

Guidelines for MAFMA Final Report
Final Reports due 3 months after completion of project
(4-5 pages)

Project Title: Role of soy proteins in colon cancer etiology

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Academic Institution: Iowa State University

Award Date: 2004

Please complete all questions below and attached form

1. Objective Summary (1-2 sentence summary)

The overall hypothesis of this project was: Soy protein processing alters the ability of soy proteins to reduce colon tumor growth. Through processing of soy, the concentrations and structural composition of bioactive compounds are modified. Some processing procedures will generate effective soy proteins for cancer protection whereas others will reduce the effectiveness. The project included 2 specific aims:

Aim 1: Perform a mouse colon tumorigenesis study in which 4 differently processed soy proteins are compared for their ability to inhibit colon tumor initiation and progression.

Aim 2: Identify biological parameters in the mice that are responsive to the dietary treatments.

2. Objective Accomplishments

(If objectives were not met, what extenuating circumstances contributed to that factor?)

Convey all of your progress on this project including that obtained with the industry and other matching funds.

The goal of this study was to further explore the role of soy protein in colon cancer. In previous research we extensively have used the AOM mouse model with CF7Black/J mice. However, we have noticed that this model gave us inconsistent tumor incidence and was therefore not useful for studying dietary effects. The current project was originally designed to use this model, but we discovered a newer model in the literature that we thought might be more consistent, and require less time. We discussed this with Kathy Greaves and she approved changing to the new model which involved using CD-1 mice given only one dose of AOM followed by DSS in the drinking water. Because we had no previous experience with this model we did not know what to expect relative to tumor development. We were quite surprised by the rapid and extensive tumor growth in these animals. As shown in Table 4, 100% of mice in all groups had colon tumors. The model was very specific as we did not find tumors in any other tissue. At this time, we have not completed the histological assessment of the tumors obtained from the animals, and therefore cannot be sure of their pathology. However, we are fairly confident that the tumors greater than 5 in volume were most likely adenomas. We plan to verify this in the near future with a veterinary pathobiologist.

We found no evidence for a protective effect of soy proteins on tumor growth at this point in the data analysis. This finding disagrees with our previous study in which soy protein reduced the tumor burden compared to a casein diet. The previous study was done with the

AOM only protocol and therefore may not be directly comparable. We are excited about this new model however, particularly as the treatment causes such a rapid development of colon tumors.

We did quantify the isoflavone and saponin content of the diets, even though these were not the focus of the study. There was one diet (Supro 670) with higher levels of isoflavones than the others but this did not seem to explain any of the results and likely the differences were not great enough to impact the results. We have not yet completed the serum analysis of isoflavones due to a health problem in Dr. Berhow's group. However, given the lack of big differences in the diet we do not anticipate any significant diet effects on serum levels of isoflavones.

The core aspect of this study was to investigate if the soy protein isolates differently affected markers of colon cancer. It was our hypothesis that differently processing of soy proteins would result in changes in absorption/digestion of the soy components leading to different colonic content composition – or altered systemic factors resulting from absorbed components. These factors could then impact colon cell metabolism, growth and death and thereby alter tumor development. It is well known that factors that reduce cell proliferation, or increase apoptosis inhibit tumor growth. Our results were disappointing in that none of the colon markers of proliferation (crypt height and number) or apoptosis (Caspase-3) were modified by the diet. Several factors may be involved with this result. First the treatment was very strong in inducing tumors and therefore diet effects may have been overridden. As tumor development was not reduced by diet, it seems logical that these parameters were also unaffected. Second, we did not examine areas of the colon specifically associated with tumor growth. It may be that local effects were apparent, however with no difference in tumor incidence that would be unlikely as well. Third we took our tissue samples from the mice after tumors had been well developed. Perhaps changes could have been observed at an early stage of the progression which may have been lost as the tumor growth escalated.

We used the marker protein Caspase-3 to estimate changes in apoptosis within the colon cells. We selected this protein rather than any other of the numerous markers for apoptosis because it is a more reliable and early marker of the apoptotic pathway. We found no differences in expression due to diet, however as with Caspase-3 perhaps we were measuring this protein too late in the progression of the tumor development. The lack of an effect on COX-2 was disappointing as we and others have reported reduced expression of COX-2 by dietary factors. This protein is an important regulator of colon cancer progression and the identification of a dietary factor that inhibits expression would be of great value in the prevention of colon cancer. It is possible that the differences in the diet composition were not sufficient to have an impact on this parameter, or we were looking at a time point beyond where differences occurred.

3. Unexpected findings, if any

Because we have been interested in the Wnt/B-Catenin pathway in colon cancer, we quantified this protein in the colon lysates and were most surprised to find evidence for a differential diet effect. It appears that HO 313 and casein produced low levels of B-catenin in the colon of mice fed these proteins, whereas Supro 760 fed mice had high expression. B-Catenin is a multifunctional adaptor protein/transcription factor. Wnt is a secreted glycoprotein that binds to Frizzled receptors on the cell membrane. B-Catenin, in the absence of Wnt secretion, is

inactivated by association with a degradation complex. When Wnt binds to its receptors the degradation complex is inactivated releasing B-Catenin. B-Catenin then translocates to the nucleus and activates gene expression of target genes such as myc, cyclin D1, PPAR-g, MMP-7, Axin-2 and CD44. Research has suggested that levels of B-Catenin are elevated in the cytoplasm and nucleus of colon cells during tumorigenesis and mutations in the B-Catenin gene are responsible for colon tumor development. In our study, we quantified total B-Catenin expression in crude cell lysates, and therefore cannot detect if the expression was in the cytoplasm or nucleus. We were careful to remove tumors from the colon segment before scraping cells, however it is possible that some tumor cells were present in the samples and could explain the differences we observed. However, it would seem unlikely that this would have occurred only in some groups as the tumor expression was similar in all animals. This observation is intriguing and warrants further investigation.

In reviewing the results of the current study, we are interested in exploring the potential effects of soy protein diets on the inflammatory response in the colon. The DSS model can be considered an inflammatory bowel model due to the response generated in the colon (the colon wall is thickened and there are increased inflammatory cells). COX-2 and B-Catenin are involved in the inflammatory response as well and are important markers of these cellular events. In the colon, both the carcinogenic and the inflammatory pathways seem to be targets for dietary factors. This model will allow us to study both pathways simultaneously. Although this study did not show a direct protective effect on tumor growth, we are very excited about using this model to further explore the role of soy in the colon. It is a very rapid model and highly effective for both inflammation and tumor development.

4. Practical impacts of research efforts. Include: implementation of accomplishments by industry partners (if any), identification of economic impacts, and any further pursuit by PI of research in area of this project whether MAFMA or not.

a. Short Term Impacts: This study provides evidence that the soy protein isolates produced by the Solae Company are similar to casein in their effect on mouse growth and body composition. In this model none of the diets showed a protective effect against colon cancer.

b. Long Term Impacts: The animal model that was developed in this study will continue to be useful for examining the impact of dietary components such as soy protein isolate on colon cells and risk of colon cancer. In addition, we identified a potential new system that may be influenced by diet, the Wnt/B-Catenin system, and plays a role in both colon cancer and inflammation.

5. If you are also making reports to other funding agencies in the course of this research work, please include a copy of that report.

An complete report of the data has been submitted to the Solae Company and is attached below.

6. If any publications resulted from the research, a copy must be included. Please note we were notified by the USDA/CSREES National Program Leader for the Midwest Advance Food Manufacturing Alliance (MAFMA) that all publications resulting from research that was funded by MAFMA must include the following wording **“The project was supported by the USDA Cooperative State Research, Education and Extension Service, special research grant number 200X-34328-xxxxx.**

Role of soy proteins in colon cancer etiology

MAFMA/Solae Project
Final Report, March 15, 2007

The overall hypothesis of this project was: Soy protein processing alters the ability of soy proteins to reduce colon tumor growth. Through processing of soy, the concentrations and structural composition of bioactive compounds are modified. Some processing procedures will generate effective soy proteins for cancer protection whereas others will reduce the effectiveness.

The project included 2 specific aims:

Aim 1: Perform a mouse colon tumorigenesis study in which 4 differently processed soy proteins are compared for their ability to inhibit colon tumor initiation and progression.

Aim 2: Identify biological parameters in the mice that are responsive to the dietary treatments.

METHODS AND EXPERIMENTAL DESIGN

Diets:

Soy proteins were obtained from the Solae Company and processed into mouse chow according to the table 1 formulations. The diets were isocaloric and isonitrogenous, and the casein diet included addition of phytate to match the estimated content in the soy proteins. The diets were prepared in-house and pelleted by extrusion at the University of Missouri.

Table 1. Diet formulations

| | CASEIN | 313 | 710 | 670 | 760 |
|-----------------------|------------|-------|-------|-------|-------|
| Ingredient | mg/kg diet | | | | |
| Choline bitartrate | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| DL-methionine | 4 | 4 | 4 | 4 | 4 |
| Vitamin mix - AIN 93G | 10 | 10 | 10 | 10 | 10 |
| Salt mix - AIN93 | 35 | 35 | 35 | 35 | 35 |
| Corn Starch | 393.5 | 396.5 | 396.5 | 396.5 | 396.5 |
| Cellulose BW200 | 38 | 50 | 50 | 50 | 50 |
| Phytic acid | 15 | 0 | 0 | 0 | 0 |
| Dyetrose | 132 | 132 | 132 | 132 | 132 |
| Sucrose | 100 | 100 | 100 | 100 | 100 |
| Casein | 200 | 0 | 0 | 0 | 0 |
| Soy Protein | 0 | 200 | 200 | 200 | 200 |
| Safflower oil | 20 | 20 | 20 | 20 | 20 |
| Corn oil | 50 | 50 | 50 | 50 | 50 |

Animals:

3-week-old female CD-1 (ICR) mice were purchased from Charles River Laboratories (Wilmington, MA). All animals were housed in plastic cages (4 mice/cage) with free access to drinking water and a pelleted casein control diet, under controlled conditions of humidity (40 ± 10%), light (12h/12 h light/dark cycle) and temperature (22 ± 2°C). For 6 days they were fed the casein control diet, then the mice were randomly assigned by body weight and provided the diet treatments and tap water for one week before AOM injection.

| Table 2. | Treatment protocol | | |
|-----------------|---------------------------|-----------------|------------------|
| Group | Diet | No. mice | Treatment |
| 1 | Casein | 25 | DSS/AOM |
| 2 | Soy protein 313 | 26 | DSS/AOM |
| 3 | Soy protein 670 | 26 | DSS/AOM |
| 4 | Soy protein 710 | 25 | DSS/AOM |
| 5 | Soy protein 760 | 26 | DSS/AOM |

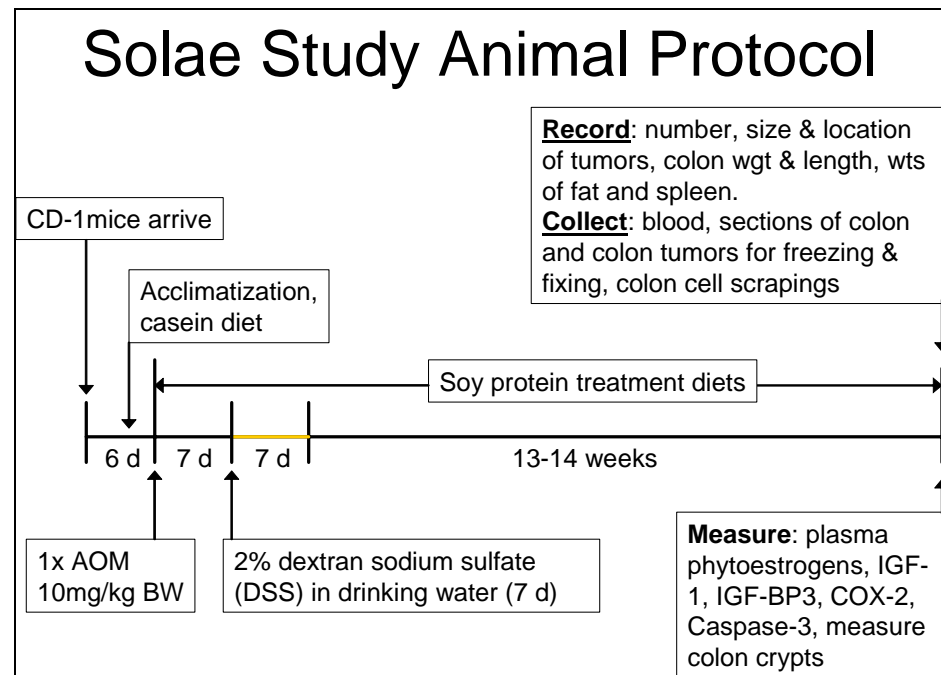


Figure 1. Study protocol

All mice were given a single intraperitoneal injection of azoxymethane (AOM, 10 mg/kg body weight). Starting 1 week after the injection, animals received 2% dextran sodium sulfate (DSS) in the drinking water for 7 days and continued on the indicated diets for 13-14 weeks. The mice were on their diets for 15-16 weeks, were terminated 14-15 weeks after AOM and were 19-20 weeks of age at termination. Mice were then euthanized and the following obtained: a) number, size and location of tumors, b) blood from cardiac puncture c) colon cell scrapings and d) sections of intact colon and colon tumors for fixing in formaldehyde. Plasma was analyzed for phytoestrogens (by HPLC), IGF-1 (by ELISA), and IGFBP-3 (by Western immunoblot). Colon lysates were analyzed for COX-2 and Caspase-3 (marker of apoptosis) by Western immunoblot. And formalin fixed, hematoxylin/eosin stained colon sections were analyzed for crypt parameters including muscle thickness, crypt height and crypt number.

Prior to initiating this study, we performed a small trial to evaluate the impact of DSS on the mice. Four groups of mice were used to observe the effects of 7 days of 2% DSS on mice noting any blood in the rectum or feces, diarrhea and body weight loss. The DSS was well tolerated by the mice with no observable differences between untreated and treated mice, hence the larger study was carried out using this protocol. AOM was purchased from Midwest Scientific (St Louis, MO). DSS with a molecular weight of 36,000–50,000 was purchased from MP Biomedicals, (Cat. No. 160110, Irvine, CA). Soy protein isolates were obtained from the Solae Company.

The statistical analysis used was one-way ANOVA with adjusted multiple comparisons performed using the Tukey-Kramer test, using the SAS software.

References for the DSS protocol:

Carcinogenesis vol.27 no.1 pp.162–169, 2006. Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice. Rikako Suzuki, Hiroyuki Kohno, Shigeyuki Sugie, Hitoshi Nakagama and Takuji Tanaka.

AND

Cancer Sci Nov. 2003; vol. 94, no. 11, pp. 965–973. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Takuji Tanaka, Hiroyuki Kohno, Rikako Suzuki, Yasuhiro Yamada, Shigeyuki Sugie and Hideki Mori

RESULTS

The focus of the study was to investigate the impact of differently processed soy proteins on colon cancer risk factors in a mouse model. The experimental design did not include any descriptive comparison of diet by isoflavone or saponin concentration. However, as we have done many studies investigating the role of isoflavones and saponins on the colon, we were curious as to the concentration of these components. Dr. Mark Berhow, USDA Peoria, ran an analysis of the diets for these components. His analysis included all of the metabolites of these compounds. We are showing the isoflavone equivalents in figure 2A which represents the sum of all related metabolites, total isoflavones in 2B and total saponins in 2C. In general, all of the soy isolate diets were low in isoflavones. One of the diets, Supro 670 stood out from the rest however as having higher levels of isoflavones. No differences in total saponins were observed across the diets. The casein diet was free of both isoflavones and saponins.

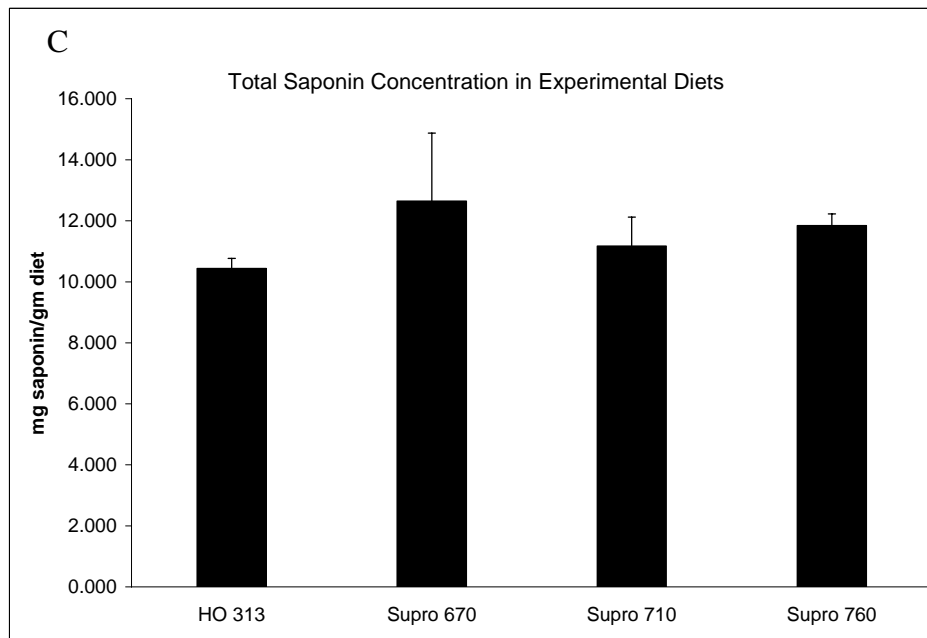
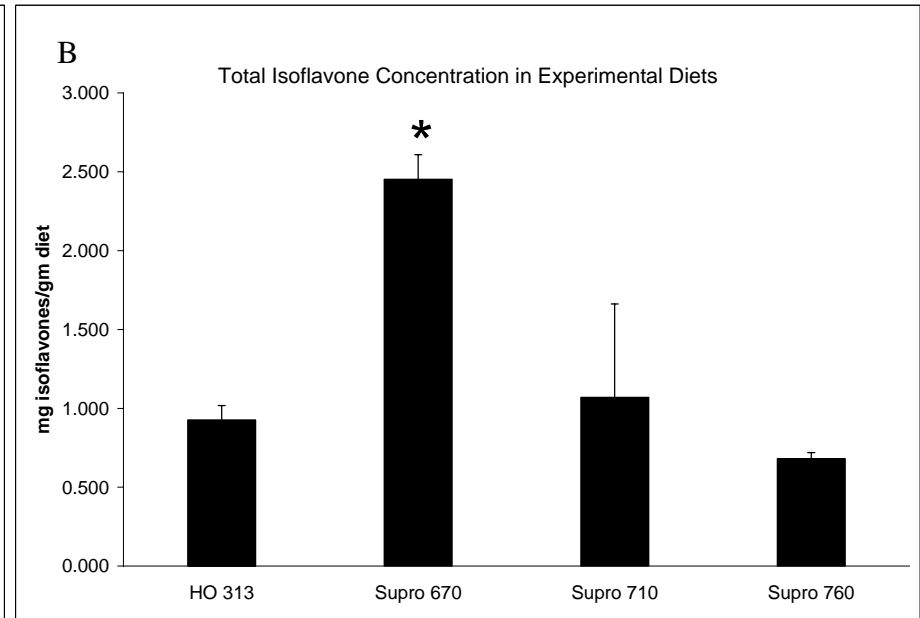
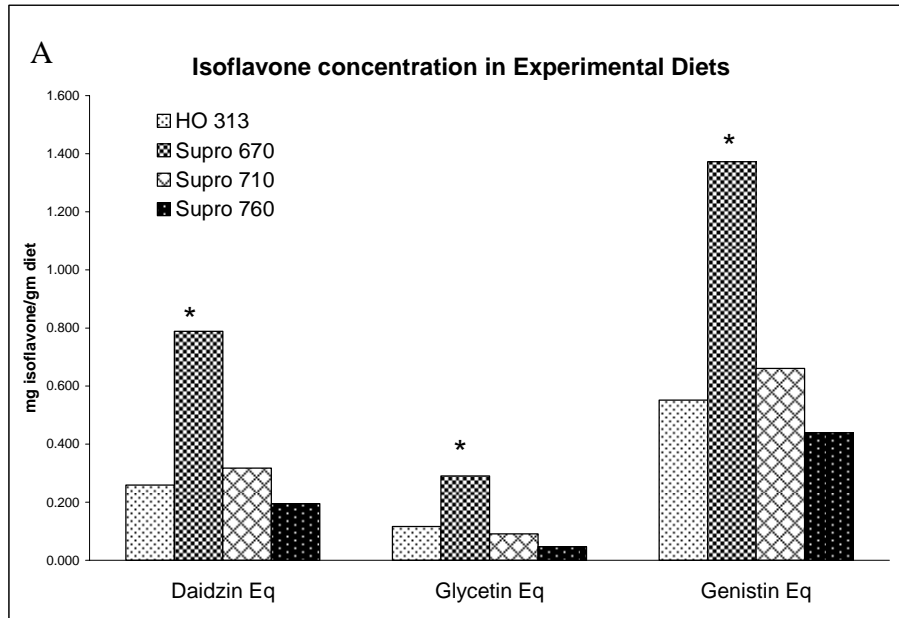


Figure 2. Phytoestrogen and saponin concentration in experimental diets. Bars represent means \pm SD of three determinations by HPLC analysis of A, isoflavone equivalents for daidzin, glycetin and genistin, B, total isoflavones and C total saponins expressed as mg/g of diet. The casein diet contained no detectable levels of any of the isoflavones or saponins (data not shown). Asterisks show the isoflavones in which the Supro 670 diet was significantly different from all other diets ($P < 0.05$). Saponin concentration was not different across the diets.

The mice consumed the diets well throughout the study, with the exception of during the DSS treatment – and this was not different by diet group. No differences in final body weight were observed due to treatment across the dietary groups (Table 3). There was an overall trend for the mice fed the 760 diet to have more adipose tissue in the renal, lingual and abdominal pads compared to the other diets. The renal fat pad/bw value for the 760 group was nearing statistical significance (p=0.06) compared to the casein group. Interestingly this was the diet lowest in total isoflavones. We measured the spleen weight as an indicator of inflammation/sepsis in the animals and found no difference in spleen weight by treatment (close for casein vs 670, P = 0.065).

| | Diet | body weight (g) | colon length (cm) | colon | colon/BW | Renal fat pad | RFP/BW | lingual fat pad | LFP/BW | abdominal fat pad | AFP/BW | spleen | SPL/BW |
|---------|--------|-----------------|-------------------|-------|----------|---------------|--------|-----------------|--------|-------------------|--------|--------|--------|
| AVG | Casein | 33.54 | 8.96 | 0.36 | 1.11 | 0.42 | 1.14 | 0.37 | 0.98 | 0.75 | 2.05 | 0.16 | 0.50 |
| std err | | 1.23 | 0.18 | 0.03 | 0.10 | 0.07 | 0.16 | 0.08 | 0.17 | 0.11 | 0.24 | 0.01 | 0.04 |
| AVG | 310 | 33.27 | 8.80 | 0.34 | 0.98 | 0.43 | 1.15 | 0.38 | 1.00 | 0.73 | 2.01 | 0.22 | 0.66 |
| std err | | 1.13 | 0.18 | 0.03 | 0.08 | 0.07 | 0.16 | 0.07 | 0.17 | 0.10 | 0.21 | 0.02 | 0.04 |
| AVG | 670 | 35.92 | 8.78 | 0.33 | 0.97 | 0.60 | 1.50 | 0.46 | 1.13 | 1.06 | 2.67 | 0.25 | 0.75 |
| std err | | 1.28 | 0.16 | 0.03 | 0.10 | 0.12 | 0.24 | 0.11 | 0.21 | 0.21 | 0.44 | 0.03 | 0.11 |
| AVG | 710 | 36.82 | 8.69 | 0.35 | 0.98 | 0.59 | 1.52 | 0.66 | 1.66 | 0.93 | 2.46 | 0.24 | 0.69 |
| std err | | 1.17 | 0.20 | 0.02 | 0.06 | 0.10 | 0.22 | 0.16 | 0.33 | 0.14 | 0.31 | 0.02 | 0.06 |
| AVG | 760 | 36.44 | 8.67 | 0.30 | 0.86 | 0.78 | 1.95 | 0.70 | 1.72 | 1.27 | 3.18 | 0.19 | 0.55 |
| std err | | 1.22 | 0.25 | 0.02 | 0.05 | 0.11 | 0.25 | 0.11 | 0.25 | 0.18 | 0.39 | 0.02 | 0.07 |

The tumor incidence in the mice using this method was surprisingly high (Table 4). By termination, all mice had tumors in all treatment groups, and it was necessary to terminate some mice early due to high tumor burden. This protocol was much more effective than our previous one using 6 weeks of AOM treatment without DSS. The DSS model allows a much faster induction of colon tumors, however the treatment may be so severe as to override dietary protections.

Table 4. Percent of mice with tumors

| # of tumors | < 5 | 5-10 | >10 | Total |
|-------------|-----|------|-----|-------|
| Casein | 20 | 56 | 24 | 100 |
| 310 | 12 | 73 | 15 | 100 |
| 670 | 16 | 56 | 28 | 100 |
| 710 | 8 | 71 | 21 | 100 |
| 760 | 19 | 65 | 15 | 100 |

In the group of mice fed the 310 or 760 soy protein diets, there were fewer mice exhibiting more than 10 tumors per mouse compared to the other groups (15% compared to 24, 28, and 21%; Table 4). This may suggest a slight delay in tumor growth in these animals, although this study does not verify that finding.

Table 5. Mice killed early

| | | | | | | | | | | | TOTAL |
|---------------|---|---|---|---|---|----|----|----|----|---|-------|
| wks after AOM | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | | |
| Casein | | | | | | 2 | 1 | | 1 | 4 | |
| 310 | | | | | 2 | 1 | 1 | 1 | 3 | 8 | |
| 670 | 1 | | | | 3 | 2 | | 1 | 1 | 8 | |
| 710 | | | | 1 | 1 | 1 | 1 | 2 | 2 | 8 | |
| 760 | | | | | 3 | | 1 | | 1 | 5 | |

It was necessary to terminate early 31 out of 125 (25%) of the mice due to prolapse rectum or severe bleeding which were indicative of heavy tumor burden. As shown in Table 5 there did appear to be any strong differences in the distribution of mice with rapid tumor growth associated with dietary treatment

Table 6. Number of Tumors per mouse

| | | | |
|--------|------|---|------|
| Casein | 7.84 | ± | 0.88 |
| 310 | 8.19 | ± | 0.85 |
| 670 | 9.00 | ± | 1.01 |
| 710 | 8.29 | ± | 0.56 |
| 760 | 7.62 | ± | 0.72 |

No differences in number of tumors per mouse were observed due to the dietary treatments (Table 6).

Table 7. Tumor number and volume

| | Tumor Number by Location | | | Tumor Volume by Location | | |
|--------|--------------------------|------|------|--------------------------|------|------|
| | 1 cm | 2 cm | 3 cm | 1 cm | 2 cm | 3 cm |
| Casein | 3.1 | 2.1 | 1.9 | 2.0 | 1.5 | 1.4 |
| 310 | 2.8 | 2.0 | 1.8 | 2.0 | 1.2 | 1.4 |
| 670 | 3.2 | 2.0 | 2.2 | 1.8 | 1.6 | 1.7 |
| 710 | 3.4 | 2.3 | 1.8 | 2.9 | 1.8 | 1.8 |
| 760 | 2.6 | 2.3 | 1.7 | 1.5 | 1.6 | 1.1 |

Measured from the rectum

Volume calculated from widthxheightxlength measured using a caliper

No differences in tumor number or volume by location in the colon were observed due to the dietary treatments (Table 7).

To determine if the diet provided any protection of the colon from the AOM and DSS treatment we assessed the histological parameters of the colon crypts using standard quantification methods. Fixed rings of colon were stained with hematoxylin and eosin and the muscle thickness and crypt height measured, and the number of crypts counted. The results were no differences in crypt height or number of crypts by diet (Figure 3). The muscle thickness tended to be lower in the mice fed Supro 760 and 670 compared to casein or Supro 710, although these did not reach statistical significance.

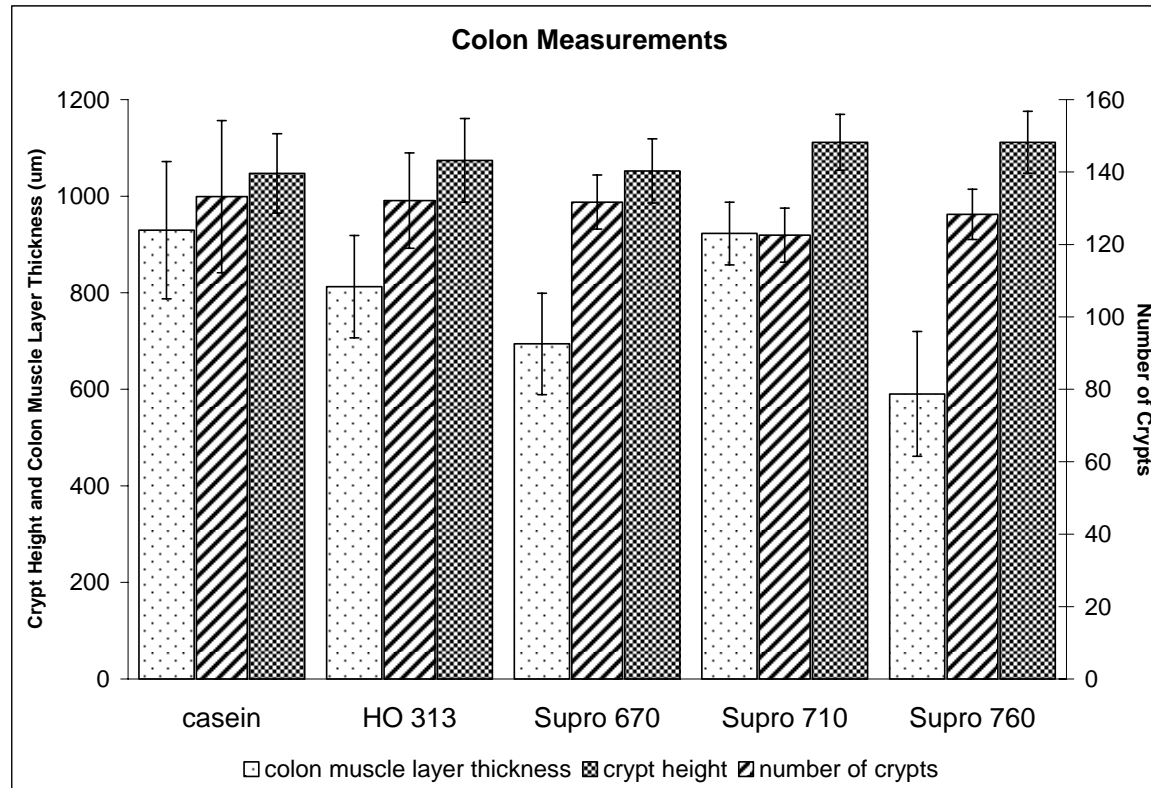
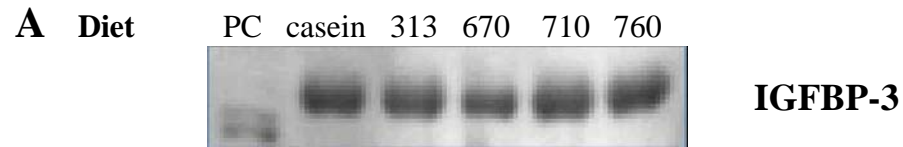


Figure 3. Bars represent means \pm SE of 5-9 mice per diet group where the left y-axis scale is for crypt height and colon muscular layer thickness measured in μm and the right y-axis scale is for the number of colon crypts per circular colon cross section. Measurements were made on formalin fixed mouse colons that were paraffin-embedded on edge and stained with hematoxylin and eosin (H&E) yielding “ring-shaped” cross sections. One-way ANOVA by SAS software showed no significant effect of diet for number of crypts, crypt height or colon muscular layer thickness.

As a measure of the impact of the diets on growth, we assessed the plasma concentrations of IGFBP-3 and IGF-I (Figure 4). Circulating levels of IGFBP-3 regulate availability and turnover of IGF-I and therefore can be a useful marker of changes in this hormone. No differences due to diet were observed in either the binding protein or hormone. This suggests that the diets did not alter growth parameters in the animals, and despite the presence of high numbers of colon tumors, IGF-I levels were within the normal range.



B

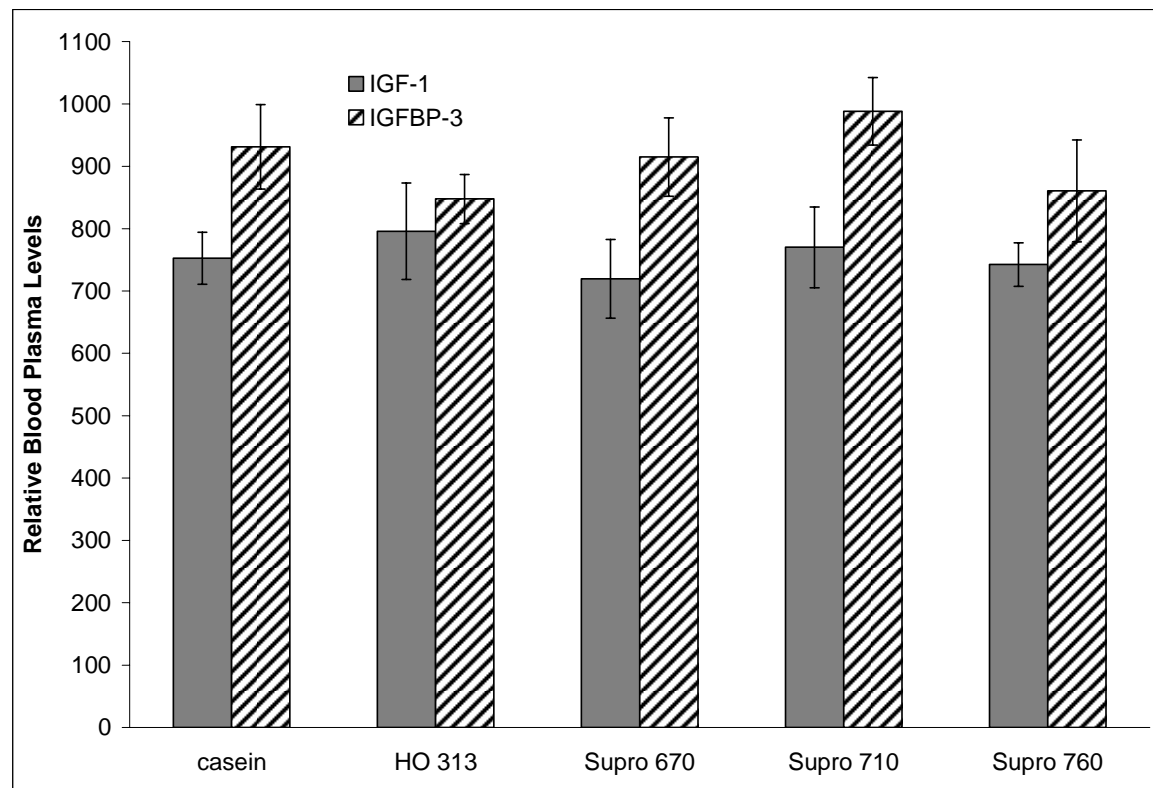


Figure 4. Effects of diet on mouse plasma levels of IGF-1 and IGFBP-3. A. Example of Western immunoblot image for IGFBP-3 protein where the first lane (PC) contains the positive control according to the manufacturer and other lanes each contain 0.1ul of blood plasma from one CD-1 mouse. B. Bars represent means \pm SE of 8 mice per diet group. IGF-1 blood plasma levels were measured using R&D Systems, Inc (Minneapolis, MN) Quantikine kit #MG100 and are expressed as ng/ml. IGFBP-3 levels were quantitated by QuantityOne software from Western Blot images and are expressed as intensity \times mm² (\times 100) normalized across 4 blots. One-way ANOVA by SAS software showed no significant diet effects on IGF-1 or IGFBP-3 levels.

Caspase-3 is a primary component in the cell that induces apoptosis via proteolytic cleavage of key proteins such as PARP. Activation of Caspase-3 leads to further activation of the caspase cascade which is responsible for the apoptotic response in cells. To determine if changes in apoptosis occurred in the mouse colon we used a Western immunoblot to assess Caspase-3 in colon lysates. B-tubulin was analyzed on each gel as a loading control (B-tubulin should be unchanged by diet conditions) and the data are expressed as a ratio. There were no differences in expression of Caspase-3 due to diet (Figure 5).

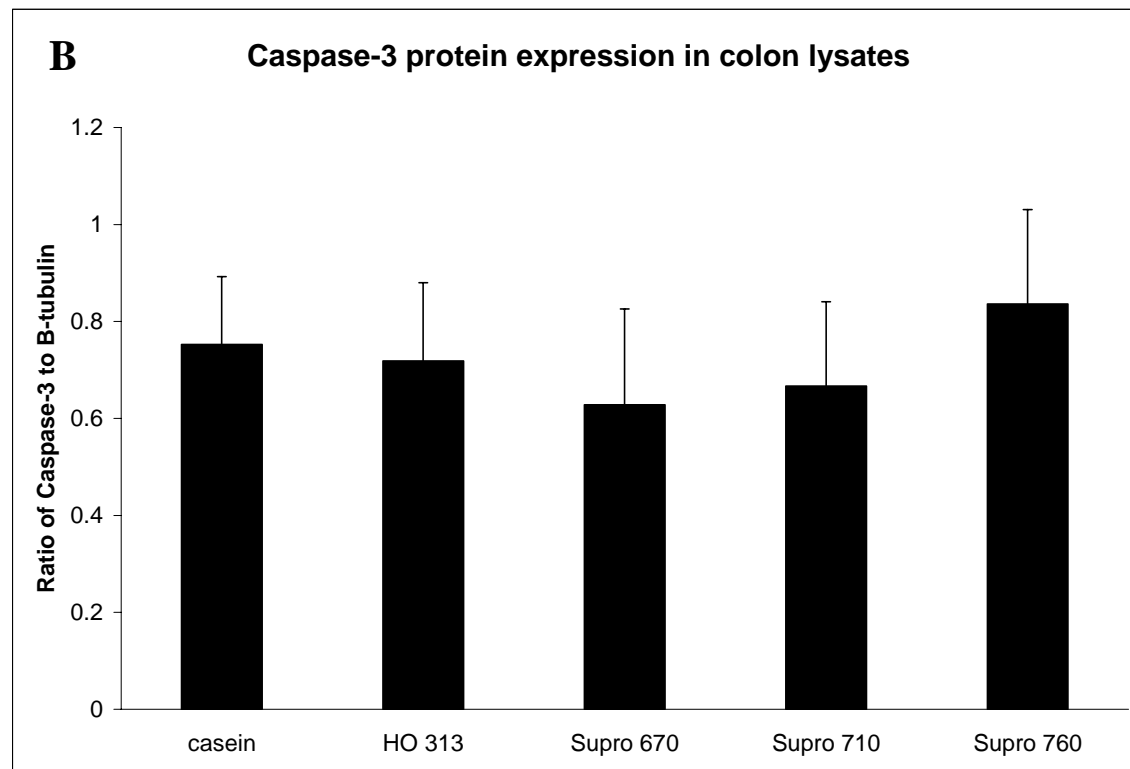
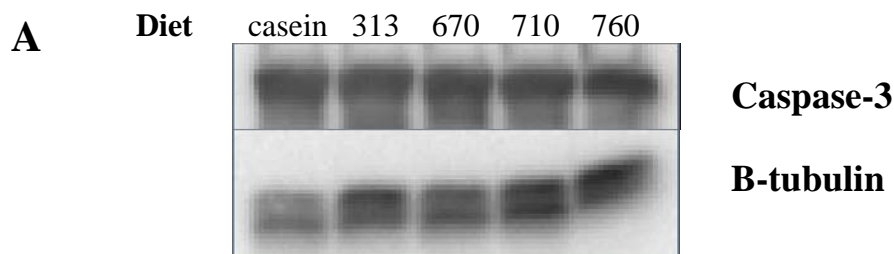


Figure 5. **Effects of diet on mouse colon levels of Caspase-3 protein levels in mouse colon lysates.** A. Examples of Western immunoblot images for Caspase-3 proteins where each lane contains 50ug CD-1 mouse colon whole cell lysate from one mouse exposed once to 10mg/kg body weight of AOM followed by 1 week of 2% dextran sodium sulfate in the drinking water and provided with the indicated diets. B. Bars represent means \pm SE of 8 mice per diet group for Caspase-3. Caspase-3 protein levels were quantitated by QuantityOne software from Western Blot images and are expressed as a ratio of the intensity \times mm² for the protein to that of B-tubulin. One-way ANOVA by SAS software showed no significant diet effects on Caspase-3 protein levels.

Induction of COX-2 expression is considered to be an early event in colon cancer progression. In humans, prevention of COX-2 has been found to be highly effective in preventing colon cancer in patients with familial adenomatous polyposis. To determine if the diet composition would impact COX-2 levels, we measured this protein using Western immunoblot in colon lysates. Although there was a trend for lower COX-2 in the mice fed Supro 760, this did not reach statistical significance (Figure 6).

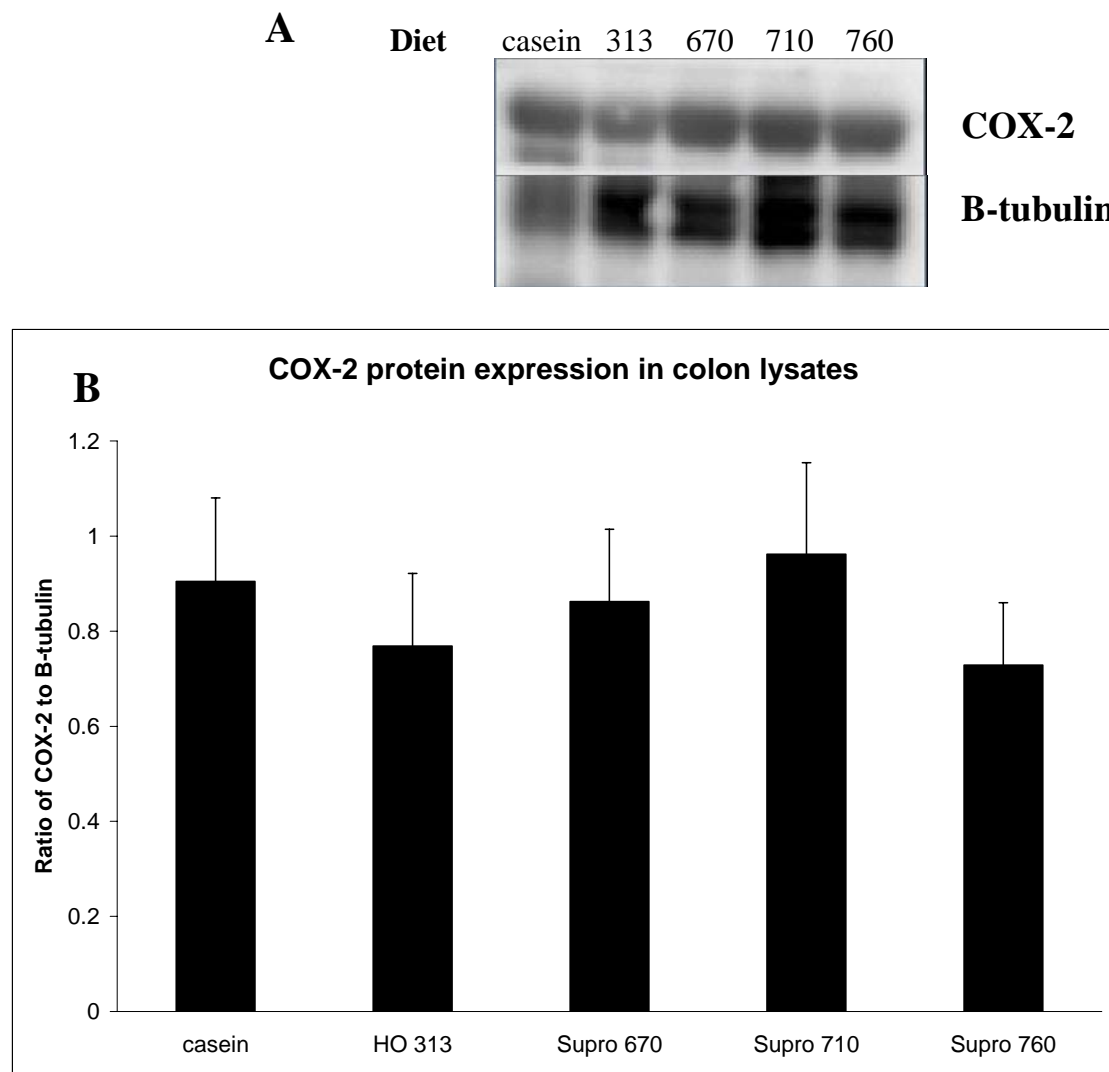


Figure 6. Effects of diet on mouse colon levels of COX-2 protein levels in mouse colon lysates. A. Examples of Western immunoblot images for COX-2 proteins where each lane contains 50ug CD-1 mouse colon whole cell lysate from one mouse exposed once to 10mg/kg body weight of AOM followed by 1 week of 2% dextran sodium sulfate in the drinking water and provided with the indicated diets. B, Bars represent means \pm SE of 15 mice per diet group for COX-2. COX-2 protein levels were quantitated by QuantityOne software from Western Blot images and are expressed as a ratio of the intensity \times mm² for the protein to that of B-tubulin. One-way ANOVA by SAS software showed no significant diet effects on COX-2 protein levels.

The Wnt/B-Catenin pathway is involved in integration of signals from many cell regulatory pathways including retinoic acid, FGF, TGF- β and BMP. B-catenin is deregulated in many forms of cancer, including colon cancer. We were curious as to whether the dietary proteins would impact this system in our model, and therefore use Western immunoblot to quantify expression in colon lysates. Due to relatively high variation in the data there was no statistical significance in the expression by diet (Figure 7). However it is clear that mice fed the casein or HO 313 diets tended to have low expression of B-catenin, whereas mice fed Supro 760 had high expression. This observation warrants further investigation and may provide a new avenue for research on the impact of soy proteins on colon cell metabolism.

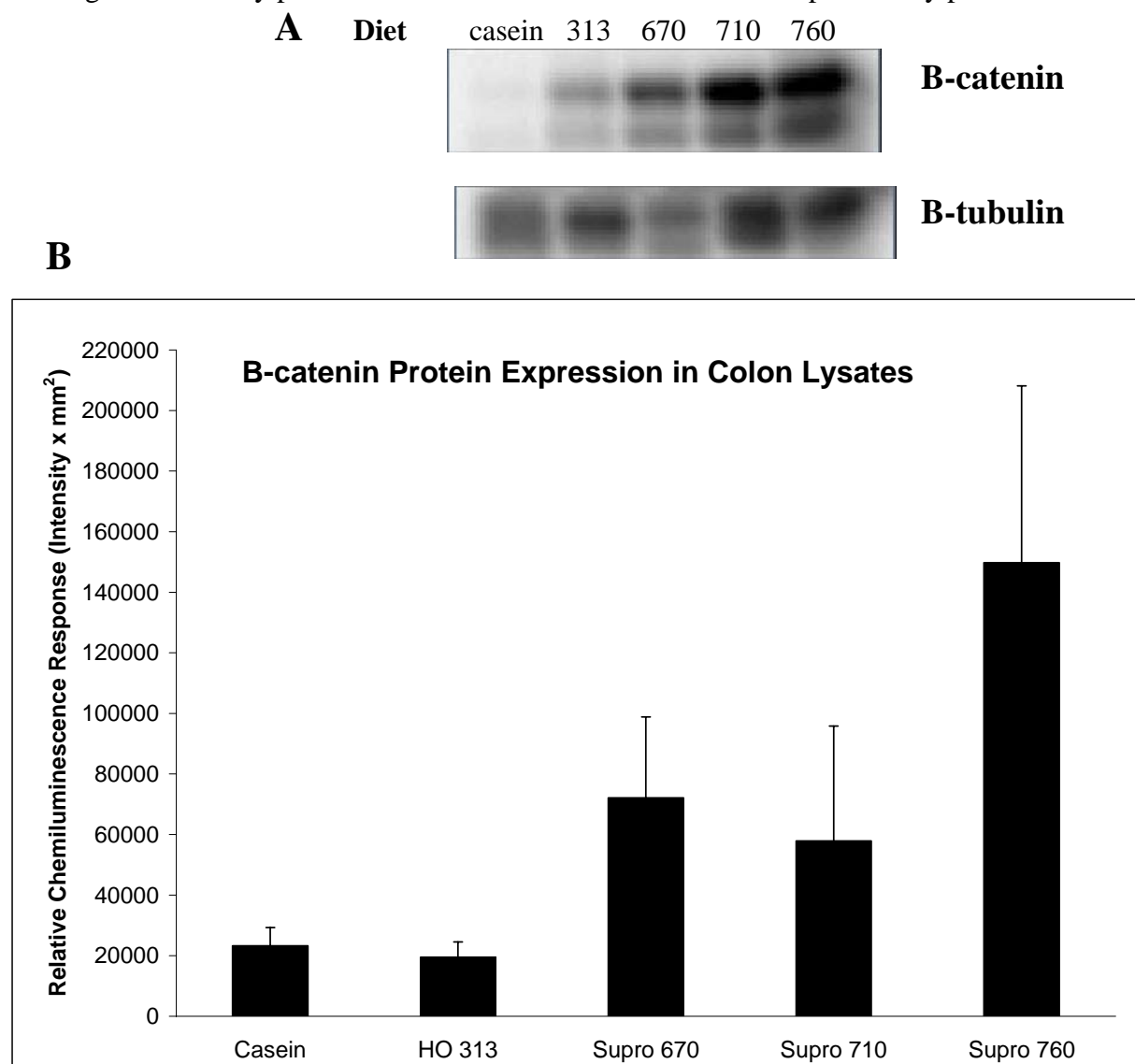


Figure 7. **Effects of diet on mouse colon levels of B-catenin protein levels in mouse colon lysates.** A. Examples of Western immunoblot images for B-catenin protein where each lane contains 50ug CD-1 mouse colon whole cell lysate from one mouse exposed once to 10mg/kg body weight of AOM followed by 1 week of 2% dextran sodium sulfate in the drinking water and provided with the indicated diets. B, Bars represent means \pm SE of 8 mice per diet group. B-catenin protein levels were quantitated by QuantityOne software from Western Blot images and are expressed as the intensity x mm². One-way ANOVA by SAS software showed marginal significance for Supro 760 diet versus both casein and HO313 diets ($P < 0.10$).

DISCUSSION

The goal of this study was to further explore the role of soy protein in colon cancer. In previous research we extensively have used the AOM mouse model with CF7Black/J mice. However, we have noticed that this model gave us inconsistent tumor incidence and was therefore not useful for studying dietary effects. The current project was originally designed to use this model, but we discovered a newer model in the literature that we thought might be more consistent, and require less time. We discussed this with Kathy Greaves and she approved changing to the new model which involved using CD-1 mice given only one dose of AOM followed by DSS in the drinking water. Because we had no previous experience with this model we did not know what to expect relative to tumor development. We were quite surprised by the rapid and extensive tumor growth in these animals. As shown in Table 4, 100% of mice in all groups had colon tumors. The model was very specific as we did not find tumors in any other tissue. At this time, we have not completed the histological assessment of the tumors obtained from the animals, and therefore cannot be sure of their pathology. However, we are fairly confident that the tumors greater than 5 in volume were most likely adenomas. We plan to verify this in the near future with a veterinary pathobiologist.

We found no evidence for a protective effect of soy proteins on tumor growth at this point in the data analysis. This finding disagrees with our previous study in which soy protein reduced the tumor burden compared to a casein diet. The previous study was done with the AOM only protocol and therefore may not be directly comparable. We are excited about this new model however, particularly as the treatment causes such a rapid development of colon tumors.

We did quantify the isoflavone and saponin content of the diets, even though these were not the focus of the study. There was one diet (Supro 670) with higher levels of isoflavones than the others but this did not seem to explain any of the results and likely the differences were not great enough to impact the results. We have not yet completed the serum analysis of isoflavones due to a health problem in Dr. Berhow's group. However, given the lack of big differences in the diet we do not anticipate any significant diet effects on serum levels of isoflavones.

The core aspect of this study was to investigate if the soy protein isolates differently affected markers of colon cancer. It was our hypothesis that differently processing of soy proteins would result in changes in absorption/digestion of the soy components leading to different colonic content composition – or altered systemic factors resulting from absorbed components. These factors could then impact colon cell metabolism, growth and death and thereby alter tumor development. It is well known that factors that reduce cell proliferation, or increase apoptosis inhibit tumor growth. Our results were disappointing in that none of the colon markers of proliferation (crypt height and number) or apoptosis (Caspase-3) were modified by the diet. Several factors may be involved with this result. First the treatment was very strong in inducing tumors and therefore diet effects may have been overridden. As tumor development was not reduced by diet, it seems logical that these parameters were also unaffected. Second, we did not examine areas of the colon specifically associated with tumor growth. It may be that local effects were apparent, however with no difference in tumor incidence that would be unlikely as well. Third we took our tissue samples from the mice after tumors had been well developed. Perhaps changes could have been observed at an early stage of the progression which may have been lost as the tumor growth escalated.

We used the marker protein Caspase-3 to estimate changes in apoptosis within the colon cells. We selected this protein rather than any other of the numerous markers for apoptosis because it is a more reliable and early marker of the apoptotic pathway. We found no differences in expression due to diet, however as with Caspase-3 perhaps we were measuring this protein too late in the progression of the tumor development. The lack of an effect on COX-2 was disappointing as we and others have reported reduced expression of COX-2 by dietary factors. This protein is an important regulator of colon cancer progression and the identification of a dietary factor that inhibits expression would be of great value in the prevention of colon cancer. It is possible that the differences in the diet composition were not sufficient to have an impact on this parameter, or we were looking at a time point beyond where differences occurred.

Because we have been interested in the Wnt/B-Catenin pathway in colon cancer, we quantified this protein in the colon lysates and were most surprised to find evidence for a differential diet effect. It appears that HO 313 and casein produced low levels of B-catenin in the colon of mice fed these proteins, whereas Supro 760 fed mice had high expression. B-Catenin is a multifunctional adaptor protein/transcription factor. Wnt is a secreted glycoprotein that binds to Frizzled receptors on the cell membrane. B-Catenin, in the absence of Wnt secretion, is inactivated by association with a degradation complex. When Wnt binds to its receptors the degradation complex is inactivated releasing B-Catenin. B-Catenin then translocates to the nucleus and activates gene expression of target genes such as myc, cyclin D1, PPAR-g, MMP-7, Axin-2 and CD44. Research has suggested that levels of B-Catenin are elevated in the cytoplasm and nucleus of colon cells during tumorigenesis and mutations in the B-Catenin gene are responsible for colon tumor development. In our study, we quantified total B-Catenin expression in crude cell lysates, and therefore cannot detect if the expression was in the cytoplasm or nucleus. We were careful to remove tumors from the colon segment before scraping cells, however it is possible that some tumor cells were present in the samples and could explain the differences we observed. However, it would seem unlikely that this would have occurred only in some groups as the tumor expression was similar in all animals. This observation is intriguing and warrants further investigation.

In reviewing the results of the current study, we are interested in exploring the potential effects of soy protein diets on the inflammatory response in the colon. The DSS model can be considered an inflammatory bowel model due to the response generated in the colon (the colon wall is thickened and there are increased inflammatory cells). COX-2 and B-Catenin are involved in the inflammatory response as well and are important markers of these cellular events. In the colon, both the carcinogenic and the inflammatory pathways seem to be targets for dietary factors. This model will allow us to study both pathways simultaneously. Although this study did not show a direct protective effect on tumor growth, we are very excited about using this model to further explore the role of soy in the colon. It is a very rapid model and highly effective for both inflammation and tumor development.

Special thanks to MAFMA and the Solae Company for providing the funding and soy protein isolates for this research.