whole and refined bread) was associated with reduced risk of colorectal cancer (22, 31), whereas intake of rice, pasta and pastries was associated with increased risk of colorectal cancer (23, 24). The effect of bread was analyzed mainly by the processing of the wheat as either whole or refined. The effect of color of the wheats, red versus white, was not considered when a comparison was made. As whole or refined wheat bread and crispy bread are made mostly from red wheat, whereas white bread, waffles and pancakes are made from white wheat, it can be concluded that in these studies the actual effect of wheat processing might be confounded by the effect of wheat color. The results obtained in these epidemiological studies may represent an association between colon cancer incidence and consumption of hard red wheat.

While epidemiological studies support health-promoting benefits of whole grains, there are few experimental studies that have examined the effect of whole grains consumption on colon cancer risk (43). In contrast, there have been a number of experimental studies examining individual biologically active components of whole grains on reducing colon cancer risk (39-41). Of the whole grains, wheat is the world's largest crop, and the principal cereal grain in the United States (218). A variety of wheat fractions or components present in wheat have been shown to protect against colon cancer risk when fed in isolated forms (28, 46, 219). Of these fractions wheat bran has received the most attention. Dietary fiber from wheat bran has been shown to reduce colon cancer risk in several studies (44, 220, 221). In addition to dietary fiber, other components in wheats, such as lignans (53), ferulic acids (49, 50, 222), phytic acids (49, 179), lutein (223, 224), anthocyanidin and flavonoids (48, 56) have been shown to offer chemoprevention.

To date evidence from epidemiological and experimental studies support a relationship between consumption of whole grains, including wheats, and a reduced risk of colorectal cancer (225). It is not clear which constituents in whole grains offer the most significant protection against colon cancer, and it has been speculated that the benefits of the whole grains may be greater than any individual component (18, 40, 226). However, there are few experimental studies that have investigated the effect of intact whole and refined grains on colon carcinogenesis. A previous study in this laboratory (227) compared the effect of whole and refined wheat diets made from red or white wheat flour on colon carcinogen-treated rats. Rats fed red wheat flour, either whole or refined, had significantly fewer colonic pre-cancerous lesions, termed aberrant crypts, than those fed diets containing white wheat flour, regardless of the state of refinement. Consistent with these results, Maziya-Dixon and

colleagues (42) found reduced tumor number in carcinogen treated mice fed red wheat compared to white wheat, regardless of processing state. These animal studies suggest that red wheat has a greater chemopreventive effect than the white wheat. Based on these observations we chose to investigate the effect of diets containing wheats with different characteristics on reducing colon cancer risk in carcinogen treated rats. Aberrant crypt foci (ACF), a pre-neoplastic lesion, and sialomucin producing aberrant crypt foci (SiM-ACF), which are considered to be more dysplastic and more advanced towards tumorigenesis, were measured as the major endpoints at 7 weeks after carcinogen treatment, which we define as the early promotional stage of colon cancer.

Materials and Methods

Animals and Diets

Male Wistar rats, age 3-4 weeks, were purchased from Harlan Sprague Dawley (Indianapolis, IN). All rats were housed individually in wire cages in rooms maintained at $20 \pm 2^\circ\text{C}$ with a relative humidity of $50 \pm 10\%$, and a 12 hour light/dark cycle. Throughout the study food and water were available *ad libitum*. The study was approved by the University of Minnesota Committee on Animal Care and Use. Diet ingredients except the wheat flours were purchased from Harlan Tekland (Madison, WI). Whole and refined hard red wheat was a gift of General Mills (Minneapolis, MN). Whole soft red wheat was milled locally. Refined soft red wheat was purchased from Minnel Milling Co. (Fostoria, OH). Whole and refined hard white wheats were purchased from North Dakota State University (Fargo, ND). Whole and refined soft white wheats were purchased from King Milling Co. (Lowell, MI). The basal diet was a modification of the AIN-93G, a purified rodent diet (228). The 8 experimental diets contained flours made from one of the following wheats:

- Whole hard red
- Refined hard red
- Whole hard white
- Refined hard white
- Whole soft red
- Refined soft red
- Whole soft white
- Refined soft white

This allowed for a systematic comparison of three wheat characteristics color (red vs white), hardness (hard vs soft) and processing state (whole vs refined). The flours were stored at -20°C until incorporated into the diets.

Table 2-1 shows the proximate analysis of each wheat flour. All the experimental diets contained 61.5% of wheat flour and the wheat based diet formulation was based on the AIN-93G diet. Based on the composition of the wheat flours, all diets were matched for carbohydrate, protein, total dietary fiber, and fat. Diets were freshly prepared every 2 weeks and kept refrigerated. Table 2-2 shows the composition of each wheat flour based diet.

Experimental Design

Rats were adapted to the basal diet for one week upon arrival. The colon carcinogen 1, 2-dimethylhydrazine (DMH) was then administered subcutaneously twice, a week apart, at a dose of 50 mg/kg body weight. During carcinogen treatment all rats were fed the same basal diet. Five days after the last dose of carcinogen, rats were randomly divided into nine groups of 12 animals each, as described above. One group continued to be fed the basal diet (control group) and the remaining 8 groups were fed the experimental wheat diets. Rats were fed for an additional 7 weeks prior to sacrifice. Body weight and food intake was recorded weekly throughout the study.

Colon Sample Preparation

Rats were anesthetized with isoflurane, opened by laparotomy, and colons removed. Colons were flushed with PBS (pH 7.4). The colons were gently slid onto a 2 ml glass pipette

starting from the cecal end and completely submerged in 10% formalin in PBS for 5 minutes. Colons were then cut open longitudinal and washed with PBS. A five cm section of the distal colon, 2 cm apart from the anal end, was fixed flat between filter paper. The colon was then submerged in 10% formalin-PBS for overnight at 4°C. The colon tissues were coded in order to allow unbiased enumeration of aberrant crypts foci (ACF).

Determination of Aberrant Crypt Foci (ACF)

The formalin fixed colon tissue was stained with 0.2% methylene blue (Sigma Chemical Co, St Louis, MO) for 5 min with gentle shaking. Colon tissue was then washed with distilled water, transferred to a clean dish and kept moist with distilled water. The mucosal side of the tissue was examined under a stereomicroscope at a magnification of 100X (Olympus SZX, Olympus Optical Co, Tokyo, Japan). The total number of AC and ACF were counted by a modification of the method of Bird (97). After counting the total AC and ACF, colons were stored at 4°C in 10% formalin solution until analyzed for mucin production.

Determination of Mucin Production

Mucin production was analyzed by staining the colon tissue with a high-iron diamine alcian blue stain (HID-AB) (122). The formalin-fixed colon tissue previously stained with methylene blue for ACF visualization was rinsed in distilled water for 5 minutes and then transferred to a staining dish containing freshly prepared iron-diamine solution. Colon tissue was incubated in the dark for 18 hours in iron-diamine solution. After incubation colon tissue was rinsed three times in distilled water and stained for 30 minutes with 1% alcian blue (Sigma) in 3% acetic acid solution. Colon tissue was then rinsed three times in 80% ethanol followed by distilled water. The HID-AB stained colon tissue was examined at 100X

magnification under a microscope (Olympus BX 40, Olympus Optical Co, Tokyo, Japan). Scoring was carried out according to the criteria described by Caderni and Giannini (122).

Statistical Analyses

All diet groups together were analyzed by one-way analysis of variance using SAS system for windows, release 8.2 (SAS Institute, Cary, NC). All p values reported were adjusted for multiple comparisons using Duncan's multiple range test. Three way analysis of variance (ANOVA) was used to interpret a systematic examination of three wheat characteristics- processing state (whole vs refined), hardness (hard vs soft), and color (red vs white).

Results

Body Weight and Food Intake

The initial and final body weights and average food intakes are summarized in Table 2-3. Weekly recorded body weight (data not shown) including final body weight did not differ significantly among the nine animal groups. Food intake was similar in all diet groups throughout the study, indicating that the animals fed the wheat diets consumed diet and gained weight equivalent to the basal diet group.

Effect of Wheat Diets on AC and ACF Number

All wheat-fed groups showed a numerical reduction in the number of AC (Figure 2-1) and ACF (Figure 2-2) per cm² of colon tissues. Groups fed whole and refined hard red, whole and refined soft white and refined soft red showed a statistically significant reduction in AC and ACF relative to the basal diet (AIN-93G) group. Table 2-4 shows the number of AC and ACF in all diet groups. Although there was a trend for whole and refined hard white and whole soft red towards fewer ACF compared to the control group, these differences were not statistically significant. There were no significant differences among groups in large ACF, defined as ACF with 4 or more AC in a focus. Three-way analysis of variance (ANOVA) indicated a significant interaction between color and hardness of the wheat flour. That is, the effect of color was dependent on the hardness of the wheat, indicating that when the wheat was hard and red it was more protective than when the wheat was soft and white. Figure 2-3 shows interaction plots of total ACF of the eight experimental wheat diet groups. Processing state had no influence on the indicators of colon cancer risk. Probability values for the main effects of the wheat flour- based experimental diets are shown in Table 2-5.

Effect of Wheat Diets on Sialomucin Producing ACF (SiM-ACF) Number

In all wheat fed groups, total number of sialomucin producing ACF (SiM-ACF), which are suggested to be more advanced towards tumorigenesis, was significantly fewer compared to the basal diet group (Figure 2-4). This suggests that all types of wheat flour reduce colon cancer risk, regardless of their color, hardness, or processed state when fed in the early promotion stage of carcinogenesis. Table 2-6 shows the effect of wheats with different characteristics on the total number of SiM-ACF and a combination of sialo and sulfomucin producing ACF. Statistical analysis by three way analysis of variance indicated that there were no significant main effects of the wheat diets on reducing SiM-ACF (Table 2-5).

Discussion

Whole grains are rich in nutrients and phytochemicals, which are thought to be protective against a number of chronic diseases such as cancer, cardiovascular disease, diabetes and obesity. Epidemiological studies have observed that diets with high whole grain intakes are associated with a reduced risk of several types of cancer including colorectal cancer. In epidemiological studies, it is difficult to assess whole grain intake because existing instruments for measuring food frequency are not designed to measure an individual dietary component. Thus, to identify whole grain intake, various phrases have been used such as whole grain bread, whole grain cereal, high fiber cereal, dark bread, brown bread, whole grain pasta and whole-meal bread (16, 17). Based on these classifying categories researchers have attempted to distinguish frequent consumers of whole grain products from occasional consumers of whole grain products.

Among the grains, wheat is the principal grain produced worldwide and accounts for one-third of the total cereal grain produced. In the United States, wheat contributes about 71% of total grain consumption. There are six major classes of wheats found in the United States. They fall into three major categories such as hard wheats, soft wheats and durum wheats. Hard wheats include hard red winter, hard red spring and hard white spring. Hard wheats are high in protein and have the ability to absorb large amounts of water. They are the preferred wheat class to make rolls and yeast breads. Soft wheats include soft white winter and soft red winter. They have lower content of protein and are the preferred class for making pastries, cakes, cookies, and snack foods.

In a meta-analysis of whole grain intake and colon cancer risk, whole grain foods were defined as whole-grain bread, whole-grain bread or pasta, whole-meal bread, whole grains and whole-grain foods. The majority of the case-control studies included in the meta-analysis collected eating frequency and quantified food preferences and food intake based on whole and refined grains food. The intake of whole grain food was mainly recorded as whole wheat and crispy bread or whole grain hot cereal, whereas refined grain bread was recorded based on intakes of white bread, pancakes, waffles and spaghetti. In the case-control studies which monitored the relation between food types and colorectal cancer, bread was the major source of whole grains intake of the participants. Overall high whole grains intake was inversely associated with colon cancer risk. However, given the categories used to classify whole and refined foods, it may be that the chemopreventive association with high whole grains intake is more related to high bread consumption than either the whole or refined processing state of the grain. As hard red wheat is the preferred class of wheat to make bread and rolls, from the results of case-control studies, it can be summarized that the association of whole grain consumption on reducing colorectal cancer risk is equally likely be an association with hard red wheat (21, 22, 43).

In regards to whole grains and colon cancer, most animal studies examined a single component of the grains such as fiber, phytic acids, and phenolic compounds as opposed to the whole grains, to understand the chemopreventive mechanism (217, 229, 230). Unlike epidemiological studies, there are only a few animal studies that have investigated the effects of whole grains with respect to wheats on colon cancer risk. The first study, conducted by Maziya-Dixon and colleagues in 1994 (42), examined the effect of different classes of wheat in the chemically induced colon tumor in the CF1 mouse model. The mice were fed diets

containing 50% of either whole or refined hard red winter and whole and refined hard white winter wheat during the initiation and progression stage of colon carcinogenesis. The study found that mice fed either whole or refined red wheat had a significantly reduced tumor incidence (50%) compared to mice fed either whole or refined hard white wheat diets (72%). However, there was no effect of processing of wheat in reducing tumor incidence in mice.

Consistent with these results, a previous study in our laboratory by Iovinelli and Gallaher (227) observed a similar effect of hard red wheat diet on carcinogen treated rats. The study examined the effect of wheat class and processing on reducing precancerous lesions during initiation and early promotion stage of colon carcinogenesis. Rats treated with a colon carcinogen were fed either whole or refined hard red winter or soft white winter wheat diets. The study observed that rats fed diets containing hard red wheat flour, whether whole or refined, had significantly fewer AC and ACF than those fed whole or refined soft white wheat diets. The hard red wheat diet groups also had reduced crypt multiplicity compared to the soft white groups. Thus, using different animal models and endpoint measures, both studies identify wheat class, not processing, as a significant factor in reducing colon cancer risk.

Another study by Yu and colleagues investigated the effect of only soft white wheat flour in its unrefined (whole) and refined forms compared to the AIN-93G diet in the carcinogen treated F344 rat. Diets containing refined wheat or unrefined (whole) wheat were examined for their ability to inhibit IQ-induced colonic aberrant crypt foci (ACF) in the Fischer 344 rat model. The study failed to determine any significant difference between the unrefined (whole) and refined soft white wheat diets and AIN-93G diet in terms of AC and ACF numbers per colon tissue (43). In regards to soft white wheat diet, no significant effect in

reducing precancerous lesions was observed in this animals model, indicating soft white wheat, either whole or refined, does not reduce colon cancer risk. On the other hand, results from the above mentioned studies show hard red wheat significantly reduce the number of precancerous lesions and thus offers a chemopreventive advantage.

This raises the question as to what is present in red wheat that is providing chemoprevention. Several studies evaluated the chemo-preventive properties of phytochemicals in different cultivars of wheats (2, 47, 223). These studies found that the concentration of phytochemicals, which have been found to be chemo-preventive, varies among wheat cultivar but the difference in phytochemical concentrations was not influenced by the color of wheat. Total flavonoids, ferulic acids, carotenoids and total lipophilic antioxidant activity were significantly higher in one white wheat than the two red wheats examined. In contrast, lutein content and total hydrophilic antioxidant activities were higher in red wheat than white wheat. Both epidemiological (55) and animal studies (224) showed dietary lutein to be inversely related to colon cancer risk. Further analysis of phytochemicals and structural differences between wheat classes may help to elucidate underlying chemo-preventive mechanisms of wheats.

In the present study, we measured AC and ACF as precancerous lesions in the early promotion stage of colon carcinogenesis in a rat model (97, 122). ACF have been widely used as early markers of colon cancer risk since they were first reported by Bird (231). In our study, the total numbers of AC and ACF were lower in the groups fed whole and refined hard red, whole and refined soft white, and refined soft red wheat diets. This effect was not influenced by the processing state of wheat flours. The data obtained from this study is consistent with other animal studies (42, 227) where a hard red wheat diet was shown to be

protective, based on fewer ACF number, compared to a soft white wheat diet, regardless of processing. Our statistical analysis of ACF number indicates that there was an interaction between the color and hardness of the wheat, suggesting that when the wheat is hard and the color is red it was more protective than when the wheat is soft and white.

ACF have been identified as either hyperplastic or dysplastic depending on their crypt structure and nuclear features (105, 123). Hyperplastic ACF show an increase in the number of cells per crypt, however they have normal goblet cell formation, and normal cell and nuclear differentiation. In contrast, dysplastic ACF show loss of goblet cell differentiation, distortion of crypt structure, and extensive nuclear stratification (105, 123). Several researchers have suggested that other morphological markers be used in addition to ACF, such as those indicating the higher degree of dysplasia. Recently, sialomucin producing ACF (SiM-ACF), a morphological marker easily identified in whole mounts of colon, have been used by several researchers as a colon cancer marker (122, 123). These SiM-ACF are dysplastic in nature and have been suggested to be more indicative of ACF that are likely to progress to tumors. Several studies suggest that dysplastic ACF, which represent only a small fraction of total ACF, are direct precursors of adenomas and carcinomas (115, 132). In the present study, it was observed that all the wheat diets significantly reduced the number of SiM-ACF compared to the control diet. There were no main effects of the wheat diets on SiM-ACF, suggesting that no one characteristic of wheat was more important than other.

In conclusions, this study suggests that hard red wheat consumption may reduce the risk of colon cancer in early promotion stage of carcinogenesis, based on ACF number. Statistical analysis shows that whole wheat does not differ from refined wheat in reducing morphological markers of colon cancer risk. Statistical analysis of ACF number shows a

significant interaction between color and hardness of wheat. This indicates the hard red wheat is more protective than the soft white wheat. The underlying mechanism of this protective effect of hard red wheat needs further investigation. Further experimental studies are required to identify grain consumption in relation to wheat class and to elucidate why hard red wheat has a greater chemo-preventive effect relative to soft white wheat.

Table 2-1. Proximate Analysis of Wheat Flours ^a

Flour	Carbohydrate (%)	Protein (%)	Fiber (%)	Fat (%)
Whole Hard Red	74.5	11.9	11.6	2.37
Refined Hard Red	74.4	10.4	3.2	1.38
Whole Hard White	72.5	13.1	11.3	2.50
Refined Hard White	74.1	12.0	2.2	1.09
Whole Soft Red	74.3	9.5	9.0	2.64
Refined Soft Red	77.4	8.33	2.8	1.55
Whole Soft White	72.8	9.71	12.6	3.02
Refined Soft White	77.0	8.62	3.0	1.70

^a Proximate analysis of wheat flours was performed by Medallion Laboratories (Golden Valley, MN)

Table 2-2. Diet Composition (g/kg)

Diet	Basal	Whole Hard Red	Refined Hard Red	Whole Hard White	Refined Hard White	Whole Soft Red	Refined Soft Red	Whole Soft White	Refined Soft White
Flour	0	615	615	615	615	615	615	615	615
Sucrose	100	91.8	55.4	90.1	57.3	91.8	38.7	102.3	45.2
Corn Starch	448.2	0	0	0	0	0	0	0	0
Casein	200	126.9	136	136	126.2	141.5	148.8	140.3	147
Cellulose	80	8.7	60.3	10.5	66.5	24.7	62.8	2.5	61.5
Mineral Mix	35	35	35	35	35	35	35	35	35
Vitamin Mix	10	10	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Butylated Hydroxytoluene	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Soybean Oil	120	105.8	81.5	113.1	83.3	75.3	82.9	88.1	79.6
Cholesterol	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
% CHO	55	55	51	55	51.3	55	51.4	55	51.8
% Protein	20	20	20	20	20	20	20	20	20
% Fiber	8	8	8	8	8	8	8	8	8
% Fat	12	12	9	12.8	9	9	9.2	10.6	9

Table 2-3. Body Weight and Average Food Intake of Animals ^a

Diet Group	Initial Body Weight (g)	Final Body Weight (g)	Food Intake (g/day)
Basal	70.6 ± 0.9	458.8 ± 12.9	26.7 ± 0.4
Whole Hard Red	62.1 ± 0.9	488.8 ± 12.9	22.9 ± 0.2
Refined Hard Red	59.0 ± 1.3	491.3 ± 9.5	20.3 ± 0.2
Whole Hard White	61.9 ± 1.5	483.5 ± 8.5	22.2 ± 0.2
Refined Hard White	59.8 ± 4.9	485.4 ± 8.5	26.6 ± 0.4
Whole Soft Red	63.0 ± 4.1	483.5 ± 15.9	25.3 ± 0.6
Refined Soft Red	61.0 ± 0.9	478.7 ± 11.8	29.2 ± 0.6
Whole Soft White	60.1 ± 1.5	494.2 ± 14.4	28.4 ± 0.7
Refined Soft White	62.2 ± 1.4	479.2 ± 14.2	26.4 ± 0.4

^a Values are reported as mean ± SE, n=12 per group

Table 2-4. Effect of Different Varieties of Wheat on AC and ACF Number of Rat Colon

^a

Diet Group	AC/cm²	ACF/cm²	Large ACF^b/cm²
Basal	17.6 ± 3.1	8.1 ± 4.9	1.1 ± 0.5
Whole Hard Red	9.9 ± 1.6	4.5 ± 0.7*	0.7 ± 0.1
Refined Hard Red	7.7 ± 1.4*	3.6 ± 0.6*	0.4 ± 0.1
Whole Hard White	12.3 ± 1.9	5.3 ± 0.8	0.9 ± 0.2
Refined Hard White	10.9 ± 1.4	4.9 ± 0.6	0.6 ± 0.1
Whole Soft Red	11.9 ± 1.6	5.2 ± 0.6	0.8 ± 0.5
Refined Soft Red	9.7 ± 1.1*	4.4 ± 0.4*	0.6 ± 0.1
Whole Soft White	8.8 ± 1.3*	3.9 ± 0.5*	0.6 ± 0.1
Refined Soft White	8.6 ± 1.8*	3.9 ± 0.7*	0.6 ± 0.2

^a Values are reported as mean ± SE, n=12

^b Four or more AC in a foci considered as large ACF

* Values are significantly different from basal diet group (p<0.03)

Table 2-5. Main Effects on Cancer Risk Markers of Different Varieties of Wheat

Main Effects	AC/cm²	ACF/cm²	Large ACF/cm²	SiM/cm²
p value				
Process	0.179	0.253	0.084	0.314
Hardness	0.689	0.600	0.869	0.628
Color	0.756	0.805	0.672	0.789
Process*Hardness	0.799	0.836	0.483	0.143
Process*Color	0.527	0.471	0.712	0.951
Hardness*Color	0.028	0.036	0.127	0.899
Process*Color*Hardness	0.793	0.903	0.822	0.996

Table 2-6. Effect of Different Varieties of Wheat on SiM Producing ACF Number of Rat Colon ^a

Diet Group	SiM-ACF/cm²	(SiM+SuM)-ACF/cm²
Basal	1.6 ± 0.4	1.6 ± 0.3
Whole Hard Red	0.30 ± 0.08*	1.1 ± 0.3
Refined Hard Red	0.47 ± 0.1*	0.9 ± 0.2
Whole Hard White	0.29 ± 0.07*	1.1 ± 0.3
Refined Hard White	0.46 ± 0.07*	1.1 ± 0.2
Whole Soft Red	0.45 ± 0.11*	1.2 ± 0.2
Refined Soft Red	0.41 ± 0.09*	1.0 ± 0.2
Whole Soft White	0.42 ± 0.11*	1.0 ± 0.2
Refined Soft White	0.39 ± 0.13*	1.1 ± 0.3

^a Values are reported as mean ± SE, n=12. Abbreviations: SiM-ACF, sialomucin producing ACF and (SiM+SuM)-ACF, a combination of sialo and sulphomucin producing ACF

* Values are significantly different from basal diet group (p<0.0003)

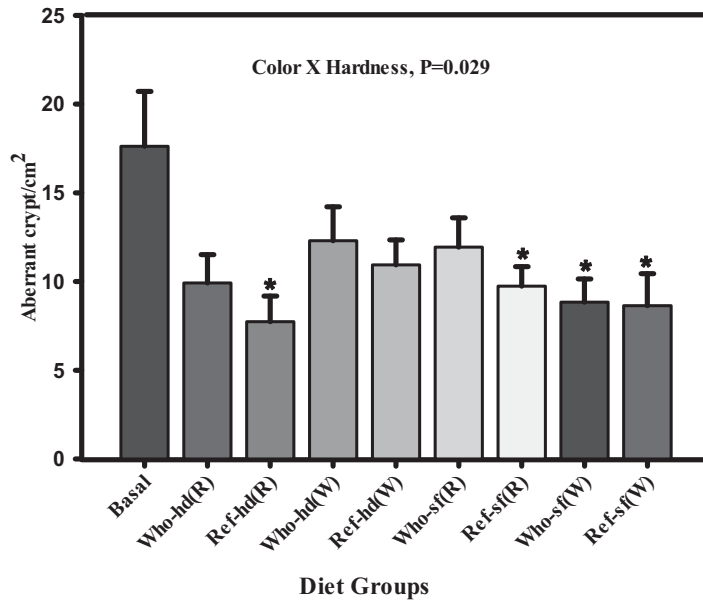


Figure 2-1. Total aberrant crypt/cm² of rat colon during the early promotion stage of colon cancer

Abbreviations: Who-hd(R), whole hard red; Ref-hd(R), refined hard red; Who-hd(W), whole hard white; Ref-hd(W), refined hard white; Who-sf(R), whole soft red; Ref-sf(R), refined soft red; Who-sf(W), whole soft white; Ref-sf(W), refined soft white

Values are represented as mean ± SE, n=12

* Bars with asterisks are identified as statistically significant different from the basal diet

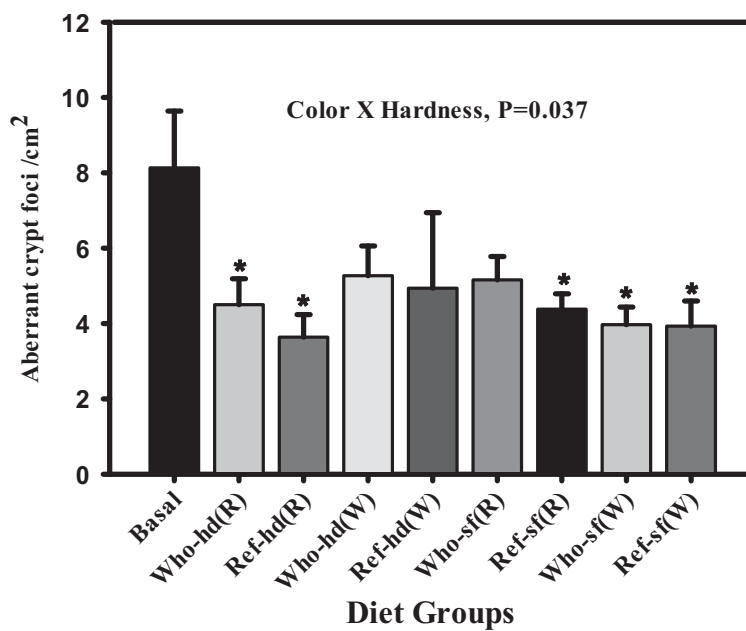


Figure 2-2. Total aberrant crypt foci/cm² of rat colon during the early promotion stage of colon cancer

Abbreviations: Who-hd(R), whole hard red; Ref-hd(R), refined hard red; Who-hd(W), whole hard white; Ref-hd(W), refined hard white; Who-sf(R), whole soft red; Ref-sf(R), refined soft red; Who-sf(W), whole soft white; Ref-sf(W), refined soft white

Values are represented as mean ± SE, n=12

* Bars with asterisks are identified as statistically significant different from the basal diet (p =0.035)

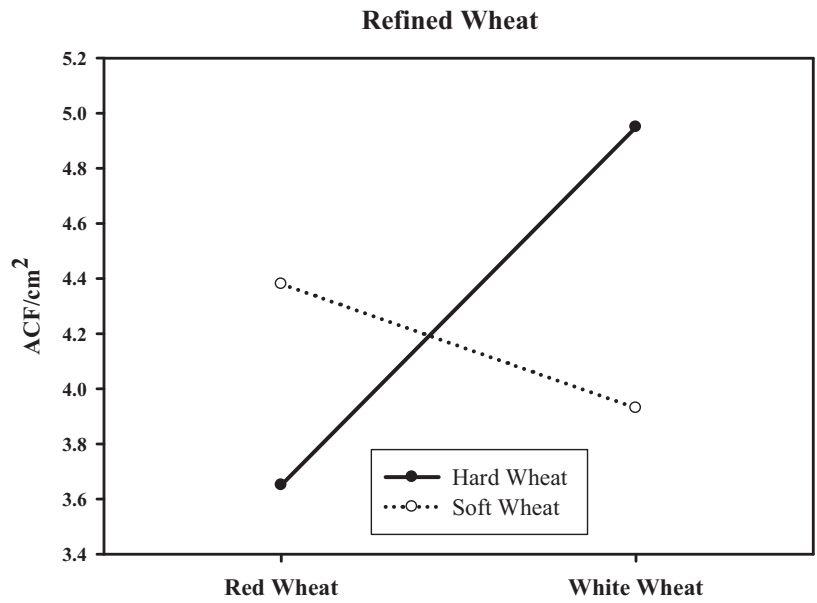
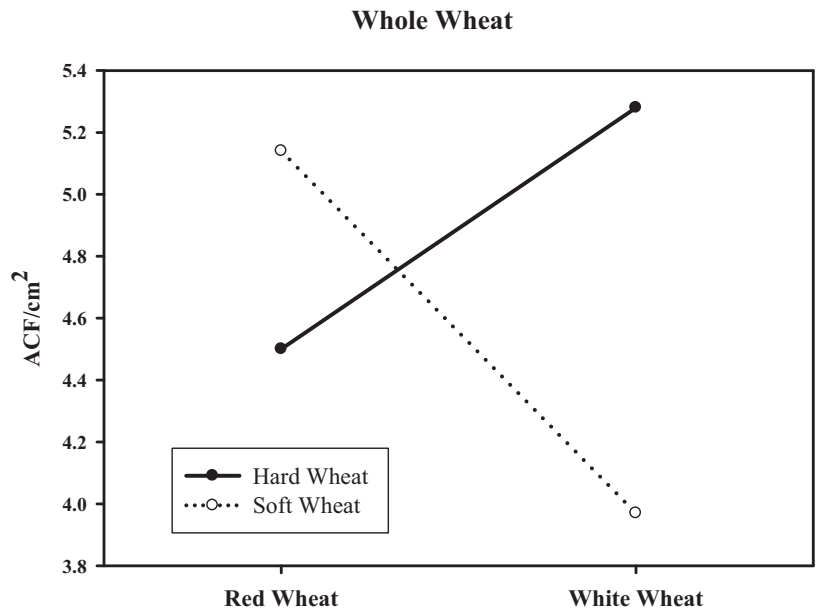


Figure 2-3: Interaction plot of total aberrant crypt foci (ACF) of the wheat diets.

Interaction plot indicates when wheats are red, hard wheat has decreased ACF and when wheats are white, hard wheat has increased ACF

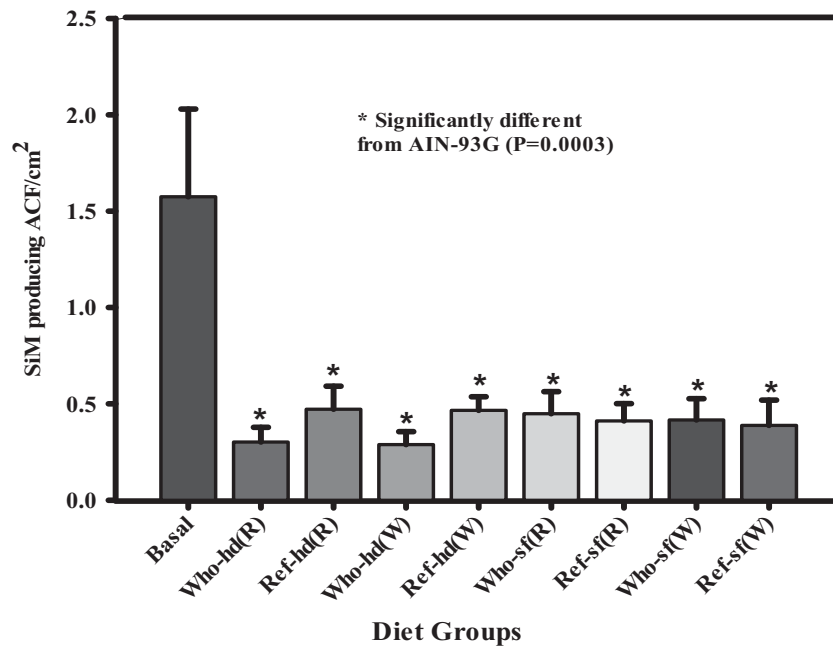


Figure 2-4: Total sialomucin producing ACF/cm² (SiM-ACF) of rat colon during the early promotion stage of colon cancer

Abbreviations: Who-hd(R), whole hard red; Ref-hd(R), refined hard red; Who-hd(W), whole hard white; Ref-hd(W), refined hard white; Who-sf(R), whole soft red; Ref-sf(R), refined soft red; Who-sf(W), whole soft white; Ref-sf(W), refined soft white

Values are represented as mean ± SE, n=12

* Bars with asterisks are identified as statistically significant different from the basal diet (p =0.0003)

Chapter-3
Second Study

**The Effect of Wheat Class and Processing State on Reducing
Colon Cancer Risk in the Late Promotion Stage**

Abstract

Previously our laboratory found hard red wheat flour to reduce colonic precancerous lesions, aberrant crypt foci, (ACF) relative to soft white wheat flour, regardless of processing (whole vs. refined), during the initiation and early promotion stage of colon cancer development. Here we report the effect of wheat class and processing on regression of established ACF and sialomucin producing ACF (suggested to have greater tumorigenic potential) in the late promotion stage of colon cancer. Rats were adapted to a basal (AIN-93G) diet for 10 days then given dimethylhydrazine twice, a week apart. Rats continued to be fed the basal diet throughout carcinogen treatment and for an additional 6 weeks. One group of rats was killed (initial basal diet group) and the remaining rats divided into 5 groups (n=15), and fed either the basal diet (final basal diet group) or diets containing 61.5% of either whole hard red, refined hard red, whole soft white or refined soft white for 8 more weeks. Sialomucin producing ACF were reduced in both hard red and soft white diet groups' compared to the final basal diet group. There were fewer ACF in both whole hard red and refined hard red wheat diets compared to either initial or final basal diet groups. Rats fed diets containing hard red wheat flour had fewer AC, ACF and sialomucin producing ACF compared to soft white wheat flour, regardless of processing state. Further, rats fed diets containing hard red wheat flour had fewer ACF than the initial basal diet group. Thus hard red wheat, whether whole or refined, caused regression of ACF in rats, suggesting that hard red wheat can reduce a state of high colon cancer risk to a lower one.

Introduction

Colorectal cancer is the third most common cancer and second leading cause of cancer deaths in the United States. It represents about 13.5% of all incident cancer in men and women. The exact etiology of colorectal cancer is not known, although major factors thought to influence the risk of colorectal cancer development include diet, life-style, family history and age. Evidence from epidemiological and experimental studies suggest a relationship between diet and colorectal cancer risk. Risk factors for colon cancer identified through epidemiological studies include reduced consumption of whole grains, fruits and vegetables (18, 24, 30, 36, 46, 219, 232, 233), and increased consumption of red meat and animal fat (234, 235). Of whole-grain foods, increased consumption of whole wheat bread or cereal has shown to be associated with reduced risk of colorectal cancer (15, 17, 25, 26).

Epidemiological studies suggest that consumption of whole grains offers protection against colon cancer (17, 18, 226). A meta-analysis of case-control studies of 20 different types of cancer and colon polyps found that those with high whole grains intake had 34% lower risk of overall cancer than those with low whole grains intake. In this meta-analysis, 43 of 45 studies had an odds ratio below one, indicating an association between whole grains consumption and reduced risk of various types of cancer. The majority of the studies included in the meta-analysis examined the relation between whole grains and colorectal cancer and/or colon polyps and in 9 of 10 studies odds ratio were less than 1, supporting a positive effect of whole grains in reducing colon cancer risk.

The case control studies examined in the meta-analysis of whole grain intake and colon cancer risk defined 'whole grains' as whole grains bread, whole grains pasta, whole-meal bread, whole grains and whole grains foods (15, 17, 22, 35, 225, 236). Bread was found to be the major source of whole grains intake in the case control studies that quantified only

whole grains and whole-grain foods intake by the subjects. Thus, whole grains consumption measured in a population may be more related to high bread consumption, whether refined or whole, versus whole grains consumption. Hard red wheat is the preferred class of wheat used to make breads and rolls because of its high protein content and ability to absorb large amounts of water, while soft wheat is the preferred class of wheat for making cookies, cakes, pastries, crackers and snack foods. Whole grain breads are usually made from hard red wheat. Thus the results of case-control studies may suggest that the association between whole grains and colon cancer risk can be attributed to hard red wheat.

The effects of individual components of whole wheat on colon cancer development have been investigated in a number of experimental studies. However, only a few experimental studies have examined the effect of different varieties of whole and refined wheat on colon cancer risk. A previous study in our laboratory by Iovinelli and Gallaher (227) investigated the effect of two varieties of wheats, hard red winter and soft white winter on colon cancer risk in carcinogen-treated rats. The study observed that rats fed hard red wheat flour, whether whole or refined, had significantly fewer precancerous lesions, aberrant crypt foci (ACF) compared to rats fed whole or refined soft white wheat flour. The hard red wheat flour group also had lower multiplicity of ACF than the soft white wheat flour group. The effect of wheat flour on reducing colon cancer development was observed as a result of wheat class but not for state of refinement. The results of this study were consistent with the finding of Maziya-Dixon and colleagues (42), who also observed an effect of a hard red wheat diet on reducing colon tumorigenesis in carcinogen-treated mice, regardless of processing. The mice were fed whole or refined hard red or hard white winter wheat. Mice fed the hard red wheat diets had a significantly lower incidence of tumor development compared to the mice fed the

hard white wheat diets. Thus, two experimental studies have observed that wheat class but not processing has a significant effect on colon cancer risk using different animal models and end point measures. These studies of colon cancer prevention have indicated that hard red wheat is protective relative to white wheat. However, from these studies, it is not known which constituents of the hard red wheat are responsible for prevention of colon cancer development. If a particular class of wheat is established to have greater chemo-preventive effect than the others, it would be a simple public health measure to increase its use in foods to increase consumption of it on a regular basis (42, 227).

Based on the finding of these two studies, we choose to further investigate the effect of hard red and soft white wheat diets, in both the whole and refined form, on reducing colon cancer risk in the promotion stage of colon carcinogenesis. The study was designed to examine the effect of wheat diets in the late promotion stage of colon carcinogenesis. During the first study of this thesis project the effect of four major classes of wheats such as hard red, hard white, soft red and soft white was evaluated during the early promotion stage of colon cancer development. Hard red wheat flour reduced the number of precancerous lesions regardless of processing. This second study of this project is focused on the late promotion stage of colon carcinogenesis and the effect of class (hard red vs. soft white) and processing (whole vs. refined) of wheat flours on aberrant crypt foci (ACF) and sialomucin-producing ACF (SiM-ACF) number.

Colon carcinogenesis involves clonal expansion of mutated colonic crypt cells generally termed as pre-cancerous lesions (104). Since the discovery of these pre-cancerous lesions, more commonly known as aberrant crypt foci (ACF) (97), they have been used by many researchers as an early biomarker of colon carcinogenesis to evaluate the effect of a variety

of chemo-preventive agents. However, in some studies, the lack of correlation of ACF with tumor development has challenged ACF as a reliable biomarker (100, 109). In contrast, other studies observed a positive correlation between number of ACF and colon tumors (103, 108, 237). These conflicting results suggest that the number of ACF alone might not be appropriate to use as a reliable biomarker of colon cancer. Therefore, in addition to ACF, several other biomarkers have been considered, of which sialomucin producing ACF (SiM-ACF) and mucin depleted foci (MDF) have received particular attention (121, 122, 125). SiM-ACF are characterized by the production of mucin containing sialic acid. MDF are characterized by the absence of mucin production. Both of these types of ACF have a higher rate of cell proliferation and higher degree of dysplasia compared to ACF that produces sulphomucins, the normal form of mucin, and therefore are suggested to be more prone to tumor development. In our present study, we compared the effect of hard red and soft white wheat diets on reducing these morphological markers in addition to ACF in the late promotion stage of carcinogenesis.

Studies have shown that after carcinogen treatment the number of ACF varies with time in the colon. The number of ACF tended to increase with time, however the increase in ACF number was not linear but instead fluctuated. Based on this observation, it has been suggested that ACF remain in dynamic state and may remodel or regress (238). Thus another objective of this study was to examine the effect of wheat type and processing on regression of established ACF, which would suggest a change from a high to low risk state of colon cancer development.

Materials and Methods

Animals and Diets

Ninety male Wistar rats, weighing 50-75 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN). All rats were housed individually in wire cages with flat wire bottom and kept in a 12 hour light/dark cycle. The rooms were maintained at $20 \pm 2^{\circ}\text{C}$ with a relative humidity of $50 \pm 10\%$. Throughout the study rats had free access to diets and distilled water. The study was approved by the University of Minnesota Committee on Animal Care and Use. All diet ingredients were purchased from Harlan Tekland (Madison, WI). Whole and refined hard red wheat flour was a gift of General Mills (Minneapolis, MN). Whole and refined soft white wheat flour was purchased from King Milling Co. (Lowell, MI). Rats were divided into 6 groups (15 rats per group). Two groups received the basal diet (AIN-93G) and the remaining 4 groups received one of following wheat flour based experimental diets:

- Whole hard red
- Refined hard red
- Whole soft white
- Refined soft white

All the wheat flours were stored at -20°C until incorporated into the diets. Table 3-1 shows the proximate analysis of each wheat flour. Each experimental diet consisted of 61.5% of wheat flour by weight and the formulation of each diet was based on the AIN-93G diet (228). Diets were freshly prepared every two weeks and kept refrigerated. Table 3-2 shows the composition of all diets.

Experimental Design

Rats were adapted to the basal diet for 10 days after arrival. Rats were then injected twice with 1, 2 dimethylhydrazine (DMH) (50 mg/kg body weight) subcutaneously one week apart. Rats continued to be fed the basal diet during and six weeks after the carcinogen treatment. One group of rats (initial basal group) was killed after six weeks of carcinogen treatment. The remaining rats were divided into 5 groups. One group continued to be fed the basal diet (final basal group) and the remaining animals were randomized into the four experimental wheat based diets for 8 more weeks prior to sacrifice. Figure 3-1 shows a schematic representation of the feeding regimen. Body weights were recorded weekly and food intake was recorded bi-weekly throughout the study.

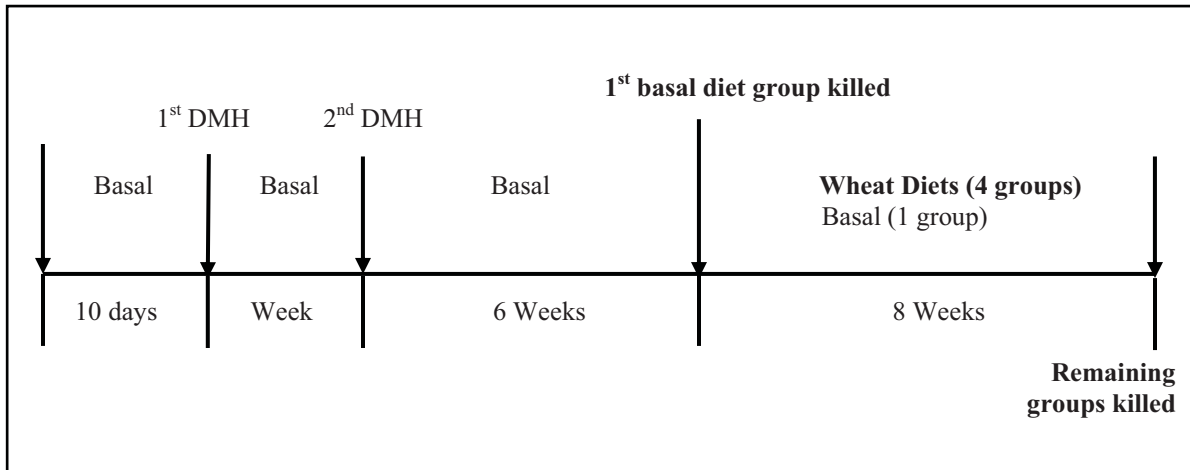


Figure 3-1. Schematic representation of the feeding regimen

Colon Sample Preparation

Rats were anesthetized using isoflurane, dissected and colons removed. Colons were flushed with cold PBS (pH 7.4). The colons were gently slid onto a 2 ml glass pipette starting from the cecal end and completely submerged in 10% formalin in PBS for 5 minutes. The colons were cut open with a razor blade longitudinally and washed with PBS buffer several times. A

two cm section of the anal end of the colon and the whole proximal end of the colon were removed. In order to fix the colon tissue, the distal colon was placed flat between filter papers saturated with 10% formalin-PBS and stored overnight in an airtight container at 4°C. All colons were individually coded to allow an unbiased enumeration of ACF.

Determination of Aberrant Crypt Foci (ACF)

After overnight fixation in formalin, colon tissue was stained with 0.2% methylene blue (Sigma Chemical Co, St Louis, MO) for 5 min with gentle shaking. Colon tissue was then washed with distilled water to remove excess methylene blue, transferred to a clean dish, and submerged into distilled water to avoid drying. The mucosal side of the tissue was examined under a stereomicroscope at a magnification of 100X (Olympus SZX, Olympus Optical Co, Tokyo, Japan). Using a modification method of Bird (97) the total number of AC and ACF were counted. Colon tissue was then stored at 4°C in 10% formalin solution until analyzed for mucin production.

Determination of Mucin Production

The formalin fixed colon tissue was rinsed in distilled water for 5 minutes to remove excess formalin. The colon tissue was incubated in the dark for 18 hours in freshly prepared iron-diamine solution. After incubation colon tissue was rinsed three times in distilled water and stained with 1% alcian blue (Sigma Chemical Co, St Louis, MO) in 3% acetic acid solution for 30 minutes. Colon tissue was then rinsed three times in 80% ethanol followed by distilled water. The colon tissue was examined under the microscope at a magnification of 100X (Olympus BX 40, Olympus Optical Co, Tokyo, Japan) and was scored according to the criteria described by Caderni (122).

Determination of Mucin Depleted Foci (MDF)

Mucin depleted foci (MDF) are characterized by the absence or inadequate production of mucin by the aberrant crypt cells. In addition to lack of mucin production, MDF can also be recognized by focal lesions that are formed by a lumen which is often distorted when compared with normal surrounding crypts. Elevation of the lesion above the surface of the crypt and a multiplicity greater than 3 are also important features of MDF for identification. MDF were counted under the microscope at a magnification of 100X (Olympus).

Statistical Analyses

Differences due to diet were analyzed by one-way analysis of variance using SAS system for windows, release 8.2 (SAS Institute, Cary, NC) with individual group differences inspected using Duncan's multiple range test. Simple linear correlations among different variables were determined. Two way analysis of variance (ANOVA) was used to examine the effect of the two wheat characteristics, processing state (whole vs. refined) and class (hard red vs. soft white). Differences were considered statistically significant when p value was less than or equal to 0.05.

Results

Body Weight and Food Intake

The initial and final body weights and the average food intakes during the final 8 weeks are summarized in Table 3-3. Weekly body weight (data not shown) including final body weight did not differ significantly among any of the diets and the final basal diet, indicating no effect of the wheat diets on body weight. No difference in food intake was observed in animal groups between the basal diet and any of the wheat diets throughout the study.

Effect of Wheat Diets on AC and ACF Number

Table 3-4 and Figures 3-2, 3-3, and 3-4 show the number of AC, ACF and large ACF in each diet groups. Among all groups, animals fed the refined hard red diet had significantly fewer AC compared to the final basal diet group and the refined soft white group. In terms of ACF number, both refined and whole hard red wheat groups and the whole soft white group were significantly different from initial and final basal diet groups. There was a trend of refined soft white towards fewer AC and ACF compared to the final basal diet group. No significant difference among groups in large ACF with 4 or more AC in foci was observed (Figure 3-4), except for the refined hard red group, which had fewer large ACF compared to the final control group. In terms of multiplicity, defined as the number of aberrant crypts in each aberrant crypt foci, there were no significant differences among diet groups (Figure 3-3). Table 3-4 shows the statistical main effects of the two wheat characteristics, class and processed state. Although there was no effect of processing of wheat, the effect of wheat class (hard red vs. soft white), was significant for every aberrant crypt marker, with animals

fed the hard red wheat diets having fewer AC, ACF, and large ACF and a lower crypt multiplicity compared to the soft white group (Table 3-4 and Figure 3-6).

Effect of Wheat Diets on Sialomucin Producing ACF (SiM-ACF) and Mucin Depleted Foci (MDF) Number

In all wheat groups, total number of sialomucin producing ACF (SiM-ACF) was significantly lower compared to the final basal diet group (Figure 3-5). There was a trend for numerically lower number of SiM-ACF in the hard red wheat diet groups compared to the initial basal diet group ($p=0.07$), however the effect was not significantly different. No mucin depleted foci (MDF) were detected in the whole hard red group. Combining both the whole and refined form of each wheat class (hard red and soft white) showed that the hard red groups were significantly different than the soft white groups in SiM-ACF and MDF number, with fewer in the hard red group for each parameter (Figure 3-7). Correlation analysis among ACF measures indicated that both total ACF and large ACF were strongly correlated with SiM-ACF and MDF and, to a lesser extent, with (SiM+SuM)-ACF, a combination of sialo and sulfo mucin producing ACF (Table 3-6).

Regression Effect of Wheat Diets on ACF and SiM-ACF

Figure 3-7 shows the change in ACF number between 6 weeks and 14 weeks. Fourteen weeks post DMH injection, the number of ACF was greater in the final basal diet group compared to the initial basal diet group, indicating the total number of ACF increased over time when the animals continued to be fed the basal diet. However, in animals switched to the hard red wheat diets at 6 weeks and fed 8 more weeks, the number of ACF significantly decreased, indicating the hard red wheat diets regressed established ACF, suggesting a

change to a lower cancer risk state. When animals were switched to the soft white wheat diets, after 8 weeks of feeding the number of ACF only slightly decreased a change that was not statistically significant. Figure 3-8 shows that in the hard red wheat diet groups the yield of SiM-ACF significantly regressed between 6 weeks and 14 weeks. On the other hand, no regression was observed with the soft white wheat diets.

Discussion

Whole grains, particularly whole wheat, are rich in a wide range of compounds with potential health promoting benefits. Whole wheat contain several compounds which have shown to be associated with improved health status, include lignans, tocotrienols, phenolic compounds (53, 54, 239), and anti-nutrients such as phytic acid, tannins, and enzyme inhibitors (179, 222). Hence, there is a dietary recommendation to increase intake of whole grains to prevent certain diseases including colon cancer. However, the exact mechanism by which a diet high in whole wheats offers protection against colon cancer development has not been fully explored (15, 16). Several mechanisms have been proposed to explain the chemopreventive ability of wheat constituents, including inhibition of cell proliferation and ACF formation (224), inactivation of carcinogens (221), and activation of apoptosis (44, 46, 49). In attempts to understand the chemopreventive effect or define a mechanism, most experimental studies have examined only a part of the wheat kernel e.g. wheat bran (39, 46, 240, 241) or wheat germ (40), while there are few experimental studies that have investigated the effect of different types of whole or refined wheat on colon carcinogenesis and related risk factors. Evidence from two experimental studies conducted by Iovinelli and Gallaher (227), and Maziya-Dixon and colleagues (42) suggest that red wheat is more protective than white wheat. These studies examined the effect of wheat class and processing in reducing colon cancer markers (ACF or tumors) in carcinogen-treated animals. Both of these studies observed that the protective effect of hard red wheat flour was due to the class of wheat, not the state of refinement. However, it has not been identified in these studies what characteristics present in hard red wheat are responsible for its protective effect in animal models. It is important to mention that in both studies, the animals were fed the wheat flour diets before, during and after the carcinogen treatment. Results of these studies do not clarify

whether the hard red wheat flour offers protection at the initiation stage or promotion stage of colon cancer or both. If protection by hard red wheat flour is found during the promotion stage, this would suggest protection is not mediated by alterations in carcinogen metabolism or DNA repair mechanisms. The effect of red wheat may be mediated through changes in gene expression which may stimulate tumor suppressor genes or inactivate proto-oncogenes. Therefore, the objective of the second study was to determine whether the chemoprotective effect of red wheat diet occurred in the late promotion stage of colon carcinogenesis.

In the first part of this thesis, we investigated the effect of wheat color, hardness and processed state on reducing precancerous lesions in the early promotion stage of colon cancer development. Of four major types of wheats, hard red and soft white significantly reduced the number of ACF and SiM-ACF compared to the basal diet group regardless of processing. There was trend for hard red wheat to reduce ACF and SiM-ACF relative to soft white wheat. Our results support the findings of Iovinelli and Gallaher (227) who observed the effect of hard red wheat in reducing colon cancer risk, determined by ACF number. Similar to the effect observed in the early promotion stage, we have now observed that hard red wheat reduced the number of ACF compared to the soft white wheat and basal diet groups in the late promotion stage of colon cancer. This effect of red wheat was also independent of processing.

The predictive value of ACF for the development of tumors at the later stage of colon cancer development has been observed to be inconsistent. Some studies found tumor number to correlate with ACF number, while others only with large ACF or multiplicity (50, 109, 110).

Although the most common parameter to measure is the number of ACF, either in whole

colon or part of the colon, multiplicity of ACF or large ACF has been suggested for use in addition to ACF (125, 144). Multiplicity of ACF leads to the formation of a large ACF over time, which has a higher degree of dysplasia. These large ACF have shown to be correlated with the number of colon tumors, and were therefore suggested to be a more reliable biomarker than ACF (113).

Our study observed that the hard red wheat diet groups, regardless of processing, had significantly fewer large ACF compared to the basal diet group. As processing of wheat did not have any influence on its physiological effect, we combined both whole and refined diet groups and further analyzed the effect of the wheat diets. Two-way analysis of variance indicates that rats fed the hard wheat diets had significantly fewer large ACF and lower multiplicity of ACF compared to the soft white diet group.

Some recent studies suggest that dysplastic ACF are direct precursors of adenomas and carcinomas although they represent only a small fraction of total ACF (114, 130).

Histological analysis has shown that only dysplastic ACF have the potential to progress into tumors, whereas hyperplastic ACF do not have that ability. In this study we have examined SiM-ACF and MDF as indicative of dysplastic ACF. The hard red wheat diets showed a statistically significant reduction in SiM-ACF compared to the final basal diet group. MDF, which have been shown to be more dysplastic than SiM-ACF (110, 121, 122, 145), were lower in the hard red wheat diet group compared to the soft white and the final basal diet groups. There was a highly significant correlation between MDF and both ACF and large ACF.

In addition to determining the effect of hard red and soft white wheat flours on morphological markers of colon carcinogenesis, we also evaluated the effect of wheat type

and processing on regressing established ACF and SiM-ACF in the late promotion stage of colon cancer. A study by Papanikolaou and colleagues (238) examined a sequential development of ACF in different strains of mice (SWR/J, A/J, AKR/J mice), differing markedly in their susceptibility to tumor development in response to the colon-specific carcinogen azoxymethane (AOM), a metabolite of dimethylhydrazine. After the carcinogen treatment, the number, size and morphological characteristics of ACF were examined in order to determine whether any of these properties explain the nature of precancerous lesions and their ability to progress into tumors in a variety of mice strains. The development of ACF was evaluated at five different time points and the most susceptible strain (A/J mice) had a significantly greater number of large ACF number at the last time point measured at 24 weeks after carcinogen administration. In general, a downward trend in ACF number was observed in all three strains of mice followed by a rebound in the number of ACF. This study supports the finding of McLellan and colleagues (242) who also observed a transient decrease in total ACF followed by rebound in AOM-treated rats. A fluctuation in ACF number indicates that ACF may undergo a remodeling process in which some disappear or regress, while others proliferate, enlarge and become more complex. Because of their dynamic nature, ACF could be a target of chemotherapeutic agents during the process of remodeling.

In our study, we also observed that rats fed hard red wheat show an ability to regress established ACF. That is, an increase in ACF number in the animal groups fed the basal diet from 6 weeks to 14 weeks was observed. But, interestingly, when the animals were switched to the hard red wheat diet after 6 weeks of basal diet feeding, the number of ACF significantly decreased, indicating hard red wheat diet regressed established ACF. This

suggests a change from a higher to a lower risk state for colon cancer development. Our study failed to find any effect of the soft white wheat diet on regressing ACF number, as after 6 weeks of basal diet feeding, the animals switched to the soft white wheat diets showed only a slightly decreasing the number of ACF, a difference that was not statistically significant. The hard red wheat was also found to regress significantly SiM-ACF compared to the basal diet. It is not known from our study why this regression occurs. It has been speculated that regression of ACF might be caused by the inhibition of proliferative activity or by the induction of apoptosis in the epithelial cells of ACF (243). Further investigation is warranted to understand this phenomenon.

In conclusion, we have observed that in the late promotion stage of colon carcinogenesis, hard red wheat flour, regardless of processing state, has a greater chemopreventive effect than soft white wheat, based on the reduction in ACF, SiM-ACF and MDF. In addition, hard red wheat flour shows an ability to regress established ACF, suggesting an ability to change the colon from a high risk to a low risk state of colon cancer development. The components in red wheat responsible for this protective effect are not clear. Further studies in this area are required.

Table 3-1. Proximate Analysis of Wheat Flours ^a

Flour	Carbohydrate (%)	Protein (%)	Fiber (%)	Fat (%)
Whole Hard Red	74.5	11.9	11.6	2.4
Refined Hard Red	74.4	10.4	3.2	1.4
Whole Soft White	72.8	9.7	12.6	3.0
Refined Soft White	77.0	8.6	3.0	1.7

^a Proximate analysis was done by Medallion Laboratories (Golden Valley, MN)

Table 3-2. Diet Composition (g/kg)

Diet	Basal	Whole Hard Red	Refined Hard Red	Whole Soft White	Refined Soft White
Flour	0	615	615	615	615
Sucrose	100g	91.8	55.4	102.3	45.2
Corn Starch	448.2	0	0	0	0
Casein	200	126.9	136	104.3	147
Cellulose	80	8.7	60.3	2.5	61.5
Mineral Mix	35	35	35	35	35
Vitamin Mix	10	10	10	10	10
L-Cystine	3	3	3	3	3
Choline	2.5	2.5	2.5	2.5	2.5
Bitartrate					
Butylated	0.014	0.014	0.014	0.014	0.014
Hydroxytoluene					
Soybean Oil	120	105.8	81.5	88.1	79.6
Cholesterol	1.2	1.2	1.2	1.2	1.2
% CHO	55	55	51.5	55	51.8
% Protein	20	20	20	20	20
% Fiber	8	8	8	8	8
% Fat	12	12	9	10.7	9

Table 3-3. Body Weight and Average Food Intake of Animals ^a

Diet Group	Initial Body Weight (g)	Final Body Weight (g)	Food Intake ^b (g/day)
Initial Basal (Basal-I)	56.3 ± 2.4	418.5 ± 9.5	26.7 ± 0.5
Whole Hard Red	54.6 ± 1.1	546.7 ± 16.8	23.3 ± 1.0
Refined Hard Red	57.9 ± 2.1	510.8 ± 22.7	25.8 ± 1.2
Whole Soft White	60.1 ± 1.8	531.6 ± 11.7	24.8 ± 1.1
Refined Soft White	62.9 ± 1.8	534.6 ± 33.0	25.2 ± 1.3
Final Basal (Basal-F)	59.1 ± 1.9	510.6 ± 17.1	22.6 ± 0.8

^a Values are reported as mean ± SE, n=15

^b Average daily food intake during the last 8 weeks of the study

Table 3-4. Effect of Different Varieties of Wheat on AC and ACF Number of Rat Colon ¹

Variables (n/cm ²)	Initial Basal	Whole			Refined		Final Basal	Main Effect
		Hard Red	Soft White	Hard Red	Hard Red	Soft White		
AC	13.8 ± 1.1 ^{bc}	10.7 ± 1.1 ^{bc}	12.9 ± 1.6 ^{bc}	9.1 ± 0.9 ^c	15.9 ± 2.3 ^{ba}	19.4 ± 1.7 ^a	Class (p=0.005)	
ACF	6.8 ± 0.5 ^a	3.8 ± 0.3 ^c	4.9 ± 0.6 ^{bc}	3.5 ± 0.3 ^c	5.9 ± 0.7 ^{ab}	7.7 ± 0.6 ^a	Class (p=0.001)	
Large ACF²	0.6 ± 0.08 ^c	1.0 ± 0.1 ^{bac}	1.2 ± 0.1 ^{ab}	0.7 ± 0.09 ^{bc}	1.4 ± 0.3 ^{ab}	1.6 ± 0.2 ^a	Class (p=0.04)	
Multiplicity	4.3 ± 0.05	4.7 ± 0.09	4.8 ± 0.09	4.6 ± 0.07	4.9 ± 0.1	4.9 ± 0.1	Class (p=0.05)	

¹ Values are reported as mean ± SE, n=15. Values with different letters are significantly different (p=<0.05)

² Four or more AC in a foci considered as a large ACF

Table 3-5. Effect of Different Varieties of Wheat on SiM Producing ACF (SiM-ACF) and Mucin Depleted ACF (MDF) Numbers of Rat Colon ¹

Variables (n/cm ²)	Initial Basal	Whole			Refined		Final Basal	Main Effect
		Hard Red	Soft White	Hard Red	Hard Red	Soft White		
SiM-ACF	17.9 ± 2.1 ^b	7.9 ± 0.9 ^b	17.1 ± 2.2 ^b	12.3 ± 2.1 ^b	17.8 ± 2.3 ^b	28.5 ± 4.7 ^a	Class (p=0.0004)	
(SiM+SuM)-ACF	13.9 ± 1.9	10.9 ± 1.0	8.8 ± 2.0	14.2 ± 2.7	10.1 ± 2.0	8.8 ± 1.6	Class (p=0.13)	
MDF	1.0 ± 0.3 ^{ab}	0 ^b	0.3 ± 0.2 ^{ab}	0.9 ± 0.3 ^{ab}	1.3 ± 0.3 ^a	1.3 ± 0.3 ^a	Class (p=0.0002)	

¹ Values are reported as mean ± SE, n=15. Values with different letters are significantly different (p<0.05).

Abbreviations: SiM-ACF, sialomucin producing ACF; (SiM+SuM)-ACF, a combination of sialo and sulfomucin producing ACF; MDF, mucin depleted foci.

Table 3-6. Correlation Coefficients among Aberrant Crypt Foci Measures ¹

r Value	(SiM+SuM) -ACF	SiM-ACF	MDF
p Value			
Total ACF	0.291	0.486	0.413
	0.0056	<0.0001	<0.0001
Large ACF	0.262	0.390	0.343
	0.0131	0.0002	0.0010

¹ Values are reported as mean ± SE, n=15

Abbreviations: SiM-ACF, sialomucin producing ACF; (SiM+SuM)-ACF, a combination of sialo and sulfomucin producing ACF; MDF, mucin depleted ACF.

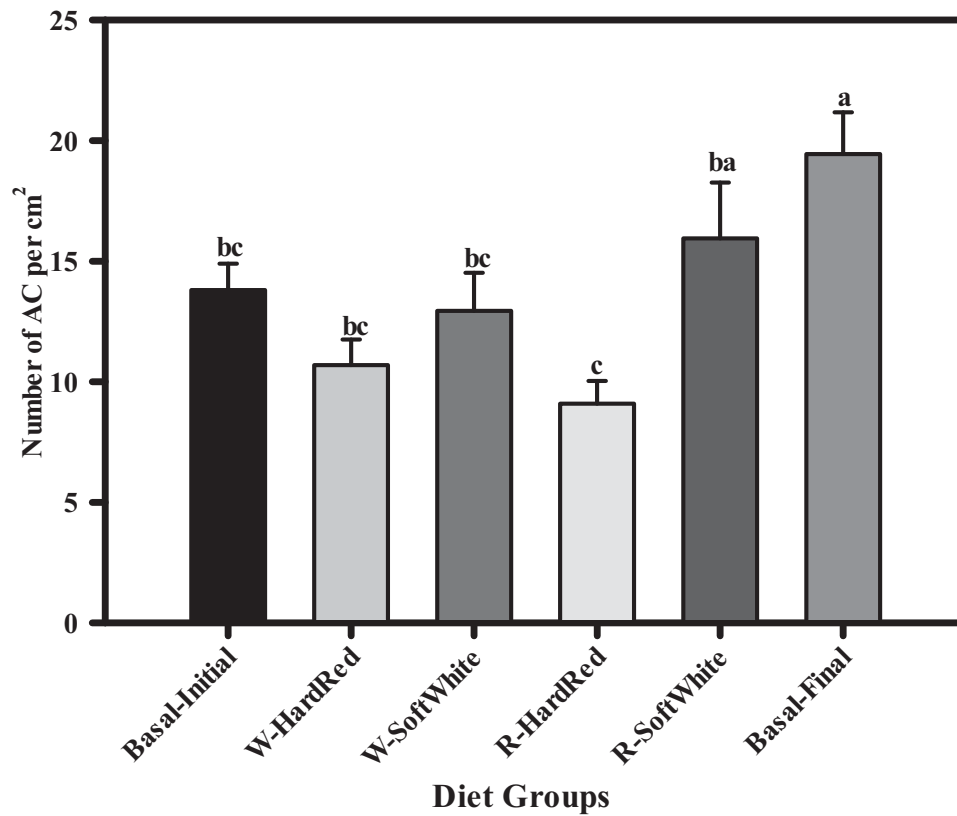


Figure 3-2. Total aberrant crypt (AC) of rat colon tissue during the late promotion stage of colon cancer

Values are represented as mean \pm SE, n=15. Bars with different letters are statistically significant ($p < 0.05$).

Abbreviations: W=Whole; R=Refined.

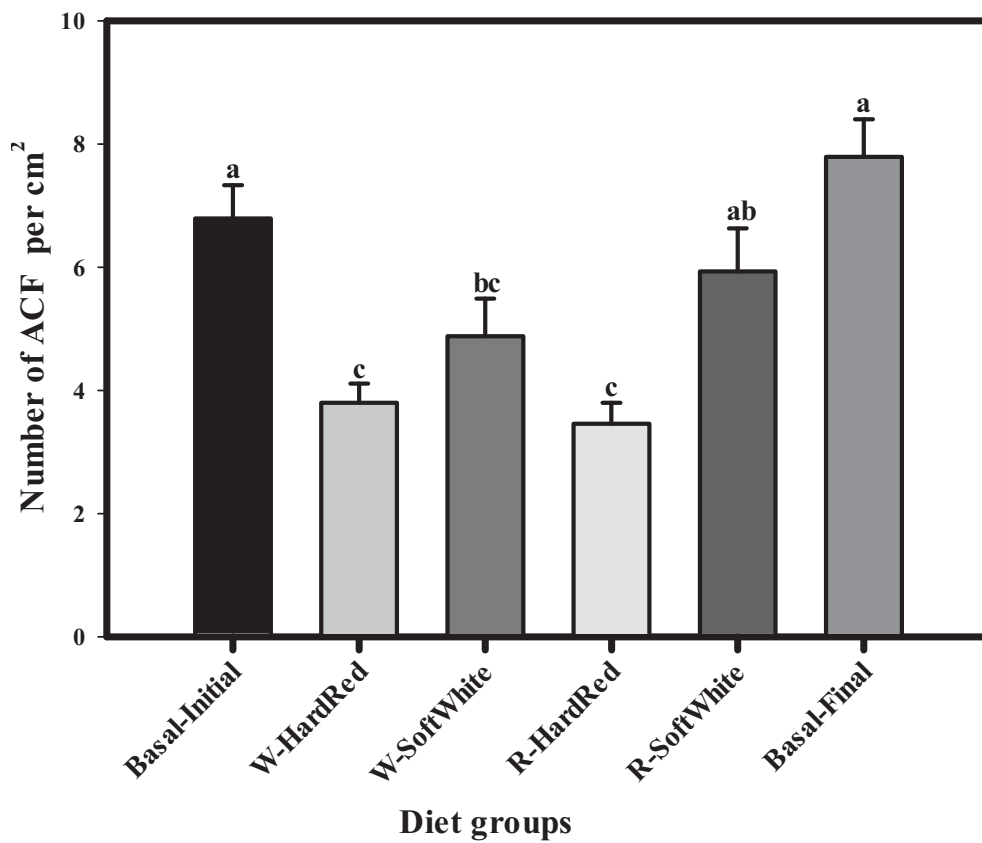


Figure 3-3. Total aberrant crypt foci (ACF) of rat colon tissue during the late promotion stage of colon cancer

Values are represented as mean \pm SE, n=15. Bars with different letters are statistically significant (p < 0.05).

Abbreviations: W=Whole; R=Refined

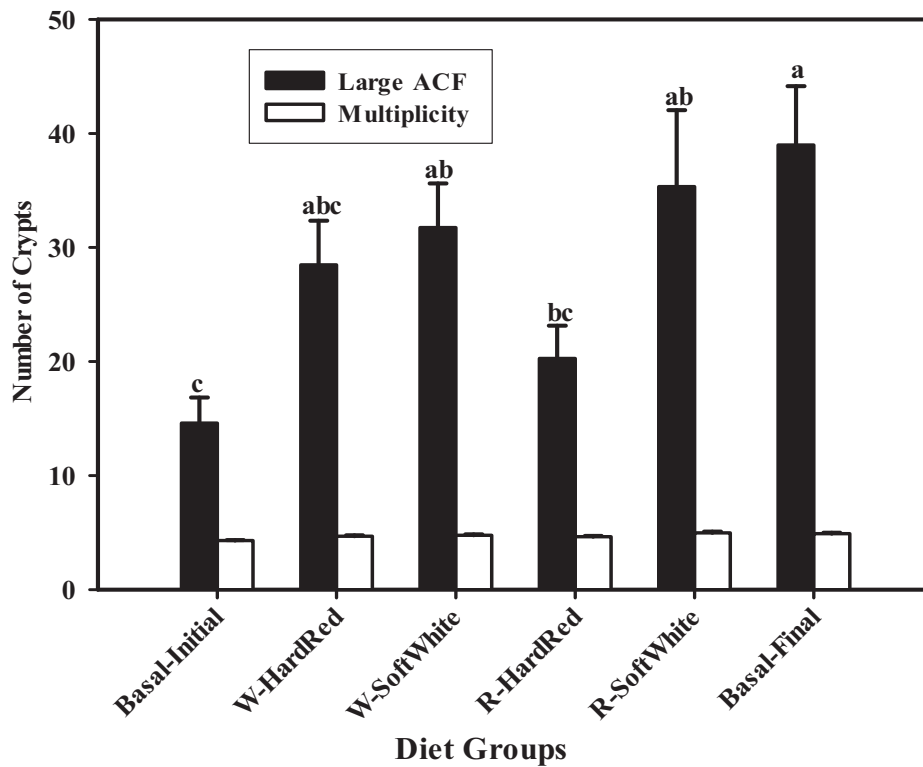


Figure 3-4. Total large aberrant crypt foci (large ACF) of rat colon tissue and multiplicity of aberrant crypt foci during the late promotion stage of colon cancer

Values are represented as mean ± SE, n=15. Bars with different letters are statistically significant (p <0.05).

Abbreviations: W=Whole; R=Refined.

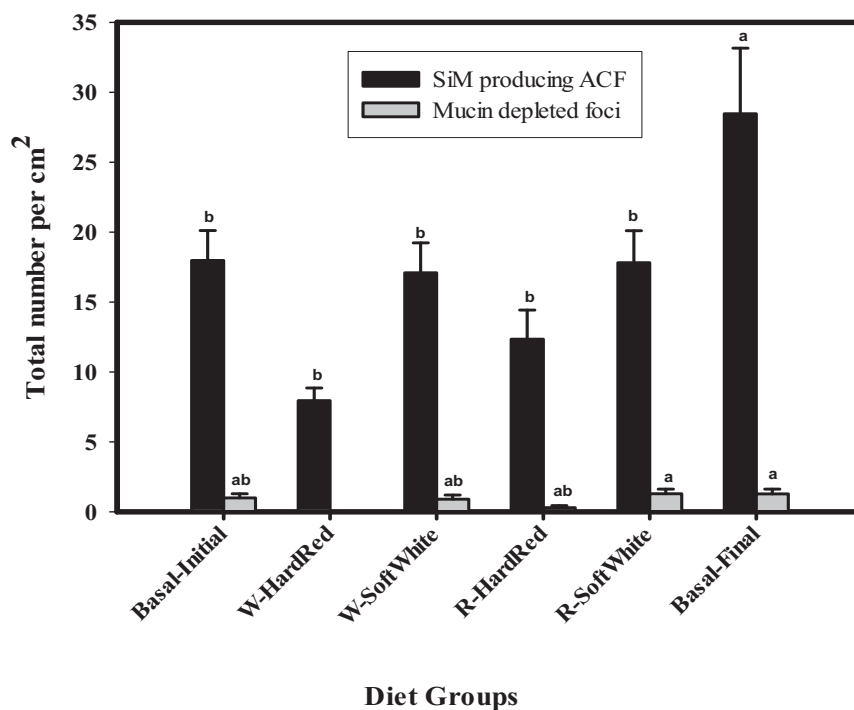


Figure 3-5. Total sialomucin producing ACF (SiM-ACF) and mucin depleted foci (MDF) of rat colon tissue during the late promotion stage of colon cancer

Values are represented as mean ± SE, n=15. Bars with different letters are statistically significant (p <0.05).

Abbreviations: W=Whole; R=Refined.

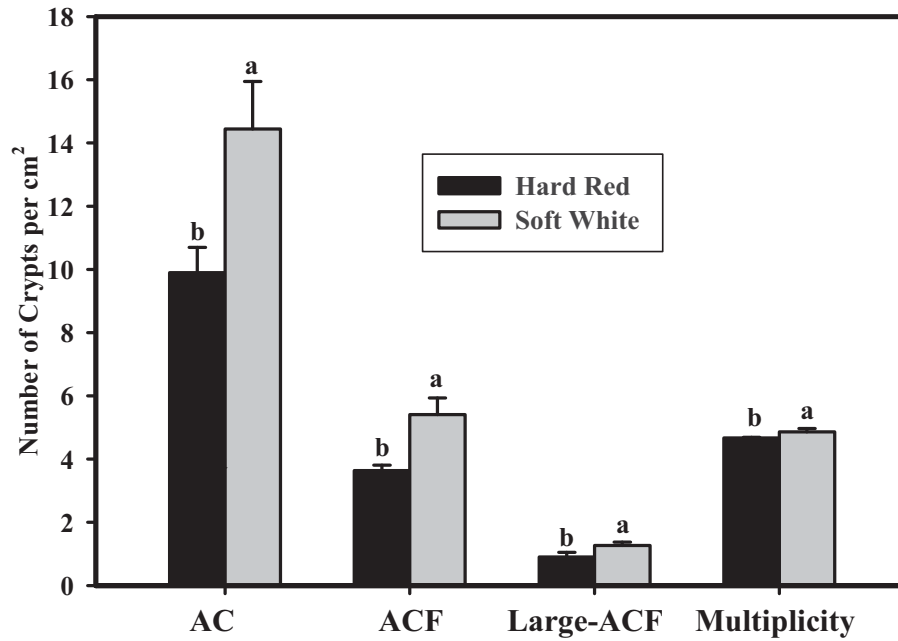


Figure 3-6. Effect of hard red (combined whole and refined) and soft white (combined whole and refined) wheat on aberrant crypt number and multiplicity

Values are represented as mean ± SE, n=15. Bars with different letters are statistically significant (p <0.05).

Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci.

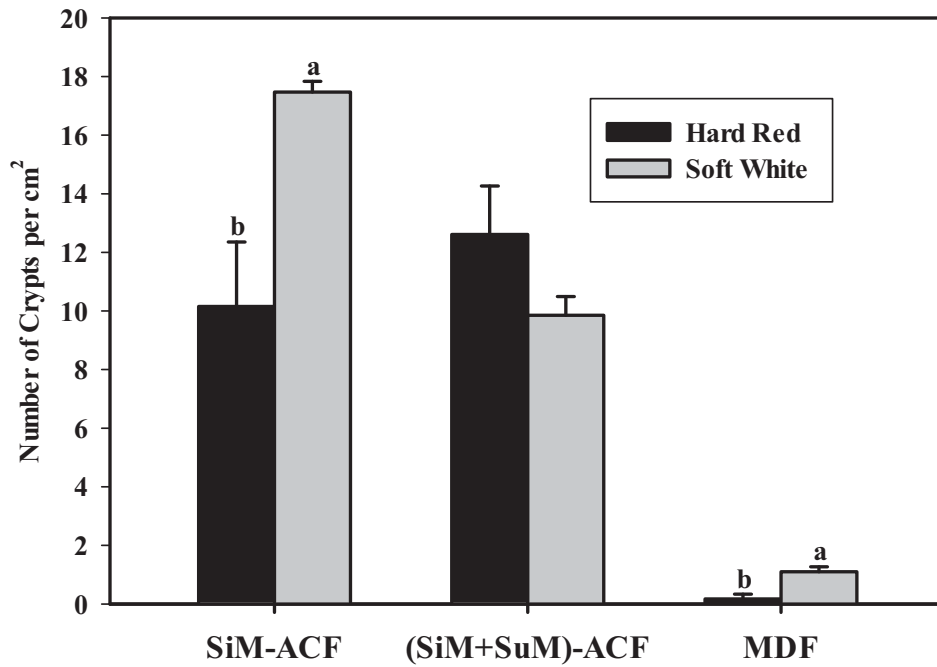


Figure 3-7. Effect of hard red (combined whole and refined) and soft white (combined whole and refined) wheat on dysplastic aberrant crypt numbers

Values are represented as mean \pm SE, n=15. Bars with different letters are statistically significant (p < 0.05).

Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci.

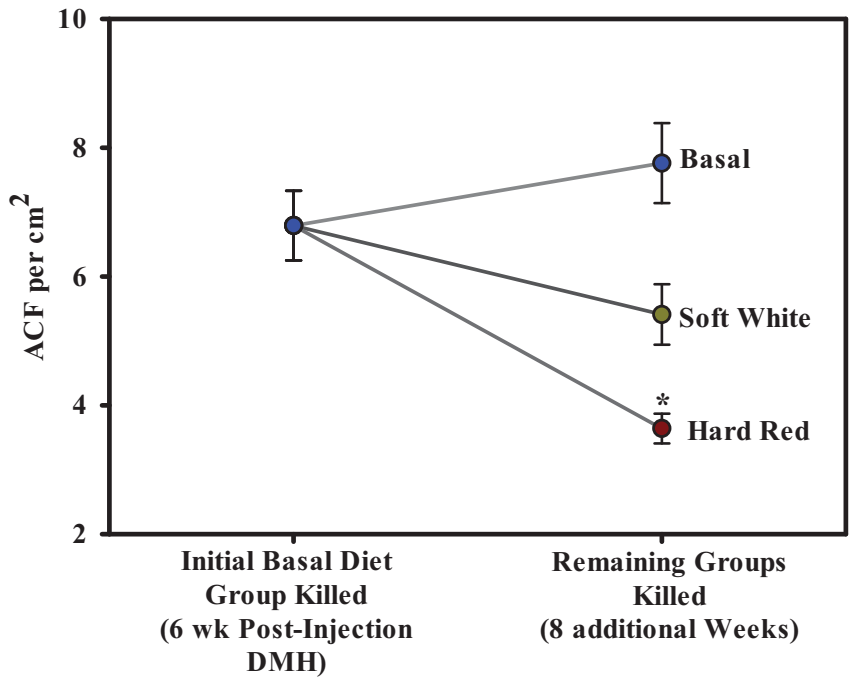


Figure 3-8. Regression of aberrant crypt foci by hard red (combined whole and refined) and soft white (combined whole and refined) wheat flour

Values are represented as mean \pm SE, n=15

* Significantly different than initial basal diet (p<0.05)

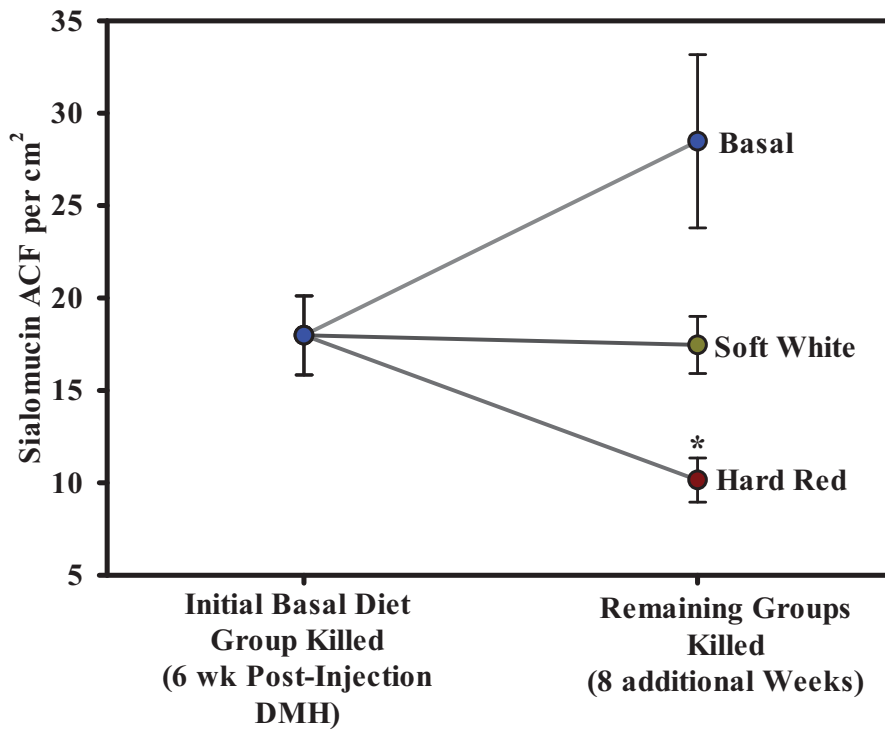


Figure 3-9. Regression of marker of dysplasia by hard red (combined whole and refined) and soft white (combined whole and refined) wheat flour

Values are represented as mean \pm SE, n=15

* Significantly different than initial basal diet (p<0.05)

Chapter-4
Final Study

**The Effect of Wheat Class on Reducing and
Regressing Colon Cancer Risk**

Abstract

A previous study in our laboratory found that hard red wheat flour, regardless of processing state, is more effective than soft white wheat flour in reducing colon cancer risk, based on reduction in aberrant crypt foci (ACF), sialomucin producing ACF (SiM-ACF) and mucin depleted foci (MDF). In addition, hard red wheat flour showed an ability to regress a high risk state of colon cancer to a low risk state. Here we examined the effect of wheat diets on reducing colon cancer risk in the early and late promotion stages, as judged by changes in the number of morphological, biochemical and stem cell mutation markers of colon carcinogenesis. Rats were adapted to a basal diet (AIN-93G) for 10 days, then given dimethylhydrazine, once a week, for two consecutive weeks. After the last dose of carcinogen, rats were divided into six groups (n=15). Of the six groups, four groups continued to be fed the basal diet and two groups were fed either refined hard red or soft white wheat-based diet. After six weeks of feeding (early promotion stage) one basal, and the hard red and soft white group were killed. Of the remaining three basal groups, one group continued to be fed the basal diet, one group was switched to the hard red wheat diet, and one group was switched to the soft white wheat diet. The animals were fed for an additional 8 weeks (late promotion stage). Hard red and soft white wheat diets significantly reduced morphological markers (ACF) compared to the basal diet in both the early and late promotion stage. Both hard red and soft white wheat diets reduced dysplasia markers (SiM-ACF and MDF) during the late promotion stage. Animal groups fed the hard red and soft white wheat diet had significantly fewer β -catenin accumulated crypts (BCAC), a biochemical marker of colon carcinogenesis during the early and late promotion stage. The hard red wheat diets significantly reduced the number of metallothionein-positive crypts, a

marker of stem cells in both stages. However, soft wheat diet had no effect on metallothionein-positive crypts. Overall, hard red wheat flour lowered cancer risk more than soft white wheat flour, and the differences between wheat flours were more pronounced in late promotion stage.

Introduction

Colon carcinogenesis is a multistep process which leads to morphological, biochemical and molecular changes in colonic mucosal cells. This multistep process involves a sequential transformation of normal colonic epithelial cells into precancerous lesions termed aberrant crypt foci (ACF), which then progress into adenomas, carcinomas and finally a metastatic tumor (122, 168, 232). To date, a number of morphological and biochemical markers of colon carcinogenesis have been identified in animals and in humans. The significance of these biomarkers is mainly to understand the biological process of colon carcinogenesis and to investigate the role of chemopreventive agents in prevention of colon cancer.

ACF were first identified as minute, precancerous lesions in carcinogen-treated mice. These ACF were detected based on their enlarged structure and thick epithelium compared to the surrounding normal crypts (97). Later, ACF were identified microscopically in a variety of other species including rats, hamsters and dogs (98, 99, 244). However, the question remains as to whether carcinogen induced ACF in animal models share the similar features of colorectal adenomas, which are considered as precancerous lesions in humans. Later, ACF were observed in human colonic mucosa and were found to share many common features with ACF induced by carcinogen in rodents (103). Large numbers of ACF were observed in patients with familial adenomatous polyposis (FAP) and in patients with or without sporadic colorectal carcinoma (103, 119, 120).

Although ACF are present in human colons, they can not be quantified easily *in vivo*.

Therefore, the use of ACF is largely limited to investigations in animal models. ACF have been used extensively in animal models as a colon cancer biomarker to provide a quantitative

assessment of the stepwise development of colon carcinogenesis (245-247). However, there are inconsistencies between the association of ACF and tumor number in animal models. Some studies have shown a strong correlation between ACF and tumor number (103, 108, 109). However, other studies fail to find a correlation between the number of ACF detected at an early stage and tumors development during later stage of colon carcinogenesis, although there was a correlation between large ACF or ACF multiplicity and tumor incidence (109, 110, 248). The number and size of ACF usually increase over time, eventually forming large ACF consisting of 4 or more aberrant crypts in a focus. These large ACF (103) and crypt multiplicity (109), which is the average number of crypt per foci, demonstrate a stronger association with tumor development compared to ACF, and therefore are suggested to be better predictors of colon tumor formation.

Evidence from several animal and human studies suggest that only a subset of ACF are likely to progress into colon tumors. Histological analyses have shown that ACF appear as either hyperplastic or dysplastic. Dysplastic ACF show mild to moderate cellular atypia to severe dysplasia (102, 119, 242), which increases over time following a carcinogen treatment. Dysplasia of ACF was identified by mild to severe grading of nuclear stratification and elongation, and increased crypt height with severe dilation (242). The dysplastic ACF are considered to be more predictive of formation into tumor compared to the hyperplastic ACF in both humans and animals and are thus considered as an indicator of increased risk for progression to cancer (114, 115, 129).

In addition to alterations in cellular morphology, the alteration in mucin production from sulfomucin to sialomucin in the goblet cells of colonic mucosa has been interpreted as an

early event in colon cancer development (122, 129). In ACF, the degree of dysplasia is suggested to be related to an alteration of mucin in the colonic crypts with a switch towards sialomucin. Therefore ACF that express sialomucin (SiM-ACF) are suggested to have a greater degree of dysplasia (123). Recently, mucin depleted foci (MDF) have been observed in the whole mount of colons of rats. A complete or almost complete loss in mucin production in the colonic crypt is the major feature for identification of MDF. The total number of MDF in colon tissue is very limited. However, MDF appear to be a subset of ACF that are highly dysplastic and show a stronger association with tumors compared to the total number of ACF (121, 125). Thus SiM-ACF and MDF, as markers of dysplasia, may be better markers of colon cancer risk than ACF alone.

Crypts that lack mucin usually have accumulation of β -catenin protein (126, 145). These crypts are known as β -catenin accumulated crypts (BCAC) and are characterized by increased expression of the *β -catenin* gene, with accumulation of β -catenin protein in the membrane, cytoplasm and nucleus (115, 116, 142). BCAC do not appear raised above the mucosal surface like ACF, and are only identified in histological sections by *en face* preparation. Histological analysis has shown that BCAC have a higher grade of dysplasia, with disruption of cellular morphology and greater proliferative activity, compared to ACF. Consequently, they are considered more likely to progress into tumors more than ACF. Thus, BCAC are likely to be a useful early biochemical marker of colon carcinogenesis.

During colon cancer development, the colonic epithelial tissue accumulates a series of mutations over years. Due to the rapid turnover of colonic epithelial tissue, most of the cells in this tissues are replaced within a week, therefore any mutations that acquire in these cells

are lost (181, 249). It has been hypothesized that tumorigenic mutations occur in a small numbers of cells, which are colonic crypt stem cells that reside long term in tissue. Crypt stem cells are located at the bottom of the crypt, and their normal function is to maintain the homeostasis of the colonic epithelial tissues. These crypt stem cells are characterized by their self-renewal ability and high proliferative activity. By self-renewing, colonic crypt stem cells reside in the colonic crypts for long period of time instead of undergoing terminal differentiation and migrating to the lumen. Therefore the crypt stem cells are more likely to acquire multiple mutations necessary for the development of colon carcinogenesis (14).

Self-renewal of normal stem cells populations is regulated by the Wnt/ β -catenin signaling pathway. In colonic crypts, the Wnt signaling pathway drives the formation of a Tcf-4/ β -catenin complex, which maintains the proliferative activity of crypt stem cell (250, 251). It is known that this pathway become dysregulated during colon cancer development. As a result, crypt stem cells lose their normal regulatory controls on cell division and undergo proliferation and neoplastic transformation. Recent studies have reported that changes in crypt stem cells population are involved in carcinogenesis. Histological evidence from adenomas in APC Min^{+/-} mice (252) and colon carcinomas in rodents show that the intestinal cells that turn into tumors have the property of stem cells (253). Recently colon cancer stem cells have been identified using a cell surface marker, CD133. It has been observed that in colon cancer, tumors have a high density of CD133-positive cells and transplantation of these CD133-positive cells reproduced tumors in the immunodeficient mice (171-173). Based on studies such as this and others, it has been suggested that colon cancer develops from a small number of undifferentiated tumorigenic cells that are cancer stem cells. Therefore, studying the cellular and molecular changes involved in the regulation

of crypt stem cells might help to understand the histological changes during tumor progression (250) and these cancer stem cells might be a target of chemotherapeutic strategy.

Recently, cellular over expression of metallothionein (MT) has been observed in various types of cancers including colorectal cancers (211). Studies have reported that the presence of MT-positive crypts in colonic tissue is an indicator of colon crypt stem cell mutations (199, 211, 212). In animals, the frequency of crypt-restricted immunopositivity for MT increases proportionately with the dose of mutagen administration. Initially individual crypts contain both the positive and negative phenotype. However, later, some crypts become entirely MT-positive, indicating this phenotype may increase over time after mutagen administration. The frequency of MT-positive crypts stabilizes after a few weeks and remains at the same level for 6 months, depending on the type of mutagen exposure (211, 212). Thus, quantification of this marker would likely be useful to study the effect of chemopreventive agents on formation of cancer stem cells.

Two previous studies on colon cancer prevention (42, 227) have indicated that hard red wheat, either as whole or refined, is protective against colon cancer development relative to soft white wheat in carcinogen-treated animals. The effect of hard red wheat on reducing colon cancer risk was evaluated based on its ability to reduce the total number of ACF in the colonic mucosal tissues and tumor number. Why red wheat shows a stronger protective effect than white wheat on colon carcinogenesis unclear. In both studies, the wheat flour diets were fed during the initiation and promotion stage of colon cancer. It was not clear whether the effect of red wheats was predominant at the initiation or the promotion stage of colon cancer development or was effective in both stages. A recent study in our laboratory by Yang and

Gallaher (254) observed that red wheat significantly reduced the number of large AC when fed only at the initiation stage of colon cancer development. There was a trend for a lower degree of dysplasia and less cytoplasmic and nuclear expression of β -catenin relative to white wheat. Red wheat did not reduce colonic DNA damage and apoptosis, indicating that the effect of red wheat at the initiation stage was not mediated through changes in DNA repair or increase in apoptosis after carcinogen treatment.

Based on the finding of our previous two studies (42, 227), we further investigated the effect of hard red and soft white wheat diets using both whole and refined form on reducing colon cancer risk in the early and late promotion stage of colon carcinogenesis. Our first two experiments in this thesis support the protective effect of hard red wheat flour, based on its effect of reducing the number of morphological markers such as ACF and large ACF and a dysplasia marker, sialomucin producing ACF, regardless of processing state. The effect of red wheat, either whole or refined, on reducing ACF was significantly greater than that of white wheat in the late promotion stage of colon cancer. Our study also showed the ability of hard red wheat to regress an established high risk state to a lower risk state of colon cancer using ACF as the marker of risk.

In the final experiment of this thesis, we examined the effect of two commercially available wheat flours, refined hard red and refined soft white, on reducing colon cancer risk in the early and late promotion, which also allowed for examining regression from a high risk state to a lower risk state. To evaluate risk, we utilized changes in the number of a stem cells mutation marker, and morphological and biochemical markers of colon carcinogenesis.

Materials and Methods

Animals and Diets

Ninety male Wistar rats with an initial body weight between 50 and 75g were purchased from Harlan Sprague Dawley (Indianapolis, IN). All rats were housed individually in stainless steel wire-bottomed cages and kept in a 12 hour light/dark cycle. The room was maintained at $20 \pm 2^{\circ}\text{C}$ with a relative humidity of $50 \pm 10\%$. Throughout the study rats were given diet and distilled water *ad libidum*. The study was approved by the University of Minnesota Committee on Animal Care and Use. All diet ingredients except wheat flours were purchased from Harlan Tekland (Madison, WI). Refined hard red and refined soft white wheat flour were a gift of ConAgra Foods (Commerce City, CO). The wheat flours were stored at -20°C until incorporation into diets. Table 4-1 shows the proximate analysis of each wheat flour. Formulation of each wheat based experimental diet was based on the AIN-93G, purified rodent diet (228) and consisted of 61.5% of wheat flour by weight. The basal diet contained no wheat flour. Diets were freshly prepared every two weeks and kept refrigerated. Table 4-2 shows the composition of the diets.

Experimental Design

Rats were adapted to the basal diet for 10 days upon arrival. After adaptation, the colon carcinogen dimethylhydrazine (DMH) was administered at a dose of 50 mg/kg body weight subcutaneously, two times, a week apart. Rats were fed the basal diet before and during the carcinogen treatment. Five days after the carcinogen treatment rats were divided into 6 groups (n=15). One group of rats was fed the refined hard red wheat diet and another group the refined soft white wheat diet. The remaining 4 groups continued to be fed the basal diet.

After six weeks of feeding the hard red and soft white wheat groups and a basal diet group (initial basal group) were killed to investigate the effect of wheat diets on biomarkers of colon cancer during the early promotion stage. The remaining 3 groups were divided into a basal diet group (final basal group), a refined hard red wheat group and a refined soft white wheat group and were fed their corresponding diets for 8 more weeks until sacrificed. Figure 4-1 shows a schematic representation of the animal feeding regimen. Body weights were recorded weekly and food intake was recorded bi-weekly throughout the study.

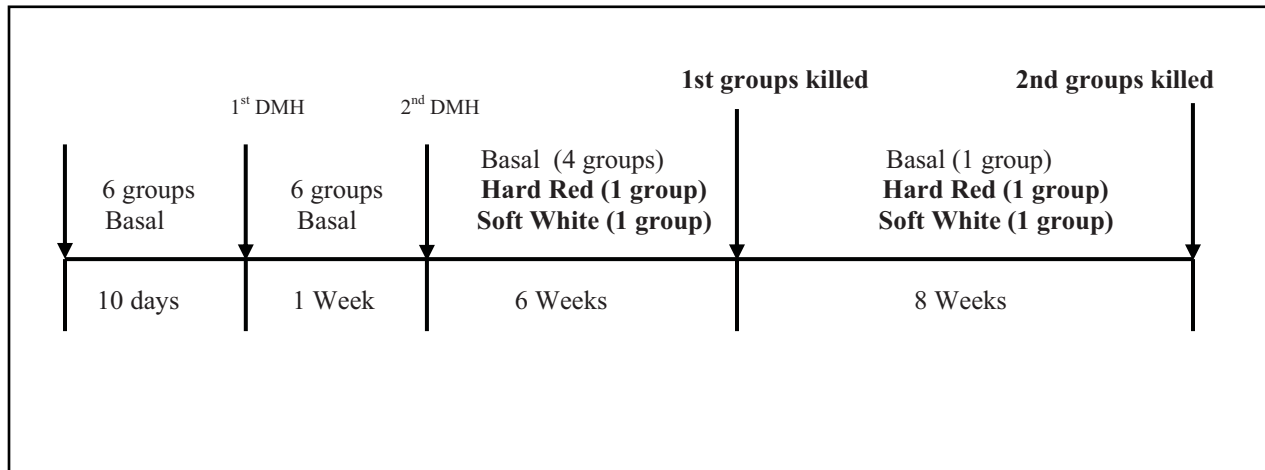


Figure 4-1: Schematic representation of the animal feeding regimen

Colon Sample Preparation

Rats were anesthetized using isoflurane, opened by laparotomy, and colons removed. The colons were flushed with cold PBS (pH 7.4). The colons were gently slid onto a 2 ml glass pipette starting from cecal end and completely submerged in 10% formalin-PBS for 5 minutes. The colons were cut open longitudinally with a razor blade and washed with PBS several times. A 2.5 cm section from the distal end of the colons and the proximal end of the

colons was removed. The whole distal colons were placed flat between filter paper and submerged in 10% formalin in PBS and fixed overnight at 4°C. These 2.5 cm portion of colon tissues removed from the distal end were used for histological analysis. Each 2.5 cm section was divided into half (approximately 1.25 cm of each) and embedded in paraffin. Four µm thick serial sections of an *en face* orientation were prepared. The colons were coded to allow for unbiased evaluation.

Determination of Aberrant Crypt (AC) and Aberrant Crypt Foci (ACF)

To count the total number of aberrant crypt (AC) and aberrant crypt foci (ACF), formalin fixed colon tissue was stained with 0.2% methylene blue (Sigma Chemical Co, St Louis, MO) for 5 min with gentle shaking. Colon tissue was then washed with distilled water to remove excess methylene blue stain, transferred to a clean dish and submerged with distilled water to avoid drying. The mucosal side of the tissue was examined under a stereo microscope at a magnification of 100X (Olympus SZX, Olympus Optical Co, Tokyo, Japan). ACF and the number of AC in each focus was recorded according to a modification of the method of Bird (97). The colons were stored at 4°C in 10% formalin-PBS until analyzed for mucin production.

Determination of Sialomucin Producing ACF (SiM-ACF) and Mucin Depleted Foci (MDF)

Formalin fixed colon tissue was stained with high iron diamine alcain blue (HID-AB) solution to determine presence or absence of mucin and the type of mucin produced. Colon tissue was rinsed in distilled water for 5 minutes to remove excess formalin and then transferred to a staining dish containing freshly prepared iron-diamine solution. Colon tissue

was incubated in iron-diamine solution in the dark for 18 hours at room temperature. After incubation colon tissue was rinsed three times in distilled water and stained with 1% alcian blue (Sigma) in 3% acetic acid solution for 30 minutes at room temperature. Colon tissue was then rinsed three times in 80% ethanol followed by distilled water. The HID-AB stained colon tissue was examined at 100X magnification under a microscope (Olympus BX 40, Olympus Optical Co, Tokyo, Japan) and was scored according to the criteria described by Caderni (122). Sialomucin producing ACF (SiM-ACF) were identified by either dark or bright blue staining and mucin depleted foci (MDF) were identified by either very little staining or no staining.

Immunohistochemical Determination of β -Catenin Accumulated Crypts (BCAC)

Paraffin embedded sections were heated at 65°C for 30 min, deparaffinized in xylene three times for 5 min, and rehydrated through graded alcohol solutions at room temperature. Antigen retrieval was carried out by heating sections in a pressure cooker in antigen unmasking solution (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's instruction. To prevent nonspecific staining, the sections were treated with 2% serum albumin for 30 min at room temperature. Sections were incubated with mouse monoclonal anti- β -catenin antibody (BD Transduction Laboratories, Lexington, KY) at a dilution of 1:300 in a humidified chamber at 4°C overnight. Negative control sections were incubated with normal horse serum at the same dilution as primary antibody. After overnight incubation with primary antibody, the sections were washed in PBS, incubated for an hour with secondary antibody, biotinylated anti-mouse immunoglobulin, at a dilution of 1:200 (Vector Laboratories Inc., Burlingame, CA), and thereafter treated with 0.3% hydrogen peroxide to quench endogenous peroxidase activity. Staining of the tissue was performed

using avidin-biotin reagent (Vectastain ABC reagent, Vector Laboratories) for an hour. Horseradish peroxidase activity was visualized by treatment with hydrogen peroxide and diaminobenzidine (Vector Laboratories) for 7 min. The sections were counter-stained with hematoxylin. β -Catenin expression was evaluated based on the presence of staining in membrane and/or nuclei of mucosal cells.

Determination of Metallothionein-positive (MT-positive) Crypts

The paraffin embedded sections were deparaffinized in xylene three times for 5 min, and rehydrated through graded alcohol solutions at room temperature for 10 min. Antigen retrieval was carried out by microwave treatment for 10 min in freshly prepared 0.01 M citrate buffer. The sections were incubated in 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. The tissue sections were then incubated with anti-metallothionein primary antibody (Dako, Carpinteria, CA) at a dilution of 1:50 in a humidified chamber at 4°C overnight. Negative control sections were incubated in the absence of antibody, in normal mouse serum. After overnight incubation, the tissue sections were incubated with secondary antibody, rabbit anti-mouse immunoglobulin (Dako), for an hour at room temperature at a dilution of 1:100. The horseradish peroxidase activity was visualized by staining with diaminobenzidine for 9 min. The tissue sections were counterstained with hematoxylin. The percentage of metallothionein-positive (MT-positive) colonic crypt cells was obtained in each section at 40X magnification under a microscope (Olympus BX 40, Olympus Optical Co, Tokyo, Japan). Scoring was made based on 10%, 20%, 40% and >50% of positive staining of MT in the colonic crypts. The percent of MT-positive was determined by the intensity of staining of the crypts. For example when the

crypts were almost entirely positive for MT staining, the crypts were score over 50% of MT-positive.

Statistical Analyses

The data were analyzed by one-way using SAS system for windows, release 8.2 (SAS Institute Cary, NC). Two way analysis of variance (ANOVA) was used to examine the effect of diets and of promotion stage. Differences among group means were inspected using the Student-Newman-Keuls test. Pearson correlation analysis was used to determine the associations between measurements. A probability of $p \leq 0.05$ was used as the critical level of significance.

Results

Body Weight and Food Intake

The initial and final body weights and average food intakes are summarized in Table 4-3 and Table 4-4. Weekly body weight (data not shown) including final body weight showed no significant differences among animal groups. There were no differences in food intake among animal groups fed either basal diet or wheat diets throughout the promotion stage, indicating the wheat diets did not have any influence on body weight or food intake.

Effect of Wheat Diets on Morphological Markers

Table 4-5 shows the number of AC, ACF, large ACF and multiplicity of ACF in all diet groups during the early and late promotion stage. The hard red and soft white wheat diets significantly reduced the number of AC, ACF and large ACF compared to the basal diet in both the early and late promotion stages. However, there was no significant difference in ACF multiplicity among the diet groups. In almost all cases hard red wheat diet group had fewer of the markers compared to the soft white group; however, the difference did not achieve statistical significance (Figure 4-2).

When the effect of the hard red and the soft white wheat diets was compared between the early and late promotion stage, it was observed that the reductions in morphological markers by the wheat diets was slightly greater during the late promotion stage. There was an increase in ACF multiplicity between 6 weeks and 14 weeks. Animal groups fed hard red or soft white wheat diet had significantly fewer ACF at 14 weeks (late promotion stage) compared to 6 weeks (early promotion stage).

Two-way analysis of variance, showed that, overall, the hard red and soft white groups had significantly fewer AC, ACF and large ACF compared to the basal diet groups. There was a trend for fewer ACF in the hard red wheat diet group relative to the soft white wheat diet group but this difference was not statistically significant. There was a significant interaction ($p=0.05$) between diet and promotional stage.

Effect of Wheat Diets on Dysplastic Markers

Table 4-6 shows the number of dysplastic markers in carcinogen-treated rats fed the wheat diets or the basal diet during the early and late promotion stage. In the early promotion stage, total number of sialomucin producing ACF (SiM-ACF) was significantly lower in the hard red wheat diet group relative to the basal diet. The soft white wheat diet group was not different from the basal or hard red wheat groups. No significant difference among groups in flat ACF, MDF or their combination was observed in the early promotion stage. In the late promotion stage, total number of SiM-ACF, flat ACF and MDF was least in the hard red wheat diet group, followed by the soft white wheat diet and the basal group. These differences were statistically significant (Figure 4-3).

When results from the early and late promotion stage were combined, it was observed that the hard red wheat diet groups had significantly fewer SiM-ACF and MDF compared to the groups fed soft white wheat diet. Overall, relative to the basal diet group, the hard red wheat diet group reduced markers of dysplasia to a greater extent than did the soft white wheat diet. The total number of flat ACF and MDF increased over time, whereas the total number of SiM-ACF remained unchanged between 6 weeks (early promotion stage) and 14 weeks (late promotion stage).

Effect of Wheat Diets on a Biochemical Marker, β -Catenin Accumulated Crypt (BCAC)

In *en face* paraffin embedded sections of the colon tissues, β -catenin expression was detected immunohistochemically either in the membrane (M-BCAC) or both in membrane and nuclei (MN-BCAC) and in the majority BCAC, β -catenin was detected only in membranes. Table 4-7 shows the number of BCAC in animals fed the wheat diets or basal diet.

In the early promotion stage, both the hard red and soft white wheat diet groups had significantly fewer M-BCAC and MN-BCAC compared to the basal diet group. Compared to the early promotion stage, the number of M-BCAC and MN-BCAC was lower in the late promotion stage (Figure 4-4). In the late promotion stage, the hard red wheat diet group had fewer M-BCAC and MN-BCAC than the soft white wheat diet and the basal groups, and this difference was statistically significant. Overall in the promotion stage, the effect of hard red wheat was predominant in late promotion stage. A colon section stained immunohistologically for β -catenin is shown in Figure 4-5.

Effect of Wheat Diets on a Stem Cell Marker, Metallothionein (MT)

Table 4-8 shows metallothionein positive (MT-positive) crypts in both the early and late promotion stage. During the early promotion stage, both the total and average number of MT-positive crypts were significantly fewer in the hard red wheat diet group compared to the soft white wheat group and the basal group. A similar effect of hard red wheat was also observed in the late promotion stage. No effect of the soft white wheat diet was observed on MT-positivity in the colonic crypts either in the early or late promotion stage (Figure 4-6). Similar to BCAC, the total number of MT-positive crypts was lower in the late promotion stage compared to the early promotion stage indicating expression of MT could be an early

event in colon cancer development. A colon section stained immunohistologically for MT is shown in Figure 4-7.

Regression Effect of Wheat Diets on AC and SiM-ACF

Figure 4-8 shows the change in AC number between 6 weeks and 14 weeks. Fourteen weeks post DMH injection, the number of AC was slightly greater in the final basal diet group compared to the initial basal diet group, indicating the total number of AC remain unchanged or slightly decreased over time when the animals continued to be fed the basal diet.

However, in animals switched to the hard red or soft white wheat diets at 6 weeks and fed 8 more weeks, the number of AC significantly decreased, indicating that both wheat diets regressed established AC, suggesting a change to a lower cancer risk state. This decrease in AC number in the wheat diets compared to the initial basal group was statistically significant.

Figure 4-9 shows that in the hard red wheat diet group the yield of SiM-ACF significantly regressed between 6 weeks and 14 weeks. Regression was also observed with the soft white wheat diets. However, the regression due to hard red wheat was greater than with the soft white wheat.

Correlations among Colon Cancer Risk Markers

Table 4-9 shows correlation coefficients among the colon cancer risk markers studied. There was a significant correlation between ACF and SiM-ACF, MN-BCAC, Total-MT and average MT. However, except for SiM-ACF, the correlations were weak. Large ACF strongly correlated with all dysplasia markers (SiM-ACF, MDF, and flat ACF). However, large ACF did not correlate with either BCAC or MT-positive crypts.

Discussion

Colorectal cancer is one of the most common causes of death from cancer in the western world. It has been demonstrated that environmental factors, including diet, are one of the major contributors to its prevalence. Evidence from epidemiological and experimental studies suggest that whole grains and whole-grain foods might be a promising dietary intervention for prevention of colon cancer. Among whole grains, wheat is the principal cereal grain and the one most commonly consumed in the United States. In our previous studies we systemically assessed the chemopreventive effect of wheat flours differing in color, processing and hardness during the early promotion stage of colon carcinogenesis. We observed that the hard red wheat flour reduced the number of aberrant crypt foci (ACF) and sialomucin producing ACF (SiM-ACF) in the early promotion stage compared to the basal diet regardless of processed state. Three-way analysis of variance indicated that whole wheat did not differ from refined wheat in its effect on the early morphological markers of colon cancer risk. There was a statistically significant interaction between color and hardness of wheat flour, indicating that hard red wheat had fewer ACF compared to soft white wheat. Similarly, we found in the late promotion stage that, regardless of processing state, hard red wheat flour, had a greater effect than the soft white wheat on reducing colon cancer risk, based on fewer ACF, SiM-ACF and mucin depleted foci (MDF) in rats fed hard red wheat diets. Interestingly, feeding hard red wheat flour regressed animals from a high risk to a low risk state of colon cancer development. Our results on the chemopreventive effect of hard red wheat are consistent with the findings of studies conducted by Maziya-Dixon et al. (42), and Iovinelli and Gallaher (227). Both studies identified the effect of wheat class but not

processing, as a significant factor in colon cancer prevention, using different end point measures of colon cancer risk, in different animal models.

In the present study, we enumerated the total number of ACF at both 6 weeks (early promotion) and 14 weeks (late promotion) after the carcinogen treatment. The animals were fed refined hard red and soft white wheat diets throughout the early and late promotion stage. We observed that both the hard red and soft white wheat diets significantly reduced the number of ACF compared to the basal diet during the early and late promotion stage of colon cancer. There was a trend for fewer ACF in hard red and soft white wheat diet groups in the late promotion stage compared to the early promotion stage, indicating a decrease in ACF number over time in the animal groups fed wheat diets relative to basal diet.

In the process of colon cancer development, the number and size of ACF increase over time, eventually forming large ACF, defined as consisting of 4 or more aberrant crypts in a focus. These large ACF and the multiplicity of ACF are suggested to be better predictors of colon tumor development than total ACF, particularly during later stage of colon carcinogenesis. In our study, both hard red and soft white wheat significantly reduced the large ACF number in the early and late promotion stage. However no significant effect of hard red and soft white wheat was observed on reducing ACF multiplicity.

During colon cancer progression, ACF acquire phenotypic changes which include an increase in the degree of dysplasia and alterations in mucin production. We evaluated the effect of wheat diets on several markers of dysplasia, such as SiM-ACF, flat ACF and MDF. We observed that the hard red wheat diet reduced the number of dysplasia markers throughout the promotion stage, and this reduction was greater in the late promotion stage. There was a strong correlation between ACF and SiM-ACF. Interestingly, large ACF was highly

correlated with all measures of dysplastic ACF. It was also observed in our study that the hard red and soft white wheat regressed ACF and SiM-ACF from a higher risk to a lower risk of cancer development. The regression effect of hard red was greater than the soft white wheat diet.

Alterations in β -catenin gene expression are regarded as an early event in colon carcinogenesis. Studies have reported that β -catenin accumulated crypts (BCAC) in rat colonic mucosa predispose to colon cancer. The accumulation of β -catenin protein in the colonic crypts may be due to the frequent mutations in the β -catenin gene. Increased β -catenin in ACF with higher degree of dysplasia suggests that they have malignant potential to tumor. Increased nuclear and cytoplasmic expression of β -catenin suggests that these phenotypic alterations reflect a role of β -catenin in the progression of ACF (144, 148, 255-257). In our study, we have investigated the possibility of application of BCAC to a short term bio-assay for the assessment of colon cancer risk. We also examined whether BCAC can be used as an early biochemical marker for evaluation of the effect of wheat during the early and late promotion stage of colon carcinogenesis.

In our study we enumerated the total number of β -catenin accumulated crypts (BCAC) at 6 weeks and 14 weeks after the carcinogen treatment. We observed that after 6 weeks of carcinogen treatment, both the hard red and soft white wheat diet groups had significantly reduced membranous and nuclear expression of β -catenin compared to the basal diet group. There was no significant difference between the hard red and soft white wheat diet groups. However, after 14 weeks of carcinogen treatment (late promotion stage) hard red wheat diet group had significantly fewer BCAC compared to the soft white wheat diet and the basal diet group.

Studies have reported that BCAC frequently consisted of small crypts rather than large crypts and are lesions independent from typical ACF (144, 148, 255-257). We observed a correlation between nuclear BCAC and total ACF ($p=0.02$). We did not find any correlation of nuclear BCAC with large ACF. Our analysis was based on the total number of BCAC detected from a small portion (2.5 cm) of distal colon which represented only 20% nuclear BCAC of the total BCAC. It may be that use of a whole colon sample for determination of total number of BCAC might provide more statistical power to predict a correlation between ACF and BCAC.

Persistent over expression of the protein metallothionein (MT) within single crypts has been tested as a biomarker of colonic crypt stem cell mutation. Expression of MT protein has been associated with colon cancer development (199, 211, 212). In our study, we assessed MT-positivity in the colonic crypts after 6 weeks and 14 weeks of carcinogen administration to animals. The purpose was to enable assessment of development of cancer stem cells in the colonic crypts. We observed that the hard red wheat diet group has significantly fewer MT-positive crypts compared to soft white wheat diet and the basal diet groups after both 6 weeks and 14 weeks of carcinogen treatment. No effect of soft white wheat diet was observed on reducing MT-positive crypts.

A study by Donelley and colleagues (212) observed a linear correlation between total MT-positive crypts number and ACF number ($r=0.732$, $p<0.01$) or large ACF ($r=0.84$, $p<0.01$) in balb/c mice treated with dual mutagen until 20 weeks. Consistent with this study, we also found a correlation between total MT-positive crypts and ACF, although not as strong as reported by Donelley et al. (212). As for BCAC, enumeration MT-positive crypts in larger sections of colon tissue increase the strength of the correlation.

In conclusion, our study indicates that feeding hard red wheat significantly reduced the number of morphological markers, and a biochemical and stem cell mutation marker compared to the basal diet group. Overall, the effect of the hard red wheat diet was greater in the late promotion stage compared to the early promotion stage. This reduction in markers of colon cancer risk is consistent with the results from other studies. In those studies, hard red wheat diet reduced the number of ACF or tumor incidence when compared to refined wheat or a basal wheat-free diet (42, 227). These studies and our own support a protective effect of red wheat diet towards colon cancer risk. However why red wheat shows a stronger chemopreventive effect than white wheat is not clear yet.

The concentration of compounds known to be chemopreventive differ among wheat varieties (47, 223), which might explain why the chemopreventive potential of one wheat is greater than the others. Among these components, the concentration of lutein and hydrophilic antioxidants are higher in red wheat than white wheat. Lutein, the major yellow pigment of red wheat has been shown to decrease colonic cell proliferation and ACF formation (47, 54, 224). To date, it remains unclear which component in red wheat is responsible for the chemopreventive effect of red wheat relative to white wheat.

In the present study we used two commercial wheat flours, pastry flour (soft white wheat flour) and all purpose bread flour (red wheat flour) to investigate the effect of these flours on reducing a number of established colon cancer biomarkers. Due to the limited information regarding differences in composition of chemopreventive agents in these two commercial flours, it is not clear why one wheat would be more protective than the other. Further research is needed to identify the compounds present in these wheat flours which are offering

chemoprevention. Knowledge about the bioavailability of these compounds and their biological mechanism would also help to elucidate their role in colon cancer prevention.

Table 4-1. Proximate Analysis of Wheat Flours ^a

Composition (%)	Refined Hard Red	Refined Soft White
Carbohydrate	74.4	77.0
Protein	10.4	8.6
Fiber	3.2	3.0
Fat	1.4	1.7

^a Proximate analysis was done by Medallion Laboratories (Golden Valley, MN)

Table 4-2. Diet Composition (g/kg)

Diet	Basal	Refined Hard Red	Refined Soft White
Flour	0	615	615
Sucrose	100	55.4	45.2
Corn Starch	448.2	0	0
Casein	200	136	147
Cellulose	80	60.3	61.5
Mineral Mix	35	35	35
Vitamin Mix	10	10	10
L-Cystine	3	3	3
Choline Bitartrate	2.5	2.5	2.5
Butylated hydroxytoluene	0.014	0.014	0.014
Soybean Oil	120	81.5	79.6
Cholesterol	1.2	1.2	1.2
% CHO	55	51.5	51.9
% Protein	20	20	20
% Fiber	8	8	8
% Fat	12	9	9

Table 4-3. Body Weight of Animals in the Early and Late Promotion Stage ¹

Diet Group	Early Promotion Stage		Late Promotion Stage	
	Initial Body Weight	Final Body Weight	Initial Body Weight	Final Body Weight
	(g)	(g)	(g)	(g)
Basal	65.5 ± 1.6	432.0 ± 15.9	69.9 ± 1.5	541.1 ± 19.2
Hard Red	64.8 ± 1.1	433.3 ± 15.4	65.9 ± 1.2	502.0 ± 18.6
Soft White	67.7 ± 1.1	457.1 ± 13.9	66.8 ± 1.5	525.8 ± 14.1

¹ Values are reported as mean ± SE, n=15

Table 4-4. Average Food Intake of Animals in the Early and Late Promotion Stage ¹

Diet Group	Average Food Intake	
	Early Promotion Stage	Late Promotion Stage
	(g/day)	(g/day)
Basal	22.8 ± 0.6	24.3 ± 0.3
Hard Red	25.6 ± 0.7	22.6 ± 0.2
Soft White	25.3 ± 0.5	24.4 ± 0.3

¹ Values are reported as mean ± SE, n=15

Table 4-5. Effect of Hard Red and Soft White Wheat on Morphological Markers in Rat Colon ^{1, 2, 3}

Morphological Markers (n/cm ²)	Early Promotion Stage			Late Promotion Stage		
	Basal	Hard Red	Soft White	Basal	Hard Red	Soft White
AC	19.4 ± 1.6 ^a	10.9 ± 1.0 ^b	13.6 ± 1.6 ^b	20.3 ± 1.6 ^a	9.5 ± 0.9 ^b	10.7 ± 0.9 ^b
ACF	9.2 ± 0.7 ^a	5.3 ± 0.4 ^b	6.5 ± 0.7 ^b	7.3 ± 0.4 ^a	3.6 ± 0.2 ^b	4.1 ± 0.3 ^b
Large ACF ²	1.0 ± 0.2 ^a	0.4 ± 0.08 ^b	0.6 ± 0.1 ^b	1.8 ± 0.2 ^a	0.8 ± 0.1 ^b	0.8 ± 0.1 ^b
ACF Multiplicity	4.4 ± 0.04	4.3 ± 0.05	4.4 ± 0.06	5.2 ± 0.2	4.8 ± 0.1	5.1 ± 0.2

¹ Values are reported as mean ± SE, n=15

² Four or more AC in a foci considered as a large ACF

³ Values in a row, within a stage, with different superscripts are significantly different (p<0.05)

Table 4-6. Effect of Hard Red and Soft White Wheat on Dysplasia Markers in Rat Colon ^{1,2}

Dysplastic Marker (n/cm ²)	Early Promotion Stage			Late Promotion Stage		
	Basal	Hard Red	Soft White	Basal	Hard Red	Soft White
SiM-ACF	3.47 ± 0.39 ^a	2.21 ± 0.20 ^b	3.02 ± 0.35 ^{ab}	3.78 ± 0.25 ^a	1.62 ± 0.14 ^c	2.51 ± 0.24 ^b
MDF	0.06 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	0.21 ± 0.02 ^a	0.06 ± 0.01 ^c	0.13 ± 0.02 ^b
Flat ACF	0.03 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.16 ± 0.02 ^a	0.01 ± 0.01 ^b	0.08 ± 0.02 ^b
MDF + Flat	0.10 ± 0.02	0.07 ± 0.02	0.09 ± 0.02	0.37 ± 0.03 ^a	0.07 ± 0.02 ^c	0.17 ± 0.02 ^b

¹ Values are reported as mean ± SE, n=15. Abbreviations: SiM-ACF, sialomucin producing ACF; MDF, mucin depleted foci

² Values in a row, within a stage, with different superscripts are significantly different (p<0.05)

Table 4-7. Effect of Hard Red and Soft White Wheat on Number of β -Catenin Accumulated Crypts (BCAC) in Rat Colon^{1, 2}

BCAC (n/cm ²)	Early Promotion Stage			Late Promotion Stage		
	Basal	Hard Red	Soft White	Basal	Hard Red	Soft White
M-BCAC	9.27 \pm 1.85 ^a	4.09 \pm 0.53 ^b	5.32 \pm 0.64 ^b	6.17 \pm 0.83 ^a	1.82 \pm 0.46 ^c	4.38 \pm 0.47 ^b
MN-BCAC	3.88 \pm 0.95 ^a	0.93 \pm 0.26 ^b	1.45 \pm 0.42 ^b	1.20 \pm 0.32 ^a	0.06 \pm 0.04 ^b	0.88 \pm 0.21 ^a

¹ Values are reported as mean \pm SE; n=15. Abbreviations: M-BCAC, membranous β -catenin accumulated crypts; MN-BCAC, both membranous and nuclear β -catenin accumulated crypts

² Values in a row, within a stage, with different superscripts are significantly different (p<0.05)

Table 4-8. Effect of Hard Red and Soft White Wheat on Metallothionein Expression (Stem Cell Mutation Marker) in Rat Colon ^{1,2}

Metallothionein Expression	Early Promotion Stage			Late Promotion Stage		
	Basal	Hard Red	Soft White	Basal	Hard Red	Soft White
MT1	2.59 ± 0.47	1.22 ± 0.35	2.57 ± 0.48	2.47 ± 0.45 ^a	0.77 ± 0.17 ^b	2.01 ± 0.35 ^a
MT2	0.76 ± 0.17 ^a	0.24 ± 0.07 ^b	1.06 ± 0.21 ^a	0.36 ± 0.07	0.18 ± 0.05	0.40 ± 0.10
MT3	0.22 ± 0.09 ^{ab}	0.02 ± 0.01 ^b	0.41 ± 0.08 ^a	0.12 ± 0.03	0.08 ± 0.03	0.08 ± 0.04
MT4	0.28 ± 0.12	0.02 ± 0.01	0.19 ± 0.04	0.06 ± 0.03	0.02 ± 0.01	0.09 ± 0.04
Total MT	3.56 ± 0.79 ^a	1.51 ± 0.41 ^b	4.19 ± 0.68 ^a	3.01 ± 0.51 ^a	1.05 ± 0.19 ^b	2.59 ± 0.4 ^a
Average MT	0.16 ± 0.04 ^a	0.05 ± 0.01 ^b	0.18 ± 0.03 ^a	0.09 ± 0.01 ^a	0.04 ± 0.006 ^b	0.09 ± 0.02 ^a

¹ Values are reported as mean ± SE, n=15. Abbreviations are as follows: MT-1, MT-2, MT-3, MT-4 represent 10 %, 20%, 40% and over 50% of metallothionein positive crypts; Total-MT, total number of metallothionein positive crypts and Ave-MT, average metallothionein positive crypts

² Values with different superscripts are significantly different (p<0.05)

Table 4-9. Correlation Coefficients among Colon Cancer Risk Markers ¹

r Value	SiM-ACF	MDF	Flat	MN-BCAC	Total-MT	Ave-MT
p Value			ACF			
ACF	0.643	0.186	0.182	0.268	0.228	0.268
	<0.0001	0.079	0.085	0.012	0.035	0.013
Large ACF	0.518	0.448	0.423	0.084	0.186	0.186
	<0.0001	<0.0001	<0.0001	0.442	0.087	0.087

¹ SiM-ACF, sialomucin producing ACF; MDF, mucin depleted ACF; MN-BCAC, membranous and nuclear β -catenin accumulated crypt; Total-MT, total number of metallothionein positive crypt; Ave-MT, average metallothionein positive crypt

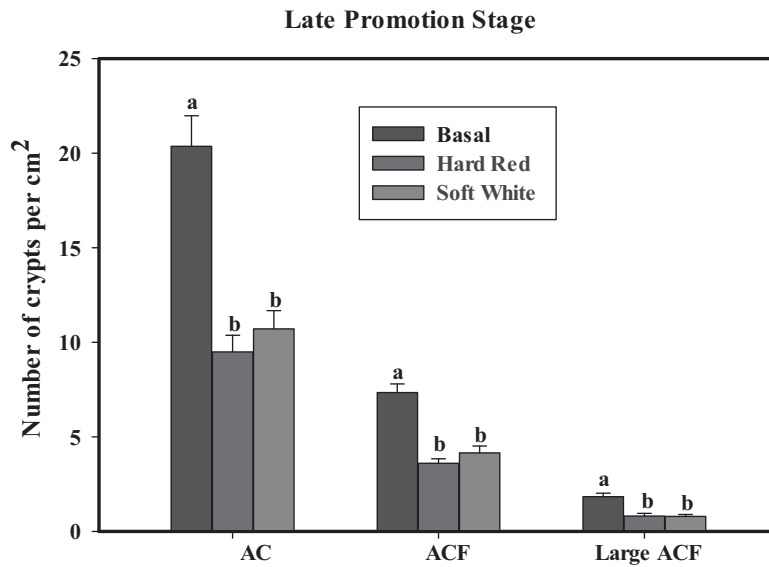
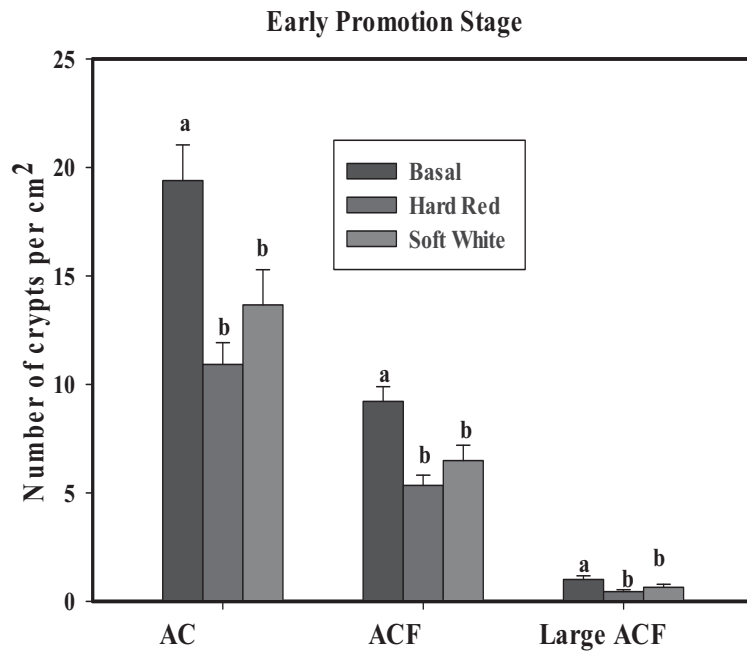


Figure 4-2. Effect of hard red and soft white wheat on morphological markers at the early and late promotion stage of colon carcinogenesis

Values are means \pm SE, n=15. Bars with different letters are significantly different (p<0.05).

4 or more AC in a foci considered as large ACF.

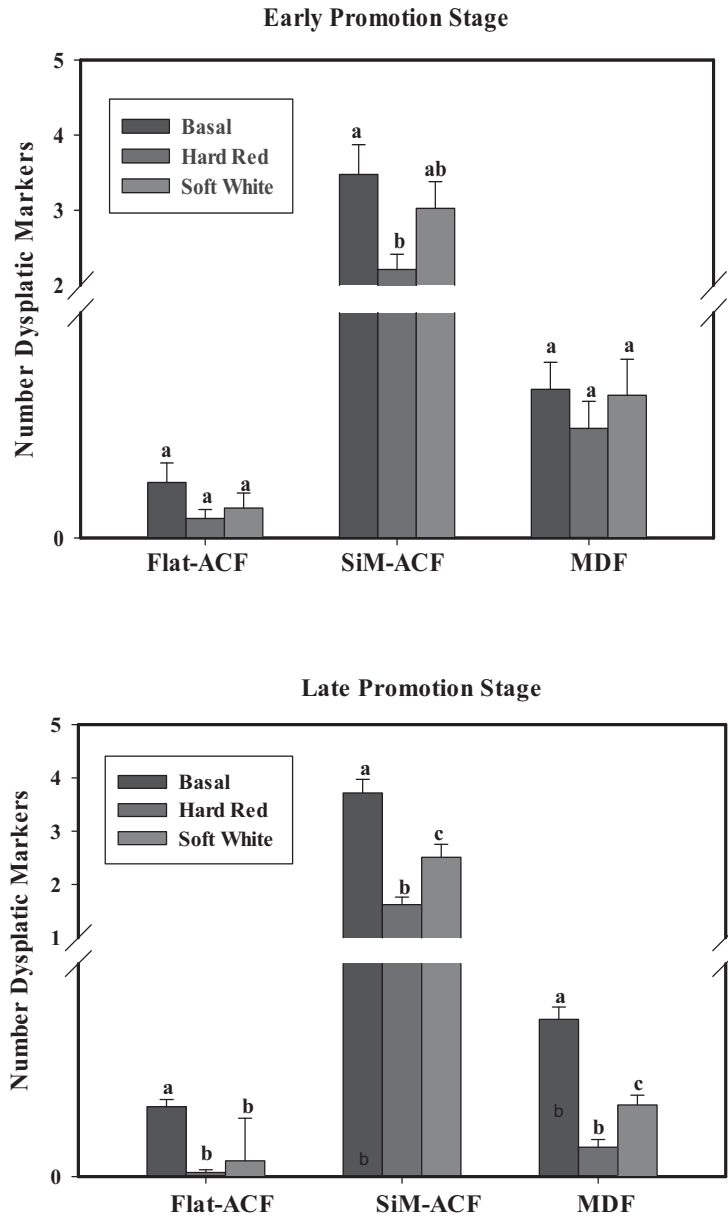


Figure 4-3. Effect of hard red and soft white wheat on dysplastic markers at the early and late promotion stage of colon carcinogenesis

Values are means \pm SE, n=15. Bars with different letters are significantly different ($p < 0.05$).

Abbreviations: SiM-ACF, sialomucin producing ACF, MDF, mucin depleted foci.

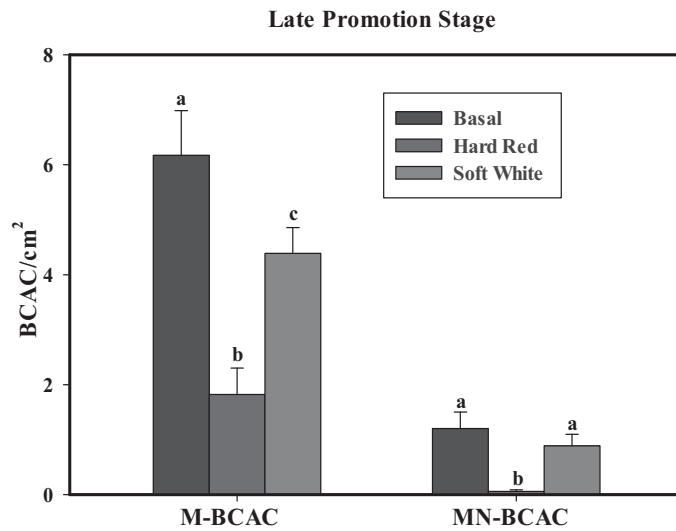
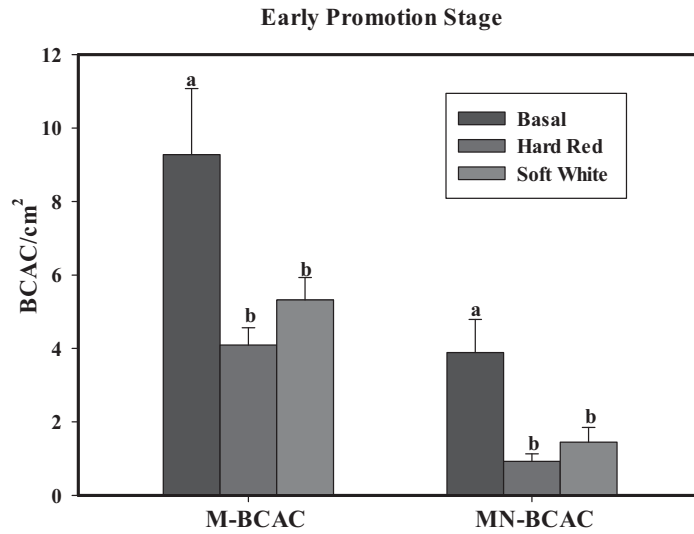


Figure 4-4. Effect of hard red and soft white wheat on biochemical markers at the early and late promotion stage of colon carcinogenesis

Values are means \pm SE, n=15. Bars with different letters are significantly different ($p < 0.05$).

Abbreviations: M-BCAC, membranous β -catenin accumulated crypt; MN-BCAC, membranous and nuclear β -catenin accumulated crypt.

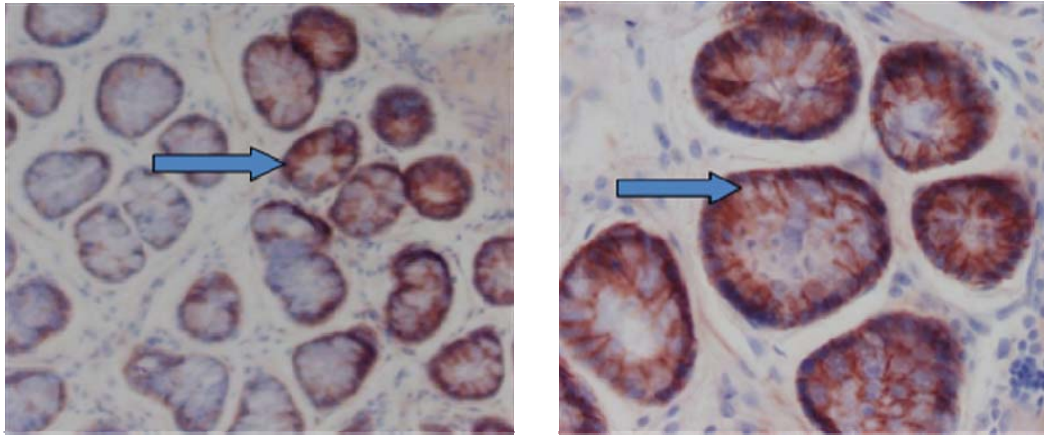


Figure-4-5: Immunohistochemical staining of β -catenin in colonic mucosa.

The membranous, cytoplasmic or nuclear immunostaining of β -catenin was determined under light microscope (400X). Adjacent normal crypts show no immunostaining for β -catenin. Arrowheads indicate nuclear accumulation of β -catenin.

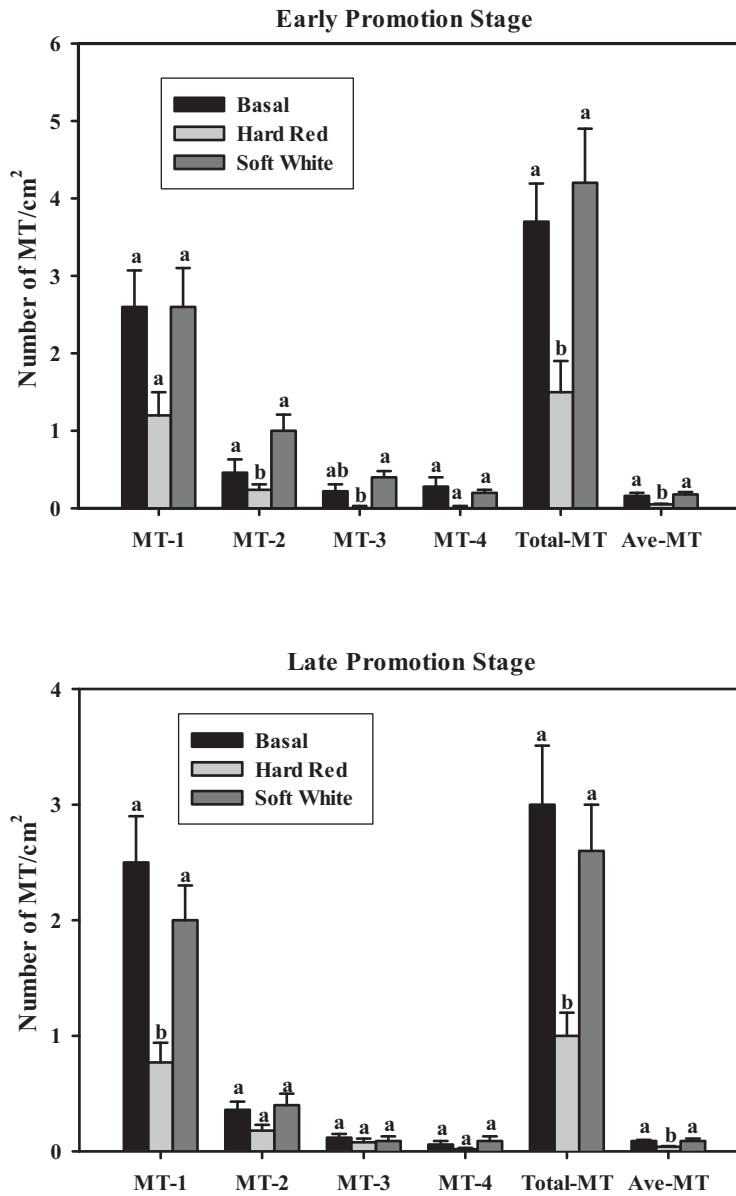


Figure 4-6. Effect of hard red and soft white wheat on stem cell markers at the early and late promotion stage of colon carcinogenesis

Values are means \pm SE, n=15. Bars with different letters are significantly different (p<0.05).

Abbreviations : MT-1, MT-2, MT-3, MT-4 represent 10 %, 20%, 40% and over 50% of MT- positive crypts; Total-MT, total number of metallothionein positive crypts and Ave-MT, average metallothionein positive crypts.

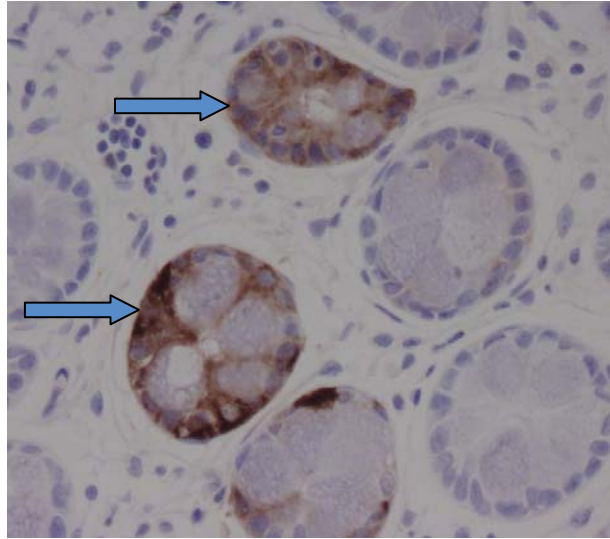


Figure-4-7: Immunohistochemical staining of metallothionein (MT) in colonic mucosa.

The immunostaining of metallothionein (MT) was determined under light microscope (400X). Adjacent normal crypts show no immunostaining for MT. Arrowheads indicate MT-positive crypts.

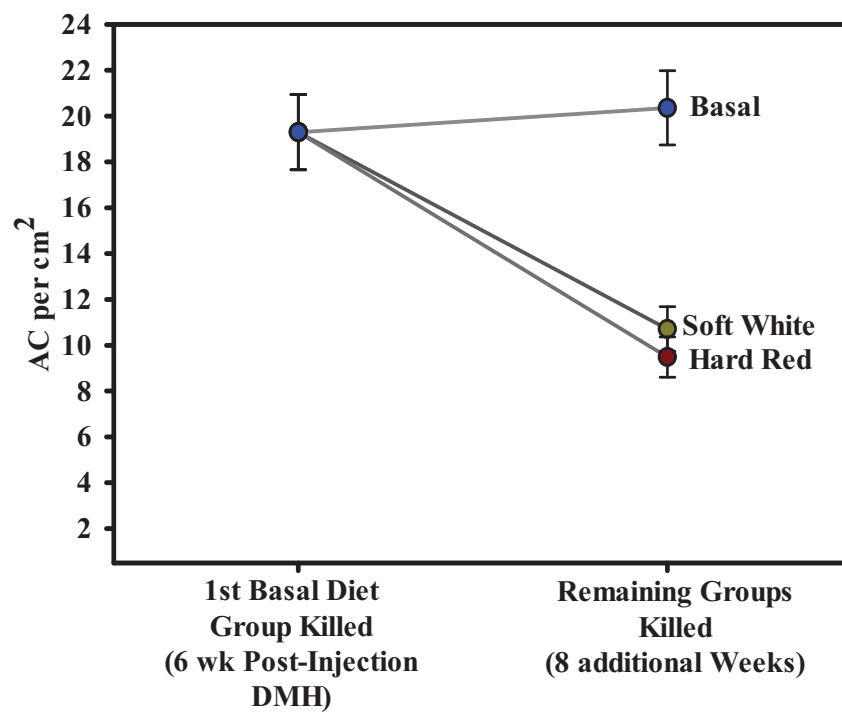


Figure 4-8. Regression of aberrant crypt (AC) by the hard red and soft white wheat flour

Values are represented as mean \pm SE, n=15

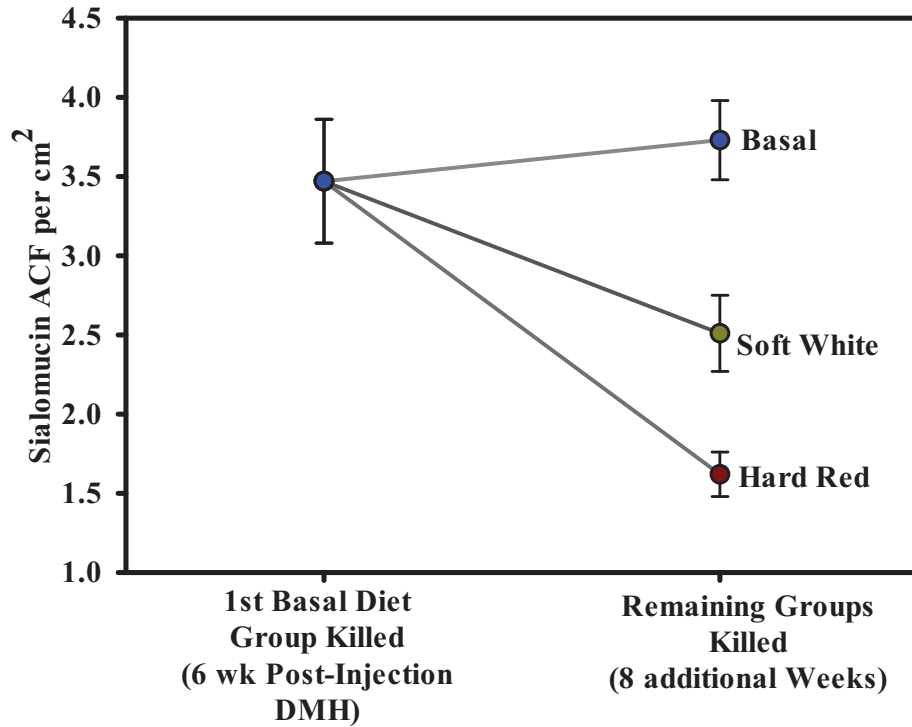


Figure 4-9. Regression of sialomucin producing aberrant crypt foci (SiM-ACF) by the hard red and soft white wheat flour

Values are represented as mean \pm SE, n=15

Chapter-5
Project Summary

Colorectal cancer is one of the most common cancers and the second leading cause of cancer deaths in the United States. It remains a major public health problem in the US, as well as in other western countries. The American Cancer Society (ACS) Guidelines on Nutrition and Physical Activity for Cancer Prevention recommends to eat at least five servings or more of fruits and vegetables each day and to choose whole grains (especially whole grain rice, bread, pasta, and cereals) in preference to processed (refined) grains and sugars. These recommendations were based on epidemiological studies which suggest high whole grains consumption decreased the risk of colon cancer, while refined grains increased the risk of colon cancer. There are few experimental studies which have examined the chemoprotective effect of whole grains, especially whole wheat, instead of individual components.

This project examined the effect of wheat class and refining state on reducing biomarkers of colon cancer risk. In the first experiment, rats treated with colon carcinogen were fed diets made from the four major classes of wheat flour. The effects of different characteristics of wheat (e.g., color, hardness and processing) were examined in reducing pre-cancerous lesions, i.e., aberrant crypt foci (ACF), during the early promotion stage of colon cancer development. It was observed that all animal groups fed wheat diets showed a numerical reduction in the number of ACF. Groups fed whole and refined hard red, whole and refined soft white, and refined soft red showed a statistically significant reduction in ACF compared to the basal diet group. Three-way analysis of variance (ANOVA) indicated a significant interaction between color and hardness of the wheat flour based on the ACF number, i.e., the effect of color depended on the hardness of the wheat. Processing state had no influence on the ACF number during the early promotion stage of colon cancer. SiM-ACF, which are suggested to be more advanced toward tumorigenesis, were significantly lower in all wheat

groups compared to the basal diet group, regardless of their color, hardness or processed state.

In the second experiment, rats treated with colon carcinogen were fed diets made from whole or refined hard red and soft white wheat during the late promotion stage of colon cancer development. Using wheat class and processing as the main effects, two-way ANOVA indicated that there was a statistically significant effect of wheat class, but not processing, on reducing the ACF number. ACF were significantly lower in hard red wheat diet groups (either whole or refined) compared to the soft white wheat (either whole or refined) and the basal diet groups. All wheat diet groups had significantly fewer SiM-ACF compared to the final basal diet groups. No mucin depleted foci (MDF), which are more dysplastic and more tumorigenic than SiM-ACF, were detected in the whole hard red wheat diet group. Rats fed diets containing hard red wheat flour, either whole or refined, had significantly fewer AC, ACF, large ACF, SiM-ACF, MDF and lower ACF multiplicity than those fed diets containing soft white wheat flour. The results suggest that hard red wheat flour, regardless of processing state, has a greater effect on reducing colon cancer risk in the late promotion stage of colon cancer than soft white wheat.

Our first two studies have shown that red wheat is more effective in reducing colon cancer risk relative to white wheat in the early and late promotion stage. The purpose of our final experiment was to further investigate the effect of wheat class on colon cancer risk, using a number of markers risk, such as morphological markers, a biochemical marker and a stem cell mutation marker. We observed that both hard red and soft white wheat diets significantly reduced morphological markers compared to the basal diet in the early and late

promotion stage. The hard red and soft white wheat diet groups had a significant reduction compared to the basal diet group in the markers of crypt dysplasia only in the late promotion stage. Animal groups fed the hard red and soft white wheat diet had significantly fewer β -catenin accumulated crypts (BCAC), a biochemical marker of colon carcinogenesis, during both the early and late promotion stage. The total and average metallothionein-positive (MT-positive) crypts, a marker of colonic crypt stem cell mutation, were significantly fewer in the hard red wheat diet group. In contrast, the soft wheat diet had no effect on MT-positive crypts. There were significant correlations between ACF and SiM-ACF, BCAC and MT-positive crypts.

In conclusion, our study found no difference between whole and refined wheat flour in reducing biomarkers of colon cancer. Red wheat tended to be more effective than white wheat. Further research is needed to examine why the hard red wheat flour is more chemoprotective than soft white wheat.

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