Prevention of precancerous colonic lesions in rats by soy flakes, soy flour, genistein, and calcium

Deepa G Thiagarajan, Maurice R Bennink, Leslie D Bourquin, and Firdevs A Kavas

ABSTRACT

The main purpose of this research was to determine whether diets containing soy products would inhibit the early stages of azoxymethane-induced colon cancer in F344 rats. Additional objectives were to determine whether feeding starch instead of sucrose, feeding additional calcium (0.5% compared with 0.1%), or feeding a low-fiber powdered enteral formula would influence early colon carcinogenesis. Colon cancer was initiated with 2 injections of azoxymethane (15 mg/kg body wt) and a 12-wk dietary treatment period was started 1 wk after the second injection. Precancerous colon lesions were assessed as foci with aberrant crypts (FAC). The mean numbers of FAC were 133 [soy concentrate (low concentration of phytochemicals), 111 (starch substituted for sucrose), 98 [full-fat soy flakes (whole soybeans)], 87 (defatted soy flour), 77 (0.015% genistein), and 70 (0.5% Ca). The soy flour and full-fat soy flake diets contained 0.049% genistein derivatives (primarily glycosides), but were less effective in inhibiting the formation of FAC than the diet containing 0.015% genistein (as the aglycone). Eating soybeans and soy flour may reduce the early stages of colon cancer.

INTRODUCTION

Colon cancer is a serious health problem in most developed countries and is the third leading cause of cancer mortality throughout the world (1). It is the second most common cause of cancer mortality in the United States with 54,900 deaths and 133,500 new cases annually (2). Epidemiologic data suggest that populations that regularly consume soyfoods have a lower incidence of colon cancer than do populations that do not (3). Several phytochemicals in soybeans have been reported to have potential anticancer properties. For example, Pereira et al (4) found that dietary genistein decreased precancerous colonic lesions in rats treated with the colon carcinogen azoxymethane. Feeding a soybean extract containing the Bowman-Birk protease inhibitor (5) or feeding the purified Bowman-Birk protease inhibitor (6, 7) decreased chemically induced colon cancer in rats and mice. Raich et al (8) reported that 0.2% dietary β-sitosterol reduced colon cancer in rats; others showed an inverse relation between dietary phytosterol intake and risk of colon cancer in humans (9, 10). Pretlow et al (11) and Shamsuddin et al (12–14) reported that colon cancer was reduced in rats and mice when phytate was provided in the drinking water.

These studies show that isolated soy phytochemicals fed in large amounts reduce experimental colon cancer. However, research to date does not show that feeding soyfoods inhibits chemically induced colon cancer. Two studies compared soy protein with beef protein in 1,2-dimethylhydrazine-induced colon carcinogenesis and found that feeding soy protein did not reduce the incidence of colon cancer (15, 16). The form of soy protein was not identified, but on the basis of the protein content, it appears that soy protein isolate was used in both studies. The amount of phytochemicals in soy protein isolate is much lower than that in soybeans and may explain why soy did not have a protective effect in these studies. The main objective of this study was to determine whether phytochemicals in soybeans and soy flour are present in sufficient amounts to reduce precancerous colon lesions [foci with aberrant crypts (FAC)] in rats injected with azoxymethane.

MATERIALS AND METHODS

Materials

Azoxymethane was from Ash-Stevens, Inc (Detroit). Soy concentrate, defatted soy flour, and full-fat soy flakes were from Central Soya (Ft Wayne, IN). Genistein was synthesized as described previously (17).

Animals

Male Fischer 344 rats were obtained at 3 wk of age from Harlan Sprague-Dawley Inc (Indianapolis). The rats were housed 3 per plastic cage with sawdust bedding. The animal room was maintained at 21–22 °C and 45% ± 10% relative humidity with a 12-h light-dark cycle. Diet and water were provided ad libitum. This study was approved by the Michigan State University Committee on Animal Use and Care.

1 From the Department of Food Science and Human Nutrition, Michigan State University, East Lansing.
2 Supported by the Michigan Soybean Promotion Committee and the Michigan State University Agricultural Experiment Station. Genistein was a generous gift from MG Nair, Michigan State University.
3 Address reprint requests to MR Bennink, 106 G M Trout Building, Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824-1224. E-mail: mbennink@pilot.msu.edu.
Diets

Diet compositions are listed in Table 1. The diets contained fat (14.8%), protein from either soy or casein, and 0.1% or 0.5% Ca. The ratio of protein, minerals, and vitamins to energy was kept similar to the AIN-93G diet (18). The soy-concentrate diet contained negligible amounts of isoflavones (Table 2) and was the control diet. This study comprised 2 consecutive experiments. A low-residue enteral beverage mix was fed in both experiments. Food consumption by rats eating the enteral beverage mix in the beginning of the first experiment was low and weight gain was reduced. To improve the food consumption and weight gain, the enteral beverage mix was supplemented with AIN 93 mineral and vitamin mixes (18) beginning at week 4 of experiment 1. Because the composition of the low-residue diet was altered during experiment 1, the enteral mix was supplemented with AIN 93 vitamin and mineral mixes for the full 12-wk dietary treatment period in experiment 2. Experiment 2 also included a traditional Turkish soup mix and the soy-concentrate diet (control) from experiment 1 for comparative purposes. The composition of the Turkish soup mix is listed in Table 1.

Experimental design

Preliminary experiments indicated that 13 rats per treatment would provide 90% power ($\alpha = 0.05$) to detect a 10% reduction in the number of FAC. Each dietary treatment group consisted of 15 rats except the starch group, which included 13 rats. All rats except negative controls were injected subcutaneously with azoxymethane (15 mg/kg body wt) at 21 and 28 d of age. Because the time of day when azoxymethane injections are made is important (4), the injections took place between 0800 and 1000. The negative control group received saline injections instead of azoxymethane. Rats were fed the control diet from days 21 to 35, were placed on dietary treatments at 35 d of age, and were killed 12 wk later by overexposure to carbon dioxide followed by exsanguination.

Pathology

Colons were removed, cut open longitudinally, rinsed with tap water to remove debris, placed in phosphate-buffered saline (PBS, pH 7.4) until fixed (10–15 min), pinned flat to cardboard, and fixed in 2% paraformaldehyde-PBS (pH 7.4) at 4°C for 1 h.

### Table 1

<table>
<thead>
<tr>
<th>Diet composition</th>
<th>Soy concentrate</th>
<th>Soy flakes</th>
<th>Soy flour</th>
<th>Calcium</th>
<th>Genistein</th>
<th>Starch</th>
<th>Turkish soup mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>% by wt</td>
<td>28.45</td>
<td>—</td>
<td>—</td>
<td>28.45</td>
<td>28.45</td>
<td>28.45</td>
<td>19.71</td>
</tr>
<tr>
<td>Full-fat soy flakes</td>
<td>—</td>
<td>46.07</td>
<td>35.79</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Turkish soup mix</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>51.24</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>10.63</td>
<td>2.85</td>
<td>3.36</td>
<td>9.64</td>
<td>10.63</td>
<td>40.63</td>
<td>2.31</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40.00</td>
<td>40.00</td>
<td>40.00</td>
<td>40.00</td>
<td>40.00</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>1.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

1 Low-fiber diet not listed. It was a powdered enteral formula and contained (from the manufacturer's label) 15.9% fat, 60% carbohydrate, 0% fiber, 15.9% protein (13.37% casein, 2.52% soy isolate), and 0.26% calcium. Twenty-two days after the start of dietary treatment in experiment 1 and for the duration of experiment 2, the low-fiber diet was changed to 3.5% AIN-76 mineral mix, 1% AIN-93G vitamin mix, and 95.5% enteral formula. The soy-concentrate diet was the control diet.

2 Additional ingredients not listed were 1.13% AIN-93G vitamin mix, 3.95% AIN-93G mineral mix, 0.23% methionine, 0.34% L-cystine, 0.23% choline chloride, and 0.003% butylated hydroxytoluene.

3 Proximate composition of tarhana soup is 12.2% protein, 4.4% fat, 56.4% carbohydrate, 1.9% dietary fiber, 16.6% ash, and 0.685% calcium.

### Table 2

<table>
<thead>
<tr>
<th>Isoflavone concentrations in rat diets</th>
<th>Soy concentrate, starch, and calcium</th>
<th>Soy flakes</th>
<th>Soy flour</th>
<th>Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg aglycone/kg diet</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

1 Expressed as mg of the aglycone, not the glycoside weight.

2 None detected.
Fixed colon were stained with methylene blue (0.1% in PBS) for 3 min as described previously (19), rinsed in PBS, and stored in 0.4% formaldehyde-PBS (pH 7.4) at 4°C. Azoxymethane-induced lesions appeared as darkly stained, enlarged, and slightly elevated crypts that could be distinguished from the lightly stained normal mucosa. The lesions could appear as single aberrant crypts or as foci containing >2 aberrant crypts. The number of FAC and the number of aberrant crypts per focus were visualized by stereomicroscopy. Foci with >10 aberrant crypts (too many aberrant crypts per focus to count accurately) were recorded as foci containing >10 aberrant crypts. Two persons counted the aberrant crypts and the data on FAC are reported as averages. The CV for the measurement of FAC was <5%.

**Isoflavone analysis**

Isoflavones were extracted from the soy flakes, soy flour, and soy concentrate by mixing a 1-g sample of each with 20 mL of a solution of 0.1 mol hydrochloric acid/L. Eighty milliliters of methanol was added and the mixture was sonicated for 20 min, left at room temperature for 2 h, and gravity filtered with Whatman no. 1 filter paper (Whatman, Clifton, NJ). The filtrate was centrifuged at 10 000 × g and the isoflavones in the supernate were separated and quantified by HPLC. The isoflavones were separated with a reversed-phase column (Microsorb-MV, 25 × 0.46-cm column packed with C18 silica particles of 5-μm diameter and 100-Å pore size; Rainin Instrument Co, Woburn, MA) by using a gradient mobile phase. Solvent A was 10% methanol, 89.9% water, and 0.1% acetic acid; solvent B was 99.9% methanol and 0.1% acetic acid. The amount of solvent B was increased linearly from 20% at 0 min to 30% at 2 min to 70% at 30 min. Isoflavones were detected at 260 nm and quantified by comparison with external standards.

**Statistical analyses**

Results for FAC were analyzed as a completely randomized design according to the general linear models procedure of SAS (version 6.11; SAS Institute Inc, Cary, NC). Rat weights were analyzed by repeated-measures analysis of variance according to the general linear models procedure of SAS. When significant treatment effects were detected (P ≤ 0.05), the Bonferroni method was used to compare treatment means.

**RESULTS**

The effects of dietary treatment on colon carcinogenesis are shown in Figure 1 as the total number of FAC, the number of foci with >4 aberrant crypts, and the total number of aberrant crypts per rat that had foci with 1–9 aberrant crypts. These results show that rats eating diets containing 0.5% Ca or 0.015% added genistein had the fewest colon lesions. Soy flour was somewhat more protective than full-fat soy flakes, but was less effective at inhibiting the formation of FAC than when 0.015% genistein (as the aglycone) was added to the soy-concentrate diet.

Because the low-fiber diet was modified midway through experiment 1, a second experiment was conducted. This time the enteral diet was supplemented with the AIN mineral and vitamin mixes for the full 12-wk feeding experiment and there was a significant reduction in FAC in rats eating the low-fiber diet compared with the soy-concentrate diet (Figure 2). Rats fed the low-fiber diet in experiment 2 had an identical number of FAC in their colons; rats fed the 0.76%-Ca, low-fiber diet in experiment 2 and the 0.5%-Ca diet in experiment 1 had similar incidences of FAC. Rats fed the low-fiber diet in experiment 1 had 122 FAC, whereas rats fed the low-fiber diet in experiment 2 had 71 FAC.
DISCUSSION

Some researchers assess anticancer activity as the ability to decrease the number of FAC without regard to focus size (number of aberrant crypts per focus) (4, 20, 21). Pretlow et al (11) found the best relation between tumor number and foci with \( \geq 4 \) aberrant crypts per focus. We analyzed our results as the total number of FAC and as the number of foci with \( \geq 2 \) aberrant crypts per focus, \( \geq 3 \) aberrant crypts per focus, and so forth and found consistent dietary treatment effects regardless of how the data were analyzed. Even though the tumor-promotion period in this study was only 12 wk, 9 small tumors were detected. These tumors were in rats fed the low-fiber (5 tumors), soy-concentrate (3 tumors), and soy flake (1 tumor) diets. Because of the short 12-wk promotion period and inadequate time for tumor development, the tumors were not classified histologically and we did not statistically analyze tumor numbers. However, there was a trend for animals fed the diets that produced the higher numbers of FAC to have the most tumors.

Extracting the oil- and fat-soluble components from soybeans enhanced anticancer activity (full-fat soy flakes compared with soy flour). This result is somewhat surprising because the fat content of the diets was equal and because extraction of oil from soybeans removes sterols and some saponins, which are postulated to help protect against colon cancer. Extracting soy flour with ethanol to produce soy concentrate removed anticancer activity, presumably by extracting phytochemicals such as isoflavones, Bowman-Birk protease inhibitor, and saponins. Adding the isoflavone genistein to the soy-concentrate diet caused a significant decrease in the number of FAC.

The soy flour and full-fat soy flake diets contained 0.049% genistein derivatives (primarily as glycosides), but were less effective in inhibiting the formation of FAC than when 0.015% genistein (as the aglycone) was added to the soy-concentrate diet. These data suggest that the aglycone genistein is more effective than genistein in the form of glycosides. Feeding pure genistein compared with genistin would unequivocally answer the question of whether genistein as glycosides has less anticancer activity than the aglycone form. However, genistin is expensive and difficult to synthesize. We do not have data on the Bowman-Birk protease inhibitor or saponin content of the diets.

The AIN-93G diet has a high calcium content (0.5%) to allow maximum bone and teeth mineralization in rapidly growing rodents. However, dietary calcium content can have strong anticancer activity in the colon. Others have shown that calcium additions to the already high-calcium content of AIN-93G diets decrease azoxymethane-induced FAC (4) and tumors (22) in rats. The evidence is inconclusive regarding the benefits of consuming a high amount of calcium to protect against colon cancer in humans. Some clinical trials show that supplemental calcium reduces the risk of colon cancer by decreasing cell proliferation in the upper 40% of colon crypts in people at intermediate or high risk of colon cancer (23–27). However, 3 other studies did not find a reduction in colonic mucosa proliferation rates or a downward shift in the proliferative compartment (28–30). Even though the human data are less supportive of the idea that high-calcium diets have a protective effect against colon cancer, we intentionally kept the dietary calcium content low (0.1%) so that the anticancer activity of other dietary ingredients would not be masked by the relatively high dietary calcium amounts used in other animal studies.

The source of digestible carbohydrate in the diet is regarded as unimportant by most cancer researchers. However, Caderni et al (31) showed that feeding a high-sucrose diet stimulated colon epithelial-cell proliferation, which could enhance colon carcinogenesis. The only difference between the soy-concentrate diet and the starch diet in the present study was that the soy-concentrate diet contained 40% sucrose and 10.63% cornstarch, whereas the starch diet contained 40.63% cornstarch and 10% sucrose. This study shows that a diet high in sucrose (soy-concentrate diet) promotes colon carcinogenesis compared with a high-starch diet (Figure 1).

Because high proliferation rates in colonic mucosa are associated with increased risk of colon cancer (32, 33), we wanted to
determine whether a diet that produces low proliferation rates in the colonic mucosa would inhibit colon carcinogenesis. Low proliferation rates are produced when a low-residue diet is fed (34–36). Feeding a low-fiber, powdered enteral formula did not inhibit colon carcinogenesis as we expected (Figure 1, low-fiber compared with soy-concentrate diet). During the first 3 wk, rats eating the low-fiber diet grew slowly because they ate less food. The enteral formula meets the nutrient requirements for men and women but not for young rats. Therefore, during the last 9 wk of dietary treatment, the low-fiber diet was fortified with AIN mineral and vitamin mixes, which stimulated food intake and more normal growth. The calcium content was 0.26% during the first 3 wk of dietary treatment and 0.76% during the last 9 wk. Because there was no difference in the number of FAC between rats fed the soy-concentrate diet and those fed the low-fiber diet, these results suggest that a low-fiber diet does not protect against the early stages of colon carcinogenesis. If the inhibitory effect of calcium on FAC is linear, then the low-fiber diet should have produced fewer FAC. It could be argued that the low-fiber diet facilitated the formation of FAC.

As shown in Table 3, there were differences in growth in this experiment. Even though the growth differences were significant, the differences in colon carcinogenesis cannot be attributed to growth rates. For example, rats fed the 0.5%-Ca diet gained the most weight, but had the fewest FAC. Rats fed the starch and low-fiber diets had 2 of the lower overall weight gains, but had 2 of the higher numbers of FAC.

One major difference between the 2 experiments was that during experiment 1 the low-fiber diet contained 0.26% Ca for the first 3 wk after the initiation of carcinogenesis and 0.76% Ca for the next 9 wk, whereas the low-fiber diet contained 0.76% Ca for all 12 wk in experiment 2. These results emphasize the importance of dietary calcium concentrations immediately after (and perhaps during) the initiation of carcinogenesis. Because there was a large difference in calcium content between the low-fiber diet and the soy-concentrate diet in experiment 2, we attribute the decrease in incidence of FAC in rats fed the low-fiber diet not to low fiber, but to high calcium. This research did not specifically answer the question of whether a low proliferation rate in colonic mucosa would decrease the formation of FAC. To answer this question, a 0.1%-Ca diet with and without fiber should to be fed and the number of FAC and the proliferation rate determined. Growth was more similar among treatment groups in experiment 2 (Table 4) than in experiment 1, but rats eating the mineral- and vitamin-supplemented, low-fiber diet still gained 9% less weight than did rats consuming the starch diet.

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial weight</th>
<th>Final weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy concentrate (n = 15)</td>
<td>76.8 ± 4.8</td>
<td>317.2 ± 6.1a</td>
</tr>
<tr>
<td>Saline (n = 14)</td>
<td>83.6 ± 1.2</td>
<td>324.3 ± 9.6e</td>
</tr>
<tr>
<td>Genistein (n = 15)</td>
<td>82.0 ± 2.3</td>
<td>274.0 ± 6.9a</td>
</tr>
<tr>
<td>Soy flakes (n = 15)</td>
<td>80.4 ± 3.6</td>
<td>296.6 ± 6.1b</td>
</tr>
<tr>
<td>Soy flour (n = 15)</td>
<td>80.2 ± 2.4</td>
<td>283.4 ± 3.8a</td>
</tr>
<tr>
<td>Calcium (n = 15)</td>
<td>79.4 ± 2.7</td>
<td>320.4 ± 1.8a</td>
</tr>
<tr>
<td>Starch (n = 13)</td>
<td>82.9 ± 2.3</td>
<td>263.8 ± 7.7a</td>
</tr>
<tr>
<td>Low fiber (n = 15)</td>
<td>81.4 ± 2.2</td>
<td>271.8 ± 5.6a</td>
</tr>
</tbody>
</table>

*SEM. Means with different superscript letters are significantly different, P ≤ 0.01.*

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial weight</th>
<th>Final weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>93.3 ± 4.7</td>
<td>379.2 ± 6.3</td>
</tr>
<tr>
<td>Soup mix (n = 10)</td>
<td>93.1 ± 2.0</td>
<td>372.2 ± 3.8</td>
</tr>
<tr>
<td>Low fiber (n = 11)</td>
<td>93.9 ± 2.6</td>
<td>353.2 ± 7.4</td>
</tr>
</tbody>
</table>

*SEM. There were no significant differences in initial or final weights.*

Tarhana is a traditional soup in the Turkish cuisine and is purported to protect against cancer. The soup reduces mutagenesis in the Ames assay (37), but the soup mix did not reduce the promotion stage of colon cancer (number of FAC) in this experiment (Figure 2). Because the diet with Tarhana contained 0.45% Ca (0.35% from Tarhana and 0.1% from calcium carbonate), one would expect, based on dietary calcium content, 70–75 FAC rather than 109. The higher number of FAC suggests that some constituent in Tarhana enhanced FAC formation or, alternately, attenuated the FAC-lowering effect of a 0.45%-Ca diet.

This research substantiated the concepts that calcium content and type of carbohydrate are important variables when studying early stages of colon carcinogenesis. We formulated the basal diet with low calcium (0.1%) and high sucrose (40%) to stimulate FAC formation. With the diet of 0.1% Ca and 40% sucrose, soybeans and soy flour have sufficient phytochemicals to reduce azoxymethane-induced colon carcinogenesis. When Pereira et al (4) fed 0.015% genistein and 0.5% Ca, the number of FAC decreased by 34%. When we fed 0.015% genistein and 0.1% Ca, there was a 43% reduction in the number of FAC, which suggests that it is harder to detect anticancer activity of phytochemicals when diets contain 0.5% Ca. Whether a diet with soybeans or soy flour would reduce FAC formation when the diet contains 0.5% Ca remains to be determined.

### REFERENCES

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