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THE EFFECT OF SOY PROTEIN ON COLON CARCINOGENESIS

IN VITRO AND IN VIVO

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Food Science

by

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ABSTRACT

Previous studies have suggested a beneficial effect of dietary soy against inflammation and colon cancer. Much of this has been attributed to soy isoflavones; however, soy proteins and peptides have also shown bioactivity and antioxidant activity in some models. Enzymatic hydrolysis of proteins has also been shown to increase free radical scavenging by increasing solvent accessibility of active amino acids.

We investigated the antioxidant capacity of an isoflavone-free soy protein concentrate (SPC) and an enzymatically-hydrolyzed SPC (SPH) using the oxygen radical absorbance capacity (ORAC) assay. Consistent with our expectations, SPH delayed the oxidation of fluorescein significantly longer than SPC ($p < 0.01$), indicating its superior antioxidative capacity.

The cytoprotective effects of SPC and SPH were also compared in the Caco-2 human intestinal cell model to evaluate their ability to prevent H_2O_2 -induced oxidation. Cells were cotreated with $50 \mu M H_2O_2$ and SPC or SPH (0-2 mg/ml) in PBS for 60 min or were pretreated with SPC or SPH (0-2 mg/ml) for 30 min and subsequently treated with $50 \mu M H_2O_2$ for 60 min. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Treatment with 0.5 mg/ml SPC increased cell viability from 42.8-50.0% in H_2O_2 -only treated cells to $76.1 \pm 9.0\%$ and $60.8 \pm 6.5\%$ of the PBS control in the cotreatment and pretreatment trials, respectively. Higher concentrations of SPH were required to achieve similar cytoprotective effects. At a concentration of 2 mg/ml SPH, cell viability was increased to $85.3 \pm 10.9\%$ and $62.1 \pm 3.2\%$ in the cotreatment and pretreatment trials, respectively. Photomicrographs of treated cells showed that SPC

prevented H₂O₂-induced changes in morphology and reduced intracellular reactive oxygen species (ROS). These effects were also observed for SPH treatment, but to a lesser extent.

The effect of dietary supplementation with SPC on colon inflammation and carcinogenesis in male, CF-1 mice was investigated in one 10-week and one 20-week study. In both studies, following one week pretreatment with SPC or casein as the sole source of protein (19% kcal), mice were injected with the colon carcinogen azoxymethane (AOM, 10 mg/kg body wt). One week later, mice received dextran sodium sulfate (DSS, 1.5% w/v) as their sole drinking fluid for one week to induce colon-specific inflammation. On days 3 and 7, and weeks 7 and 20 (second study only) after DSS treatment, mice were euthanized and the colons were examined for markers of inflammation and carcinogenesis. All samples were scored using the inflammation index, which measures inflammation area, severity, ulceration and hyperplasia and dysplasia. In both studies, trends in these measures appeared to be highly dependent on sacrifice time-point. With the data from both studies combined, mean polyp multiplicity was significantly reduced in mice fed SPC at Week 7 and for the study overall ($p < 0.001$ and $p < 0.01$, respectively). Hyperplasia and dysplasia, inflammation area and total inflammation index score were significantly decreased in the SPC group compared to the control at Week 7 ($p < 0.10$, $p < 0.10$ and $p < 0.05$, respectively). At 20 weeks, mice in both the control and SPC-treated groups showed colon tumors. A trend toward reduced tumor burden and multiplicity in mice fed the SPC diet was observed, but the effect was not statistically significant.

Finally, the ORAC assay was used to compare the radical scavenging capacities of SPC and casein, to elucidate any effect this may have had in the mouse studies. In comparisons of the proteins alone and of the SPC and casein-containing mouse diets, casein

was found to have significantly greater radical scavenging capacity ($p < 0.0001$). In conclusion, while these studies suggest that soy may have antioxidant activity and colon cancer preventative effects independent of isoflavone content, further research is required to confirm this finding and the underlying mechanism.

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Chapter 1: Literature Review

1.1 Soybeans

1.1.1 Composition

Soybeans (*Glycine max* L., Fabaceae) are legumes, composed of approximately 35% protein, 19% fat, 15% soluble carbohydrate, 15% fiber, 10-14% moisture and 5% minerals and ash (1, 2). Soybeans contain water-soluble vitamins such as thiamine, riboflavin, niacin, folic acid, inositol and choline; fat-soluble vitamins A and E; and minerals such as potassium, sodium, calcium, magnesium, sulfur and phosphorus (3). Soy contains several classes of compounds with potential bioactivity, including isoflavones, saponins, peptides and sphingolipids.

Isoflavones are heterocyclic polyphenols. The levels of isoflavones in soybeans have been found to be highly variable between cultivars, ranging from ~1.1-3.3 mg/g (4), and levels also depend on processing (5). Soy milk, tofu and soy flours have isoflavone concentrations of 1.3-3.8 mg/g on a dry weight basis (6). The most abundant isoflavones in soy are the glycosides genistin and daidzin, which account for as much as 90% of the total isoflavone content. The aglycone forms, genistein and daidzein, are also present as minor species (**Figure 1**) (6, 7).

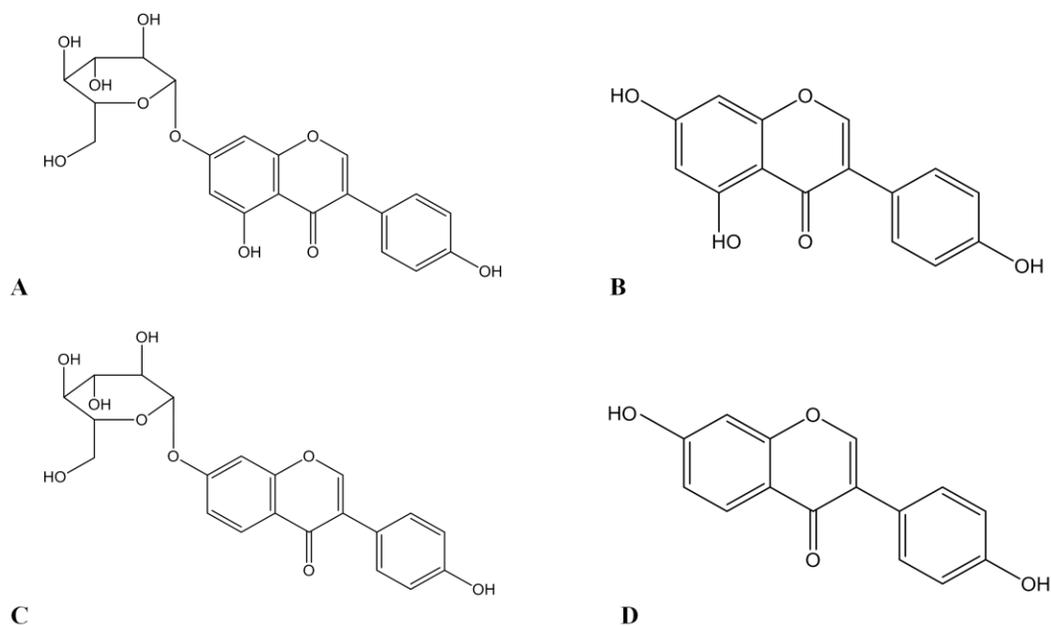


Figure 1: Chemical structures of the soy isoflavones: **(A)** genistin, **(B)** genistein, **(C)** daidzin and **(D)** daidzein.

Soybeans are also a major source of dietary saponins (0.5% w/w dry soybeans), which are thermally stable, amphiphilic glycosides (6). Processed soy products, such as soy milk and tofu, contain slightly lower levels of saponins, typically ranging from 0 to 0.4% (6, 8). The most frequently-occurring saponins are those with an oleanane-type triterpene aglycone portion (**Figure 2**), though more than 40 different saponins have been found in soy (9).

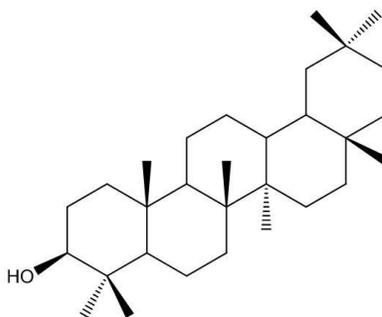


Figure 2: Chemical structure of oleanane-type pentacyclic triterpene saponins.

The focus of this research is soy protein. Soybeans are naturally high in protein and bioactive peptides, which can be present endogenously or can be derived through fermentative or enzymatic hydrolysis of soy protein (10). Glycinin and β -conglycinin account for 50 and 25% of total soybean endosperm protein, respectively (11). Glycinin is a 320-380 kDa protein containing 6 polypeptide units; β -conglycinin is a 150 kDa protein composed of three different subunits (11). Protease inhibitors make up 8-15% of soybean protein. The two most widely studied protease inhibitors are the Bowman Birk Inhibitor (BBI) and the soybean trypsin (Kunitz) inhibitor (6). BBI is a small, heat-stable polypeptide (8 kDa) with seven disulfide bridges and two homologous inhibitory sites for trypsin and chymotrypsin. The Kunitz inhibitor is a 21 kDa thermolabile polypeptide with only two disulfide bonds and inhibitory activity against only trypsin (6, 11).

1.1.2 Processing

Soybeans are not widely consumed in their raw or whole form in the United States, and extensive processing is most common. It is estimated that ca. 85% of soybeans in the US are processed into oil and meal. Soybean oil accounted for 82% of the edible fats and oils consumed by Americans in 1999 (12), and 95% of all soybean oil produced in the US is used in the food supply (13). Of the soy meal produced in the US, approximately 98% is used for animal feed; the remaining 2% is further processed into soy flour, protein isolates or protein concentrates.

In the processing of soybeans, the beans are cleaned, dehulled, cracked, flaked and steam conditioned before the oil is removed by solvent extraction (**Figure 3**) (8). From the defatted flakes, soy meal, soy protein concentrate (SPC) or soy protein isolate (SPI) can be derived. SPC is most commonly produced by removing a portion of the carbohydrates by alcohol extraction, leaving a high fiber, low fat, high protein (70%) product, which can be used in the production of processed meats, baked goods and breakfast cereals (14). This alcohol extraction also removes the isoflavones present. SPI is made by extracting and precipitating the protein, yielding a product which is *ca.* 90 percent protein. It is often used as an ingredient for protein-enriched snacks and beverages (14). In the US, soy protein has been used as an ingredient in the food industry since the late 1950s (14).

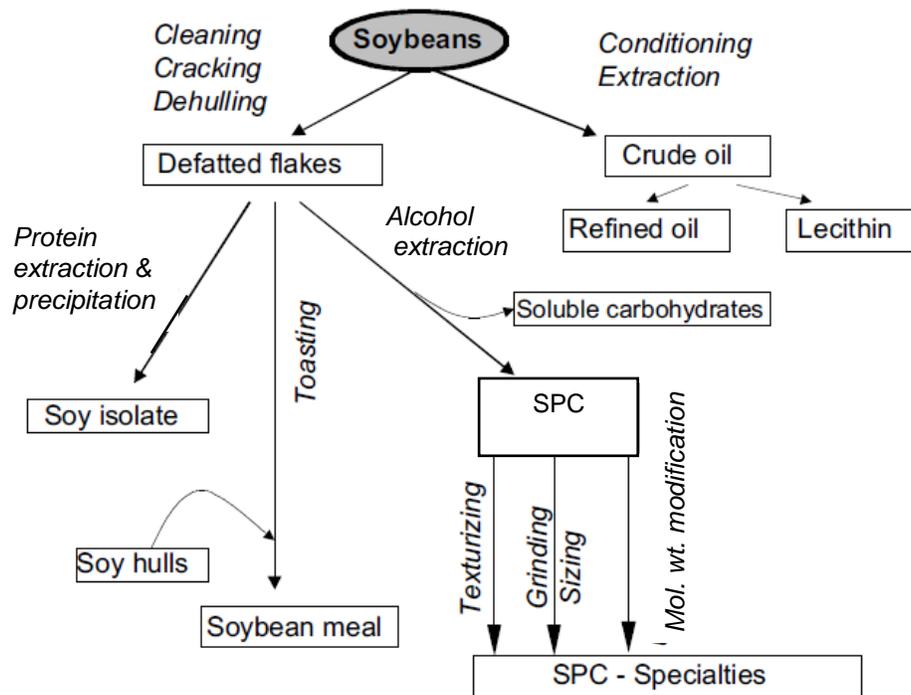


Figure 3: Manufacture of soy products. Source: Peisker, M. Manufacturing of soy protein concentrate for animal nutrition (8).

Globally, soybeans represent about 0.5% of daily energy intake per person; in Asia, soybeans account for 4.9% (15). Soybean production in the US is second only to corn; however, average per capita consumption of soy protein in the United States is only 1-3 g/day. In contrast, the average per capita soy protein intake in Asia is estimated to range from 20-80 g/day (16). Evidence suggests, however, that soy consumption is increasing in the United States, with sales of soy products increasing from \$300 million to \$4 billion between 1992 and 2008 (17). Soy consumption is especially increasing for its less processed forms (18), which may be due in part to its putative health benefits. Various studies have shown potential anti-cancer, cardiovascular, bone strengthening, cholesterol-lowering, and estrogenic health benefits (14, 19, 20).

1.2 Colon Cancer

1.2.1 Incidence and Risk Factors

Colorectal cancer was the third most commonly occurring cancer and third most common cause of cancer death in both men and women in the United States in 2009 (21). It is fatal in almost half of all cases, and is estimated to have accounted for 9% of all domestic cancer deaths in men and women in 2009 (21).

Genetics can play an important role in colon cancer risk, and 5-10% of colorectal cancer cases are a result of a hereditary conditions such as familial adenomatous polyposis (FAP) or hereditary nonpolyposis colorectal cancer (HNPCC) (15). Ulcerative colitis and Crohn's disease are also considered risk factors for colon cancer (22, 23). Diet and environment play an even larger role in colon carcinogenesis (24). Doll et al. have estimated that up to 90% of all colon cancer may be preventable with changes in diet (24). Smoking, body and abdominal fatness, factors leading to greater adult attained height, consumption of red meat, processed meat and high levels of alcohol are all considered factors with convincing evidence for increased colon cancer risk (15, 25). Physical activity decreases colon cancer risk, and garlic, milk, foods containing fiber and calcium have probable evidence for colon cancer prevention (15).

In general, cancer development is described using a three-stage model: initiation, promotion, and progression. During tumor initiation, DNA mutations occur, activating oncogenes or inactivating tumor-suppressor genes (26). Following initiation, the cells clonally expand, during the process of promotion. Finally, in the third stage, progression, increases in tumor size, invasion into other tissues, and metastasis can occur (26).

Colon and colorectal cancers generally begin as non-cancerous growths, or polyps, on the inner lining of the colon or rectum (27). Polyps can develop into cancerous growths over time, and are generally classified as inflammatory (generally not pre-cancerous), hyperplastic (generally not pre-cancerous) or adenomatous (adenomas, pre-cancerous). Dysplasia is also considered a pre-cancerous condition, and is especially common in people with chronic inflammatory diseases such as colitis and Crohn's disease. This occurs when immature cells in the epithelium of the colon or rectum expand, outnumbering mature cells (27).

Adenocarcinoma is the most common form of colorectal cancer and accounts for 95% of all cases. These tumors originate in the mucus glands of the colon and rectum (27).

Although early stage tumors are localized in the colonic epithelium, they can invade the basal layer of the colon wall and then metastasize to blood and lymph vessels (**Figure 4**). The American Joint Committee on Cancer (AJCC) Staging System is based on a three letter system: T (describes depth and extent of the growth of the tumor), N (describes the extent of spread to lymph nodes), and M (metastasis). Stage grouping is used to combine the ratings for each letter category, and the overall stage from 0 (no growth beyond inner lining) to IV (spread to one or more distant organs) is assigned (27). Generally, disease treatment and lethality are strongly dependent on the stage of the cancer at the time of detection. If detected in Stage I, the five-year survival rate for people with colon cancer is 74%. In contrast, if detected at Stage IV, the five-year survival rate is only 6% (28). As a result, early detection and prevention are vital to reduce colon cancer incidence and increase survival.

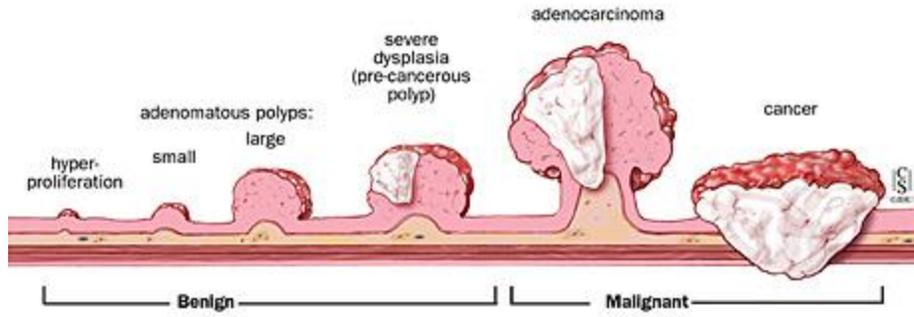


Figure 4: Progression of a colon polyp from benign to malignant. Progression of pre-cancerous dysplasia is shown in white. Source: Johns Hopkins Gastroenterology & Hepatology (29).

1.2.2 Inflammation and Oxidative Stress in Carcinogenesis

The link between inflammation and cancer has been recognized since the nineteenth century (30). For example, epidemiological evidence has shown that people with inflammatory bowel disease, a disease characterized by inflammation in the colon, have a lifetime risk of colon cancer approximately 15% greater than the general population (31). While acute inflammation is a beneficial immune response to infection or trauma, chronic inflammation can result in both DNA damage and cancer promotion (15). In general, cancer risk increases as the duration of chronic inflammation increases (32).

Two pathways are used to describe the link between inflammation and cancer: extrinsic and intrinsic (30). The extrinsic pathway includes inflammatory conditions, such as inflammatory bowel disease, or other external sources of irritation. The intrinsic pathway is that driven by genetic mutations, such as oncogene activation, which can cause inflammation (30). Mutation of the oncogenes of the RAS family are the most common in human cancers and result in the production of the tumor-promoting inflammatory cytokines and chemokines (30). As a result of either pathway, activation of transcription factors, such as nuclear factor kappa-B (NF- κ B), in tumor cells can occur. This leads to the production of chemokines, cytokines and prostaglandins by tumor cells, which recruit macrophages, eosinophils, neutrophils and mast cells to the site (30). Transcription factors are consequently activated in these cells, further propagating the cycle of production of inflammatory mediators and recruitment of inflammatory cells. This cycling results in cancer-related inflammation, which can cause cell proliferation, tumor migration and invasion, inhibition of adaptive immunity and other cancer-promoting activities (30).

Oxidative stress is also often implicated as a causative agent of carcinogenesis. Free radicals are unstable, highly reactive molecules, which contain an unpaired electron in their outer orbital. Reactive oxygen species (ROS) are comprised of at least one oxygen atom and are known to promote the oxidation of many biological molecules. As a category, ROS include both radical (*e.g.*, superoxide, hydroxyl, peroxy, alkoxy and hydroperoxy radicals) and non-radical (*e.g.*, hydrogen peroxide, peroxynitrite) species (33). ROS are normally produced in the body to act as signaling molecules in various physiological processes, and the body balances the production of ROS with their removal, with both enzymatic and non-enzymatic mechanisms. The superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase and catalase systems attenuate free radical damage enzymatically. Non-enzymatic antioxidants, including reduced glutathione (GSH), exogenous antioxidant nutrients, including proteins, lipids, vitamins, and minerals, and non-nutritive phytochemicals are also known to prevent radical damage (33).

Oxidative stress occurs when the production rate of free radicals exceeds their clearance rate. Free radicals can inhibit enzymes or directly oxidize proteins, free amino acids, lipids and DNA, which can lead to cell injury, mutation or cell death (33). DNA damage can include breaks, base modifications, intrastrand adduct formation, dimerization and crosslinking (34). These structural alterations and resulting genetic mutations can result in cancer initiation. Free radicals are also thought to affect cancer progression, by causing abnormal gene expression, blocking cell communication and modifying secondary messenger systems, which can result in an increase in cell proliferation or decrease in apoptosis (32). In the progression stage, oxidative stress may cause the accumulation of additional damaging DNA alterations which confer invasive or metastatic phenotypes (32). In addition to their

direct physiological effects during all three stages of cancer development, free radicals can also activate carcinogens to electrophilic, DNA-damaging molecules (35, 36).

Oxidative stress and inflammation are interconnected states, which together can lead to carcinogenesis. Free radicals are produced by all cells; however, inflammatory cells produce them at a much higher level (36). Inflammatory cells secrete cytokines and chemokines, which stimulate ROS and reactive nitrogen species (RNS) production (34). Neutrophils and macrophages have both been found to produce high levels of superoxide, hydrogen peroxide and hydroxyl radicals following stimulation (36). ROS and RNS also recruit other inflammatory cells, thereby amplifying the effect. This can create a highly oxidative environment in the afflicted area. A simplified schematic of the relationship between inflammation, oxidative stress and carcinogenesis can be seen in **Figure 5**

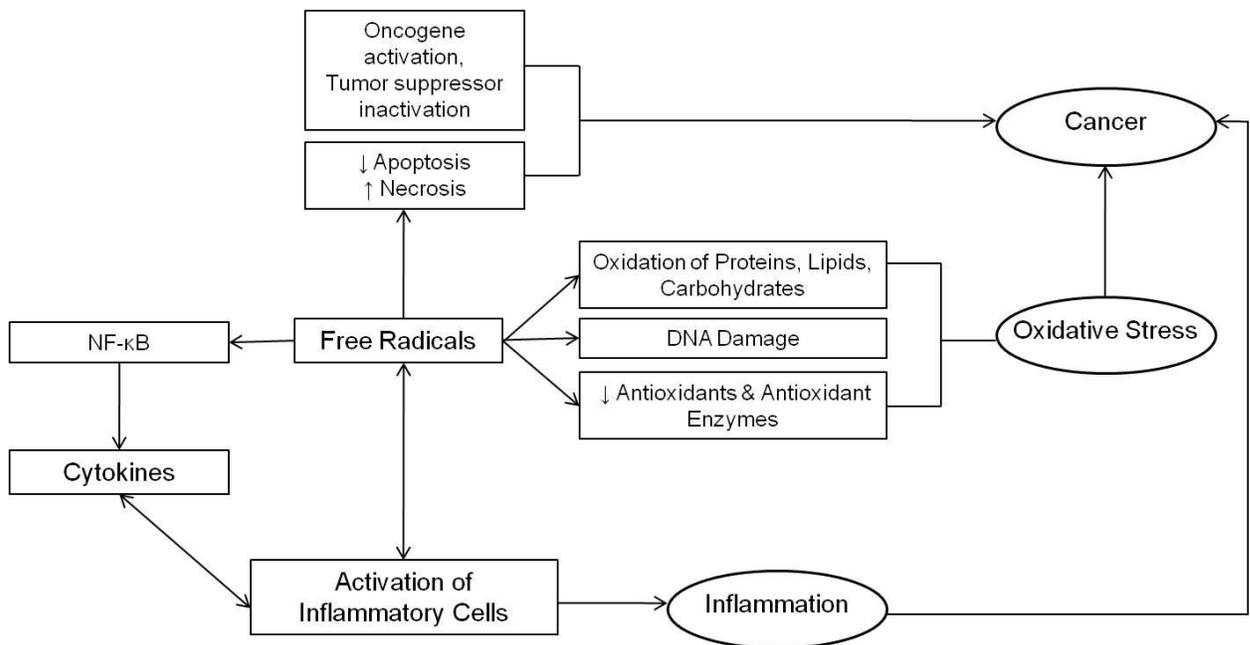


Figure 5: Schematic of the relationship between inflammation, oxidative stress and cancer. Adapted from Federico et al. (34).

Laboratory based evidence has shown the link between reductions in oxidative stress and improved clinical outcomes. For examples, in a murine model of colon cancer, it was found that three distinct antioxidants (*S*-adenosylmethionine, green tea polyphenols, and 2(*R,S*)-*n*-propylthiazolidine-4-(*R*)-carboxylic acid) were able to attenuate progression of colitis (37). Anti-inflammatory treatments and therapies have also been found to have positive clinical effects on cancer prevention and cancer treatment (32).

1.2.3 AOM/DSS Mouse Model of Colon Carcinogenesis

Animal models enable researchers to study the effect of dietary components on colon cancer in a controlled, economical and efficient way. Numerous genetic and carcinogen-based murine models of colon cancer exist, which recapitulate the pathology of human colorectal cancer to varying degrees. Because oxidative stress and inflammation are known to play such an important role in colon carcinogenesis, one useful model of colon cancer is the azoxymethane/ dextran sodium sulfate (AOM/DSS)–treated mouse model (38).

Other models of inflammatory bowel disease traditionally use long-term or repeated administration of DSS; however, the incidence and multiplicity of tumors are relatively low in these models (39). The AOM/DSS model was developed to overcome these limitations, as well as to emulate inflammation-related colon carcinogenesis in humans. The model was developed in CD-1 mice by treatment with a single intraperitoneal dose of 10 mg/kg body weight AOM, a colon-specific carcinogen, followed by a one-week administration of 2% DSS, a colon-specific irritant in the drinking water (39). DSS induces colitis by disrupting the intestinal barrier and exposing lamina propria macrophages to enteric bacteria, leading to activation of the nuclear factor kappa B (NF- κ B) pathway in the macrophages. This subsequently results in the production of pro-inflammatory cytokines and free radical generation by the macrophages, as well as activation of inflammatory pathways in intestinal epithelial cells (26). In the study, the mice were maintained on a casein-based diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo) (39).

In developing the model, Tanaka et al. found that the AOM/DSS treatment resulted in a 100% incidence of colonic adenocarcinomas after 20 weeks (39). Aberrant crypt foci (ACF) were also measured and are a commonly examined marker of colon cancer

pathogenesis in animal models. ACF are preneoplastic lesions which are morphologically distinguishable from normal colon crypts due to their increased size, elliptical shape and thicker lining of epithelial cells (40). ACF may also display dysplasia and express APC gene and RAS oncogene mutations (40); therefore, ACF may go through the multi-step process leading to the formation of microadenomas (41). Some microadenomas will develop into adenomas, and some of these will then develop into adenocarcinomas (41). Significant correlations have been found between the number of ACF and number of adenomas and between the number of adenomas and the size of the foci in human colons (42).

Because ACF are easily identifiable histologically and may be early markers of colon carcinogenesis, they are commonly used endpoints in colon cancer studies; however, adenomas and adenocarcinomas may be more relevant markers. The AOM/DSS mouse model was concluded to be a good model for investigating colitis-related colon carcinogenesis and for identification of putative anti-carcinogenic compounds (39).

1.3 Prevention of Colon Cancer by Dietary Soy

1.3.1 Human Studies

Several epidemiological and human intervention studies have investigated the effect of soy consumption on colon cancer and related diseases. In a meta-analysis of 38 epidemiological studies, soy consumption was associated with reduced overall risk for colon cancer (Pooled Risk Estimate (PRE)=0.70, $p<0.001$) (43). In a review of seven case-control studies, an inverse association was found between soy intake and colon and colorectal cancers, though only two of these studies were statistically significant (44). In these case-control studies, an inverse association was found specifically between adenomas and soy consumption (44). In six of seven studies of rectal cancer reviewed, an inverse association with consumption of unfermented soy foods was found; the results of two of these studies were statistically significant (44). One unpublished, case-control study in the review found an odds ratio (OR) of 0.53 for colon cancer for people consuming tofu or soybeans once or more per month compared to people eating less than one serving per month (44).

A more recent review on the association between soy and colorectal cancer risk examined four cohort and seven case-control studies (45). The analysis by Yan et al. did not find an overall significant inverse relationship between soy consumption and colorectal, colon or rectal cancer risk. However, when stratified by gender, a decreased risk of colorectal cancer was observed in soy-consuming women (combined risk estimate (CRE)=0.79, $p=0.026$). No significant association was observed in men (CRE=1.10) (45). Of the studies examined in the review, one prospective cohort study of 68,412 women found that with each 5 g/day (dry weight) increase in soy food intake, there was an 8% reduction in colorectal cancer risk (46). A study of 83,063 Japanese men and women found that, in

general, increased soy intake had no substantial effect on the risk of colorectal cancer in men and women, but did find that the risk of proximal colon cancer in men was decreased with increasing soy food and isoflavone consumption (Hazard Ratio 0.51, $p=0.009$, and 0.55, $p=0.007$, respectively) (47). The most recently published case-control study on soy consumption and colorectal cancer found energy-adjusted intakes of soy foods (dry weight) were inversely associated with colorectal cancer risk in men and postmenopausal women (OR= 0.65, $p=0.03$ and OR=0.60, $p=0.053$, respectively). Epidemiological studies of soy food consumption and colorectal cancer have shown positive trends, though the effect is often dependent on gender, clinical outcome and the type of soy food consumed.

Several human intervention studies have been conducted to examine the effect of various soy foods on markers of oxidative stress and inflammation. In a crossover study of 42 hypercholesterolemic subjects, plasma antioxidant concentrations were significantly higher after supplementation with isoflavones (50 mg/1000 kcal, $p=0.0001$) (48). Plasma antioxidant concentrations were measured using HPLC, and plasma total antioxidant performance (TAP) was determined using a method which measures the rate of oxidation of a lipid-soluble fluorescent probe. In subjects receiving dietary soy protein, TAP was 10% higher than those on animal protein diets ($p=0.0003$); however, this increased TAP did not significantly alter most other markers of oxidative stress (48). A similar study by Swain et al. found no differences in total plasma antioxidant status in a double-blind, parallel trial of diets of soy protein with isoflavones, isoflavone-depleted soy protein or whey protein (49).

A study by Zemel et al. also found no effect of soy supplementation on oxidative stress markers and inflammatory markers (50). When comparing the effect of a dairy or soy protein isolate smoothie in twenty overweight and obese men and women, they found that

only the dairy smoothie significantly reduced markers of oxidative stress (plasma malondialdehyde, 22%; 8-isoprostane- F_{2a} , 12%) and inflammation (tumor necrosis factor- α , 15%; interleukin-6, 13%; monocyte chemoattractant protein-1, 10%) compared to baseline measures (50). Beavers et al. found no significant difference in plasma inflammation and oxidative stress markers in thirty-one female subjects treated with either dairy or soy milk (51). However, in a study of post-menopausal women with metabolic syndrome, supplementation with soy protein for 8 weeks increased plasma total antioxidant content (5.8%) and decreased plasma lipid peroxides (9.4%) compared to women on a control diet (52). In general, the results of intervention studies focusing on the effect of soy food consumption on antioxidant status and oxidative stress markers have been mixed.

Intervention studies focusing more specifically on the effect of dietary soy treatment on biomarkers of human colon cancer have been limited and yielded mixed findings. In 2005, Adams et al. completed a 12-month randomized controlled trial to examine the effect of soy protein on colon epithelial cell proliferation in 50-80 year old men and women with a history of adenomatous polyps (53). The treatment group, on a diet of soy protein plus isoflavones (83 mg/d), had increased colonic epithelial cell proliferation compared to the isoflavone-free diet control group (11% increase, $p=0.01$). The number of labeled nuclei per 100 μm of crypt height also increased in the isoflavone-supplemented group compared to the isoflavone-free group (53).

Other human studies have examined the effect of soy extracts on gastrointestinal disease. Lichtenstein et al. examined the effect of Bowman-Birk inhibitor concentrate (BBIC), a soy extract with high protease inhibitor activity, in a randomized, controlled trial in patients with ulcerative colitis (54). The disease activity, response and remission of the 28

patients was assessed using the Sutherland Disease Activity Index after supplementation with either 800 chymotrypsin inhibitor (CI) units of BBIC or a placebo (54). No adverse effects or toxicity were observed, and the index scores of patients on the BBIC treatment were significantly decreased compared to patients on the placebo ($p=0.067$) (54). Additionally, 50 and 36% of patients on BBIC had a partial response or went into remission, respectively. In contrast, only 29 and 7.1% of patients on the placebo achieved a partial response or remission, respectively (54). This was the first trial of BBIC as a treatment for inflammatory bowel disease, and the results suggest that this soy extract may be an effective treatment for colitis. Intervention studies examining the effect of dietary supplementation with soy protein with isoflavones and BBIC have showed promising results; however, the number of studies is limited and more are necessary.

1.3.2 Animal Models

Studies of soy in various mouse and rat models of colon cancer and oxidative stress have shown promising, though mixed, results. Fournier et al. reviewed five *in vivo* studies of soy, and four of the studies analyzed found soy constituents to be protective for colon cancer; these studies focused largely on soy saponins, isoflavones and BBIC (6). In a review by Messina et al. neither of two studies which specifically examined colon carcinogenesis in rats found a protective effect of SPI compared to a control diet (55).

Several studies have been done to test the effect of a SPI-supplemented diet in colon cancer models when compared to a casein control diet. One study in male Sprague-Dawley rats found that the incidence of AOM-induced colon tumors was 76% lower in rats fed a 20% SPI diet compared to those on an AIN-93G diet with casein as the sole protein ($p < 0.05$), though no significant difference in tumor volume or multiplicity was seen (56). In a study by Linz et al., a 20% SPI diet significantly reduced the incidence of the largest class (≥ 5 crypts/focus) of ACF in AOM-treated male Sprague-Dawley rats (57). This study examined the effect of lifetime, pre-weaning, or post-weaning exposure to dietary SPI, and generally found similar reduction in ACF incidence in all cases. Mice fed a casein diet had an 83% incidence of the largest ACFs (5 crypts) in the distal colon, whereas mice on the SPI diet had only an 18% incidence ($p = 0.003$) (57). The protection from ACF by soy protein exposure during gestation and weaning may suggest that shorter exposure to soy protein early in life may protect against colon cancer (43). Several other studies have similarly found that the consumption of soy protein isolates may inhibit AOM-induced colon tumor formation (40, 58, 59).

More recent studies have examined gene expression changes in the colon following dietary supplementation with soy protein compared to casein. Xiao et al. found that colonic fatty acid synthase (FASN) and serum insulin levels were decreased in male Sprague-Dawley rats when fed 200 g/kg diet SPI for 30 days before initiation with 15 mg/kg body weight AOM. SPI also appeared to decrease AOM-induced phosphorylation of p53 tumor suppressor in colonic crypts, which suggested that SPI was able to reduce FASN-mediated anti-apoptotic effects (60). Bises et al. found that a 20% soy meal diet decreased pro-apoptotic Bak gene expression and increased anti-apoptotic Cox-2 and ER- α expression, particularly in females, which suggests a potentially detrimental effect of soy on tumor progression (61). However, this study was relatively small in scale and was done in healthy, not carcinogen-induced, animals. Depending on the model, diet and markers examined, results from gene expression studies have been mixed.

A number of studies have also explored alternate forms of soy, particularly miso (a fermented soy product) on ACF in rats. Masaoka et al. found that 10 and 20% dietary dry red miso significantly decreased the number of ACF/colon by 37.9 and 51.9% respectively, when compared to a sodium chloride control, in F344/DuCrj rats after 3 weekly injections of 15 mg/kg body weight AOM ($p < 0.01$) (62). Ohara et al. conducted a similar study and found that supplementation with 10% long-fermented dry red miso decreased the number of ACF/colon by 22% compared to the control; however, the number of aberrant crypts per focus was increased in the miso group (63). In a study in male Wistar SPF rats, Silva et al. found that giving 3 ml/kg body weight fermented soy yogurt by gavage had no significant effect on ACF after initiation with 1,2-dimethylhydrazine (DMH, 50 mg/kg body weight) (64). The effect of fermented soy products on ACF formation in rat colons is still unclear,

and the mechanism of action may differ from unfermented products due to the changes in protein structure, size and other chemical components which occur during fermentation.

Because of the association between oxidative stress and cancer, many studies have looked at the effect of soy supplementation on markers of oxidative stress in mice and rats. Paraquat (PQ) is known to cause oxidative stress in animals by generating superoxide radicals (65). In one study, Aoiki et al. found that supplementation with soy protein, but not soy isoflavones or saponins, reduced PQ-induced stress in rats (66). These findings agree with those of Hagen et al. who found an isolated soy protein diet (20%) decreased oxidative stress markers and lipid peroxidation, and increased antioxidant activity in the heart muscle of male Wistar rats with induced myocardial infarction (67). Takenaka et al. examined the activity of intact soy protein, soy peptides and amino acids in male Wistar rats with paraquat-induced oxidative stress. The results showed that both 20% dietary soy protein and soy peptides prevented elevation of serum thiobarbituric acid reactive substances (TBARS) concentrations and prevented lung weight elevation (a marker of oxidative lung injury), while the amino acid mixture resembling soy protein composition had no such effects (65).

In contrast to those studies, which have used directly induced oxidative stress, several have examined the effects of soy on oxidative stress in healthy or exercising animals. In one study, male C57Bl/6J mice fed 20% soy protein for two weeks had lower levels of oxidative parameters and higher antioxidant capacity than mice fed casein (68). Another study also measured similar parameters in a study of exercise-induced oxidative stress in male NMRI mice supplemented with 20% dietary casein, soy or whey protein (69). Only the whey-supplemented diet was able to decrease oxidative stress; however, the total protein level of the diet was relatively high (40%), as the preformulated diet contained 20% unspecified

protein before supplementation (69). This protein level may have led to results similar to that found by Gu et al., who found that high protein diets (60%) increased oxidative stress compared to normal protein diets (20%) in mice (68). One additional study, in female Sprague-Dawley rats, found that superoxide dismutase (SOD) and catalase (CAT) activities were significantly higher and TBARS levels were also significantly lower in rats fed a black soybean-supplemented diet (35%) compared to a casein-supplemented diet (35%) (70).

Several studies have also investigated the effect of BBIC as an anticarcinogenic agent. In a review of the anti-cancer properties of BBIC, three studies found that BBIC-supplementation suppressed DMH-induced colon carcinogenesis in mice (71). Another study found that BBIC suppressed intestinal tumorigenesis in the APC^{Min/+} mouse model. This genetic model is similar to human familial adenomatous polyposis and develops large numbers of intestinal adenomas (71). Two more recent studies found similar results. A 0.5% BBIC-supplemented diet significantly reduced tumor multiplicity and incidence in DMH-treated male Sprague-Dawley rats. If the diet was supplemented with autoclaved BBIC, the effect was lost, likely due to the loss of protease inhibitor activity (72). In the DSS model of ulcerative colitis, mice supplemented with 0.5% BBIC had significantly lower mortality, longer delay of mortality, reductions in three of four histopathological colonic inflammation criteria (extent of inflammation, severity of inflammation, amount of necrosis, and regeneration) and decreased total histopathological colon scores (73). This study proposed that BBIC is able to inhibit the proteases released from inflammatory cells as well as the release of superoxide anion radicals from leukocytes, resulting in reduced oxidative damage in the colon (73). While these mechanisms may have a similar end result as an antioxidant, it is important to note that BBIC does not function as a free radical scavenger (71).

It is also important to note that it is now recognized that protease inhibitors from soy, such as BBIC, are no longer thought to cause significant growth-suppressing or anti-nutritional effects (71). Based on epidemiological evidence in populations consuming high levels of soy foods, the previously reported negative effects of soybean protease inhibitors on rat pancreases are not expected to occur in humans. Potentially dangerous levels of protease inhibitors are also much less likely to be achieved in the human diet than in rodent dietary models (71). Levels of trypsin inhibitors in soy meal, SPC and SPI are also only about 2-4% of that found in unprocessed soybeans (8). Moreover, negative effects on rat pancreases are likely caused by the ability of protease inhibitors to affect trypsin activity, whereas the anticarcinogenic activity of BBIC is more related to inhibition of chymotrypsin (71). In BBIC, the chymotrypsin-inhibitory activity is greatly increased relative to the trypsin-inhibitory activity, when compared to raw soybeans (71). This suggests that BBIC may be useful as an anticarcinogenic drug in humans with minimal concern for pancreatic or anti-nutritional side effects.

Many other putative bioactive components from soy have also been tested for anticarcinogenic activity in animal models. Studies of soy isoflavones, particularly purified genistein and daidzein, have yielded positive, negative, and neutral results on various biomarkers of oxidative stress and cancer progression (7, 44, 74). Soy saponins have been found to have hypocholesterolemic, immunostimulatory, anticarcinogenic, and free-radical-scavenging activity (6). Koratkar & Rao found that an AIN-76A diet containing 3% soy saponin extract significantly decreased aberrant crypt foci incidence in a 14 week study of AOM-injected CF-1 mice (75). It is hypothesized that saponins act to inhibit colon cancer by increasing sequestration and excretion of bile acids (9). Sphingolipids are another class of

compounds in soy which have been examined for anticancer activity. Fyrst et al. found that administration of 25 mg/kg body weight sphingadienes by oral gavage suppressed intestinal tumorigenesis in the APC^{Min/+} mouse model (76). Another animal study found that supplementation of an AIN-76A diet with either 0.025 or 0.1% (wt/wt) soy glucosylceramide significantly reduced colonic cell proliferation in the upper half of colonic crypts in mice treated with DMH by 50 and 56% respectively. The number of ACF per mouse was also significantly reduced by 38 and 52% for 0.025 and 0.1% sphingolipid, respectively (77). Evidence exists that many components of soybeans may have bioactivity and anticarcinogenic effects in laboratory models; however, it is still unclear how these effects translate to humans.

1.3.3 Cell Culture

Cell culture studies of inflammatory responses and colon cancer have commonly focused on individual compounds derived from soy, such as lunasin, BBI, saponins or sphingadienes. For example, soy sphingadienes and saponins have both been shown to inhibit HT-29 human colon cancer cell growth *in vitro* (76, 78). Extracts of fermented soybean paste (Doenjang) have also been found to inhibit HT-29 cells (79). Because of their focus on single bioactives, it is difficult to extrapolate and relate mechanistic data from cell culture models to human colon carcinogenesis and soy consumption data. The simplicity of cell models and the lack of understanding of the active components of soy in human studies are both issues that hinder our understanding of soy's potential anticancer properties. Nevertheless, these studies provide hypotheses for future animal and human studies.

Several purified soy peptides have been found to have anticancer and anti-inflammatory effects *in vivo*. Lunasin has recently been shown to have anticancer activity due to its ability to disrupt histone acetylation and deacetylation, resulting in selective cell death (80). Lunasin was found to cause dose-dependent inhibition of HT-29 cells, with 62.8% inhibition at 100 μM (81). Additionally, after 18 h of treatment, 10 μM of lunasin significantly increased apoptosis and enhanced G2/M cell cycle arrest. It was concluded that lunasin may activate mitochondrial apoptotic pathways, leading to cell death (81). Lunasin also appears to have anti-inflammatory activity, as demonstrated by its ability to significantly reduce the production of ROS by lipopolysaccharide (LPS)-induced RAW 264.7 macrophages (82). In the same study, treatment of macrophages with lunasin led to a dose-dependent reduction in the production of the pro-inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-6, which are commonly up-regulated in inflammatory

diseases including cancer (82). These effects appear to be related to suppression of the NF- κ B pathway (83). Dia et al. also found that treatment of RAW.264 macrophages with lunasin inhibited the COX-2 and nitric oxide synthase (iNOS/NO) pathways, thereby suppressing of inflammation, a known factor in tumor progression and cancer risk (10).

BBI has also been tested in a similar colon cancer cell model. In HT-29 cells, two of the major constituent isoinhibitors of BBI (IBB1 and IBBD2) were found to significantly inhibit cell proliferation, despite their differences in selectivity for trypsin or chymotrypsin inhibition (84). IBB1 inhibits both trypsin and chymotrypsin, whereas IBBD2 inhibits only trypsin; yet the inhibitory concentrations in HT-29 cells were not different in this study (84). Chemically-inactivated BBI had no significant effect on the cells, and overall the results of this study suggested that the activity of BBI may be related to both trypsin and chymotrypsin inhibitory activity (84).

The effect of soy peptides on the human intestinal epithelial-like Caco-2 cell line has also been previously examined. Zhu et al. found that treatment of Caco-2 cells and HT-29 cells with soy protein shake extracts (6.5 mg/ml protein in extract, 0-10% vol/vol) resulted in cell morphological changes, such as vacuolization, and reductions in cell density (85); Satsu et al. also tested the effects of a soy peptide fraction (1 mg/ml) on the TNF- α induced production of IL-8. No significant effect by the soy peptide fraction was seen; however, an isoflavone fraction (1, 5 and 10 mg/ml) suppressed IL-8 secretion in a dose dependent manner (86). Interestingly, the isoflavone fraction tested was composed of 50% isoflavones, 10-15% saponins and 15% proteins, so the effect cannot be attributed solely to the isoflavones (86). Based on these results, the effect of soy protein on human intestinal cells, such as Caco-2 cells, is still unclear.

1.4 Antioxidant Activity of Proteins

High levels of free radicals and oxidative stress have been implicated as possible causative agents of disease. Dietary consumption of antioxidative compounds may help attenuate oxidative stress and resultant diseases, such as cancer (33). Proteins, peptides and amino acids have been found to act as antioxidants. Some proteins are capable of inhibiting oxidation by chelating pro-oxidative metals, scavenging free radicals, reducing hydroperoxides, and/or eliminating oxidants enzymatically (87). For example, 10 mg/ml SPI is capable of binding 405 μ moles of iron (87). Several amino acids are also thought to have antioxidant activity, including tyrosine, cysteine, homocysteine, methionine, histidine, lysine, tryptophan, arginine, and glycine (33, 88, 89).

The antioxidative activity of a given protein is highly dependent on both amino acid composition and tertiary structure. Certain amino acids may be capable of chelating metals or scavenging free radicals, but if buried within the hydrophobic core of a protein, these amino acids will be unable to react with hydrophilic pro-oxidant species (87). In general, hydrolysis can increase a protein's antioxidant activity, and this mimics digestion of protein by gastric and pancreatic proteases (pepsin, trypsin and chymotrypsin) (90). Tertiary structure disruption via heat treatment or enzymatic hydrolysis is a common approach to increase antioxidative activity of proteins *in vitro*. Pena-Ramos et al. examined the effect of hydrolysis on the antioxidant activity of SPI hydrolysates and found that chymotrypsin-hydrolyzed SPI exhibited significantly stronger antioxidant activity than non-hydrolyzed SPI in a TBARS lipid oxidation model (91). Park et al. found similar increases in antioxidant activity (against linoleic acid oxidation in an emulsion system) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging ability using soy protein hydrolysates, which was

likely due to increased solvent exposure of key amino acids and increased metal-chelating capacities (92). However, paradoxically, free amino acids are not effective antioxidants, and studies have shown that severe proteolysis leads to decreased antioxidant activity of the protein (87).

With soybean proteins specifically, work has been done to identify the resultant active peptides produced from hydrolysis. Chen et al. isolated six antioxidative (in a linoleic acid oxidation system) peptide fragments from β -conglycinin, a major constituent of soybean protein, hydrolyzed using protease S from a *Bacillus sp.* (93). The researchers identified and sequenced the antioxidative peptides and then carried out chemical syntheses of 28 peptides structurally related to the smallest of the previously identified peptides in order to investigate the residue-activity relationship (88). Antioxidant activity of each of the synthesized peptides was measured using the ferric thiocyanate method, and they found that Pro-His-His was the most antioxidative portion of the peptide sequence (88). Chen et al. carried out an additional study and found that the affinity of a protein for metal ions increased with the number of histidine residues (94). They also found that the peptide functions as a hydroxy-radical scavenger during oxidation of linoleic acid. Collectively, these findings suggested that histidine-containing peptides can act as a metal-ion chelators, active-oxygen quenchers and hydroxyl-radical scavengers; however, none of these properties alone could fully explain the antioxidative activity of the peptides (94).

The antioxidant activity of peptides is sequence-dependent and therefore protease-dependent (91). While work such as that done by Chen et al. is informative, hydrolysis of proteins by enzymes which more closely simulate human protein digestion would give more

information about potential bioactivity *in vivo*. Additionally, how the radical scavenging activity of these peptides translates to an *in vivo* system is unknown.

Despite the thorough examination of the antioxidant activity of proteins, peptides and amino acids in cell-free models, studies have yet to translate this into *in vitro* and *in vivo* systems. Furthermore, the relationship between antioxidative proteins and reduced cancer risk in animal models has not been fully explored.

1.5 Purpose and Significance

While some animal and human studies have suggested a positive correlation between soy consumption and reduced oxidative stress and colon carcinogenesis, the mechanistic details and bioactive components that are responsible for these effects remain unclear. Work done in cell-free models has shown that soy protein can act as a metal chelator and radical scavenger; however, further *in vitro* and *in vivo* studies are needed to validate these findings. It is necessary to investigate whether soy protein can act as an antioxidant *in vivo* to decrease oxidative stress, inflammation, and colon tumorigenesis.

Understanding if and how soy protein has a beneficial effect on inflammation or colon cancer is vital. Colorectal cancer is one of the top ten causes of death in general in the US (95), yet it is estimated that 90% of colon cancer may be preventable through changes in diet (24). In addition, it is estimated that approximately one million Americans suffer from inflammatory bowel disease (96), a disease which has been identified as a risk factor for colon cancer. Evidence suggests that increased intake of dietary antioxidants and other bioactive compounds, such as those found in soy, can reduce colon carcinogenesis and inflammation (6). Increasing soy protein consumption in the diet may therefore represent a cost-effective method of reducing colon cancer incidence (6).

Understanding the potential health benefits of soy protein is also of importance to soybean producers. The United States is one of the top soybean growers in the world, with 5.7 trillion pounds of soybeans produced in 2007 (97). Economically, soy protein is considered a waste product in the soybean oil refining industry, with the vast majority of the soy meal used for animal feed (13). Whereas soybean oil constitutes only 18-19% of the

soybean weight, protein accounts for ~35% (98). Diverting soy meal to the production of value-added products for human consumption could add value to the soy industry in the US.

1.6 Hypothesis and Objectives

Soy protein has been shown to have antioxidant activity and may have beneficial effects on oxidative stress and inflammatory diseases, including colon cancer. Previous studies have found an inverse relationship between soy consumption and colorectal cancer (43, 44), yet the direct antioxidant effect of soy protein *in vivo* is still unknown. This study will examine the antioxidant activity of soy protein concentrate (SPC), a low isoflavone soy protein product, in a human intestinal cell (Caco-2) cytoprotection model. Additionally, the effect of SPC on colon inflammation and cancer in a CF-1 mouse model will be examined.

I hypothesize that soy protein concentrate will protect Caco-2 cells against hydrogen peroxide-induced oxidation and will protect CF-1 mice from azoxymethane/dextran sodium sulfate-induced inflammation and carcinogenesis.

I propose to test this hypothesis by determining:

- a. The cytoprotective effect of SPC against hydrogen peroxide-induced oxidation in Caco-2 cells.
- b. The efficacy of a SPC-supplemented diet in reducing markers of colonic inflammation in mice.
- c. The efficacy of a SPC-supplemented diet in reducing colon carcinogenesis in mice.

Chapter 2: Materials & Methods

2.1 Soy Protein Concentrate and Hydrolysate Preparation

Chemicals:

Soy protein concentrate (Arcon SJ #066-408) was provided by the Archer Daniels Midland Company (Decatur, IL). The composition can be seen in **Table 1**. Analysis by the company showed reduced levels of isoflavones in this product, with only 0.099 mg aglycone equivalents/g concentrate. Standard soy protein isolates contain approximately 1.5-3 mg/g isoflavones (99, 100). Pepsin (3802 units/mg protein) and pancreatin (8 x USP specifications) were obtained from Sigma-Aldrich (St. Louis, MO).

Table 1: Average Composition of Arcon SJ Soy Protein Concentrate.

Component	%
Moisture	5
Protein	70
Fat	2
Ash	6
Dietary Fiber	17

Typical Amino Acid Composition	(g/100g protein)
Aspartic Acid	11.2
Threonine	3.8
Serine	5.1
Glutamic Acid	17.9
Proline	5.2
Glycine	4.2
Alanine	4.2
Cystine	1.3
Valine	5.0
Methionine	1.3
Isoleucine	4.7
Leucine	7.7
Tyrosine	3.4
Phenylalanine	5.0
Histidine	2.6
Lysine	6.5
Arginine	7.2
Tryptophan	1.2

Proximate composition and average amino acid composition data provided by Archer Daniels Midland.

Solubilization of Soy Protein Concentrate:

SPC was solubilized for use in the ORAC and cell culture assays. SPC was suspended in deionized water (1:20 w/v). The pH was adjusted to 11 under constant agitation to increase solubility. After 2 h, the pH was slowly adjusted back to 7.4. The solution was centrifuged at 3200 x g for 20 min at 20°C. The resulting supernatant was lyophilized (Freezone 2.5, Labconco, Kansas City, MO) and stored at -80°C until use.

Soy Protein Concentrate Hydrolysis:

The enzymatic hydrolysis of SPC was carried out using an adaptation of a previously published method (101). Briefly, 20 g SPC was suspended in deionized water (1:20 w/v) and was heated to 80°C for 5 min to reduce the bacteria population and denature lipoxygenase. The pH was adjusted to 2, pepsin was added (1:100 w/w, enzyme/SPC), and the solution was incubated at 37°C for 3 h. The pH was then adjusted to 7.5, pancreatin was added (1:20 w/w, enzyme/SPC) and was incubated at 37°C for 3 h. Hydrolysis was stopped by heating to 75°C for 20 min. The hydrolysate (SPH) was centrifuged at 25,000 x g for 15 min, and the resulting supernatant was lyophilized (Freezone 2.5, Labconco, Kansas City, MO) and stored at -80°C.

Nitrogen Analysis:

The Dumas method was used to analyze the total nitrogen in the SPC and SPH samples to allow comparisons in later experiments. In the Dumas method, all nitrogen-containing species in the sample are combusted at 800-1000°C to reduce them to nitrogen gas, which is then measured using a thermal conductivity detector. The analysis was carried

out using a Leco FP528 Nitrogen Analyzer (St. Joseph, MI). Samples (0.2 g) were pelleted in tin foil capsules and analyzed using an ethylenediaminetetraacetic acid (EDTA) standard.

2.2 Oxygen Radical Absorbance Capacity: SPC and SPH

The oxygen radical absorbance capacity (ORAC) assay was used to determine the antioxidant capacity of SPC and SPH. The ORAC assay is based upon the inhibition of the peroxy radical-induced oxidation of a fluorescent probe, fluorescein. The oxidation is initiated by the thermal decomposition of the azo-initiator, 2,2'-azobis-2-methylpropanimidamide, dihydrochloride (AAPH). The presence of peroxy radical scavenging species can delay the oxidation of fluorescein, and samples with greater radical scavenging capacity will extend fluorescence to a greater extent over time.

Chemicals:

Fluorescein sodium salt and AAPH were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of the highest grade commercially available.

Antioxidant Capacity Measurement:

A Fluoroskan Ascent FL fluorescent plate reader (Thermo Scientific, Waltham, MA) was used for all ORAC measurements. Pre-solubilized SPC and SPH were dissolved in phosphate buffer (10 mM, pH 7.4) at various concentrations (0-3000 mg/L). In each well of a 96-well black opaque plate, 25 μ l of sample or phosphate buffer (“blank”) was added to 150 μ l of fluorescein (10 nM) in triplicate. The plate was incubated for 30 min at 37°C in the plate reader without shaking. Following incubation, fluorescence was measured (Ex. 485 nm, Em. 520 nm) every 90 seconds for 3 cycles to determine the background signal. AAPH (25 μ l, 240 nM) or phosphate buffer (for “Fluorescein only” control) was then added to the wells and fluorescence was measured every 90 seconds for 90 min.

Data Analysis:

For each sample or control, the fluorescence values at each time point (F) were compared to the initial fluorescence reading (F_0), thus indicating the percentage of the initial fluorescence remaining over time. The experiment was run in duplicate, and values presented represent means \pm standard deviation (SD). Graphs of the decay of fluorescence over time were prepared and analyzed using GraphPad Prism (San Diego, CA). F/F_0 values over time were compared using Two-way ANOVA. At selected time points, mean F/F_0 values for SPC and SPH were compared using the student's t-test. Statistical significance was achieved at $p < 0.05$.

2.3 Caco-2 Cytoprotection Studies

Materials:

Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's Modification of Eagle's medium (DMEM, 1x), 0.25% trypsin (with 2.21 mM EDTA), and penicillin-streptomycin (10,000 IU/ml and 10,000 µg/ml, respectively) were purchased from Mediatech, Inc. (Manassas, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Hydrogen peroxide (35% w/v) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO) was obtained from EMD Chemicals (Gibbstown, NJ). 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCDHFDA) was purchased from Invitrogen (Carlsbad, CA). Solubilized SPC and SPH were prepared in phosphate buffered saline (PBS, pH 7.4). All other chemicals were of the highest grade commercially available.

Cell Culture & Cytotoxicity:

Caco-2 cells were cultured in DMEM, supplemented with 10% (v/v) FBS and 1% penicillin-streptomycin at 37°C under 5% CO₂/95% air. At 80% confluence, cells were passed using 0.25% trypsin. The effect of H₂O₂, SPC and SPH treatment on cell viability was determined. At 80% confluence, Caco-2 cells were seeded to a 96-well plate (5x10³ cells/well) and allowed to attach for 24 h before treatment. Cells were treated with H₂O₂ (0-400 µM), SPC (0-1 mg/ml) or SPH (0-1 mg/ml) in PBS for 60 min at 37°C, after which cell viability was assessed using the MTT assay. The MTT assay is based on the reduction of MTT to purple formazan crystals by mitochondrial enzymes in metabolically active cells.

After incubation for 30-60 min with 1 mg/ml MTT, the absorbance was measured at 540 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific, Waltham, MA).

Cell viability was expressed as a percentage of the cells treated with only PBS.

Cytoprotection Assays:

To determine the effect of co-treatment and pre-treatment of cells with SPC or SPH on H₂O₂-induced cell death, Caco-2 cells were passed at 80% confluence to a 96-well plate (5x10³ cells/well) and allowed to attach for 24 h prior to treatment. For the co-treatment experiment, cells were simultaneously exposed to 50 μM H₂O₂ and varying concentrations of SPC or SPH (0-2 mg/ml) in PBS for 60 min at 37°C. For the pre-treatment experiment, cells were incubated with varying concentrations of SPC or SPH (0-2 mg/ml) for 30 min at 37°C. The cells were then washed with PBS, and treated with 50 μM H₂O₂ for 60 min at 37°C. After each experiment, cell viability was assessed using the MTT assay. Cell viability was expressed as a percentage of the control, which was treated only with PBS. To examine morphological changes in the cells, images of the cells cotreated with 50 μM H₂O₂ and/or 0.5 mg/ml SPC or SPH were taken under the microscope (VistaVision microscope, DV-130 digital camera, LissView software 10.4, VWR). The cells were allowed to recover in media for 48 h at 37°C, after which the MTT assay was performed to determine cell viability, as a percent of the cells treated with only PBS

Effect of SPC and SPH on Cellular ROS

Caco-2 cells were passed at 80% confluence to petri dishes (20.8 cm², 15,625 cells per cm²). Cell density was equivalent to 5x10³ cells/well on a 96-well plate. After a 48 h

attachment period, cells were exposed to PBS alone, 50 μM H_2O_2 , 1.0 mg/ml SPC + 50 μM H_2O_2 or 1.0 mg/ml SPH + 50 μM H_2O_2 in PBS for 60 min at 37°C. Cells were washed with DMEM and incubated with 10 μM DCDHFDA, a fluorogenic ROS indicator at 37°C for 30 min. Cells were then washed twice with PBS and once with DMEM. The fluorescence was observed under a Olympus BX-51 Fluorescence Microscope at the excitation and emission wave lengths of 490 nm and 525 nm. 8-10 representative visible light and corresponding fluorescent images were taken for each treatment.

Statistical Analysis:

Graphs were prepared and analyzed using GraphPad Prism (San Diego, CA). One-way ANOVA with Tukey's post-test was used to compare the cytotoxicity of varying doses of H_2O_2 , SPC and SPH, cell viability following cotreatment and pretreatment with SPC and SPH at varying concentrations, and cell viability following treatment with 48 h recovery. The efficacy of preincubation and coincubation were compared using a paired t-test with matched concentrations of SPC or SPH. Statistical significance was achieved at $p < 0.05$.

2.4 Mouse Carcinogenesis Studies

Experimental Diet:

Diets were prepared by Research Diets, Inc. (New Brunswick, NJ) and are detailed in **Table 2**. The control diet was AIN-76A rodent diet, which contained casein as the protein source. The SPC diet was equivalent in macronutrient composition to the AIN-76A diet, with SPC replacing casein as the protein source. Azoxymethane (AOM) was purchased from MRI Global Chemical Carcinogen Repository (Kansas City, MO). Dextran sodium sulfate (DSS) was purchased from MP Biomedicals, LLC (Solon, OH). All other chemicals were of the highest grade commercially available.

Table 2: Composition of Mouse Diets.

	Control	SPC
Macronutrient Composition		
Protein (<i>% of energy</i>)	19	19
Carbohydrate (<i>% of energy</i>)	69	69
Fat (<i>% of energy</i>)	12	12
Energy (<i>kcal/g</i>)	3.8	3.8
Ingredient (g/kg)		
Casein	200	0
DL-Methionine	3	3
Corn Starch	150	150
Sucrose	500	500
Cellulose	50	2
Corn Oil	50	42.3
Mineral Mix (cat# S10001)	35	35
Vitamin Mix (cat# V10001)	10	10
Choline Bitartrate	2	2
Soy Protein Concentrate	0	255
FD&C Blue Dye #1	0	0.05

Diet composition data provided by Research Diets, Inc.

Animals and Treatment – 10 Week Study:

Male CF-1 mice 5 weeks old from Jackson Laboratories (Bar Harbor, ME) were maintained on a 12 h light/dark schedule and had access to food and water *ad libitum*. Mice were housed in shoebox cages on corn cob bedding with five mice per cage. All experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #29544). Mice were allowed to acclimate for one week prior to being randomly divided into three groups: AIN-76A diet with no AOM/DSS (“Neg”, n=15), AIN-76A diet with AOM/DSS (“Pos”, n=30), and soy protein concentrate-supplemented diet

(“SPC”, n=30). The entire study spanned 10 weeks and body weight and food consumption were monitored weekly throughout the study.

After one week on the diets, mice were injected with AOM (10mg/kg body wt. *ip.*) or saline (0.9% *ip.*). One week later, mice received DSS (1.5% w/v) as their sole drinking fluid *ad libitum* for one week. DSS consumption was monitored during this time. Mice in the Neg group were continued on regular drinking water.

Mice were euthanized at days 3, 7 and week 7 after the DSS treatment ended. Blood was taken by cardiac puncture from anesthetized mice. Plasma was obtained by centrifugation at 700 x g for 15 min and was stored at -80°C prior to analysis. Spleens were removed, weighed, and fixed in formalin. Colons were harvested and the fecal contents were collected and frozen at -80°C. The colons were then split longitudinally, rinsed and preliminary counts of visible polyps (diameter <1mm) were taken. The colons were cut crosswise: the proximal colon was snap frozen at -80°C and the distal colon was rolled using the Swiss roll technique (102) and fixed in 10% formalin.

Animals and Treatment – 20 Week Study:

A 20 week-long study, with increased sample size, was carried out using an identical design to the 10 week study. Male CF-1 mice were allowed to acclimate for one week before being randomly assigned to one of three groups: AIN-76A diet with no AOM/DSS (“Neg”, n=60), AIN-76A diet with AOM/DSS (“Pos”, n=60), and soy protein concentrate-supplemented diet (“SPC”, n=60). AOM and DSS treatment were the same as that in the 10 week study.

Mice were euthanized at days 3, 7 and week 7 after the DSS treatment ended. Blood, fecal, spleen and colon collection followed the same procedure as previously described. After colons were split and rinsed, preliminary counts of visible polyps and tumors were taken. Polyps were defined as <1mm diameter; tumors had diameters >1mm. Tumors were measured, and tumor area (length x width) was calculated.

Histopathology:

Formalin-fixed distal colon Swiss rolls were paraffin-embedded, sectioned and stained using hematoxylin and eosin (H&E). Samples were blinded and were evaluated for adenomas and by the inflammation index by a veterinary pathologist (Timothy K. Cooper) (103). Histopathologically-confirmed adenomas with dysplasia were classified as mild, moderate, severe, or as adenocarcinomas (invasive/noninvasive). Representative microscope images of colons from each treatment group were taken.

The inflammation index was calculated as the sum of the scores of four independently measured parameters: inflammation severity, inflammation area, ulceration and hyperplasia and dysplasia. Scoring was completed following the procedure of Ju et al. (103).

Inflammation severity was scored as 0 (normal colon mucosa), 1 (mild, focal or widely separated multifocal inflammation limited to the basal one third of the mucosa with lost crypts), 2 (moderate, multifocal or locally extensive inflammation and/or fibrosis up to two third of the crypts), or 3 (severe, mucosal ulcers with monocytes and polymorphonuclear leukocytes infiltrated into the mucosa, submucosa, muscularis propria and/or subserosa). Ulceration was scored as 0 (absent) or 1 (present) and was defined as an area of mucosa where the epithelial lining was missing. Inflammation area was scored as 0, 1, 2, 3, or 4,

which corresponded to 0%, 1-25%, 26-50%, 51-75% or 76-100% of the surface area examined, respectively. Hyperplasia and dysplasia (H&D) were scored as 0 (normal), 1 (mild hyperplasia, epithelial cells lined normally, but crypts 2-4 times thicker than normal crypts), 2 (low-grade dysplasia, 2-4 times thicker epithelium, hyperchromatic cells, fewer goblet cells and scattered crypts developing an arborizing pattern), or 3 (high grade dysplasia, >4 times thicker epithelium, hyperchromasia, few or no goblet cells, highly mitotic cells in the crypts with arborizing pattern and crypts extended to muscularis mucosa or submucosa).

Morphological abnormalities such as gut-associated lymphoid tissue (GALT) hyperplasia, crypt herniation, necrosis of submucosal fat, near complete ulceration, abundant foamy macrophages, focal squamous metaplasia, possible microinvasion, focal microinvasion or necrotizing arteritis in the mesentery were also noted.

Statistical Analysis:

All plots show the mean \pm standard error of the mean (SEM). Two-way ANOVA with Bonferroni's post-test was used to compare body weight and food consumption between treatment groups over time. Average DSS water consumption between groups was compared using the student's t-test. One-way ANOVA with Tukey's post-test was used to compare spleen-to-body weight ratios at each time point. Polyp multiplicity at each time point was compared between the Pos and SPC groups using students' t-test. Tumor number and tumor area at Week 20 were compared using students' t-test. Statistical significance was achieved at $p < 0.05$, unless otherwise noted. Inflammation index scores were analyzed using Chi-

square analysis to compare Pos and SPC at each time point, with statistical significance noted at $p < 0.10$. All analyses were performed using GraphPad Prism (San Diego, CA).

2.5 Oxygen Radical Absorbance Capacity: SPC, Casein and Mouse Diets

Chemicals:

Fluorescein sodium salt and AAPH were purchased from Sigma-Aldrich (St. Louis, MO). Sodium caseinate was obtained from NZMP (Lemoyne, PA). Diets evaluated were SPC and AIN76A, which were obtained from Research Diets, Inc. (New Brunswick, NJ). All other chemicals used were of the highest grade commercially available.

Antioxidant Capacity Measurement:

The procedure used to compare the antioxidant capacities of SPC, casein and the mouse diets is detailed in Section 2.2.

SPC and casein were compared on an equal protein basis. The concentrations evaluated were 350, 700 and 1400 mg/L protein, which were dissolved in phosphate buffer (10 mM, pH 7.4).

For analysis of the diets, which both contain 20% protein, levels were adjusted to use comparable levels of protein as in the previous assay. The concentrations evaluated were 700 and 1400 mg/L protein (3.5 and 7 g diet/L, respectively), in phosphate buffer (10 mM, pH 7.4).

Data Analysis:

Graphs of the decay of fluorescence over time were prepared and analyzed using GraphPad Prism (San Diego, CA). F/F_0 values over time were compared using Two-way ANOVA. At selected time points, mean F/F_0 values for SPC and SPH were compared using the student's t-test. Statistical significance was achieved at $p < 0.05$.

Chapter 3: Results and Discussion

3.1 Oxygen Radical Absorbance Capacity: SPC and SPH

The levels of nitrogen in SPC and SPH were determined by the Dumas assay. SPC and SPH contained 11.48 and 10.75% nitrogen, respectively. All subsequent analyses and experiments were carried out on an equal nitrogen basis.

The peroxy radical scavenging activities of SPC and SPH were measured using the oxygen radical absorbance capacity (ORAC) assay. In the assay, compounds with radical scavenging capacity will delay the degradation of fluorescein, a fluorescent probe that loses its fluorescence upon oxidation, in the presence of the peroxy radical generator, AAPH. Thus, compounds with greater antioxidant capacity will prevent a decrease in F/F_0 over time. The blank sample, which contained neither SPC nor SPH, lost $93.4 \pm 2.8\%$ of its initial fluorescence after 60 min (**Figure 6**), whereas the fluorescein-only (no AAPH) control decreased by only $0.78 \pm 3.87\%$ after 60 min. Although both SPC and SPH possessed peroxy radical scavenging activity in the ORAC assay, SPH extended fluorescence significantly longer than SPC. For example, after 60 min, 750 mg/L SPC and SPH retained $34.2\% \pm 6.9$ and $82.8\% \pm 1.2$ of their initial fluorescence, respectively ($p < 0.01$).

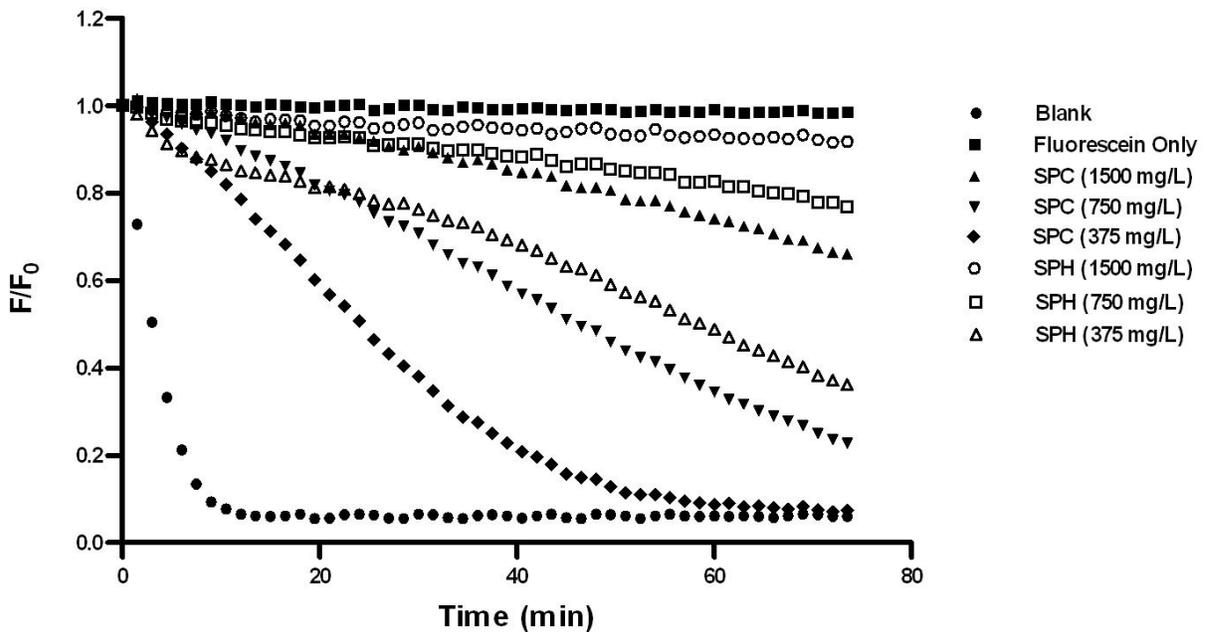


Figure 6: Oxygen radical absorbance capacity of SPC and SPH. Changes in relative fluorescent intensity of fluorescein (E_m , 485 nm; E_x , 520 nm) in the presence of AAPH and 375-1500 mg/L SPC or SPH at 37°C. Fluorescence values (F) are given relative to the initial time values (F_0). The blank was prepared without any antioxidant, and the fluorescein-only control was prepared without AAPH or antioxidant. Data points represent the mean of two replicates. Error bars were eliminated for clarity. Fluorescence values were different between treatments over time (Two-way ANOVA, $p < 0.0001$).

Previous research has found that hydrolysis can increase the antioxidant activity of proteins by increasing solvent exposure of amino acids (87). For example, Darmawan et al. found that alcalase hydrolysis of 27 defatted soy flours resulted in ORAC values of 57.0-81.1 $\mu\text{mol Trolox equivalents (TE)/g flour}$ (104). These values are higher than those found in past studies of non-hydrolyzed soy flours, which ranged from 35.1-44.2 $\mu\text{mol TE/g}$ (105, 106). Zhang et al. used microbial proteases to hydrolyze SPI and found the 12 resultant hydrolysis fractions to have TE values ranging from 23.8-83.8 $\mu\text{mol TE/g}$, which they noted is in excess of that found for many vegetable antioxidants by weight (107). One limitation of these studies is the lack of direct comparison in each aforementioned study of the hydrolysates to

unhydrolyzed soy flours or SPI. Furthermore, soy flour and SPI typically contain isoflavones, which may influence the absolute TE values. In our study, we directly compared isoflavone-free SPH and SPC, and found that SPH had greater antioxidant capacity than SPC.

3.2 Caco-2 Cytoprotection Studies

Cytotoxicity:

In order to determine the appropriate experimental medium, the stability of H₂O₂ in PBS and DMEM cell culture media was determined. When 500 μM of H₂O₂ was added to DMEM and DMEM + 10% FBS, 100-150 μM was lost immediately. By 60 min, nearly all of the H₂O₂ was lost. By contrast, H₂O₂ was stable in PBS over the time frame of interest (**Figure 7**). Previous work also reported the instability of H₂O₂ in DMEM, finding more than 90% loss within 30 min (108).

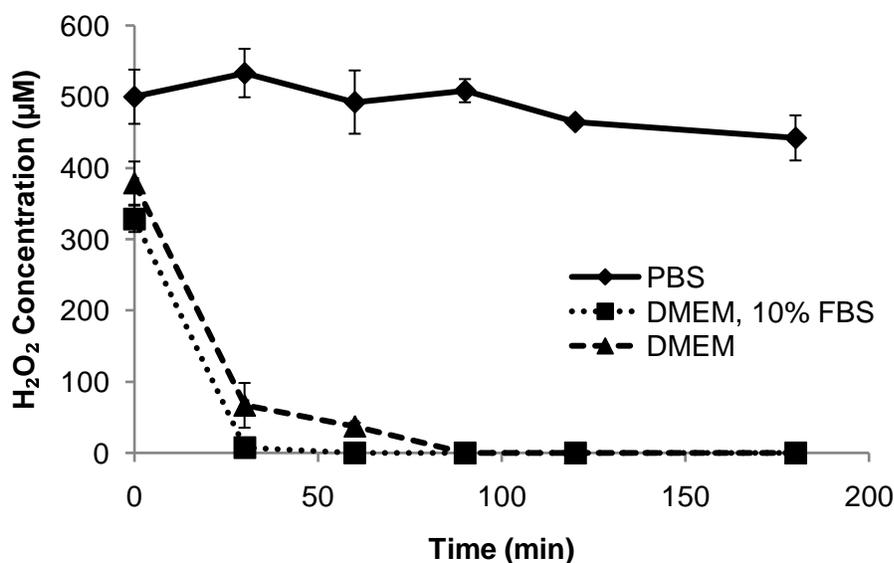


Figure 7: Stability of H₂O₂ in PBS, DMEM and DMEM + 10% FBS over time by the Fox assay. 500 μM H₂O₂ was added to all solutions at time 0, and the H₂O₂ concentration was measured every 30 min. Values represent means ± SEM.

The effect of increasing doses of H₂O₂ on Caco-2 cells was investigated in order to determine an appropriate dose to use for future cytoprotection studies. H₂O₂, a ROS, can be formed *in vivo* by dismutation from superoxide anion or can be spontaneously formed from

molecular oxygen (32). Relative to other ROS, H_2O_2 is less reactive; however, it plays an important role in carcinogenesis because it is capable of crossing cell membranes, entering the cytoplasm, diffusing throughout the mitochondria and causing cell damage (32).

Cytoprotection studies use H_2O_2 as an oxidant to measure the ability of test compounds to prevent cellular oxidative damage and death.

Cell viability, as determined by the MTT assay, decreased dose-dependently after 1 h treatment with H_2O_2 (**Figure 8**). At a concentration of $50 \mu M H_2O_2$, cell viability was reduced to $40.3\% \pm 3.5$ of the vehicle control ($p=0.004$). In a 1 h treatment with immediate assessment of cell viability, concentrations exceeding $50 \mu M$ caused no further significant cytotoxicity; therefore, $50 \mu M$ was chosen as the effective dose for all subsequent experiments. This concentration is consistent with that used to achieve similar Caco-2 cytotoxicity in previous works (109).

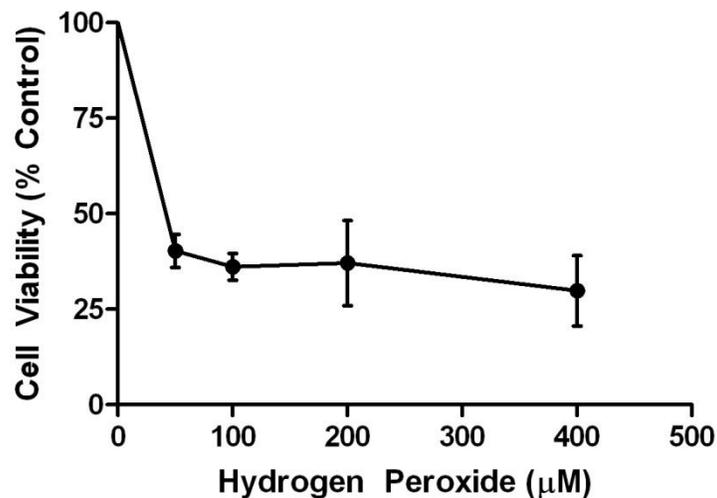


Figure 8: The effect of H_2O_2 on Caco-2 cell viability. Cell viability was measured immediately following treatment using the MTT assay. $50 \mu M H_2O_2$ reduced cell viability nearly 60% after 1 h. Values represent means of two replicates \pm SEM.

The effect of SPC and SPH treatment on Caco-2 cell viability was also determined. No cytotoxicity was seen after treatment with SPC or SPH; by contrast, both SPC and SPH treatments dose-dependently increased Caco-2 cell viability (**Figure 9**). Cell viability was significantly higher when cells were treated with 0.25, 0.5 or 1.0 mg/ml SPC or SPH compared to treatment with the vehicle control alone ($p < 0.05$). SPC and SPH likely provide nutrients and trace elements to the cells, increasing viability, but the effect plateaus at concentrations above 0.25 mg/ml. The increases in cell viability are expressed relative to the control, which was treated only with PBS. Based on subsequent findings, treatment with PBS alone for 60 min may stress cells and decrease viability (**Figure 13**). As a result, the apparent increased viability due to SPC and SPH may also be due to the PBS control having decreased viability.

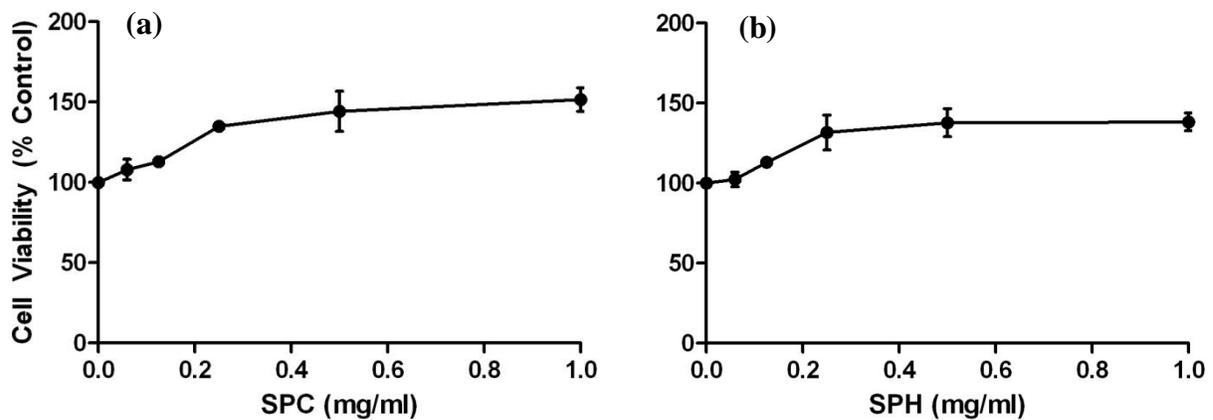


Figure 9: The effect of (a) SPC and (b) SPH on Caco-2 cell viability. Cell viability was measured immediately following 1 h treatment using the MTT assay. Values represent means of three (a) and four (b) replicates \pm SEM. Cell viability was significantly higher for cells treated with 0.25, 0.5 or 1.0 mg/ml SPC or SPH, when compared to those treated with PBS alone.

Cytoprotection:

Caco-2 cells were treated with H_2O_2 in the presence of SPC or SPH to determine the

ability of SPC and SPH required to protect the cells from H₂O₂-induced oxidative damage and death. Cells were either co-incubated with SPC/SPH and H₂O₂ (cotreatment) or were incubated with SPC/SPH prior to exposure with H₂O₂ (pretreatment). In general, cotreatment was more effective at protecting the cells than pretreatment in both the SPC and SPH trials (p=0.006 and p=0.018 for SPC and SPH, respectively) (**Figure 10 & 11**).

The effect of varying concentrations of SPC on cell viability was significant in both the cotreatment and pretreatment experiments (p=0.005 and p=0.022, respectively). Compared to control cells treated only with PBS, cells treated only with H₂O₂ had viability reduced to 50.0 ± 6.1% and 42.8 ± 6.0% in the cotreatment and pretreatment trials, respectively (**Figure 10**). Treatment with 0.5 mg/ml SPC increased cell viability back to 76.1 ± 9.0% and 60.8 ± 6.5% of the control in the cotreatment and pretreatment trials, respectively (p<0.05).

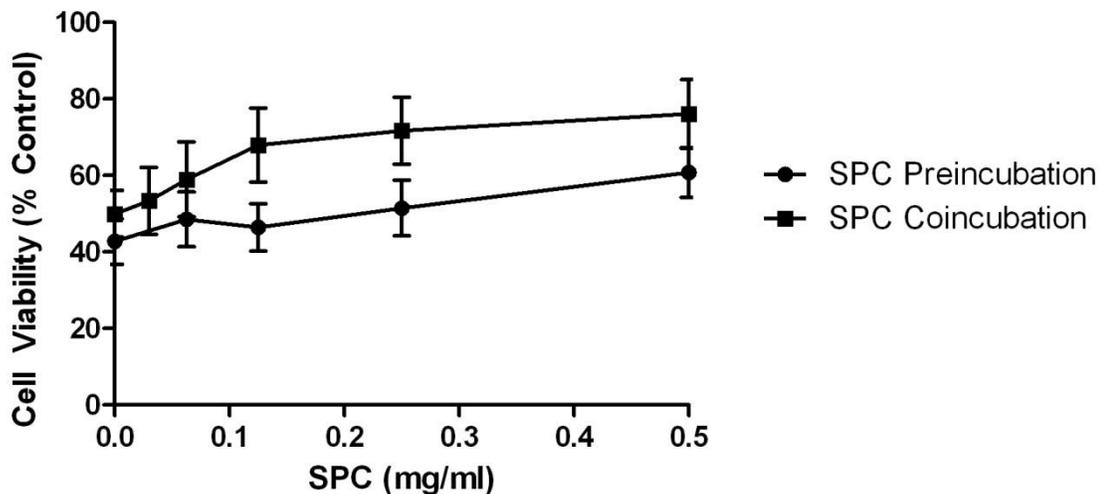


Figure 10: The effect of preincubation and coincubation of SPC on the viability of Caco-2 cells treated with H₂O₂. Values are means ± SEM and are expressed as cell viability, as compared to a control treated only with PBS. Preincubation was replicated 9 times; coincubation was replicated 6 times.

To achieve similar cytoprotective effects, higher concentrations of SPH were necessary. The effect of increasing the concentration of SPH on cell viability was only significant in the cotreatment experiment ($p=0.0004$). At a concentration of 2 mg/ml SPH, cell viability was increased to $85.3 \pm 10.9\%$ and $62.1 \pm 3.2\%$ in the cotreatment and pretreatment trials, respectively (**Figure 11**). The increase in cell viability seen in the cotreatment of cells with 1 mg/ml or 2 mg/ml compared to the vehicle control was significant ($p<0.05$).

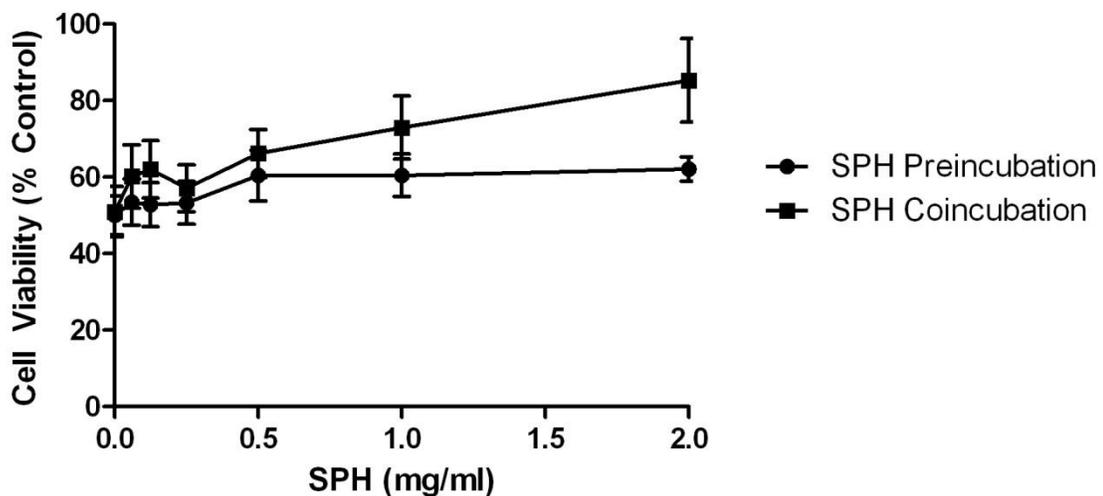


Figure 11: The effect of preincubation and coincubation of SPH on the viability of Caco-2 cells treated with H_2O_2 . Values are means \pm SEM and are expressed as cell viability, as compared to a control treated only with PBS. Preincubation was replicated 7 times; coincubation was replicated 10 times.

For both SPC and SPH, coincubation was a more effective treatment than preincubation. This result was expected, as coincubation allows for direct interaction

between the protein and H₂O₂, potentially resulting in direct radical scavenging.

Preincubation was generally still effective, and may have been a result of the activity of protein which remained attached to or embedded in the cells, even after the protein treatment solution was removed. In the future, antioxidant enzyme activity or gene expression in the cells should also be measured, as some previous studies have indicated that soy protein and peptides may affect antioxidant gene expression. Mice on a 20% soy protein diet had increased superoxide dismutase, catalase and glutathione peroxidase activity in their digestive organs (68). In cell culture, lunasin, a soy-derived peptide, has been shown to dose-dependently decrease ROS generation, TNF- α and IL-6 levels in LPS-stimulated macrophages (82).

To further elucidate the effect of SPC and SPH on Caco-2 cell viability with and without H₂O₂, cells were incubated for 1 h with PBS, H₂O₂, SPC, SPC + H₂O₂, SPH or SPH + H₂O₂. The treatment was removed and replaced with media, and the cells were allowed to recover for 48 h. Cell viability relative to the PBS control was determined using the MTT assay (**Figure 12**).

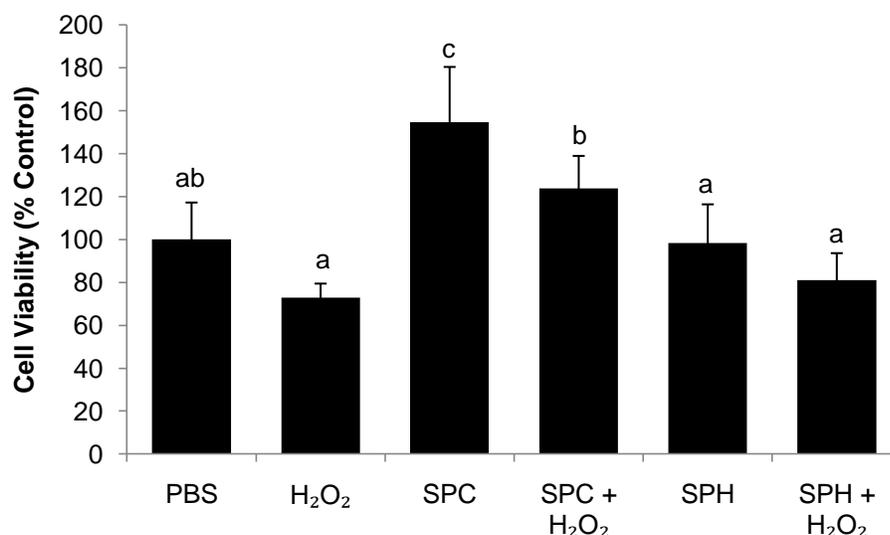


Figure 12: Caco-2 cell viability following 1 h treatment 48 h recovery of Caco-2 cells. Values are means \pm SD. Bars with a letter in common are not significantly different from each other (ANOVA, $p < 0.0001$). Cells were treated with PBS or 0.5 mg/ml SPC or SPH \pm 50 μ M H₂O₂.

After 48 h recovery, cell viability was decreased in the cells treated with H₂O₂, albeit not significantly compared to cells treated with PBS alone. Cells treated with SPH or SPH + H₂O₂ recovered to levels similar to that of cells treated with PBS. In contrast, cells treated with SPC alone had significantly higher recovered cell viability compared to the PBS control ($p < 0.05$). Cells treated with SPC + H₂O₂ were also able to recover to levels higher than that obtained by the PBS control, though not significantly so (**Figure 12**).

Cell viability after 48 h in cells treated with SPC was similar to that seen previously (**Figure 9a**), reaching about 150% of the PBS control. This suggests that the effect after 1 h is in fact an increase in cell viability and not a mere increase in mitochondrial activity. A cytoprotective effect is seen when cells are treated with SPC in the presence of H₂O₂. Cells treated with SPH and allowed to recover for 48 h do not reach the same levels of cell viability as previously seen (**Figure 9b**). This could be due to variation between experiments

or due to an increase in mitochondrial activity during the 1 h treatment, which is subsequently lost if the cells are allowed to recover. In the presence of H₂O₂, SPH did not confer any additional cytoprotective effect; albeit, the kill achieved by H₂O₂ alone may not have been great enough to see effects after 48 h recovery. In future studies incorporating 48 h recovery periods, higher doses of H₂O₂ should be used to maximize the difference between control and H₂O₂-treated cells.

Morphological Changes

Following cotreatment of Caco-2 cells with H₂O₂, SPC + H₂O₂ or SPH + H₂O₂, photomicrographs were collected to visualize any effect of treatment on cell morphology. Cells treated with SPC and SPH were visually distinguishable from cells receiving only PBS or H₂O₂, appearing flatter, more securely attached to the plate surface and generally more characteristic of healthy Caco-2 cells (**Figure 13**). Based on these results, it appears that treatment of cells with PBS alone for 1 h induces moderate stress, likely due to inadequate nutrient content.

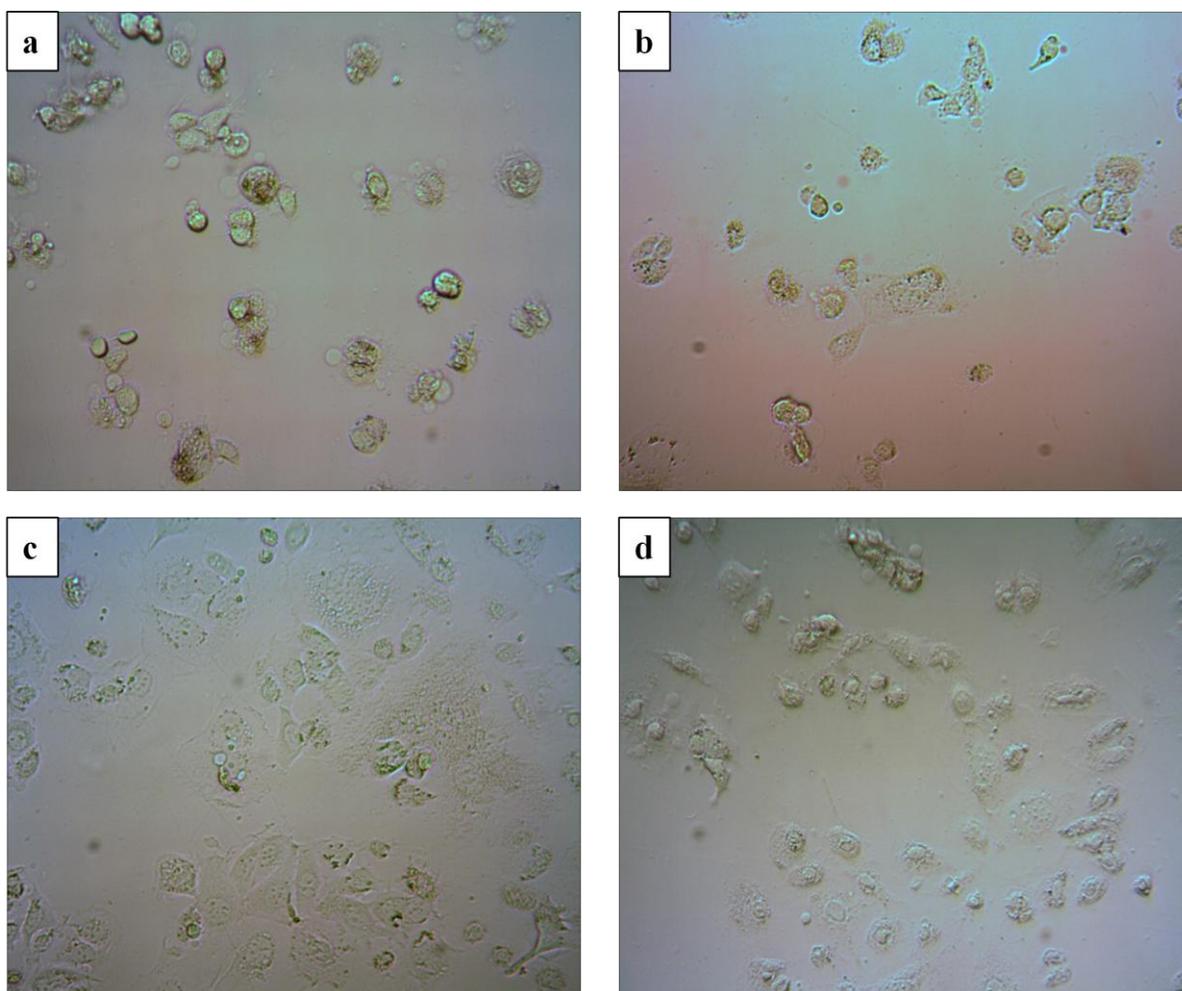


Figure 13: Cell morphology images following 1 h treatment with (a) PBS, (b) 50 μM H_2O_2 , (c) 0.5 mg/ml SPC + 50 μM H_2O_2 or (d) 0.5 mg/ml SPH + 50 μM H_2O_2 .

After 48 h recovery in media, the MTT assay was performed to determine cell viability (**Figure 14**). Compared to cells treated only with PBS, cells treated with H_2O_2 were $75.0 \pm 3.5\%$ viable ($p < 0.05$). Cells treated with SPC were as viable as cells treated only with PBS, with $98.8 \pm 3.3\%$ cell viability. This was significantly higher than the cell viability seen for cells treated with SPH ($75.8 \pm 2.2\%$, $p < 0.05$). These results are consistent with those seen previously (**Figure 12**); SPC has greater cytoprotective activity than SPH.

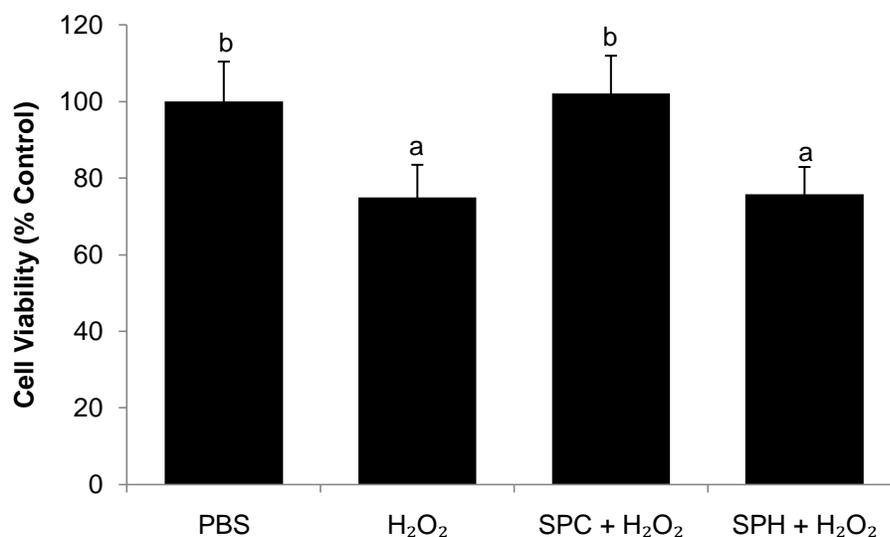


Figure 14: Cell viability following 1 h treatment with PBS, H₂O₂, SPC + H₂O₂, and SPH + H₂O₂ and 48 h recovery. Values are means ± SEM. Bars with a letter in common are not significantly different from each other (ANOVA, p<0.0001).

Effect of SPC and SPH on Cellular ROS

Following a 1 h cotreatment of Caco-2 cells with SPC or SPH and H₂O₂, the cells were incubated with 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCDHFDA), a compound that is taken up by cells, hydrolyzed by esterase and becomes fluorescent upon oxidation. Photomicrographs were taken to determine the relative levels of ROS in the cells, as the intensity of the fluorescence is proportional to the amount of ROS. The intensity of fluorescence in cells treated with H₂O₂ alone was greater than in cells cotreated with SPC/SPH and H₂O₂ (**Figure 15**). SPC appears to more potently decrease fluorescence than SPH. Although this data is not quantitative, it supports the results of the previous experiments and the hypothesis that the cytoprotective effect is due to SPC decreasing H₂O₂-induced oxidative damage.

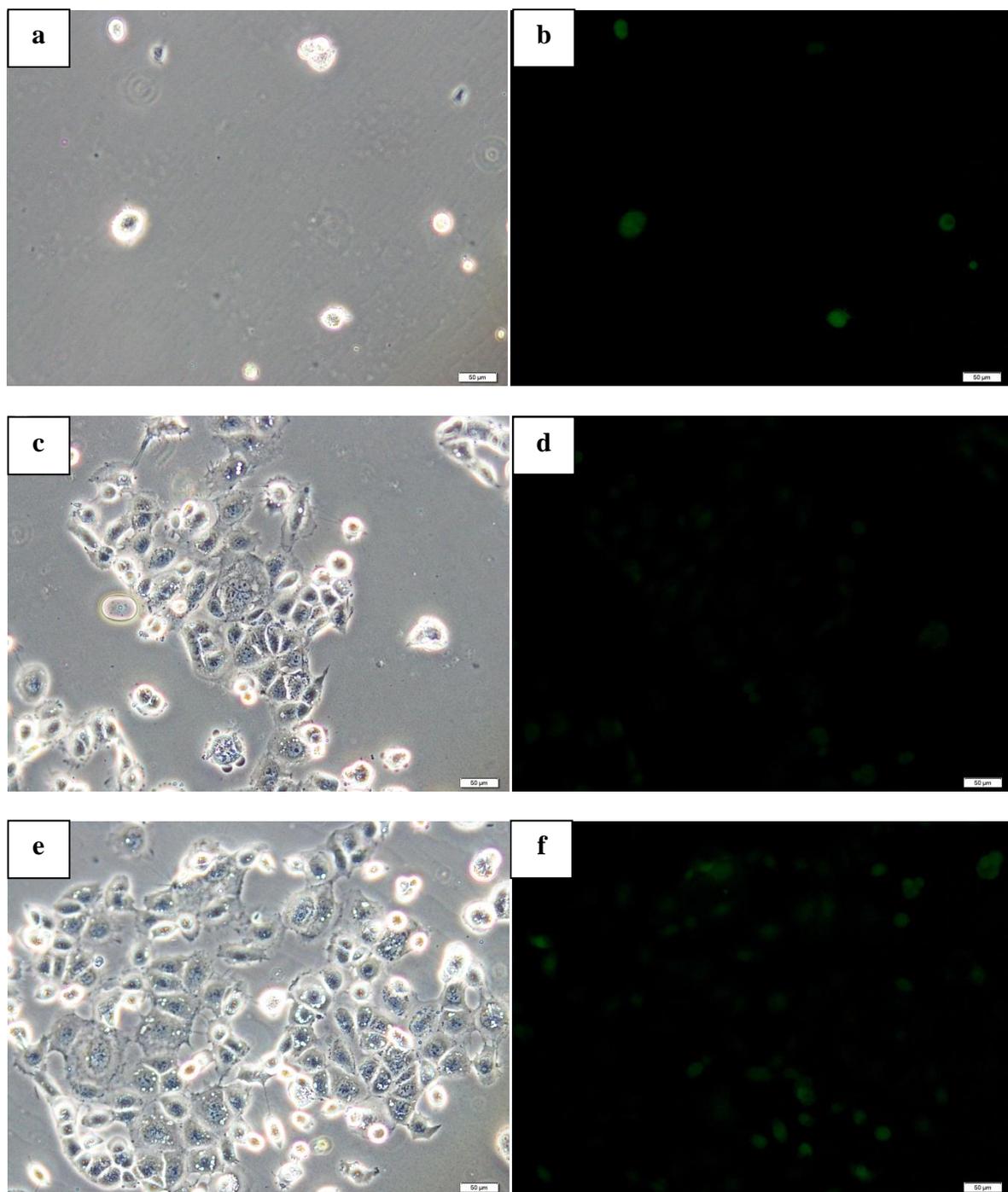


Figure 15: Intracellular ROS visualization using fluorescent dye (DCDHFDA). Paired, randomly selected, representative visible light and fluorescent images of cells treated with (a, b) H_2O_2 , (c, d) SPC + H_2O_2 and (e, f) SPH + H_2O_2 are shown.

Together, the results of these cell culture experiments suggest that SPC more potently cytoprotects Caco-2 cells from H_2O_2 than SPH. Based on the results of the ORAC assay, it

was expected that SPH would have greater antioxidant and cytoprotective activity in a cell culture model; however, in these experiments, this hypothesis has not been supported.

Translation of either of these models to the mouse or human colon is difficult, and both *in vitro* models have their limitations. It is difficult to draw conclusions about which results, those from the ORAC assay or those from the cytoprotection assays, most accurately predict what would occur *in vivo*. To our knowledge, no other studies on the comparative cytoprotective effects of SPC or SPH have been done, which limits our comparisons with previous work.

In these studies, SPH was expected to have greater radical scavenging and cytoprotective activity due to increased solvent accessibility of amino acids following hydrolysis. The fact that SPC performed better in the cytoprotective assays than SPH may therefore be problematic *in vivo*, as undigested SPC will not reach the colon. The hydrolysis procedure in this study was performed so as to mimic *in vivo* protein digestion. However, the *in vitro* digestion of SPC to produce SPH only simulates, but does not identically replicate, that which occurs *in vivo*. In this study, SPC was hydrolyzed using pepsin and pancreatin. *In vivo*, proteins are digested with the enzymes pepsin, trypsin and chymotrypsin. The acidic pH of the stomach denatures protein and activates pepsin, which cleaves peptide bonds adjacent to carboxyl ends of many amino acids. In the small intestine, trypsin, chymotrypsin and peptidases from the intestinal brush border cleave peptide bonds adjacent to enzyme-specific amino acids. In contrast, pancreatin contains a mixture of trypsin, amylase and lipase; which will result in a different final peptide and amino acid mixture than an *in vivo* digestion. As a result, the SPH produced in this study may not have the same activity as SPH

which has been hydrolyzed *in vivo*. In the future, several hydrolysis protocols using different enzyme combinations could be compared for their effect on cytoprotective activity.

Previous studies have found that hydrolysis of SPI increases antioxidant activity, and that this effect was not dependent on the time of hydrolysis (91); however, antioxidant activity is dependent on the amino acid sequences of peptides, which is affected by the type of enzyme used for digestion and its protease specificity (93). In the future, the degree of hydrolysis of the protein should be measured to compare the extent of hydrolysis of the protein to that done in previous studies.

Further modifications to the cytoprotection studies should be made in the future. Cytoprotection studies done previously have focused solely on cell viability (MTT assay) or on DNA damage (Comet assay); however, more direct measures of oxidation and ROS should be done to confirm the mechanism of action. For example, an adaptation of the thiobarbituric acid reactive substances (TBARS) assay would enable examination of lipid oxidation. Electron paramagnetic resonance (EPR) or immuno-spin trapping, with adequate procedural modification, may be able to directly measure radicals in the system. Flow cytometry using the fluorescent ROS dye, DCDHFDA, can provide quantitative support for the photomicrographs. Additionally, Western Blotting for cellular markers of oxidative damage, such as γ H2A.X, a marker of double stranded DNA breaks, would give more mechanistically informative data. Inhibition of IL-8 production could also be measured *in vitro*, as treatment of Caco-2 cells with H₂O₂ has been shown to induce its production (110).

In this study, it became clear that treatment of cells with PBS alone induced some stress and morphological changes in the cells. PBS was chosen as the treatment solution based on the stability of H₂O₂. Other alternatives may exist, such as PBS supplemented with

free amino acids and minerals which would also be present in the SPC and SPH solutions. Although free amino acids are not expected to be effective antioxidants, such an assumption would need to be verified. Over the course of treatment in the future, H_2O_2 concentrations should be measured in the supernatant. Decreases in H_2O_2 over time in the SPC or SPH solutions may imply quenching of H_2O_2 . Likewise, it would be interesting to measure H_2O_2 in the cells treated with PBS alone, as previous studies have found relatively high levels of H_2O_2 production by unstimulated human tumor cell lines in culture (111).

3.3 Mouse Carcinogenesis Studies

Body Weight, Food and DSS Consumption:

The mice in both studies were generally healthy, although three mice in the Pos group and two mice in the SPC group died before the end of the 10-week study, and one mouse in the Neg group, three in the Pos group and one in the SPC group died before the end of the 20-week study. The majority of the deaths occurred in the days following AOM injection, suggesting they were the result of acute AOM toxicity. The Neg mouse's death occurred following a cage flood. Acute AOM toxicity was not expected, as no toxicity or death was noted in the study which developed the AOM/DSS model (39); however, both of the current studies were larger in size, which may have increased the chance of variation in response to AOM. Over the course of both studies, body weight increased gradually and did not differ significantly between treatment groups (**Figure 16**). Body weight gain was therefore not affected by the type of dietary protein consumed or by AOM/DSS treatment.

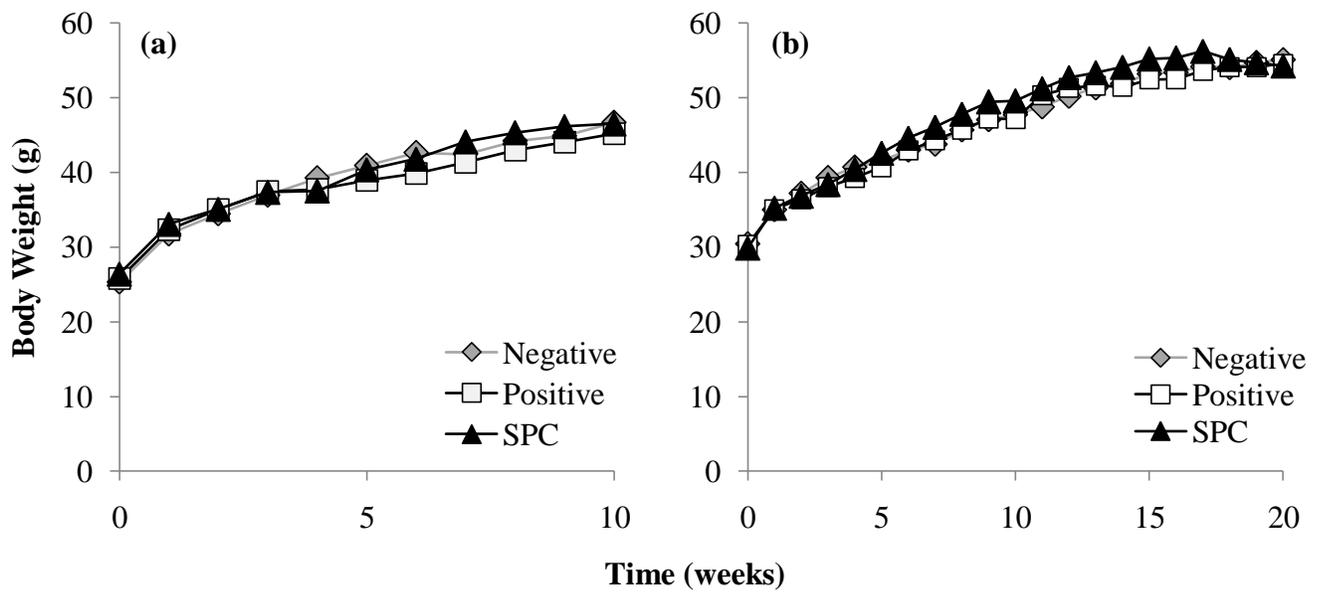


Figure 16: Body weight per mouse over time for the (a) 10-week and (b) 20-week studies. The 10-week (a) and 20-week (b) studies began with 75 and 180 male CF-1 mice, respectively. Values represent the mean; error bars were omitted for clarity.

Average food consumption per mouse was more variable than body weight, but did not differ significantly over time between treatment groups in either the 10 or 20-week study (Figure 17). Although food consumption over time and between groups varied slightly, body weight was constant between groups. Variation in food consumption between groups may have been due some of the diet falling through the cage grates. Variation over time (Figure 17b) may have been a result of measuring the food remaining in the cages at different times of the day each week.

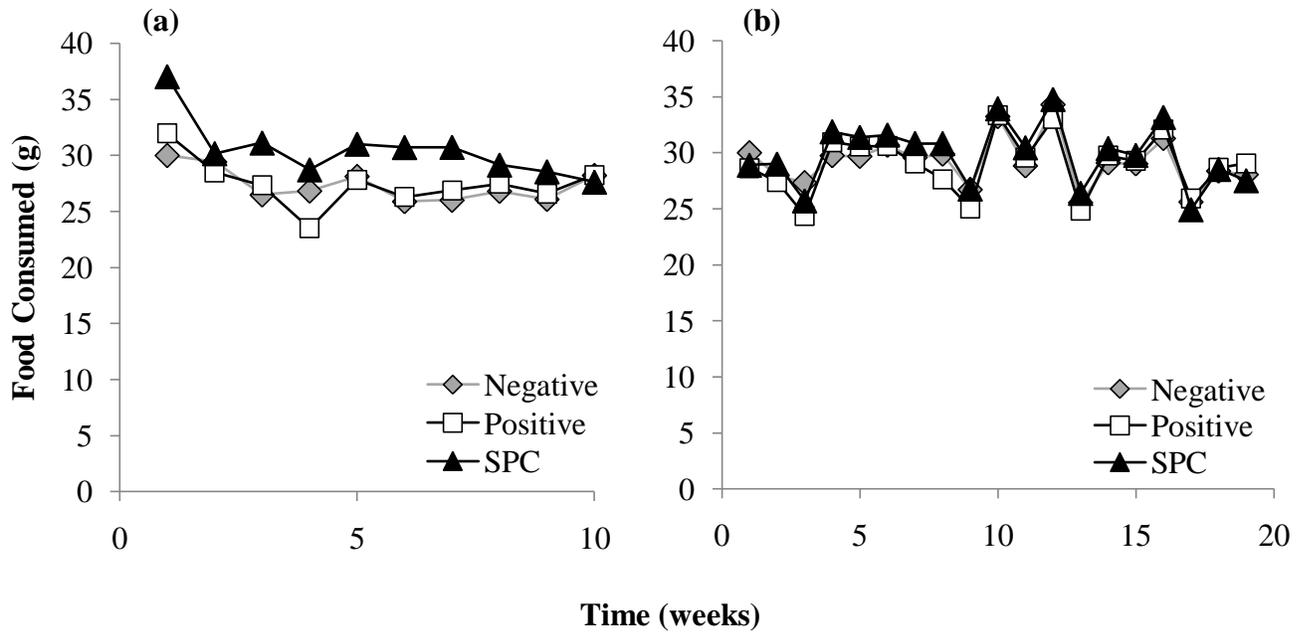


Figure 17: Food consumption per mouse over time for the (a) 10-week and (b) 20-week studies. Values represent the mean; error bars were omitted for clarity.

There was no significant difference in average DSS water consumption per mouse per day between the Pos and SPC groups over the course of treatment in either study (10-week study = 9.6 ± 0.6 and 9.8 ± 0.5 ml; 20-week study = 8.2 ± 1.4 and 9.2 ± 1.6 ml for Pos and SPC mice, respectively). Some of the variation in consumption may be attributed to minor leaking from the water bottles when cages were shifted. Overall, the exposure to DSS was the same for both treatment groups.

Spleen-to-Body Weight Ratio:

The spleen plays an important role in the immune and endothelial systems, as a reservoir for blood and destruction site for red blood cells (7). An increase in the size of the spleen, normalized to body weight, may be an indicator of inflammation in mice (112, 113). Although the relationship between spleen size and colitis has not been well researched, a

correlation between splenic atrophy and complications in colitis patients has been seen (7, 8). An example image of an enlarged and a normal mouse spleen can be seen in **Figure 18**.



Figure 18: Image of an enlarged (left) and normal (right) mouse spleen harvested from mice in the 20-week study. Spleens were harvested and weighed following sacrifice.

The average spleen-to-body weight ratios for mice in the 10-week study were not significantly different between treatment groups at any time point; however, a suggestive trend was seen for decreased spleen-to-body weight ratios in SPC mice compared to Pos mice (**Figure 19**).

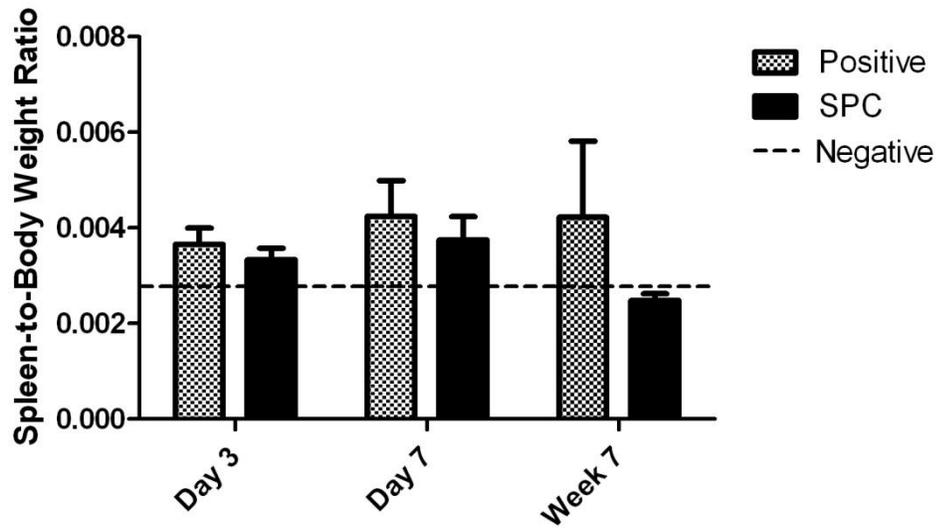


Figure 19: Spleen-to-Body Weight Ratios for 10-week study. Values represent means \pm SEM. Means were not significantly different between treatments at each time point.

In the 20-week study, the average spleen-to-body weight ratios for mice in the Pos and SPC groups were not significantly different at any time point. At Day 3, Day 7 and Week 20, the SPC mice had significantly different spleen-to-body weight ratios compared to the Neg mice; the Pos and Neg mice were not significantly different from each other at any time point (**Figure 20**).

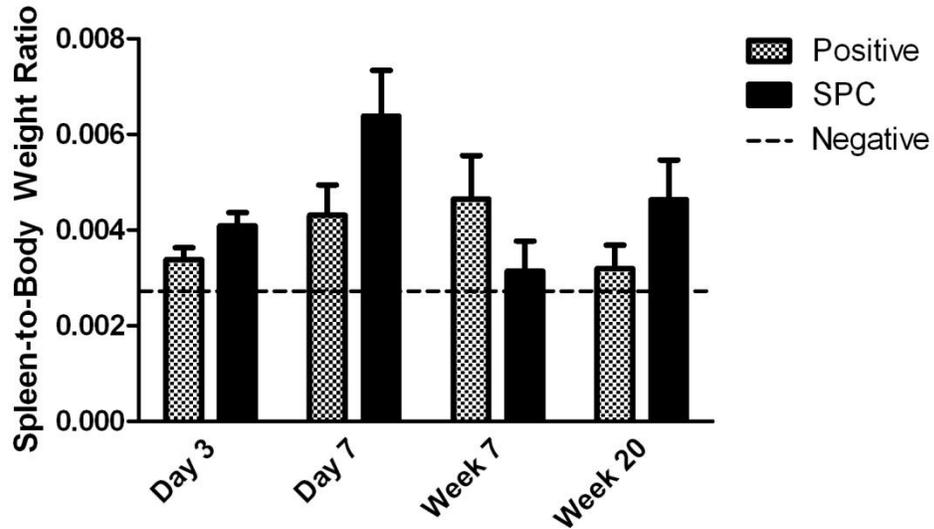


Figure 20: Spleen-to-Body Weight Ratios for 20-week study. Values represent means \pm SEM. At Day 3, Day 7 and Week 20, the ratios for the SPC group were significantly different from those for the Neg group ($p < 0.05$). No other significant differences were found.

Following the 10-week study, a trend for decreased spleen-to-body weight ratio was seen in SPC mice compared to Pos mice (**Figure 19**); however, with the exception of the Week 7 time point, the trend in the 20-week study was the opposite. Mice at Week 7 of the 20-week study showed decreased spleen-to-body weight ratio in the SPC group, and this is the time point at which similar decreases were found for polyp multiplicity and in the inflammation index (**Table 3, Figure 30**) however, the variation within groups and across studies suggests that overall the measure of spleen-to-body weight may be too crude and variable of a measure to draw meaningful conclusions about inflammation. Because visual differences in spleen size were obvious in mice which displayed clear signs of colonic illness (**Figure 18**) and because spleen-to-body weight is a rapid measure to generate preliminary data, in the future, spleen-to-body weight should still be used as a preliminary measure of the effectiveness of a treatment, but should be supplemented with additional splenic and immune

system measures. For example, complete peripheral blood counts and fluorescence-activated cell sorter (FACS) analysis of the blood for levels of circulating B cell, T cell, CD-4 and CD-8 populations may be more informative than spleen weight alone.

Polyp Multiplicity:

The visible polyps (<1mm) in each mouse colon were counted immediately after sacrifice. The number of polyps per mouse was higher in the Pos mice than the SPC group for all time points in the 10-week study, with significant differences arising at Week 7 (**Figure 21**). The average numbers of polyps per mouse at Week 7 were 6.2 ± 0.9 and 2.9 ± 0.8 , for the Pos and SPC groups, respectively ($p < 0.01$).

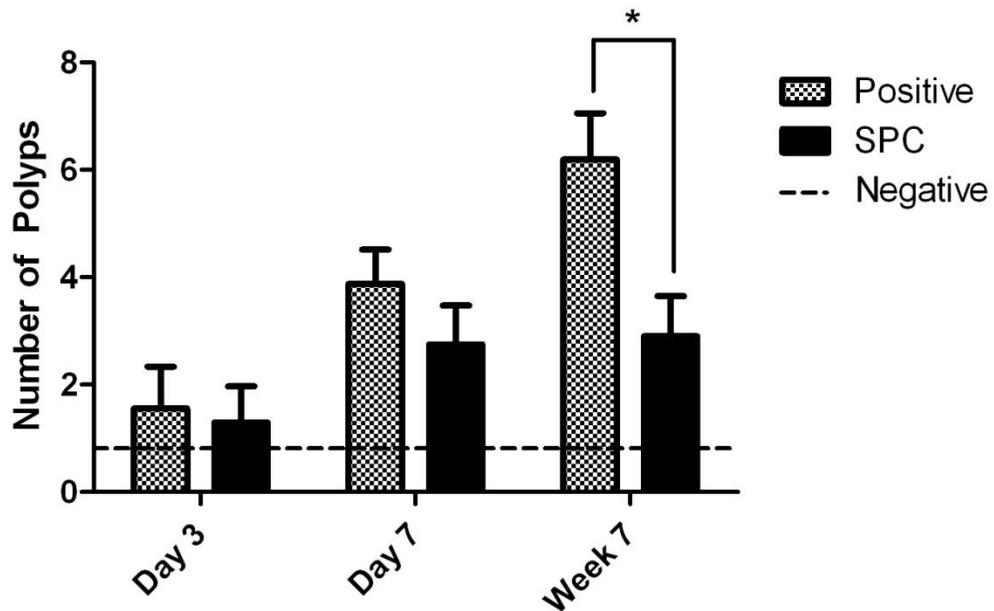


Figure 21: Polyp multiplicity in the 10-week study. Values represent means \pm SEM. Values marked with * are significantly different ($p < 0.01$).

In the 20-week study, the average number of polyps per mouse was greater in the Pos mice than the SPC group for all time points, except at Day 7 (**Figure 22**). This increase in polyps in the Pos mice compared to SPC was significant at Week 7 (Pos = 13.4 ± 2.3 , SPC = 5.3 ± 1.1 , $p < 0.01$).

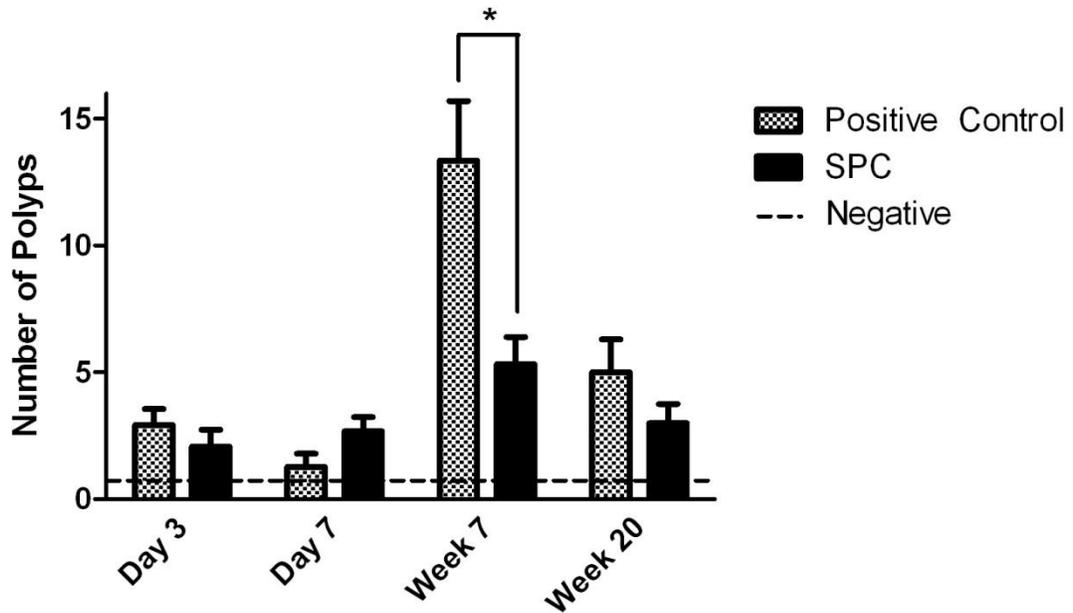


Figure 22: Polyp multiplicity in the 20-week study. Values represent means \pm SEM. Values marked with * are significantly different ($p < 0.01$).

Because there was no obvious difference in mouse strain, age, sex, carcinogen dose or volume administered, the data from both studies were combined and re-analyzed. The polyp counts for both studies were also averaged over all time points. The average number of polyps was significantly reduced in the SPC group compared to Pos at Week 7 and for the study overall (**Table 3**).

Table 3: Combined polyp multiplicity for the 10 and 20 week studies.

Treatment/Time point	Day 3	Day 7	Week 7	Week 20	Total
Positive Control	2.4 ± 0.5 a	2.2 ± 0.5 a	10.4 ± 1.6 b	4.4 ± 1.3 a	5.0 ± 0.7 b
SPC	1.8 ± 0.5 a	2.7 ± 0.4 a	4.4 ± 0.7 a**	3.6 ± 0.8 a	3.0 ± 0.3 a*

All values are combined mean number of polyps (diameter <1mm) per mouse ± SEM from the 10-week and 20-week studies, with the exception of the values from Week 20. Within a column, values with a letter in common are not significantly different. * = p<0.01, ** = p<0.001.

In both studies, the number of polyps was notably higher at the Week 7 time point than at all others. It is possible that there were less countable polyps later in the study (Week 20), due to progression of some polyps into tumors or recovery of non-cancerous polyps. With all time points combined in the studies, there was a significant reduction in the number of polyps in SPC mice compared to Pos mice (**Table 3**). Polyp multiplicity is a preliminary measure of colon carcinogenesis, and the results suggest a beneficial effect of SPC. While no other studies to our knowledge have specifically measured colon polyps or have measured similar outcomes at multiple time points following treatment, other studies have found similar decreases in ACF number. Murillo et al. found that dietary soy flour decreased ACF in mice by 58% compared to a control, casein diet (p<0.001) (40); this study also used diets with equal isoflavone contents, suggesting the effect may have been due to a soy bioactive other than isoflavones.

Tumor Multiplicity and Area

In the 20-week mouse study, colon tumors (diameter >1mm) were counted and measured at sacrifice (**Figure 23**). Tumor incidence was very similar between treatment groups: 5 of 14 mice and 6 of 15 mice had measurable tumors in the Pos and SPC groups,

respectively. Previous studies have found a greater effect of soy protein-based diets on tumor incidence: Hakkak et al. found a 76% reduction in tumor incidence in Sprague-Dawley rats on a SPI diet compared to those on a casein-based diet forty weeks after AOM injection ($p<0.05$) (56). Xiao et al. also found that dietary SPI decreased tumor incidence in rats by 47%, twenty weeks post-AOM injection, compared to dietary casein ($p<0.05$) (59).

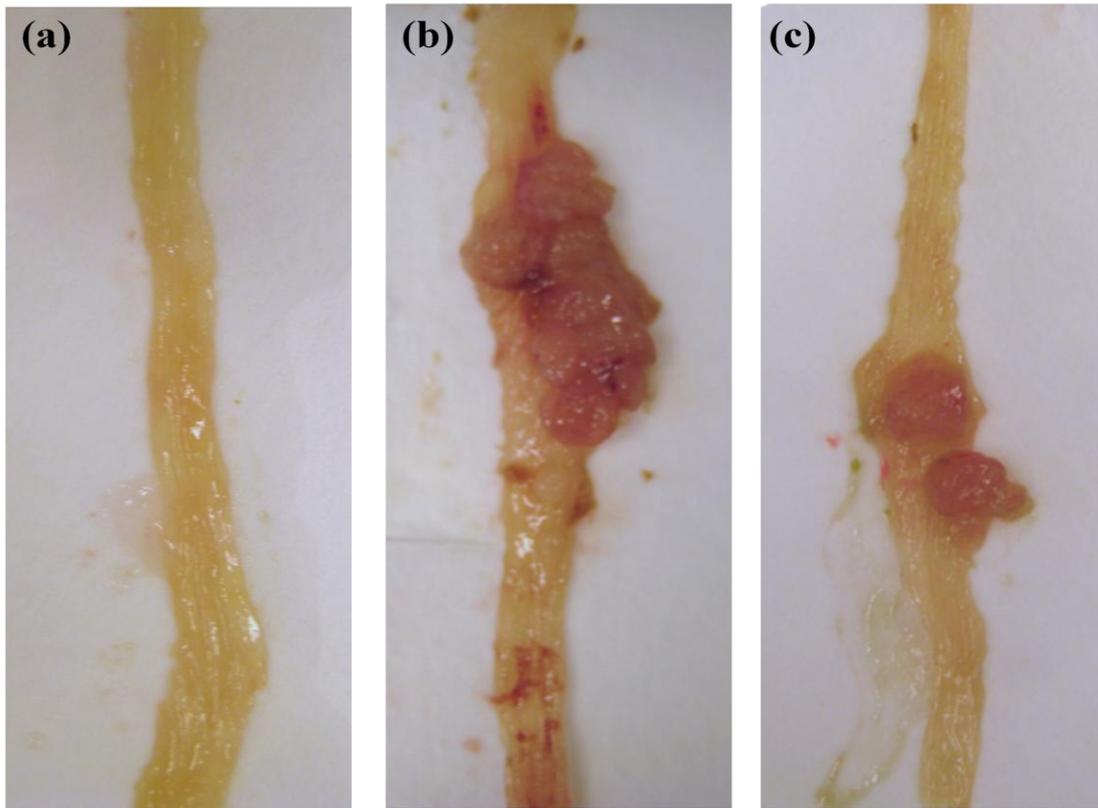


Figure 23: Images of colons from the (a) Neg, (b) Pos and (c) SPC groups at Week 20. Tumors were counted and measured at Week 7 and Week 20.

The average number of measurable tumors per mouse was calculated for the mice sacrificed at Weeks 7 and 20. The mean number of tumors per mouse was slightly higher in the Pos mice than the SPC mice, but the difference was not significant ($\alpha=0.10$) when calculated per tumor-bearing mice (**Figure 24a**) or per total mice (**Figure 24b**).

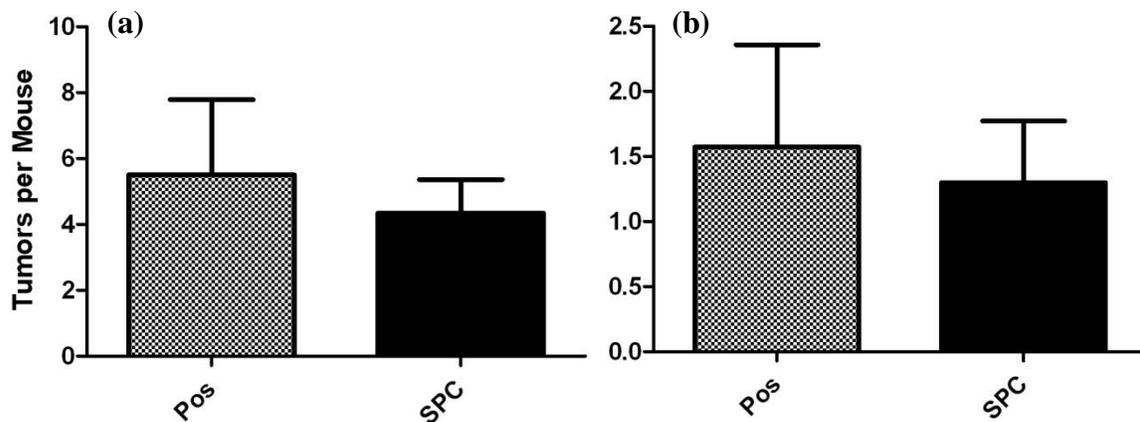


Figure 24: Average number of tumors per mouse for mice at Weeks 7 and 20. Values are mean number of tumors (diameter >1mm) per (a) tumor-bearing mouse and (b) total mice \pm SEM. The difference between Pos and SPC was not significant in either comparison.

The average tumor area was calculated for those mice with tumors. The average tumor area per mouse was greater in the Pos mice compared to the SPC group; however, the difference was not statistically significant ($p=0.127$, **Figure 25**). In previous work, Hakkak et al. also saw no effect on dietary SPI on decreases in tumor multiplicity or area (56).

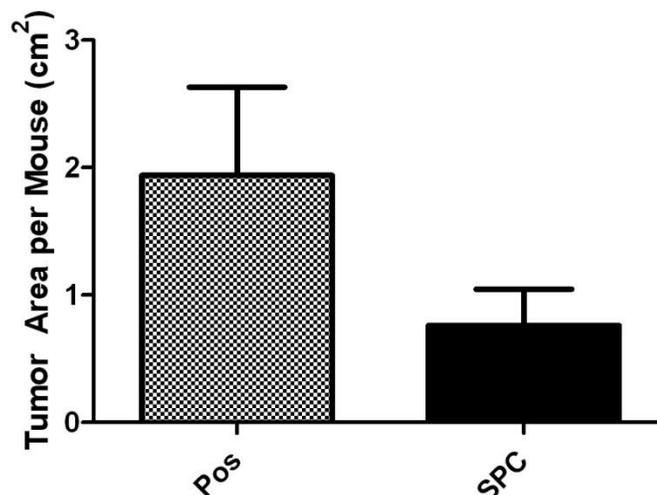


Figure 25: Average tumor area for mice in 20-week study. Values are mean tumor area (for mice with a tumor) \pm SEM. The difference between Pos and SPC was not significant.

No significant differences were found between treatment groups for tumor incidence, tumor multiplicity or tumor burden. Overall, the incidence of colon tumors was low and the variation in tumor multiplicity and burden was high, which resulted in non-significant results. Despite the large initial sample size of the 20-week study, only 14 and 15 mice were sacrificed at Week 20 for the Pos and SPC groups, respectively. In the future, modifications to the experimental procedure may help in elucidating more conclusive results about dietary treatments. To examine colon cancer specific endpoints, it may be beneficial to sacrifice mice only at Week 20, eliminating the earlier time points. Other studies on colon tumorigenesis have extended 40 or 52 weeks following AOM injection to increase the number of tumors detected (56).

Tanaka et al. found 100% incidence of colon adenocarcinomas with 5.6 ± 2.4 multiplicity at 20 weeks after 10 mg/kg body weight AOM and 1 week oral exposure to 2% DSS (39). The higher dose of DSS (2% versus 1.5% in this study) may have accounted for some of the differences seen between these studies; however, increased severity of colitis early on in the study may result in death of some mice before 20 weeks. In the future, multiple, lower-dose injections of AOM may help increase incidence, multiplicity and burden without associated acute AOM toxicity.

Colon Pathology:

Inflammation was scored histopathologically in the distal colon using the inflammation index. Colons were scored for area of inflammation, severity of inflammation, presence of ulceration and extent of hyperplasia and dysplasia. The sum of these four scores is the inflammation index. The trends for the individual markers and total inflammation

score were similar for both the 10- and 20-week studies, so the data were combined to increase statistical power.

Colonic ulceration was scored as either absent (0) or present (1), with ulcers defined as areas of the mucosa in which the epithelial lining was missing (103). Mean ulceration scores were not significantly different between the Pos and SPC groups at any of the sacrifice time points (**Figure 26**), but was generally higher in both groups immediately after completion of DSS treatment, at Days 3 and 7. Healing and recovery of colonic ulceration following DSS treatment is possible over time (114). Though difficult to quantify recovery, qualitatively, the absolute difference in ulceration scores between Day 7 and Week 7 is greater in SPC mice than Pos mice (**Figure 26**).

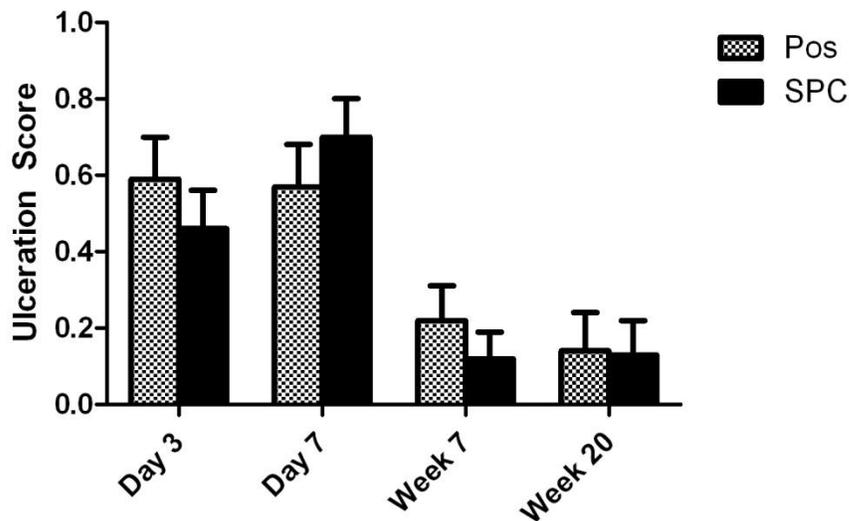


Figure 26: Ulceration scores for mice in the 10 and 20-week studies. Values are mean scores \pm SEM. Scores were not significantly different between treatment groups at each time point.

Inflammation area was scored from 0 to 4, which corresponded to 0-100% of the area examined. Inflammation area was similar at Days 3 and 7 between the Pos and SPC groups;

however, at Week 7 there was significantly higher inflammation area scores in Pos mice compared to SPC ($p < 0.10$) (**Figure 27**). A trend for reduced inflammation area in the SPC mice compared to the Pos mice was also seen at Week 20, albeit not significant.

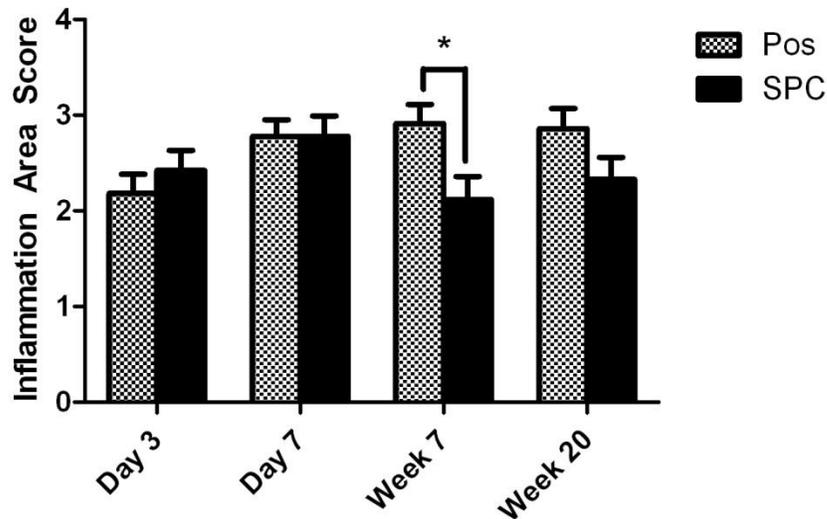


Figure 27: Inflammation area scores for mice in the 10 and 20-week studies. Values are mean scores \pm SEM. Scores were significantly different between treatment groups only at Week 7. * = $p < 0.10$.

The severity of the colonic inflammation was also scored, from 0 (normal) to 3 (severe). At all time points in both studies, the average scores for inflammation severity were similar between the Pos and SPC treatment groups (**Figure 28**). Severity scores of 2 and 3 correspond to moderate or severe inflammation, respectively, so generally, the inflammation severity scores for all mice were high throughout the studies. This suggests that the dose of DSS administered was sufficient to induce inflammation in this model.

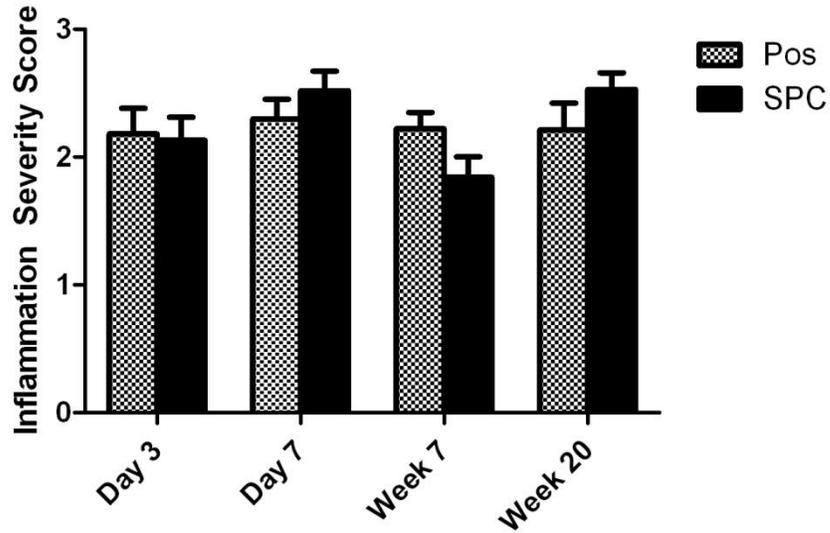


Figure 28: Inflammation severity scores for mice in the 10 and 20-week studies. Values are mean scores \pm SEM. Scores were not significantly different between groups at each time point.

Colonic hyperplasia and dysplasia (H&D) was scored from 0 (normal, no hyperplasia or dysplasia) to 3 (high-grade dysplasia). Hyperplasia is the excessive proliferation of the epithelial cells lining the colon crypts. Dysplasia is characterized by an abnormal proliferation of immature cells, which may outnumber mature, differentiated cells. H&D scores were significantly different between the Pos and SPC groups at both Day 7 and Week 7 ($p < 0.05$ and $p < 0.10$, respectively). The average H&D scores were higher in the SPC group at Day 7, but were higher in the Pos group at Week 7 (**Figure 29**). The results for H&D in this study were dependent on sacrifice time point. H&D scores in the Pos group increased over time, whereas scores for the SPC mice varied over time. Although H&D scores were significantly higher in SPC mice at Day 7, colon polyps and tumors were not increased in this group in the study overall; recovery may have occurred over time in the mice fed SPC.

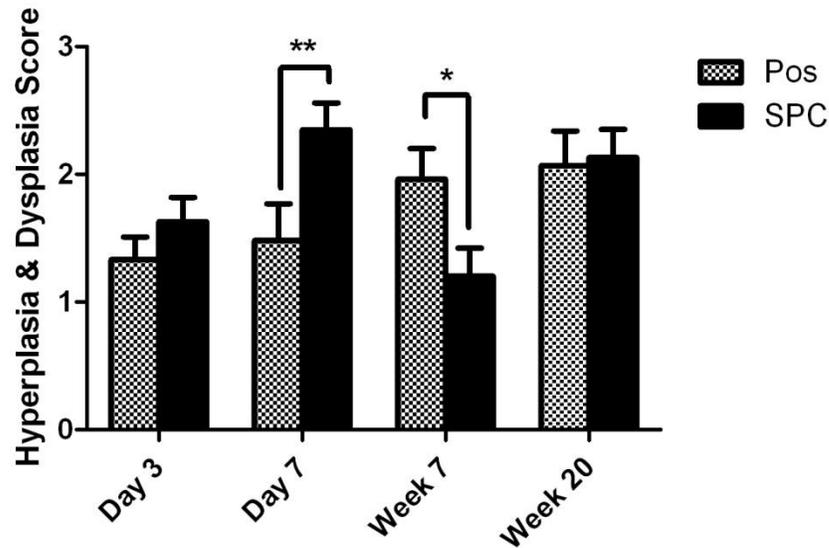


Figure 29: Hyperplasia & Dysplasia scores for mice in the 10 and 20-week studies. Values are mean scores \pm SEM. Scores were significantly different between groups at Day 7 and Week 7. * = $p < 0.10$, ** = $p < 0.05$.

The total inflammation index score was significantly reduced in the SPC mice compared to the Pos mice at Week 7 ($p < 0.05$) but was not significantly different at the other time points (**Figure 30**). As the sum of all other inflammation markers, the total index score is more informative than any other measure alone. Inflammation was not different between treatment groups at Day 3 or Day 7 following DSS treatment. By Weeks 7 and 20, most mice had developed colon cancer markers, such as polyps and tumors, which could affect scores for inflammation. This time-dependent data may suggest that an SPC diet may not significantly reduce DSS-induced colon inflammation early in the model; however, SPC may protect against cancer-related inflammation later on. Because the effect is lost at Week 20, however, it is difficult to draw conclusions about the implications for these findings in clinical cases of colon cancer.

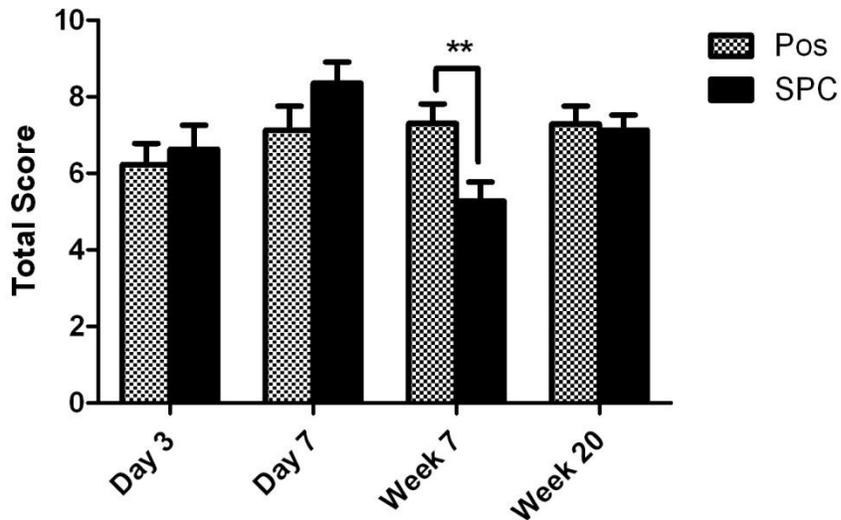


Figure 30: Total inflammation index scores for mice in the 10 and 20-week studies. Values are mean scores \pm SEM. Scores were significantly different between treatment groups at Week 7. ** = $p < 0.05$.

The variation in effects at different time points is a limitation of the AOM/DSS model in this study. Previous studies which have examined the effect of soy on colon inflammation markers are limited. One study found that mice were supplemented with 0.5% BBIC in a DSS model of colitis had reduced colon inflammation extent, severity, necrosis, regeneration and total histopathological score (73). However, to our knowledge, no studies on soy protein specifically have been done using the AOM/DSS model or inflammation index.

When present, tumors were scored as adenomas with mild, moderate, severe dysplasia, or as adenocarcinomas (invasive or noninvasive). In the 10-week study, only one mild adenoma with dysplasia was found in the Pos group. The low incidence of adenomas in the 10-week study suggests that a longer time course is necessary for adenoma development. Adenomas in the 20-week study were found by Week 7 and at Week 20 in the study. Overall, the incidence was low (37.0% and 33.3% of mice in the Pos and SPC groups, respectively, had any type of adenoma), which again suggests that a longer time course or

higher dose of AOM is necessary. In general, a greater proportion of the tumors in the Pos group (30%) were classified as invasive or noninvasive adenocarcinomas compared to the SPC group (9.1%) (**Figure 31**). Mice in the SPC group had a greater number of adenomas with mild or moderate dysplasia than the Pos group. SPC mice tended to have less serious adenomas and adenocarcinomas than Pos mice, which suggests a trend for benefit of dietary SPC.

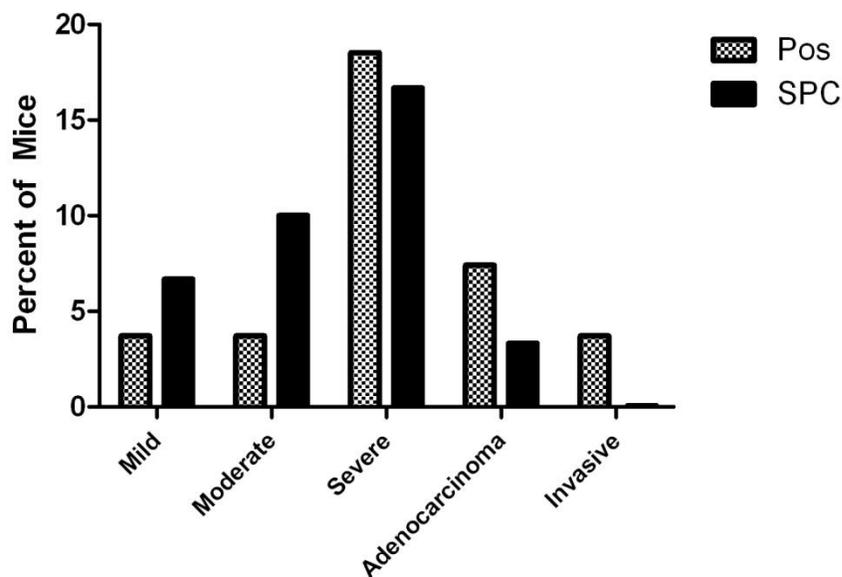


Figure 31: Percentage of mice in each treatment group at Weeks 10 and 20 with each class of adenoma with dysplasia. Adenomas were categorized as mild, moderate, severe, adenocarcinoma (noninvasive) or adenocarcinoma (invasive).

Other histopathological morphology changes in the colons were scored. In the 10-week study, the incidence of these was limited. One mouse in each treatment group displayed nearly complete colonic ulceration, and one SPC colon displayed abundant foamy macrophages. Because the incidence of these abnormalities was limited, no further analysis was carried out. In the 20-week study, the percentage of mice in each group with each marker was calculated for the whole study. Of the 10 classes of abnormalities evaluated,

four of the markers were uncommon, found in only one or fewer mice per group (nearly complete ulceration, focal squamous metaplasia, focal microinvasion and necrotizing arteritis in the mesentery). Data analysis was not performed due to the low incidence of these abnormalities. The other five markers (GALT hyperplasia, crypt herniation, necrosis of submucosal fat, abundant foamy macrophages and possible microinvasion) were more common; however, the presence of these abnormalities was equally as common in both the Pos and SPC treatment groups (**Figure 32**). GALT hyperplasia occurred in around 14-15% of mice in both groups, and occurs when the cells in the lymphoid tissue of the colon proliferate excessively. Because these abnormalities occurred equally as often in both groups, no additional conclusions on the effect of dietary SPC on colon carcinogenesis can be drawn.

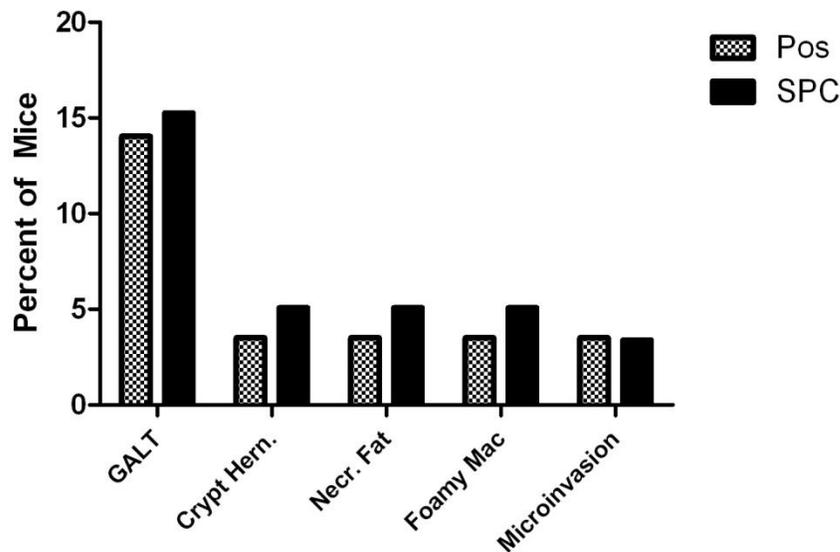


Figure 32: Percent of mice in each treatment group with each colon morphology description. The most commonly identified morphological changes were gut associated lymphoid tissue hyperplasia (GALT), crypt herniation (Crypt Hern.), necrosis of submucosal fat (Nect. Fat), abundant foamy macrophages (Foamy Mac) and possible microinvasion (Microinvasion).

Photomicrographs of colon morphology were taken and representative images are displayed in **Figures 33-35**. Normal colon crypt morphology, characterized by uniform, intact crypts, can be seen in **Figure 33**, an image of the colon of a Neg mouse from Day 7. Photographs of the classification of adenomas, ranging from mild to invasive adenocarcinomas, can be seen in **Figure 34**. Images of the five most prevalent morphological abnormalities can be seen in **Figure 35**.

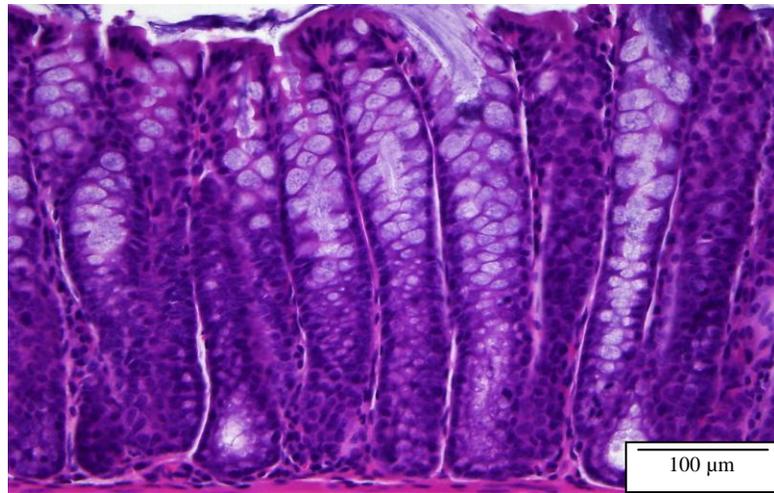


Figure 33: Image of normal colon crypt morphology. Image obtained from a mouse in the Neg group at Day 7.

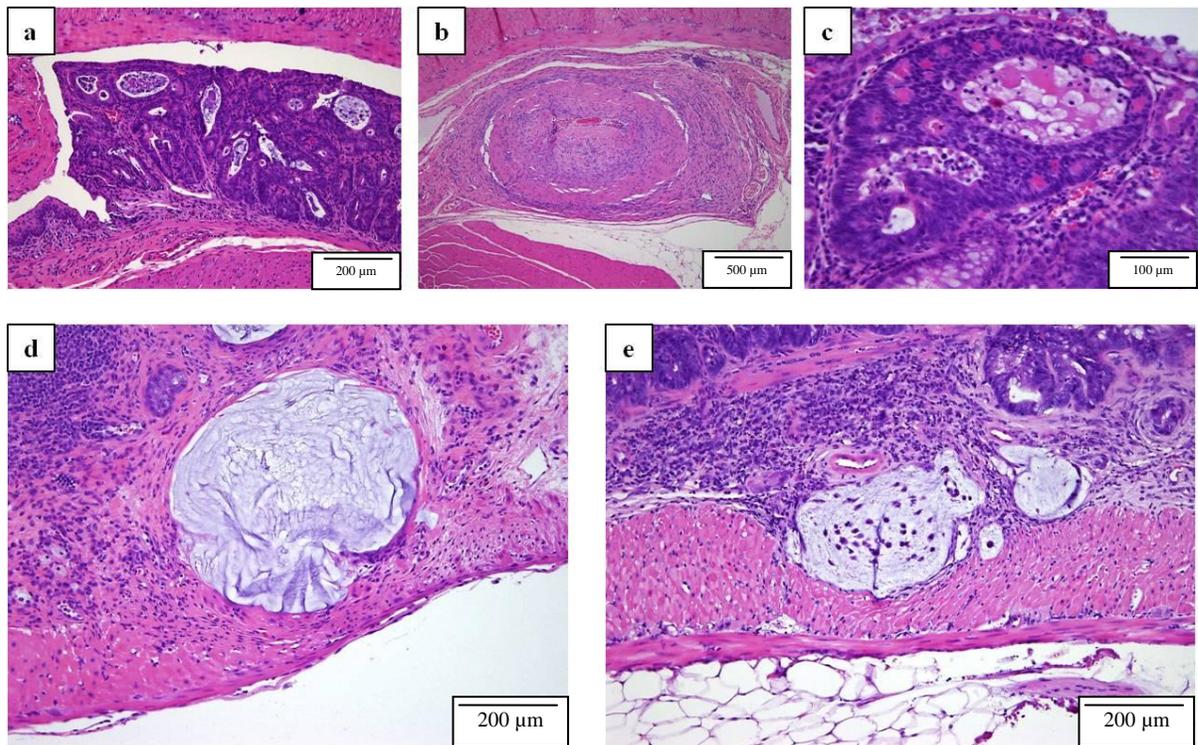


Figure 34: Representative images of the five classes of adenomas with dysplasia: (a) mild, (b) moderate, (c) severe, (d) non-invasive adenocarcinoma and (e) invasive adenocarcinoma.

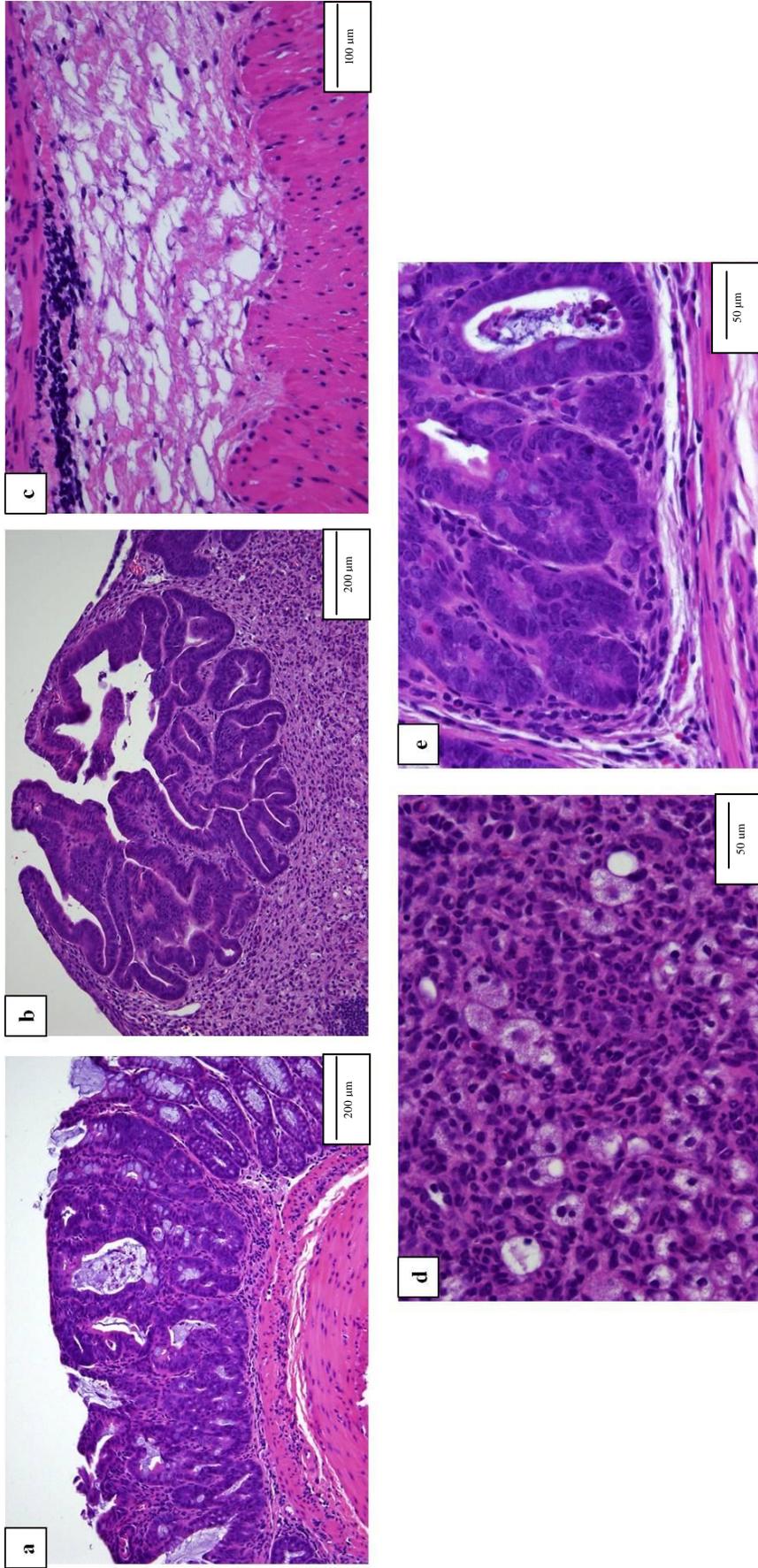


Figure 35: Images of five most common colon morphology abnormalities: (a) GALT hyperplasia, (b) crypt herniation, (c) necrosis of submucosal fat, (d) abundant foamy macrophages and (e) possible microinvasion.

Because few other studies have examined the effect of soy protein on colon inflammation and carcinogenesis in a similar way, it is difficult to compare the results of this study to previous findings. Reductions in colon polyps and inflammation index scores were significant in SPC mice; however, the effect was time point-dependent. For this reason, in the future, it may be beneficial to limit the number of sacrifice time points. Additionally, the low incidence of adenomas and adenocarcinomas suggests that changes should be made to the experimental length or doses of irritant and carcinogen.

Inflammation and colon carcinogenesis are inextricably linked; however, fully understanding the effects of SPC on both outcomes is difficult in one study. The two early sacrifice points (Days 3 and 7) may be more informative about anti-inflammatory effects, while later time points (Weeks 7 and 20) are more important to understand cancer-specific endpoints. Even with a large initial sample size ($n=70$ and 180 for 10 and 20-week studies, respectively), the number of mice sacrificed in each group at each time point is relatively small. Eliminating unnecessary time points would help ensure an adequate sample size remains at the other time points. Simplifying the model to include one early and one late time point would improve the interpretability of the model. At the early time point, endpoint measurements could focus primarily on outcomes such as the inflammation index, colon length, or oxidative stress markers. Urinary 8-isoprostanes (8-IP), colon glutathione levels, NF- κ B, TNF- α , inducible nitric oxide synthase (iNos) and other inflammatory cytokines may be useful markers to measure. At later time points, cancer markers such as ACF, should be measured. Measuring ACF is an efficient marker of colon carcinogenesis and would make it easier to compare results with other published studies. Loss of function of the p53 tumor

suppressor gene and mutations in K-Ras, a proto-oncogene, could be measured, as colon cancer is commonly associated with accumulations of these mutations (115).

In addition to changes that should be made to experimental procedures and outcomes, in the future, a different control diet should be used to assess the bioactivity of soy protein. Casein has been found to have antioxidant activity *in vitro* (116, 117), which may translate *in vivo*. In order to fully elucidate whether soy protein can act as an antioxidant *in vivo*, it must be compared to a control which does not. In the future, a control diet comprised of an amino acid mixture comparable to soy protein would be an appropriate control, as amino acids are not effective antioxidants (87). A previous study by Takenaka et al. found that dietary soy protein and peptides reduced markers of oxidative stress, while an amino acid mixture diet had no such effect (65). The effect of tertiary protein structure on antioxidant activity would be more clearly visible if SPC was compared to a diet of identical amino acid composition.

Another alternative would be to use a protein as the control such as whey, which has less antioxidant activity than casein (118). Similarly, an animal protein such as beef could be used in the control diet, although less mechanistic data could be derived than if using an amino acid mix. This may more closely mimic a human dietary intervention and the effect of switching from animal-based to plant-based protein on colon cancer.

3.4 Oxygen Radical Absorbance Capacity: SPC, Casein and Mouse Diets

The peroxy radical scavenging capacities of SPC and casein were compared using the ORAC assay, as described previously. On an equal protein basis, casein performed better in the assay by protecting fluorescein longer than SPC (**Figure 36**). After 60 minutes, only 9.82% of the initial fluorescence was lost for the 1400 mg/L casein sample. By contrast, at the same concentration of SPC, 80.8% of the fluorescence was lost after 60 minutes. For the controls, 97.4% and 4.7% of the initial fluorescence were lost after 60 minutes for the Blank and fluorescein only samples, respectively.

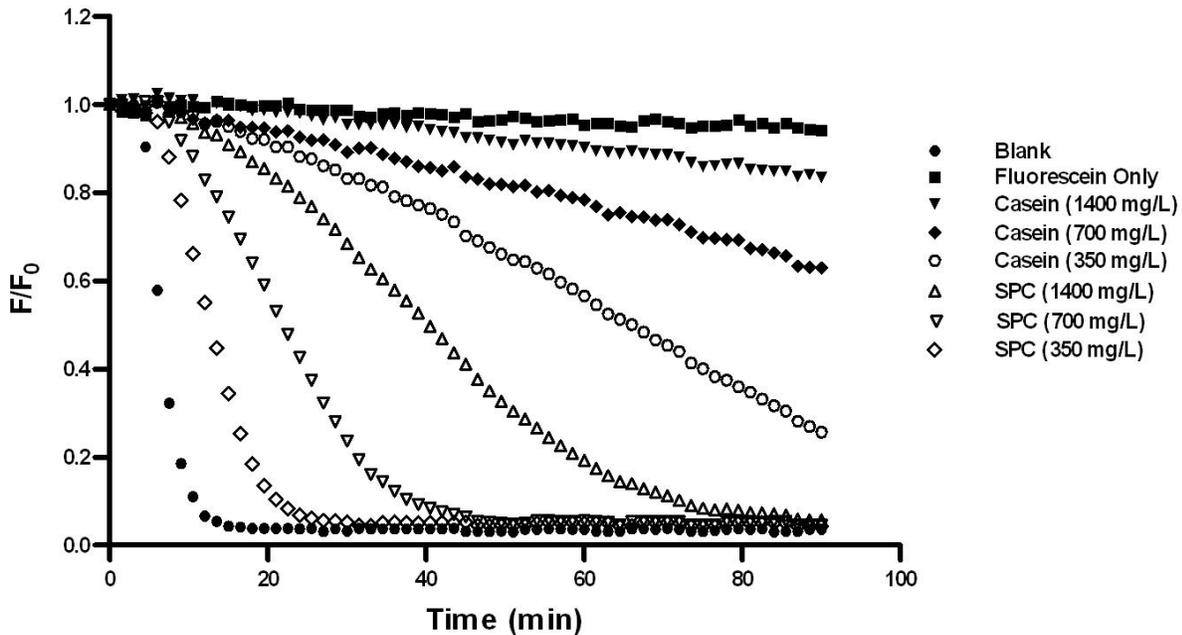


Figure 36: Oxygen radical absorbance capacity of SPC and casein. Changes in relative fluorescent intensity of fluorescein (E_m , 485 nm; E_x , 520 nm) in the presence of AAPH and 350-1400 mg/L SPC or casein at 37°C. Fluorescence values (F) are given relative to the initial time values (F_0). The blank was prepared without any antioxidant, and the fluorescein-only control was prepared without AAPH or antioxidant. Values are means of triplicate measures. Error bars were eliminated for clarity. Fluorescence values were different between treatments over time (Two-way ANOVA, $p < 0.0001$).

The peroxy radical scavenging capacities of the diets used in both mouse studies were also compared using the ORAC assay. As with the proteins alone, the diet containing casein as the protein source extended fluorescence longer than the diet containing SPC (Figure 37). After 60 minutes, at 1400 mg/L, 11.6 and 35.4% of the initial fluorescence was lost for the casein and SPC diets, respectively. The blank and fluorescein only controls lost 94.7% and 4.7% of their initial fluorescence values, respectively, after 60 minutes.

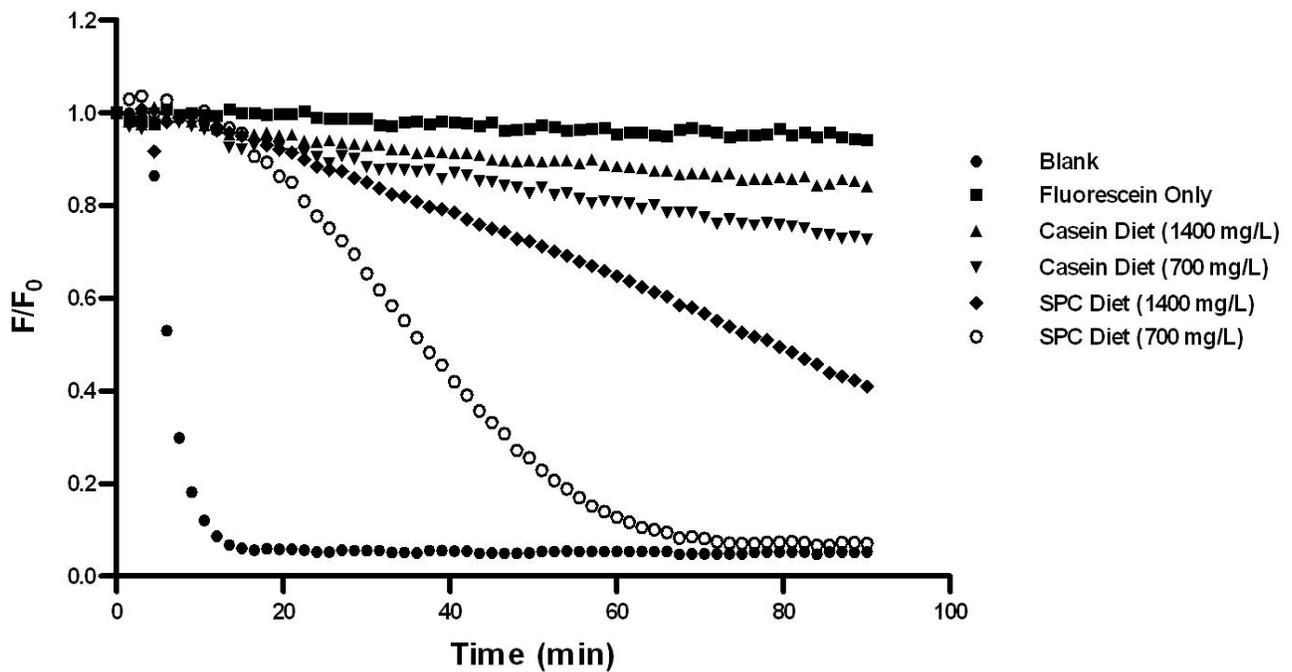


Figure 37: Oxygen radical absorbance capacity of SPC and casein diets. Changes in relative fluorescent intensity of Fluorescein (Em, 485 nm; Ex, 520 nm) in the presence of AAPH and 700 or 1400 mg/L SPC diet or Casein diet (AIN-76A) at 37°C. Fluorescence values (F) are given relative to the initial time values (F₀). The blank was prepared without any antioxidant, and the fluorescein-only control was prepared without AAPH or antioxidant. Values are means of triplicate measures. Error bars were eliminated for clarity. Fluorescence values were different between treatments over time (Two-way ANOVA, p<0.0001).

Previous studies have found caseins to possess antioxidant activity, as inhibitors of lipid peroxidation and as superoxide, hydroxyl and DPPH radical scavengers (116). Clausen

et al. examined the radical scavenging activities of milk by the ORAC and Trolox equivalent antioxidant capacity (TEAC) assays and found that caseins were the major radical scavengers in both assays, accounting for 89% of the activity (117). Interestingly, proteolytic digestion did not affect the radical scavenging activity of casein, which confirmed the investigators' hypothesis that the high radical scavenging activity of the caseins is probably related to their loose, random coil structure with good solvent exposure (117). This may help elucidate the differences seen between casein and SPC in the ORAC assay. In the future, a study comparing the antioxidant activity of hydrolyzed casein and hydrolyzed SPC may be useful.

Clausen et al. also determined the radical scavenging activity of the 20 amino acids in the ORAC assay and found that only tryptophan (Trp), methionine (Met), cystine (Cys), tyrosine (Tyr), phenylalanine (Phe) and histidine (His) were active in the ORAC assay. Trp was the most potent antioxidant, and was approximately 10 times more potent than Met (117). The approximate levels of the above three most active amino acids, Trp, Met and Cys, in SPC are 1.2, 1.3 and 1.3 g/100 g protein, which are the lowest of the amino acids listed (**Table 1**). In general, soybeans are relatively deficient in sulfur-containing amino acids, including Met, Cys and Thr (11). The differences seen in ORAC for casein and SPC are likely due to both differences in tertiary structure and amino acid composition. Despite the difficulties in translating ORAC assay activity and *in vivo* antioxidant activity, the finding that casein may possess greater radical scavenging activity in the ORAC assay may help explain why more dramatic differences between treatment groups were not seen in the mouse studies.

Chapter 4: Conclusions and Future Studies

4.1 Conclusions

We tested the hypothesis that SPC can protect Caco-2 cells against hydrogen peroxide-induced cytotoxicity *in vitro* and CF-1 mice from azoxymethane/dextran sodium sulfate-induced colonic inflammation and carcinogenesis. This was accomplished by the following objectives.

- a. To determine the cytoprotective effect of SPC against hydrogen peroxide-induced oxidation in Caco-2 cells.

In vivo digestion of SPC was simulated and the cytoprotective activity of SPC and SPH were compared. Both SPC and SPH were cytoprotective during 1 h cotreatment with H₂O₂, though SPC was more potent. Fluorescent visualization of ROS production also suggested that SPC was more effective than SPH at reducing intracellular ROS. In general, SPC was found to protect Caco-2 cells from ROS-mediated cell death.

- b. To determine the efficacy of a SPC-supplemented diet in reducing markers of colonic inflammation in mice.

Colon inflammation was measured histopathologically by the inflammation index following 10 or 20 weeks of dietary SPC after initiation with AOM and DSS. In both studies, at Week 7 following DSS treatment, total colon inflammation, inflammation area and hyperplasia and dysplasia scores were significantly reduced in mice fed SPC. Differences at

other time points were not significant between treatment groups. Trends in reductions in spleen-to-body weight ratios were suggestive of a benefit in the 10-week study, but were not seen in the 20-week study.

- c. To determine the efficacy of a SPC-supplemented diet in reducing colon carcinogenesis in mice.

In both mouse studies, the number of colon polyps in mice fed SPC was significantly lower at Week 7 and for the study overall than in mice fed casein. Non-significant decreases in colon tumor multiplicity and tumor area were also seen in mice fed SPC for 20 weeks. Additionally, the proportion of mice with adenomas classified as severe dysplastic or as adenocarcinomas was greater in the Pos group than the SPC group.

Overall, the results of these studies suggest that SPC has antioxidative and cancer preventive activities. The link between *in vitro* antioxidant activity and *in vivo* anti-cancer activity is still unclear. SPC had bioactivity in a cell culture model, protecting Caco-2 cells from H₂O₂-induced oxidative stress. SPC also reduced some markers of inflammation and colon cancer in mice in the AOM/DSS model. However, there are limitations to these studies. In the cell culture studies, direct measures of oxidative stress were limited. Additionally, the accuracy of simulated SPC digestion is unknown, which limits *in vivo* translatability. In the mouse studies, adenoma incidence was low and the results were time-point dependent, which limited our ability to draw overarching conclusions. Future studies are necessary to further understand SPC's effect on oxidative stress, inflammation and cancer outcomes in the colon.

4.2 Future Studies

The radical-scavenging, cytoprotective and cancer preventive effects of SPC which were found in this work need to be examined further. While SPC was found to have beneficial effects in reducing colonic inflammation and some markers of colon cancer, additional studies with more measurable outcomes are needed. The DSS model of colitis can be used to recapitulate acute or chronic colitis and should be used to study the effect of SPC on colon inflammation. In the current study, tumor incidence was low, which reduced statistical power. The effects of SPC on colon carcinogenesis would be clearer in a longer term study, possibly with higher or multiple doses of AOM. Future studies on dietary protein, specifically a protein such as SPC which contains other components, should also take care to match these compounds in the control diet. For example, in the current study, the levels of fiber in the diets were identical; however, we may have benefitted from having added isolated soy fiber to the control diet rather than using cellulose as the fiber in the control.

While studies on isolated soy bioactives (*eg.* BBIC) have shown promising results for people with chronic diseases (54), studies on colon cancer prevention may benefit from focusing on more commonly consumed, whole food forms of soy. Challenges exist in studying whole foods, such as how to incorporate them into the diet, what controls to use, and not fully knowing what the “active” compound may be. However, SPC is not a common human food; rather, soymilk, tofu and edamame have become more popular soy foods for human consumption. Animal studies which incorporate these foods into the diet will be more informative than studying pure compounds, yet less expensive than human intervention trials. Cell culture-based studies using these processed soy foods should also be explored. In

both cases, lyophilized powders of tofu or edamame could be evaluated; soymilk could be administered orally to mice (119). Human intervention trials on soy and inflammation and colon cancer are clearly necessary; to date, only one randomized controlled trial on soy protein and colon cancer markers has been published to our knowledge (53). The challenges of administering whole soy foods to mice or cells would be eliminated in human studies. Randomized, controlled trials of various soy foods should be carried out in men and women with a history of or at risk for adenomas or colitis. Experimental endpoints could include measurements of colon polyps, colon cell proliferation, plasma markers of inflammation or oxidative stress or the Sutherland Disease Activity Index for colitis.

Future studies exploring the antioxidant capacity of foods, particularly SPC, may benefit from using a cellular antioxidant assay (CAA), which takes into account cellular uptake of the compound of interest (120). This type of assay may serve as a link between traditional ORAC studies and Caco-2 cytoprotection studies. The results of the current ORAC, cytoprotection and animal studies are somewhat confounding, revealing the disconnect between *in vitro* and *in vivo* studies. While SPH had greater antioxidant capacity in the ORAC assay, SPC performed better in the cytoprotection studies. Likewise, casein showed greater activity in the ORAC assay than SPC, though SPC showed moderately more activity in the mouse experiments. No one single type of experiment can explain the entire story or mechanism of action. Comparing SPH, casein, and casein hydrolysates in the ORAC assay and testing casein's activity in the cytoprotection model may be interesting future experiments and provide insight into relative potency.

In addition to the knowledge gained about SPC's bioactivity, this work has pointed out future directions not directly related to the study of soy. Casein is commonly used as the

protein in control diets for animal experiments. Our ORAC results suggest that casein has more potent antioxidant activity than SPC. Therefore, future work should be done to determine whether a casein-based diet is the best choice for a control diet in studies on antioxidants and inflammation-based diseases. A study by Zemel et al. in men and women found that a dairy smoothie, but not a soy smoothie, reduced plasma markers of oxidative stress and inflammation (50). When studying soy protein, casein does not appear to be the best choice for the control protein. The results of our studies can be used to design future experiments to study not only soy, but other dietary proteins.

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