Effect of dietary estrogens on cellular markers of colon cancer in mice

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Effect of dietary estrogens on cellular markers of colon cancer in mice

by

Padma Balaji

A thesis submitted to the graduate faculty
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This is to certify that the master's thesis of

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has met the thesis requirements of Iowa State University

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# TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## ACKNOWLEDGEMENTS

## ABSTRACT

## CHAPTER

### 1. LITERATURE REVIEW

- Overview of colorectal cancer 1
- Environment and diet in the etiology of cancer 2
- Soy protein 4
- Role of estrogen in colorectal cancer 9
- Estrogen receptors 11
- Location of estrogen receptors in colon 12
- Estrogen synthesis in colonic epithelium 13
- Types of cancer 14
- Animal models in colorectal cancer 18
- Aberrant crypt foci 19
- Colorectal cancer and nonsteroidal anti-inflammatory drugs 21
- Role of prostaglandins and COX in tumorigenesis 23
- Modulation of COX-2 by estrogen 25
- Hypothesis 25
2. EXPERIMENTAL DESIGN

Animal care 27
Preparation of azoxymethane 27
Tissue collection 30
Aberrant crypt foci staining procedure 31
Protein quantification and Western immunoblotting 31
Western immunoblot procedure 32

3. MANUSCRIPT

Abstract 39
Key words 40
Introduction 41
Materials and Methods 44
Results 48
Discussion 49
Literature cited for chapter 3 56

4. DISCUSSION 69
LITERATURE CITED FOR CHAPTERS 1, 2, AND 4 80
APPENDIX 94
LIST OF TABLES

1.1. Effects of soy foods/isoflavones on ACF and colon tumors 8
2.1. Composition of the experimental diets (AIN93 based) 29
2.2. Reagents used for casting the gel for western immunoblot 33
2.3. Antibodies and reagents used for COX-2 Western immunoblot 36
3.1. Final body weight, fat and organ weights in mice fed experimental diets 62
5.1. Inter and intra-gel coefficient variances (CV) of western blotting 95
LIST OF FIGURES

1.1. Structure of soy isoflavones (phytoestrogens) and the endogenous estrogens (estradiol and estrone) 5
1.2. Genetic model of colorectal carcinogenesis 17
1.3. Arachidonic acid cascade and generation of prostaglandins by COX 22
2.1. Experimental design 28
3.1. A representative immunoblot 63
3.2. Body weight gain of mice fed experimental diets 64
3.3. Body weight of mice at 14 and 15 weeks of age 65
3.4. Abdominal fat expressed as percent of body weight 66
3.5. Mean number of aberrant crypts in mice fed experimental diets 67
3.5. Quantification of COX-2 protein expression in mice fed experimental diets 68
4.1. Aberrant crypt foci, Mucin depleted foci and β-catenin accumulated crypts 77
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Colorectal cancer (CRC) is the third leading cause of cancer deaths with an estimated 57,000 deaths per year in the United States. Evidence for a role of estrogens in colon cancer is accumulating, although the mechanism through which this is mediated is not clear. Epidemiological studies suggest that post-menopausal hormone replacement therapy (HRT) reduces CRC incidence. There is also evidence to suggest populations that consume soy and soy foods have reduced colon cancer risk. Soy contains phytoestrogens, such as the isoflavone genistein, that are similar to endogenous estrogens. Several studies have suggested isoflavones may have potential anticarcinogenic effects. In previous work, our group found a reduced colon tumor incidence in carcinogen-treated mice fed diets containing estrone (E1). Moreover, several epidemiological studies indicate a 40-50% reduction in the risk of developing colorectal cancer with chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin. It is suggested that NSAIDs provide anticancer activity by inhibiting both isoforms of the cyclooxygenase (COX) enzymes COX-1 and COX-2. Increased COX-2 activity is an early event in CRC. With emerging evidence indicating suppression of COX by estrogens we hypothesized that dietary genistein or estrone (E1) would reduce aberrant crypt foci (ACF), purported preneoplastic markers of colon carcinogenesis, and cyclooxygenase-2 (COX-2) protein levels. Ovariectomized female mice were fed diets containing casein (Casein), soy protein without isoflavones (Soy-IF), soy protein with genistein (Soy+Gen) or soy protein with estrone (Soy+E1) from 3 weeks of age. Starting at 4 weeks of age, all animals were administered weekly injections
of azoxymethane (AOM) 10 mg/kg of body weight for 6 weeks, and then terminated 6 weeks after the last dose of AOM. Colon sections stained with methylene blue were visualized under a light microscope for ACF, and COX-2 protein levels were analyzed by Western Immunoblot. A significant effect of diet on COX-2 protein levels but not ACF was observed. COX-2 protein levels were lower in mice fed Soy+E1 and Soy+Gen compared with mice fed Casein or Soy-IF. Thus, we conclude that soy protein with genistein and soy protein with estrone downregulated COX-2 levels in mouse colon suggesting a potential role in reducing colon cancer risk.
CHAPTER 1
LITERATURE REVIEW

Overview of colorectal cancer

Cancer remains one of the most common malignancies in Western countries, including the United States and other developed countries (1). Cancer accounts for approximately 23% of total deaths, ranking second only to heart disease in the United States (1). Colon cancer is the third most commonly diagnosed cancer among men and women next only to lung and prostate/breast cancer (1, 2). The incidence of colorectal cancer (CRC) is about 1 million (9.4%) world wide with no significant differences between men and women (1, 2). In the United States, the estimated deaths due to CRC were 56,290 with 28,540 and 27,750 among men and women, respectively, in 2005 (2).

Although, there is little overall gender difference in the risk of colon-cancer, the age-specific colon cancer gender ratios vary considerably. Males tend to have a higher risk than females of CRC until 35 years of age after which the mortality and incidence rates among women increases remarkably resulting in little overall gender difference in colon cancer risk (3). The observation by McMichael and Potter (3) in age-specific colon cancer ratios between sexes especially below 35 years and between 35-54 years led them to suggest a role for female sex hormones in the etiology of colon cancer. Further, the transient decline in colon cancer mortality rates in women between ages 35-44 during the early 1970’s against an overall increasing trend in other years made them to suggest a correlation to the use of oral contraceptives by women a decade earlier (3).
There is at least 25-fold variation in the occurrence of CRC worldwide with the highest incidence being in North America, followed by Australia and other developed countries (1-3). The vast geographical variation in the incidence could be attributed to the differences in environmental exposures including diet (1-3).

Epidemiological studies pertaining to the migrant Asian population to Western countries and their increase in incidence rates of CRC even in the first generation indicate the role of environment, particularly that of diet in this disease (1, 2, 4). Furthermore, a large discrepancy in the incidence and mortality rates of colon cancer between ethnic groups in the United States has also been reported. African Americans have the highest incidence rates, whereas Hispanics, Asians/Pacific Islanders, and American Indians/Alaskan Natives have the lowest, with the incidence in Whites falling in the middle (2, 5). The contribution of diet to these differences has not been well defined.

**Environment and diet in the etiology of cancer**

Many environmental factors contribute to CRC incidence rates. Some of these environmental factors include carcinogens (whether identified or not) such as man-made chemicals or naturally occurring carcinogens, viral infections, nutritional deficiencies or excesses, reproductive activities, physical activity, radiation exposure that can be controlled wholly or partly by lifestyle changes. An important role of nurture/environment in the etiology of colon cancer was first proposed by Doll and Peto (6). Some of the major avoidable causes of cancer include tobacco, alcohol, diet, food additives, reproductive and sexual behavior, occupation, pollution, medicines and medicinal procedures, infection, geophysical factors, and industrial
products (7). For many years there has been strong but indirect evidence that many of the cancers could be avoided by lifestyle changes (6). After an extensive review of literature the American Institute of Cancer Research and World Cancer Research Fund concluded that cancer is principally caused by environmental factors which include tobacco, diet and factors related to diet, including body mass index and physical activity, and occupational exposures (7). Consumption patterns of meat, fat (specifically animal fat), and fiber have been strongly correlated with the incidence of colon cancer (7-10).

Dietary factors contribute to perhaps half of the leading causes of death of Americans including cardiovascular disease, cancer and diabetes (1). There are inconsistencies linking diet with different types of cancer despite the fact that numerous studies suggest a significant role of diet (11-13). Plant-derived foods, often referred to as phytochemicals, may provide protection at several stages of the multifactorial and multistage complex nature of colon cancer (14). Examples of components that have been recognized to have a protective effect against cancer in model systems include essential nutrients such as calcium, zinc, folate, vitamin C, D, and E, non-essential bioactive food components such as carotenoids, n-3 fatty acids, conjugated linoleic acid and isoflavones (8, 15). Modifying one or more of the cancer processes such as carcinogen metabolism, hormonal balance, cell signaling, cell-cycle control, apoptosis or angiogenesis may be the mode of action of these compounds (14).

Legumes and beans are among the significant sources of protein in the traditional diets of many regions of the world (16). It is hard to imagine Asian cuisines without soy beans, lentils, black beans, chick peas, and pinto beans while
Western cuisines are based on animal protein and the associated animal fats. In addition to being a good source of dietary fiber (soluble and insoluble), these foods have been associated with decreased CRC risk (12, 17). Plant-derived foods are also good source of phytochemicals.

With the general consensus that diets high in red meat and the associated saturated fat are strongly correlated with increased colon cancer risk, substituting soy protein for red meat may decrease CRC (4, 16, 18). Soy is a common dietary component in Asian diets. Soy protein is considered a high quality protein for humans and is equivalent to milk protein based on the FDA protein digestibility corrected amino acid score method (19). With the versatility of soy to be processed in various ways along with the recent approval by the FDA for a health claim on soy based foods to reduce cardiovascular disease risk; the consumption of soy has increased substantially in the United States (20).

**Soy protein**

Soy foods and soybean components have received considerable attention for their potential to reduce cancer risk. Numerous studies have focused on soy intake and risk of breast cancer (21-23). The relationship between soy intake and other cancers, including CRC also gained attention due to the several anti-carcinogenic bioactive compounds found in soy (16). Some of which include isoflavones, phytosterols, fatty acids, fiber, and protease inhibitor (trypsin inhibitor) (18). These biologically active components may contribute individually or synergistically to the health benefits associated with soy.
Figure 1.1. Structure of isoflavones (phytoestrogens) and the endogenous estrogens (estradiol and estrone)
Isoflavones (IF) such as genistein and daidzein, are found in high concentration in soy. Due to their structural similarity to estradiol (Figure 1.1) and/or their biological activity these compounds are often referred to as phytoestrogens (16, 24). These plant-derived compounds have inherent estrogenic activity (25) or may be converted to estrogenic compounds by bacteria in the intestines (26, 27). Most often the mucosal or the bacterial β-glucosidases cleave the glucose moiety of the glucosides which are then absorbed and glucoronidated. Further metabolism of daidzin results in equol, an active metabolite that has more potent estrogenic activity than the parent compound. Genistein is metabolized to p-ethylphenol and 4-hydroxyphenyl-2-propionic acid (27). Isoflavones also possess other potentially important biological attributes in addition to their ability to bind to the estrogen receptor. Some of the cancer-related properties associated with genistein include its anti-oxidant activity, anti-promotional effect (28), inhibition of tyrosine kinase (29), aromatase (30) DNA topoisomerase (31), inhibition of cell cycle progression and growth (32), and inhibition of angiogenesis in endothelial cells (28, 29, 32, 33).

Results from studies on the effect of soy and soy isoflavones in CRC using animal models are inconsistent. Table 1.1 summarizes the conflicting results obtained with soy or the isoflavones in Aberrant crypt foci (ACF) formation and colon tumorigenesis. Thiagarajan et al (34) reported 50% reduction in ACF in male rats fed soy concentrate with genistein (167 mg/kg of diet) that were administered 2 AOM injections at 15 mg/kg of body weight. However, this report was contradicted by Gee et al (35) who used dimethylhydrazine (DMH) to induce ACF in male Wistar rats. They reported a 3-fold increase in ACF in rats fed casein-based genistein diet.
There are several differences between these studies that may provide explanation for these opposing results. Some of which include the use of casein based pure genistein diet by Gee et al (35) against the soy concentrate based genistein diet by Thiagarajan et al (34), use of different carcinogens to induce ACF such as DMH vs. AOM, the duration of the study (i.e., 6 weeks after carcinogen treatment vs 12 week after AOM) and dietary treatment before, during and after carcinogen treatment are some of the possible confounders. However, most of the long term studies on tumor incidence have indicated protective or no effect of soy or soy isoflavones. Previously we observed that oral administration of estrone with soy protein decreased tumor incidence, but not burden (tumor weight) or multiplicity in wild type and ERαKO mice (36). Though the effect of genistein was not statistically significant, this study showed that genistein reduced tumorigenesis in wild type as well as ERαKO mice. Similar results were reported in ovariectomized APC\textsuperscript{min} mice (31). Administration of estradiol/genistein reduced the tumor incidence in these mice. Also, a 20% reduction in tumor incidence in male rats fed soy protein isolate with 276 mg/kg of body weight compared to casein control was reported by Hakkak et al (37). However, Rao et al reported enhancement of noninvasive adenocarcinoma in rats fed casein based diets with genistein compared to controls. They also observed no effect of genistein on colon adenocarcinoma incidence or on the multiplicity of invasive adenocarcinoma (38). The contradictory result could be due to the use of casein based diets over soy protein based diets used by us.
### Table 1.1 Effects of soy foods/isoflavones on ACF and colon tumors

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal</th>
<th>Test compounds/diets</th>
<th>Concentration</th>
<th>Carcinogen</th>
<th>End point</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiagarajan et al. 1998</td>
<td>Male Fischer rats</td>
<td>Soy concentrate (low IF) Soy flakes (full fat) Soy flour (defatted) Soy concentrate with Gen</td>
<td>167 mg Gen/kg of diet</td>
<td>AOM</td>
<td>ACF</td>
<td>Soy concentrate with Gen and soy flour had reduced ACF compared to soy concentrate with low IF</td>
</tr>
<tr>
<td>Hakkak et al. 2001</td>
<td>Male Sprague-Dawley rats</td>
<td>Casein vs Soy protein isolate (SPI)</td>
<td>SPI + 276 mg of Gen/kg of diet</td>
<td>AOM</td>
<td>Tumor incidence</td>
<td>Tumor incidence was 31 vs. 10% in rats fed casein and SPI. Invasive adenocarcinoma was also lower in SPI group</td>
</tr>
<tr>
<td>Guo et al. 2004</td>
<td>Wild type C57BL/J6 &amp; ERαKO mice</td>
<td>Casein or soy protein with Gen or estrone</td>
<td>250 mg Gen/kg of diet 0.625 mg estrone</td>
<td>AOM</td>
<td>Tumor incidence</td>
<td>Soy+Estrone had lower tumor incidence</td>
</tr>
<tr>
<td>Sorensen et al. 1998</td>
<td>APCmin mice</td>
<td>Soy based low IF diet vs. soy based IF rich</td>
<td>Low-IF (11.5 mg Gen/kg of diet) vs. IF-rich (280.6 mg Gen/kg of diet)</td>
<td>-</td>
<td>Tumor incidence</td>
<td>No difference in colon tumors between low-IF and IF-rich diet</td>
</tr>
<tr>
<td>Gee et al. 2000</td>
<td>Male Wistar Rats</td>
<td>Casein based or soy protein based</td>
<td>250 mg Gen/kg of diet</td>
<td>DMH</td>
<td>ACF</td>
<td>Gen diet and soy protein isolate with Gen increased ACF by 3-fold and 2-fold respectively</td>
</tr>
<tr>
<td>Rao et al. 1997</td>
<td>Male F344 rats</td>
<td>Casein diet with or without Gen</td>
<td>250 mg Gen/kg of diet</td>
<td>AOM</td>
<td>Tumor incidence</td>
<td>Gen increased noninvasive adenocarcinoma multiplicity</td>
</tr>
</tbody>
</table>

Abbreviations used in Table. 1: IF – Isoflavone; SPI – Soy Protein Isolate; Gen – Genistein; ACF- Aberrant Crypt Foci DMH – 1,2-dimethylhydrazine
Human epidemiological studies also provide contradictory evidence as to the role of soy in CRC risk. A 12-month intervention trial reported a decrease in colon epithelial cell proliferation with soy supplementation compared to casein group (39). A meta-analyses of epidemiological studies associating soy intake with CRC risk indicated a reduction in mean overall risk of colon cancer (40) (16, 18). In a study conducted of the US population with 50% Caucasians as subjects, an inverse association between high intake of carotenoid vegetables, garlic, or tofu (fermented soybean product) with polyps was reported. However, a 12-month randomized intervention study on men and women recently diagnosed with adenomatous polyposis reported no decrease in colon epithelial cell proliferation when supplemented with soy protein powder containing different amounts of isoflavones (41). The study participants were supplemented with 58 g of soy protein containing 83 mg of isoflavones/day (45.6 mg of genistein, 31.7 mg of daidzein and 5.5 mg of glycitenin in aglycone units) or ethanol extracted soy protein powder containing 3 mg of total isoflavones. They reported no decrease in colorectal epithelial cell proliferation with soy protein supplementation but an increase in cell proliferation measures in the sigmoid colon. Moreover, Nagata et al (42) showed a significant positive correlation between CRC mortality rates and soy food intake after correcting for age, smoking, alcohol and animal fat intake in the Japanese population (42). With these conflicting results on the effect of soy on CRC, some showing a protective effect while others a negative effect more studies on the role of soy in CRC are needed.
Role of estrogen in CRC

The importance of estrogen in homeostatic regulation of many cellular and biochemical events is well established with the various pathophysiologic changes that occur with estrogen deficiency (24). Fraumeni et al (43) reported a high incidence of CRC among nuns along with high incidence of other hormone-associated cancers including breast, uterus and ovary. Although, there is little overall gender difference in the risk of colon cancer, there is a variation in the age-specific colon cancer gender ratio especially in the age groups of 35-54 and above 54 among men and women. Men had higher risk below 35 years of age while the risk was higher in females between 35-54 years of age. After 54 years of age the male risk again becomes higher (3). McMichael and Potter et al (3) observed a transient decline in female colon cancer risk ratio among 35-54 and 55-74 year olds in the 1960’s and early 1970’s. Peak age-specific fertility and exposure to high-dose of oral contraceptives during 1960’s were suggested to be possible reason for this transient decline in women indicating the direct influence of endogenous/exogenous estrogen in colon cancer. Several case-controlled studies and cohort-studies examining the associations between reproductive events, menstrual factors, exogenous hormones and CRC stratified by age at diagnosis, tumor site, family history and other potential risk factors also indicated the role of female sex hormones on colon cancer (44, 45). In a meta-analysis of 18 epidemiologic studies on postmenopausal HRT and CRC Grodstein et al (46) found a 20% reduction in the risk of colon cancer and a 19% decrease in the risk of rectal cancer for postmenopausal women on hormone therapy compared to women who never used hormones (46). The role of estrogen in decreasing CRC was further reiterated with
the outcome of the Women's Health Initiative trial. Two parallel randomized controlled clinical trials, one with conjugated equine estrogen (CEE) alone and the other with CEE in combination with progestin (medroxyprogesterone acetate (MPA)) were undertaken to determine the effect of HRT in reducing cardiovascular risks (47). The arm with CEE with progestin was halted in July, 2002 as the health risks of this treatment exceeded its benefits (47, 48). The primary adverse outcome included invasive breast cancer along with coronary heart disease. However, the incidences of CRC in women using CEE + progestin were lower than nonusers. Thus, suggesting a protective effect of HRT in CRC (47, 49). However, women in the HRT group who developed CRC had a greater number of positive lymph nodes suggesting a more advanced invasive type of CRC with HRT. The reason for this is not known, and demonstrates the need for more study of the role of estrogen in CRC.

**Estrogen receptors**

Estrogen mediates temporal and tissue-specific actions via estrogen receptors (ER) α and β. The ER receptors are members of the nuclear receptor superfamily of transcription factors that include thyroid receptor, vitamin D receptor, retinoic acid receptor, and other steroid receptors such as the glucocorticoid, mineralocorticoid, androgen and progesterone receptor. ERα was the first estrogen receptor that was isolated from MCF-7 human breast cancer cells followed by the ERβ from rat prostate using degenerate PCR primers (50). ERα and ERβ are localized in the breast, brain, cardiovascular system, urogenital tract and bone (50-
ERα is the main subtype in the liver, while ERβ is the main ER in the colon (50).

Ligands such as estrogen (estradiol) binding modulate the transcription of target genes. Activity of the transcription factors is regulated by a variety of factors, including phosphorylation, coregulators, and the effector pathway in addition to ligand binding. The end result of the target genes is to modulate physiological processes, such as reproductive organ development and function, bone density as well as the growth and development of breast and endometrial cancer. These biological effects are mediated via communication between many proteins and signaling pathways (50). The susceptibility of a tissue to estrogen-induced carcinogenesis might be determined by the ratio of ERα:ERβ (53). Phytoestrogens such as genistein have a greater affinity for ERβ compared to ERα by 20-fold, but activates transcription through both receptors (50).

**Location of Estrogen receptors in colon**

ERα and ERβ may also localize to distinct cellular subtypes within each tissue (50). Waliszewski et al (54) reported the location of ER to be in the stromal cells rather than in the colon epithelial cells and in situ hybridization studies also indicated the presence of ER in stromal cells above the muscularis mucosa (55). However, Xie et al (56) reported the presence of ER throughout the colon mucosa by immunohistochemistry. Controversy remains over the location of these receptors in the stroma rather than in colon epithelial cells and their role in colon cancer. But, a consistent finding is that normal human colon expressed more ERβ than ERα mRNA and expression of ERβ is low or selectively lost in human colon tumor cells. (53, 57)
Estrogen synthesis in colonic epithelium

Estrogen can be synthesized locally with tissue specificity. The precursor for estrogen is cholesterol which is converted to pregnenolone and 17-hydroxypregnenolone. Dehydroepiandrosterone (DHEA) derived from 17-hydroxypregnenolone is converted to androstenedione or androstenediol. Conversion of androstenedione into testosterone is governed by the enzyme 17β-hydroxysteroid dehydrogenase (17βHSD). The enzyme complex aromatase, cytochrome P450 arom transforms these androgens into estrogens (58). Conversion of estradiol (E2) to estrone (E1) or the reverse facilitated by 17β-HSD depends on its oxidative or reductive activity (58-60). There are at least nine different isoforms of 17βHSD whose activity differs with in tissues accounting for the differences in the varying concentrations of these steroids in different cells (61). It is speculated that the peripheral synthesis of estrogens may play a role in the non-receptor mediated effect of estrogen in the pathophysiology of cell growth (59, 61). The role of estrone and estradiol in colon cancer cells is not well defined. Oduwole et al (61) reported that 17β-HSD type 2 (estradiol to estrone) was the dominant form in human colon and was downregulated in colorectal tumors with no expression of the 17β-HSD type 1 enzyme (estrone to estradiol). However, they also reported that females with CRC that had higher 17β-HSD 2 mRNA expression had a poor survival rate thus suggesting a low expression of the 17β-HSD type 2 as an independent marker of good prognosis in females with distal colorectal cancer (62). This was contradicted by English et al (59) who reported conversion of estradiol to estrone as protective against colon cancer and loss of estrogen inactivation (i.e., conversion of active estradiol to estrone and 17-HSD type 2 enzyme) results in colon tumors. These
results suggest a reciprocal role of active and inactive estrogens (estradiol and estrone) in the etiology of colon cancer

Both endogenous and exogenous sex hormones have been associated with cancer etiology. High levels of biologically active androgens or estrogens are associated with increased risks of prostate cancer in men and ovarian and breast cancer in women (63-65). Despite the positive association of endogenous and exogenous estrogens with these cancers, plant estrogens are inversely associated with cancer (66, 67). The association of phytoestrogens with decreased cancer incidence implies a lack of estrogenicity or estrogen antagonism or differential cellular mechanisms. The growth effect of estradiol (1 and 10nM) to genistein and tamoxifen was compared on cellular proliferation in human colon cancer cell lines (HT-29, Colo320, Lovo and SW480 cells) and MCF-7 cells (67). Even at higher concentrations (100 and 500nM) the colon cancer cells were not responsive to estradiol while growth was stimulated in MCF-7 cells. However, at 10μM concentrations of genistein at which growth is stimulated in ERα positive human breast cancer MCF-7 cells there was a slight inhibition in growth of HT-29, Colo320 and Lovo colon cancer cells. Also, they reported the presence of ERβ in these cells with no ERα. Different biological effects of the phytoestrogens and endogenous estrogens as well as the differences in ratio of ERα and ERβ can be accounted for the differential effects of these compounds tissue specifically.

Types of cancer

Inherited or acquired mutations in the adenomatous polyposis coli (APC) gene or mismatch repair genes (MMR) have been associated with colon carcinogenesis (68).
Hereditary forms of colon cancer include familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (4, 69). The progression from adenomatous polyps characterized by benign neoplasms arising from glandular-type cells occurs via a multistage carcinogenesis pathway (68, 70). FAP is an autosomal, dominant inherited syndrome that affects about 1 in 7000 individuals and is caused by an inherited mutation in the adenomatous polyposis coli (APC) gene (70). The mutations can arise at different sites within APC, that invariably lead to stop codons and thus a truncated APC protein (4, 68). Hypermethylation of APC can also cause truncation of the APC protein (71). Genetic polymorphisms with a nontruncating APC have also been described (4). Although, FAP patients have more adenomas they do not necessarily turn into carcinomas. The common and early somatic event in polyps and cancer include mutations at the APC locus which could be due to a first germline hit or a somatic event. Other mutations in the transforming growth factor β receptor, k-ras oncogene and p53 tumor suppressor genes have also been described (72, 73). The predisposition to multiple primary cancers such as endometrial, ovarian, gastric, and urinary tract without intestinal polyposis along with microsatellite instability (MIN) is the hallmark of HNPCC. The silencing of APC or MMR genes leads to hereditary forms of cancer. HNPCC, another inherited autosomal dominant syndrome accounts for about 2% of CRC cases where the mismatch repair (MMR) genes are mutated but patients do not necessarily have polyps or adenomas.

Sporadic colorectal cancer: CRC arising from nongenetic origins are called sporadic cancer. The lifetime risk of CRC is around 5% with about 50% of the Western population developing adenoma by the age of 70 (2). The etiology is a
cumulative mutational activation of oncogenes coupled with inactivation of tumor-suppressor genes, and genetic alteration in a preferred sequence leading to malignant tumors (68, 74). In most cases the initiation of sporadic cancers occurs due to environmental factors, therefore this type of cancer has the greatest potential for dietary prevention.

CRC results from the progressive accumulation of genetic and epigenetic alterations that transform the normal colonic epithelium to colon adenocarcinoma. The basic tenets concerning the pathogenesis of sporadic CRC include changes at the molecular and morphologic levels leading to progression, the clonal growth advantage of the transformed cells arising from genetic and epigenetic alterations, and loss of genomic instability (73). Mutations in the APC gene that leads to truncation of the APC protein is often the initial event in tumorigenesis which results due to loss of heterozygosity (loss of both alleles of APC). Genetic and epigenetic changes of the normal epithelial cells trigger a sequence of events leading to adenoma-carcinoma formation. Figure 1.2. depicts the sequence of events leading to carcinoma and eventually to metastasis. Genetic mutations such as k-ras, SMAD4 and other alterations in addition to APC and β-catenin mutations that result in dysregulation of the wnt signaling pathway are also involved in tumorigenesis (73). Cyclooxygenase-2 (COX) protein levels as well as activity are also elevated in the early stages of colorectal carcinogenesis.
Figure 1.2. Genetic model of colorectal carcinogenesis

First hit somatic mutation (Sporadic CRC) APC

Normal epithelium

Aberrant crypt foci

Second hit somatic mutation APC

β-catenin

Axin

Cyclooxygenase-2 Genomic instability

Early adenoma

K-ras (mutation)

Intermediate adenoma

Smad4 (mutation)

Late adenoma

p53 (mutation)

Carcinoma

Other alterations

Metastasis

Animal models in colorectal cancer

The present understanding of the genetic, molecular and histopathological events that are perceived to occur during colon carcinogenesis has been possible due to the use of animal models. These models are also extremely valuable in developing strategies for chemoprevention due to their similarity in mimicking the neoplastic processes that occur in humans. The models that have been widely used in deducing the events and mechanisms associated with colon carcinogenesis include chemical carcinogen-induced animal models such as azoxymethane (AOM) or dimethylhydrazine (DMH), transgenic models, and immuno-incompetent mice xenografted with cancer cells (75). The multiple intestinal neoplasia (Min) mice, with a mutation in the APC gene are also widely used. Variations in this model include the APC<sup>Δ716</sup> knock out mice introduced by Oshima et al (76). Although, this model is similar to FAP in humans, multiple neoplasia develop in the small intestine of these mice in contrast to adenomas detected exclusively in the colon and duodenum of humans (77). Although, the APC<sup>min</sup> mice have 40-60% APC mutations, adenocarcinomas or p53 inactivation are rarely observed. The occurrence of the polyps in the small intestine rather than the colon is a major drawback in these mutant models in addition to the cost in generating these mice (78).

Aromatic amines, derivates and analogues of cycacin such as methylazoxymethanol (MAM), 1,2,-dimethylhydrazine (DMH) and Azoxymethane (AOM), as well as direct-acting carcinogens such as methylnitrosourea (MNU), and heterocyclic amines are some of the chemicals used to induce colon tumors. AOM is widely used by many investigators to induce tumors in mice. CYP2E1 belonging to the CYP-450 enzyme family metabolizes AOM to methyazoxyymethanol (MAM) by
hydroxylation of methyl groups. Oxidation of MAM to methylazoxyformaldehyde yields methylidiazoniumion which forms DNA adducts (79). The morphology, histochemical properties and the biological behavior of these AOM-induced colon carcinomas are similar to human colon carcinomas. Other similarities with AOM-induced colon carcinomas to humans which make it a good chemically induced colon carcinogenesis model include predominant colon tumors in the distal colon, oncogenic mutations at codon 12 of K-ras and H-ras, enhanced cyclooxygenase-2 and iNOS, microsatellite instability, and some APC mutations (15%) (77). Also, AOM is more potent, less expensive and more convenient to use compared to other chemicals that are used to induce tumors in animal models.

**Aberrant crypt foci**

Exposure of normal cells to chemical or viral agents results in initiation of the carcinogenic process (80). The clonal expansion resulting in altered morphology and phenotype of these initiated cells leads to promotion. Further changes in the genotype leads to malignancy and metastasis, often defined as the stage of progression. Colonocytes act as a barrier against exogenous carcinogenic substances. However the biotransformation or the activation/detoxification of the exogenous chemicals may lead to preneoplastic or precancerous lesions in the colon and rectum called aberrant crypt foci (ACF). ACF were initially identified in the colonic mucosa of rodents exposed to colorectal carcinogens and later in human colon (80, 81).

ACF are defined as single or multiple crypts with 1) altered luminal openings 2) that exhibit thickened epithelia; and 3) are larger than adjacent normal crypts (82).
ACF are thus regarded as preneoplastic or precancerous lesions in the large bowel of man as well as rodents (83). According to Bird et al the scheme of events starts with ACF that selectively go through the multistep process leading to the formation of microadenomas which selectively undergo genetic and other changes to develop into adenocarcinomas and carcinomas subsequently (80, 84). ACF can be stratified into two broad classes, hyperplastic and dysplastic based on morphology and biological behavior. It has been suggested that larger dysplastic ACF are more preneoplastic than hyperplastic ACF and are more correlated to tumors (85). ACF display a marked biological heterogeneity with respect to genotypic and phenotypic features. With increasing time and exposure a higher number of ACF exhibit dysplasia and increasing crypt multiplicity. Although, the true neoplastic potential of ACF needs to be determined, some of these dysplastic ACF progress into the adenoma- carcinoma sequence. Many studies have reported a lack of correlation between number of ACF and tumorigenesis (86, 87). Also different chemopreventive agents have differential effects. Cholic acid inhibited ACF in rats on a short term but increased tumor incidence indicating a lack of correlation with ACF and tumors (87). Different initiating or promoting carcinogens such as AOM and DMH, time of exposure to carcinogen and the dietary treatments influence the number of ACF. Furthermore, ACF that are mucin-depleted and have β-catenin accumulation are more correlated with tumors (88-90). Thus, ACF, although not fully committed to neoplasia are preliminary markers of preneoplasia that are easily identifiable and hence have been used by many investigators as a short-term marker for evaluating chemopreventive compounds against carcinogenesis.
Colorectal cancer and Nonsteroidal anti-inflammatory drugs (NSAIDs)

The best understood example of NSAID therapy in oncology involves colon cancer where multiple lines of evidence, animal and cell culture experiments, therapeutic trials and epidemiological studies, all reinforce the conclusion that NSAIDs block colon carcinogenesis at an early step (91-96). Epidemiological studies indicate a consistent reduced risk of CRC with intake of nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, by up to 50% (91, 92). Clinical trials also demonstrated that sulindac, one of the NSAIDs, caused regression of colorectal adenomas in patients with familial adenomatous polyposis (FAP) (97, 98). The well-supported chemopreventive mechanism of NSAIDs is their inhibition of cyclooxygenase (COX) enzymes in the arachidonic acid (AA) cascade. COX-1 and COX-2 are the established pharmacological targets of NSAIDs (4, 91, 92, 94, 98, 99). The pharmacology of NSAIDs and the roles of COX-1 and COX-2 are now integrated into a model suggesting that COX-1 synthesizes the housekeeping prostaglandins (PG) that protect the stomach lining from ulcers, regulate renal blood flow while COX-2, produces PGs that are associated with pain, inflammation and fever (94, 98-101) indicating the role of chronic inflammation in carcinogenesis. In general, COX-2 is not constitutively expressed in appreciable amounts by most normal tissues, but certain inflammatory cytokines, tumor promoters, growth factors and oncogenes induce COX-2 (100, 102).
Figure 1.3. Arachidonic acid cascade and generation of prostaglandins by COX

1 Endoperoxide synthase of COX
2 Peroxidase component of COX
3 Prostacyclin synthase
4 Thromboxane synthase
5 Endoperoxidase isomerase (soluble form)
6 Endoperoxidase isomerase (insoluble form)
7 Endoperoxidase reductase
8 Conversion of PGD2 to PGJ2
NE non-enzymatic conversion
MDA Malondialdehyde

Phospholipid

Phospholipase A2

Arachidonic acid
(5, 8,11, 14-eicosatetraenoic acid)

Cytochrome
P450 pathway

Lipoxygenase
pathway

Prostaglandin G2

COX-1

COX-2

NSAIDs

COX-2 selective inhibitors

Prostaglandin H2

PGI2

TXA2

NE

MDA

PGD2

PGF2α

PGE2

PGJ2

PGA2 → PGB2

TXB2

Arachidonic acid (AA) is the major prostanoid precursor. The biosynthesis of prostanoids involves a three-step sequence of stimulus-initiated hydrolysis of arachidonate from glycerophospholipids involving secretory, cytoplasmic or both types of phospholipase A2 (sPLA₂, cPLA₂); oxygenation of arachidonate yielding cyclic endoperoxide prostaglandins (PGG) and cyclization catalyzed by the endoperoxide synthase component of COX isoenzymes, to yield Prostaglandin H₂ and conversion of PGH₂ to the most biologically active end products such as PGD₂, PGE₂, PGF₂α, PGI₂ (prostacyclin) and TXA₂ (thromboxane A₂) via specific synthases (Figure 1.3).

Role of prostaglandins and COX in tumorigenesis

Certain prostaglandins especially derived by the action of COX-2 isoenzyme have been suggested to aid in carcinogenesis by altering normal cellular processes like cell proliferation by increasing polyamines and DNA synthesis via ornithine decarboxylase activity, angiogenesis, inhibiting apoptosis, immunomodulation and carcinogen metabolism (103). The NSAIDs such as aspirin, indomethacin, sulindac, ibuprofen all inhibit both isoforms of COX nonselectively. In vitro and in vivo studies indicated different mechanisms of COX inhibition by these classes of drugs. Aspirin inhibits the COX but not peroxidase activities of both COX-1 and COX-2 by acetylated a particular serine thereby blocking the channel leading to the active site resulting in irreversible inhibition of PG synthesis. Indomethacin induces an inhibitory conformational change by forming a tight, slowly dissociable complex while Ibuprofen and piroxicam are competitive inhibitors. In general most of these NSAIDs are better inhibitors of COX-1 than COX-2 (103). Although, marked reduced risk of
developing colon cancer and its nonmalignant precursor, the adenomatous polyp in FAP with NSAIDs was evidenced, the benefits have not been as obvious in sporadic colon cancer as in familial colon cancer (102). Moreover, their prolonged use has adverse effects such as nausea, dyspepsia, gastritis, abdominal pain, peptic ulceration, gastrointestinal bleeding, and/or perforation of gastroduodenal ulcers and nephrotoxicity (98).

Following the discovery of the COX-2 isoform and with the evidence indicating a positive relationship between COX-2 expression and CRC, novel COX-2 selective inhibitors such as rofecoxib and celecoxib were developed (96, 104-106). These were less likely to have the side-effects associated with the general NSAIDs including gastrointestinal mucosal defense interference. Evidence from genetic studies also demonstrated that deletion of the COX-2 gene resulted in decreased tumor formation both in the small intestine and colon of APC mutant models (76). This assessment of the role of COX-2 in colorectal tumorigenesis in animal models involving knockout mice led to further testing on animal models that suggested a regression leading to safer use and prescription of COX-2 inhibitors for FAP patients (107-109). Numerous studies suggest an initiating event such as a mutation of a gatekeeper gene such as APC results in the induction of inflammatory COX-2 while COX-1 levels remain the same in normal as well as the tumor tissue (109). Although, the reduced gastrointestinal toxicity of COX-2 selective inhibitors favors their use, they too have unfavorable side-effects. Rofecoxib, a selective COX-2 inhibitor has been shown to increase cardiovascular morbidity in some persons and was recently taken off the market (110-112)
Modulation of COX-2 by estrogen

COX-2 is induced by inflammatory cytokines, interleukins and lipopolysaccharides (LPS). There is limited but growing evidence that estrogen may influence COX-2 induction. In ovariectomized female rats Ospina et al (113) reported the suppression of interleukin (IL-1β)-mediated induction of the COX-2 pathway in rat cerebral blood vessels with estradiol treatment. Also, systemic IL-1β induced an NF-κB dependent induction of COX-2 in untreated rats that was blocked by estradiol by an ER-dependent mechanism (113). A two-fold increase in COX-2 mRNA in human uterine microvascular endothelial cells by estradiol was observed by Tamura et al (114). Mutoh et al using a β-galactidase reporter gene system measured COX-2 promoter-dependent transcriptional activity in human colon cancer DLD-1 cells with treatment of genistein and other chemopreventive compounds (115). They observed a suppression of the COX-2 promoter activity with genistein and attributed it to the resorcin moiety that is similar to the endogenous estrogen. These observations suggest the modulation of COX-2 pathway by estrogens.

Hypothesis

The basic concept of cancer prevention involves the regression, inhibition or elimination of precancerous lesions leading to reduced cancer incidence. Based on our previous findings that clearly showed the effect of dietary estrogens in decreasing tumor incidence in AOM-treated mice (36) we wanted to dissect the pathways through which estrogen might have an influence in CRC. Numerous studies reported decreased colon cancer incidence with NSAIDs and COX-2 specific inhibitors (91, 107, 115, 116). There is evidence with regard to estrogen modulating
COX-2 in cerebral blood vessels and uterine tissues from in vitro studies (113, 114). There is also evidence indicating that genistein and other chemopreventive compounds that have the resorcin moiety in their structure suppressed COX-2 promoter-depended transcriptional activity in colon cancer cells (115). An increase in COX-2 levels is one of the early events in CRC. Hence, we hypothesized that dietary estrogens, could reduce CRC risk via inhibition of COX-2. Furthermore, these compounds would reduce the formation of ACF, which are morphologically determinable lesions that occur during the initiation-promotion stage in the scheme of events associated with the multistage carcinogenesis model. We used the AOM-induced mouse model in which we previously found a positive protective effect of estrone.
CHAPTER 2
EXPERIMENTAL DESIGN

Animal care

Thirty four 3-week old ovariectomized C57BL/J6 mice purchased from Charles River, Portage, MI were maintained at the animal care facility, located in the Human Nutritional Sciences Building in a 25°C, humidity controlled, 12-hour dark-light cycle environment. Animals were housed 4 to a cage in shoe box plastic cages. Mice were acclimated with casein control diet for three days (Table 2.1). At 4 weeks of age the mice were randomized to the experimental diets with n=7-9 per group. All mice were injected intraperitoneally with azoxymethane, at 10 mg/kg of body weight once per week for 6 weeks (Figure 2.1). The AOM was administered at the same time of day and day of the week. The dose of AOM administration was arrived at based on the tolerability of the animals from previous studies and effectiveness of the dose (117, 118). Tap water and feed were provided ad libidum. Animals were weighed weekly and body weights recorded.

Preparation of Azoxymethane

Azoxymethane (AOM) was purchased from Sigma-Aldrich, St. Louis, MO. The solutions were prepared by careful handling in a fume hood. Twenty five milligrams of AOM suspension was mixed in 10 ml of distilled water and aliquots of 2 ml with a final concentration of 2.5 mg/ml were stored in sealed septum bottles in the -20°C freezer. On the day of carcinogen injections the dose amount was calculated depending on the weight of the mouse with a final dose of 10 mg/kg of body weight.
Figure 2.1. Experimental design

Female, Ovex mice

AOM
10mg/kg of body wt

6 weeks

Experimental

Diet
Casein

Diets*
Casein (n=7)
Soy protein without isoflavones (soy-IF) (n=8)
Soy protein with genistein (soy+gen) (n=9)
Soy protein with estrone (soy+E₁) (n=9)

* See Table. 2.1 for diet composition
Table 2.1. Composition of experimental diets (AIN93 based)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Casein gm/kg</th>
<th>Soy-IF gm/kg</th>
<th>Soy+Gen gm/kg</th>
<th>Soy+E1* gm/kg</th>
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<tbody>
<tr>
<td>Corn starch1</td>
<td>393</td>
<td>396</td>
<td>396</td>
<td>393</td>
</tr>
<tr>
<td>Cellulose BW2002</td>
<td>38</td>
<td>50</td>
<td>49</td>
<td>38</td>
</tr>
<tr>
<td>Phytic acid</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dyetrose3</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Sucrose1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Casein2</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soy protein w/o IF4</td>
<td>0</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Vitamin mix – AIN 93G2</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Salt mix – AIN932</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>D-L Methionine2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Choline bitartrate2</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
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<tr>
<td>Corn oil1</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Safflower oil2</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Genistein5</td>
<td>0</td>
<td>0</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>Estrone6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2*</td>
</tr>
</tbody>
</table>

* Made with a premix containing 125mg estrone in 250 gm of corn starch (dose of estrone is 0.093 mg Estrone/kg of diet). Estimated energy content 4.84 kcal/gm of diet

1 General Stores
2 ICN Biomedicals, Inc. (Aurora, OH)
3 Dyets Inc., Bethlehem, PA
4 Archer Daniels Midland Inc. (Decatur, IL)
5 LC Laboratories, Woburn, MA
6 Sigma-Aldrich, St. Louis, MO

Diets were prepared in house and pelleted by extrusion at the University of Missouri-Columbia, MO (Dr. Fu-Hung Hsieh). The diets were color-coded using color laks purchased from Monarch Food Colors, High Ridge, MO.
per mouse. The azoxymethane was administered via intra peritoneal injections using a 1 ml syringe and a 27.5 gauge needle.

**Tissue collections**

Mice were anesthetized with Nembutal (50 mg/ml with a dose of 3 mg per mouse) between 0800 and 1300 hrs, 6 weeks after the last AOM injection. A mid lateral incision was made and the thoracic cavity opened and heart exposed. Blood was then collected by cardiac puncture using heparinized (0.01% heparin) syringes and placed in 1.5ml tubes on ice until centrifuged. Serum was collected after centrifugation at 14,000 rpm for 7 min and stored in -80°C. Abdominal fat and organs including heart, lung, kidney, spleen, cecum and colon were removed and weighed. The colon was flushed twice with 1X mouse-tonicity phosphate buffered saline (MTPBS, 15.7 mM \( \text{Na}_2\text{HPO}_4 \), 4.5 mM \( \text{NaH}_2\text{PO}_4 \), 0.15 M \( \text{NaCl} \)) (119, 120) using 18-gauge, bulb-tipped gavage needles. The colon was divided into 2 sections. The distal 2 cm section of the colon was sandwiched between 2 microscope slides bound by a rubber band and fixed in 10% neutral-buffered formalin overnight. The remaining part of the colon was scraped using a microscope slide and the colonocytes collected in 5X volume lysis buffer (150mM \( \text{NaCl} \), 50mM HEPES and 1% protease inhibitor mix (that had protease inhibitors to inhibit serine, cysteine, and calpain proteases), (Amersham Biosciences, Buckinghamshire, UK) snap frozen in liquid nitrogen and stored at -80°C until further analysis by Western immunoblot.
**Aberrant crypt foci staining procedure**

The overnight formalin-fixed microscope slides were carefully split apart such that the fixed tissue was on the lower slide with the crypts facing up, excess formalin was drained with a Kim wipe and the tissue was immersed in (0.2% methylene blue stain in distilled water) (82) for 10 min. After rinsing 3X with phosphate buffered saline (PBS, 1mM Potassium phosphate monobasic, 0.15M sodium chloride, 5.6mM sodium phosphate dibasic anhydrous) for 5 min, cover slips were carefully placed on the slides and the crypts were visualized under a light microscope at 10X magnification.

The colon section was outlined on the cover slip using a fine-tip marker and the aberrant crypts were marked on the cover slip using the marker by two independent observers. Aberrant crypts were distinguished from adjacent normal crypts by their altered luminal opening that had thickened epithelia. Some of them were elongated and were distinguishable from the remaining adjacent normal crypts.

**Protein quantification and Western Immunoblotting**

Frozen colonocytes were sonicated in short bursts 3X 15 sec apart (Heat Systems W-380 sonicator), centrifuged at 120,000Xg for 30 min at 4°C (Ultracentrifuge, Beckman L8-M, Rotor number Ti 50.3) and supernatants collected. Protein concentrations of the supernatants were determined using a bicinchoninic acid (BCA) kit for protein determination (Sigma, St. Louis, MO).

Procedure for BCA protein assay: The BCA reagent was prepared by mixing 50 parts of bicinchoninic acid solution to 1 part of the copper sulfate solution. Triplicates of the sample supernatant (dilution factor 10, i.e., 1:10 1µl of lysate with 9
µl of water), Bovine serum albumin (serial dilution of 0.25, 0.5, 1, 1.5 and 2 mg/ml), dH₂O and the lysis buffer were loaded onto 96-well plate to which 200µl of the BCA reagent was added. The plate was incubated at 37°C for 30 min. The absorbance of each well was read at 562nm using the microplate reader. The absorbance values were compared to the standard curve to determine the protein concentration.

**Western immunoblot procedure**

Gels were cast on previous day of run using BioRad Mini-Protean 3 cell system (Bio-Rad, Hercules, CA). The first 4 solutions (Table 2.2) were mixed and degassed for 15 min. The cross linking agents were added immediately before transfer into the gel apparatus. A layer of water was placed over the separating gel until it was set. The water was wiped off with a Kim wipe and the stacking gel layered on top. A comb was inserted into the gel and allowed to set. The wells were rinsed with water prior to sample loading. From the determined concentrations of the supernatant sample 5µg of protein was loaded per wells. The supernatant was mixed with 6X sample buffer (1.5M Tris-HCl, glycerol, sodium dodecylsulfate (SDS), dithiothreitol (DTT), bromophenol blue) to yield a total volume of 30µl and heated at 100 °C for 1 min. The loading concentration of the supernatant was determined empirically. A COX-2 standard (positive control) (Sigma, St. Louis, MO) an internal control (a sample supernatant that was repeated in all the gels) and a molecular weight standard (Bio-Rad, Hercules, CA) were run on each gel. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% separating gel with a 4% stacking gel (Table 2.2) at 200 V until the dye (bromophenol blue) ran off the bottom.
Table 2.2. Reagents used for casting the gel for western immunoblot

<table>
<thead>
<tr>
<th>FOR 2 MINIGELS (1.5mm thick)</th>
<th>10 % resolving</th>
<th>4% stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/0.8% Bis solution¹</td>
<td>6ml</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>1.5M Tris, pH 8.8²</td>
<td>5 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris pH 6.83</td>
<td></td>
<td>1.25 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>8.10 ml</td>
<td>3.07 ml</td>
</tr>
<tr>
<td>10% SDS¹</td>
<td>200ul</td>
<td>50 ul</td>
</tr>
<tr>
<td>TEMED ¹ (N,N,N,N –tetra-methyl-ethylenediamine)</td>
<td>20ul</td>
<td>5 ul</td>
</tr>
<tr>
<td>10% Ammonium Persulphate²</td>
<td>100ul</td>
<td>25 ul</td>
</tr>
</tbody>
</table>

1 Bio-Rad, Hercules, CA
2 Fisher Scientific, Fairlawn, NJ

Typically the gel ran for about 50 min to an hour. The gel was carefully removed from the glass plates and incubated in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) along with the filter pads, filter paper and nitrocellulose membrane (Trans-Blot transfer medium, Bio-Rad, Hercules, CA) for 30 min. The electrophoresis and transfer were carried out with PowerPac™ HC Power supply (Bio-Rad, Hercules, CA) and Bio-Rad Mini Trans-Blot system (Bio-Rad, Hercules, CA). The transfer was done for 90 min at 100 V constant voltages. The membranes were then incubated for 2 hr at room temperature on a rocker in a blocking buffer (1.5% non-fat dry milk and 1.5% Bovine serum albumin (BSA) in Tris Buffered Saline
(TBS) 0.5mM Tris base, 0.9% NaCl). The membrane was cut into 2 pieces based on molecular weight using the colored standards as a guide. The upper part of the membrane was incubated with mouse monoclonal anti-COX-2 antibody (1:10,000). The lower part was incubated in mouse monoclonal anti-β-tubulin antibody (1:10,000) in block buffer overnight at 4°C on a rocker. Membranes were washed 4 X 5 min with TBS and probed with goat anti-mouse IgG-HRP conjugate (COX-2 1:50,000; β-tubulin 1:5000). Table 2.3 lists the antibodies and reagents used in these analyses. Target proteins were detected using Pierce West Femto Maximum Sensitivity substrate and quantified with a Bio-Rad GS-800 Densitometer using Quantity One software. β-tubulin served as the loading control. For each sample the density of COX-2 was normalized with the density of the corresponding β-tubulin and then a correction for intergel comparison was done using the internal sample control. To generate a correction factor the COX-2/β-tubulin ratio for the internal sample control was determined for each gel then an average derived. This average was divided by the individual gel internal sample control ratio and used as the correction factor as follows

\[
\text{Normalized COX-2}_{\text{sample}} = \frac{\text{COX-2}_{\text{sample}}}{\beta\text{-tubulin}} \times \text{Correction factor}
\]

Statistical analysis using SAS (SAS Institute, Cary, NC) was performed on this normalized COX-2 value. We used Proc mixed procedure of SAS to do one way analysis of variance (1-way ANOVA) on normalized COX-2 value and the mean number of ACF per diet group. The organ weight data as percent of body weight
was analyzed using the General Linear Model (GLM) procedure. Differences among means were determined by the least square (LS) means component. Pair wise comparison using Tukey-Kramer was done. The body weight data was analyzed using the Proc mixed procedure while the last two week differences were done after Bonferroni adjustments. Main effect and interactions were considered significant at $P \leq 0.05$ for COX-2 protein density levels and number of ACF after Tukey-Kramer adjustments. Measurements were reported as mean ±SE.
Table 2.3. Antibodies and reagents used for COX-2 western immunoblot

<table>
<thead>
<tr>
<th>Catalog No.</th>
<th>Source</th>
<th>Dilutions</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight marker</td>
<td>Bio-Rad</td>
<td></td>
<td>Kaleidoscope prestained standards control</td>
</tr>
<tr>
<td>C-0858</td>
<td>Sigma</td>
<td></td>
<td>Human, recombinant expressed in Sf21 cells</td>
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<tr>
<td>SC-19999</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>1:10,000</td>
<td>Molecular weight of COX-2 72 kDa</td>
</tr>
<tr>
<td>SC-2005</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>1:50,000</td>
<td>Goat anti-mouse IgG-HRP</td>
</tr>
<tr>
<td>SC-5274</td>
<td>Santa Cruz Biotechnology Inc.</td>
<td>1:10,000</td>
<td>Mouse monoclonal Mouse monoclonal Molecular weight of β-tubulin 55kDa</td>
</tr>
<tr>
<td>34094</td>
<td>Pierce</td>
<td>1:1</td>
<td>Super signal West Femto maximum sensitivity substrate</td>
</tr>
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CHAPTER 3
MANUSCRIPT
Suppression of cyclooxygenase-2 by dietary genistein and estrone in colon of azoxymethane-induced ovariectomized C57BL/J6 mice

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Suppression of COX-2 by estrone and genistein in mice

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* Padma Balaji weighed, helped in making diets, fed animals, collected and processes samples, counted ACF, ran western immunoblot and did data and statistical analysis. Dr. MacDonald made the diets, helped in collection of colon samples. Dr. Przybyszewski helped in collection of colon samples, data analysis and served as an independent second observer for counting ACF. Departmental statistician Man-Yu-Yum assisted in performing statistical analysis
ABSTRACT

Epidemiological studies indicate a lesser incidence of colon cancer in populations that consume fruits, vegetables and vegetable proteins such as legumes over the western diets that are rich in animal protein and fats. Soy and associated bioactive substances such as the isoflavones have been associated with lower colon cancer risk. We previously found reduced colon tumor incidence in mice fed soy protein with estrone (E1). Several epidemiological studies indicate a 40-50% reduction in the risk of developing colorectal cancer with chronic non-steroidal anti-inflammatory drugs (NSAIDS) including aspirin that inhibit both isoforms of the cyclooxygenase (COX) enzyme COX-1 and COX-2. We hypothesized that dietary genistein, or estrone (E1) would reduce aberrant crypt foci (ACF), a purported preneoplastic marker, and COX-2 protein levels in mice colon. Ovariectomized female mice were fed diets containing casein (Casein), soy protein without isoflavones (Soy-IF), soy protein + genistein (Soy+Gen) or soy protein + estrone (Soy+E1) from 3 weeks of age. Beginning at 4 weeks of age, all animals received weekly injections of azoxymethane (AOM) 10mg/kg of body weight for 6 weeks and terminated 6 weeks after the last dose of AOM. Aberrant crypt foci were visualized under a light microscope and COX-2 protein levels were analyzed by Western immunoblot. COX-2 protein levels were affected by diet ($P \leq 0.05$) but aberrant crypt foci incidence was not ($P \leq 0.05$). COX-2 levels were lower in mice fed Soy+E1 and Soy+Gen compared with mice fed the casein or Soy-IF. Thus, we conclude that soy protein with genistein ad soy protein with estrone downregulate COX-2 levels and may thereby reduce colon cancer risk.
KEY WORDS: Colon cancer, Soy protein, Genistein, Cyclooxygenase-2, Azoxymethane, Estrone
INTRODUCTION

Cancer accounts for approximately 23% of all deaths in the United States, ranking second only to heart disease. Colon cancer is the third most commonly diagnosed cancer among men and women (1, 2). In 2005 estimated new cases of colon cancer were 48,290 for men and 56,660 for women (1, 2). Colon cancer risk is influenced by various environmental factors such as exposure to chemicals, radiation, smoking, oxidative damage, and other lifestyle factors such as physical activity, and alcohol intake (3). Epidemiological data suggest that diet plays a major role in the etiology of colon cancer (4, 5). The World Cancer Research Fund and the American Institute of Cancer Research after an extensive review of literature concluded that colon cancer risk was reduced with vegetable intake, and physical activity while consumption of red meat (probably the associated saturated fat) and alcohol increased the risk (6). Colon cancer incidence rates among populations differ greatly with the lowest incidence in the Asian population (2, 7, 8). However, the incidence rates of colon cancer among the migrant Asian population to the United States reach those of the host country implicating the role of environment in the incidence of colon cancer (7-10). One plausible reason for the difference in incidence rates was attributed to the consumption of soy by Asian population (7). Soy is a good source of several phytochemicals, including the isoflavones genistein and daidzein, in addition to the high quality protein (6, 9, 11). The structural similarity of isoflavones to that of endogenous estrogen and their weak estrogenic property classified them as phytoestrogens (12, 13).
A role of hormones in the etiology of colon cancer was observed first by Fraumeni et al who noted that nuns experienced an excess of known hormone-associated cancers and also colon cancer (14). A meta-analysis of 18 epidemiological studies indicated a 20% reduction in colon cancer risk with hormone replacement therapy (HRT) (15). Furthermore, evidence from the Women's Health Initiative (WHI) also indicated that estrogen reduced the risk for colon cancer (16, 17). However, subjects in that study who developed colon cancer were diagnosed with more advanced stages of colon cancer when using HRT compared to the placebo (16).

Several epidemiological and case-controlled studies indicate an inverse association between the risk of colon cancer and intake of NSAIDs, including aspirin (18). The decrease or regression of colon carcinogenesis by NSAIDs has been attributed to the specific inhibition of the cyclooxygenase (COX) enzymes by these classes of drugs. COX catalyzes the committed step in prostaglandin synthesis. Two isoforms of COX (COX-1 and COX-2) are known (19, 20). COX-1 is a constitutively expressed enzyme and has a housekeeping role helping to maintain physiological functions such as cytoprotection and blood flow (21). COX-2 is inducible as it is dependent on extracellular and intracellular stimuli and is upregulated with inflammation (22) cellular proliferation, differentiation, and tumorigenesis (23, 24). The suppression of COX-2 induction by interleukin 1β (IL-1β) with chronic treatment of 17β-estradiol in rat cerebral vascular blood vessel suggests a role for estrogen in modulating COX-2 (25). Moreover, there is also evidence suggesting a strong link between COX/PGE2 (Prostaglandin E2) signaling
and the adenomatous polyposis coli (APC)/β-catenin/TCF pathway by estrogen whose downstream target protein includes COX-2 (26-28). There is further evidence implicating the regulation of COX-2 by the Wnt and Ras pathways suggesting a cross talk between the pathways involving estrogen in colon carcinogenesis (29). Thus, regulation/inhibition of COX-2 by estrogentic compounds would be an effective way to inhibit colon carcinogenesis in the initial stages of the multistage carcinogenesis.

Previously we showed that estrone (E1) was protective against colon tumorigenesis in mice treated with azoxymethane (30). In this study we evaluated the hypothesis that genistein reduces COX-2 and protects against azoxymethane (AOM) induced aberrant crypt foci (ACF) formation in mice.
MATERIALS AND METHODS

Chemicals: Azoxymethane (AOM) was obtained from Sigma-Aldrich (St. Louis, MO) in 10 mg isovials and stored at -20°C which was resuspended in 10ml distilled water. Genistein and Estrone (E1) were obtained from LC Laboratories (Woburn, MA) and Sigma-Aldrich (St. Louis, MO) respectively.

Animals, diet and study design: The experimental protocol was approved by the Institutional Animal Care and Use Committee, Iowa State University and followed AAALAC standards. Thirty four female ovariectomized (3wks of age) C57BL/J6 mice (Charles River, Portage, MI) were housed 4 per cage and maintained in a temperature and humidity controlled animal facility with a 12 hr light cycle. Animals were fed a modified AIN93G diet containing casein as the protein source for 3 days of acclimatization, after which they were assigned randomly to one of the four diet groups; Casein (n=8) as control, soy protein without Isoflavones (IF) (Soy-IF) (n=8), soy with genistein (Soy+Gen) (n=9) and soy with estrone (Soy+E1) (n=9) (Table 2.1). The diets were isocaloric and the soy protein was treated by the manufacturer (Archer Daniels Midland (Decatur, IL) to remove the majority of the isoflavones (IF). The same lot and batch of protein was used for all of the diets containing soy protein as the protein source. The dose of genistein (250mg/kg diet) was based on our previous finding of delayed mammary tumorigenesis in mice fed this concentration (31). The dose of E1 was extrapolated from the typical human HRT dose for Premarin (0.625 mg/d) and shown to reduce tumor incidence previously (30). Casein diet was corrected for the concentration of phytate in the soy protein diet. Beginning at 4 wks of age, the mice were injected intraperitoneally with 10 mg
Azoxymethane (AOM; Sigma-Aldrich (St. Louis, MO) /kg body weight once a week for 6 wks. AOM injections were done at the same time of day throughout the study.

**Tissue sample collection**

Mice were anesthetized with Nembutal (Iowa State University veterinary pharmacy, Ames, IA, 3 mg per mouse) between 0800 and 1300 hrs, 6 weeks after the last AOM injection. A midlateral incision was made and the intestinal tract removed. The colon was rinsed, blotted, weighed and opened longitudinally. The colon was divided into 2 sections. The distal section of 2 cm length was fixed with 10% neutral formalin sandwiched between 2 microscope slides overnight. The remaining part of the colon was scraped with a microscope slide and the colonocytes collected in lysis buffer (150mM NaCl, 50mM Hepes and 1% protease inhibitor mix) snap frozen in liquid nitrogen and stored in -80°C until used for Western blot analysis. Abdominal fat along with liver, cecum, spleen, heart and kidney were removed and weighed.

**Aberrant crypt foci**

Aberrant crypt foci (ACF) are altered luminal openings with morphologically altered crypts in AOM induced rodents (32). After fixing overnight the distal colon was dipped in 0.2% solution of methylene blue in distilled water for 10 min rinsed with phosphate buffered saline for 5 min 2X and placed on a microscope slide with the mucosal surface up. Using a light microscope at 10X magnification, aberrant crypts were distinguished from the surrounding “normal-appearing” crypts using standard criteria and counted by two independent observers.
Quantification of Cyclooxygenase-2 by immunoblot

Frozen homogenates of colonocytes were sonicated and centrifuged at 120,000 X g for 30 min at 4°C and the supernatants collected. Protein concentration of the supernatants was determined with the BCA protein assay kit (Sigma, St. Louis, MO). Protein extracts (5µg) were mixed with loading buffer, denatured and fractionated on 10% SDS-polyacrylamide gels and then electrophoretically transferred to Nitrocellulose (Bio-Rad). On each gel, were included a prestained molecular weight color marker mix (Bio-Rad, Hercules, CA), a positive control of COX-2 (Sigma, St. Louis, MO) and an internal sample control comprised of a single mouse supernatant. After transfer, the membranes were blocked in 1.5% non-fat dry milk (Blotto; Santa Cruz Biotechnologies, Santa Cruz, CA) and 1.5% Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO) for 2 hrs on a rocker at room temperature. Membranes were cut into 2 pieces based on molecular weight using the color bands from the prestained molecular weight marker. The individual strips were incubated with either mouse monoclonal anti-COX-2 antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse monoclonal anti-β-tubulin antibody (1:10,000; Santa Cruz Biotechnology) in blocking solution overnight at 4°C. Membranes were washed with Tris buffered saline (TBS) then probed with goat anti-mouse IgG-HRP conjugate (COX-2 1:50,000; β-tubulin 1:5000; Santa Cruz Biotechnology). Target proteins were detected with West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL). The membrane was scanned and quantified with a Bio-Rad GS-800 Densitometer (Bio-Rad Lab, Hercules, CA) using Quantity One software (Bio-Rad, Hercules, CA). β-tubulin served as the loading control. For each sample the ratio of sample COX-2
and the corresponding β-tubulin density was determined. A correction for intergel comparison was done using the internal sample control. To generate the correction factor, the internal sample control ratio of COX-2 and β-tubulin for each gel was determined and then an average across all gels was obtained. That average was divided by the individual gel internal sample control ratio and then used as a correction factor as follows:

\[
\text{Normalized COX-2}_{\text{sample}} = \left( \frac{\text{COX-2}_{\text{sample}}}{\beta\text{-tubulin}_{\text{sample}}} \right)_{\text{Correction}}
\]

A representative immunoblot is shown in Figure 3.1. Note the internal sample control and the COX-2 standard (std).

**Statistical analysis:** Proc Mixed procedure was used to analyze aberrant crypt foci and COX-2 protein levels by one way analysis of variance (ANOVA). The body weight was analyzed using the Proc Mixed procedure while the organ weight data was analyzed with the General Linear Models (GLM) procedure of SAS (SAS Institute, Cary, NC). Differences among means were determined by the least-square (LS) Means component. Main effects and interactions were considered significant at \( P \leq 0.005 \) for organ weights and \( P \leq 0.05 \) for COX-2 protein density levels and number of ACFs after Tukey-Kramer adjustments. All measurements are reported as the mean ±SE.
RESULTS

Mice fed the experimental diets gained weight similarly throughout the study (Figure 3.2). However, slight differences in body weight due to diet were observed at termination (Table 3.1). The differences in body weight occurred during the last 2 weeks of the study (Figure 3.2). Mice fed Soy-IF weighed slightly more than Soy+Gen at these time points. Mice fed Soy-IF had more abdominal fat than the Soy+Gen animals, which might explain this difference in body weight (Figure 3.3). When expressed as percent of body weight, colon, kidney, spleen weights were less in mice fed Soy-IF compared to Soy+E1 animals, and cecum weights were less in this Soy-IF group compared to all the diet groups (Table 3.1) \( (P \leq 0.005) \)

Aberrant crypt foci (ACF)

ACF were observed in all mice irrespective of diet groups. The mean ACF ranged from 5-8 in each group. There was no significant statistical difference in the number of ACF across the four diet groups although the Soy+Gen group tended to have more number of ACF than the other diet groups (Figure 3.4).

Cyclooxygenase-2 protein

COX-2 protein levels as determined by western immunoblot in mice fed the Soy+E1 or Soy+Gen were significantly less \( (P \leq 0.01) \) compared to the mice fed the Casein or Soy-IF diets (Figure 3.5)
DISCUSSION

The FDA recently approved a health claim for foods containing soy and recommended 25 g of soy protein per day to reduce cardiovascular disease risk (11). Some other health benefits associated with soy and soy foods include decreased bone loss and alleviation of menopausal symptoms. As a result, soy consumption is increasing in the United States. Also, many peri and postmenopausal women are using soy supplements or soy extracts that contain isoflavones to alleviate menopausal symptoms as an alternative to hormone replacement therapy (HRT). Epidemiological studies have indicated associations between HRT and reproductive history with colon cancer risk, suggesting a role for estrogenic compounds in decreasing colon cancer risk (10, 16, 33-35).

COX-2 has emerged as therapeutic and chemopreventive target for colon cancer with epidemiological and clinical studies indicating COX-2 inhibition is protective of colon cancer (22, 23, 36-39). Although the evidence is limited, COX-2 may be influenced by estrogens (25, 40). Hence the objective of this short term study was to determine if dietary genistein or estrone would affect COX-2 expression in azoxymethane-treated mouse colon. We also used ACF as a pre-tumor marker. ACF has been widely used as an early indicator of tumorigenesis by many short-term studies (41). In this study we found no diet effect on ACF. This result contradicts with two other studies. A reduction in ACF was observed in AOM-treated rats fed soy concentrate with genistein than the soy concentrate alone diet (42). And Gee et al (32) reported an increase in the number of ACF in dimethylhydrazine-treated rats fed purified genistein or genistein-rich soy protein.
We previously showed mice fed Soy+E1 had lower tumor incidence than mice fed Soy-IF (30). Therefore the lack of response in ACF in this study by Soy+E1 was surprising. However, many studies reported conflicting results on the association between ACF and tumorigenesis (43-46). Certain dietary compounds such as cholic acid and 2-(Carboxyphenyl)retinamide 2-CPR are potent inhibitors of ACF formation but cause higher incidence of tumor formation in rats leading to conflicting correlation between ACF and tumors. It is not clear if ACF are true markers of tumor development and the current study would suggest that at least in the AOM-treated C57BL/J6 mice they are not.

No difference in tumorigenesis was reported in APC\textsuperscript{min} mice fed soy protein with high or low isoflavone supplementation (47). The diets fed to these mice were high in animal fat and low in fiber and calcium similar to a western diet, which could be a possible explanation for the lack of any effect. However, the number of small intestinal tumors in these mice was significantly reduced with a low isoflavone diet containing 300ppm of sulindac, a NSAID (47). In our study the addition of genistein to soy protein did not have an effect on ACF while Thiagarajan et al (42) found a smaller dose of genistein (167mg/kg diet) with soy concentrate reduced ACF by 50% in AOM-treated rats.

The need for a better reliable preneoplastic marker is strongly emerging with these conflicting results. Yamada et al (48) proposed the use of ACF with $\beta$-catenin/APC mutations that result in $\beta$-catenin nuclearization and dysregulation of the wnt signaling pathway as a better preneoplastic marker. They proposed that the dysplastic ACF that are mucin-depleted and have $\beta$-catenin accumulation in the
nucleus are more correlated with tumorigenesis (49). We attempted to identify mucin depleted foci in the distal colon of mice from the current study using alcian blue stain in 3% acetic acid. However, we were unable to clearly distinguish the mucin depleted foci from the surrounding crypts. This may have been due to the number of ACF with mucin depletion to be only about 1-2% (refer Figure 4.1) and with the incidence of about 5-8 ACF per mouse in our current study the probability of us visualizing the mucin depleted foci is very low.

Previously we reported the reduction of colon tumorigenesis in mice fed Soy+E1 that implicated the role of estrogen in colon carcinogenesis (30). We also observed a tendency for reduction in tumor incidence in the Soy+Gen group, although not statistically significant, thus indicating a trend towards lower colon cancer incidence with estrogen-like compounds. This is supported by the finding of Javid et al (50) who reported prevention of intestinal tumorigenesis in ovariectomized APCmin mice treated with estradiol.

Our current observation of a significant reduction in COX-2 protein levels in mice fed genistein or estrone suggests a protective action of estrogens may be mediated through attenuation of COX-2 protein levels. This finding contradicts a previous report that genistein added to a casein diet had no effect on tumorigenesis in AOM-treated rats and increased tumor multiplicity (51). They also observed no effect on COX-2 activity and a significant suppressive effect on 15-PGDH (15-hydroxyprostaglandin F2α dehydrogenase) activity by genistein. Several differences in our study may provide explanation for these opposing results. Our study was done using ovariectomized female mice, whereas Rao et al (51) used
male rats (47, 51). It is possible that the response to AOM and/or the dietary treatments differs between males and females and between rats and mice. Moreover, the synergistic effect of soy protein with estrogenic compounds compared to the casein-based diet with added genistein (51) might explain the differences.

The primary therapy for patients with hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) are the selective COX-2 inhibitors (23, 28, 36, 38). Recently, COX inhibitors have been taken off the market because of serious side-effects including cardiovascular risks (52, 53). The need for alternative COX-2 inhibitors without side-effects makes this study of significance for further research. COX-2 is the rate limiting step in the generation of prostaglandins including PGE2 and PGF2α (19, 36, 54). These prostanoids mediate cellular proliferation via the wnt or Ras signaling pathways (29). Although, the precise mechanism by which estrogens affect COX-2 levels in not known, several mechanisms may be proposed. One hypothesis is that genistein and E1 mediate their protective effects via estrogen receptors ERα or ERβ (28, 30, 55). A 77% increase in tumorigenesis was reported in ovariectomized Min/+ mice compared to controls that were treated with 17β-estradiol (56). Another hypothesis is that estrogen might influence through ER-independent pathways such as the wnt signaling pathway (27).

Genistein has been extensively studied and many proposed modes of action have been proposed apart from its phytoestrogenic activity in the regulation of cell growth (57). In the present study, we observed inhibition of COX-2 protein levels in
response to short term feeding of genistein. It would be interesting to observe the effects of genistein with chronic administration and to measure COX-2 protein levels as well as activity. More studies elucidating the mode of action of genistein with regard to dosage, time of exposure in combination with other dietary factors such as soy are essential to understand the role of genistein and estrogenic compounds and their synergistic effect in colorectal carcinogenesis.

In conclusion, we found that dietary genistein and estrone significantly reduced COX-2 protein levels in AOM-treated C57BL/J6 mouse colon. The mechanism of action leading to attenuation of COX-2 protein levels remain to be elucidated. It is likely that the effect of genistein in reducing COX-2 protein levels is mediated via an ER-dependent pathway. However, the possibility of a cross talk between the wnt signaling pathways, tyrosine protein kinase pathway cannot be ruled out.
Figure legends:

Figure 3.1. A representative immunoblot in azoxymethane (AOM) treated colonic mucosa of mouse. An internal standard comprised of a sample that was run on each gel (R), as was the COX-2 standard (std).

Figure 3.2. Body weight of mice fed experimental diets

Figure 3.3. Body weight of mice at 14 and 15 weeks of age. Mice fed Soy-IF weighed more than the Soy+Gen mice at these time points. \( P \leq 0.006 \)

Figure 3.4. Abdominal fat expressed as percent of body weight in mice fed the experimental diets. Mice fed Soy+IF had more fat compared to mice fed Soy+Gen \( P \leq 0.005 \)

Figure 3.5. Mean number of Aberrant crypt foci (ACF) in mice fed experimental diets. Total number of ACF in each diet group was determined by 2 independent observers. Data expressed as mean with standard error shown in T bars. There was no significant difference (\( P \leq 0.05 \)) due to diet treatment.

Figure 3.6 Effect of experimental diets on COX-2 protein levels in azoxymethane (AOM) injected mice. Different letters on the bars represent significant differences among diets (\( P \leq 0.01 \)). There was no difference with Casein and Soy-IF diet group.
* Indicates a significant difference between casein and Soy+E1 ($P \leq 0.004$) and Casein and Soy+Gen ($P \leq 0.006$) in the least square mean of 9 rats ± SEM.
LITERATURE CITED FOR CHAPTER 3


multicenter, case-control study in the United States. Cancer Causes and Control 8: 146-158.


Table 3.1. Final body weight, fat and organ weights in mice fed experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body weight*</th>
<th>Abdominal fat</th>
<th>Colon</th>
<th>Cecum</th>
<th>Kidney</th>
<th>Heart</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n g</td>
<td>g/100 g body weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>7 27.7±0.68ab</td>
<td>7.89±0.56ab</td>
<td>0.90±0.05ab</td>
<td>1.98±0.10a</td>
<td>0.87±0.02ab</td>
<td>0.42±0.01a</td>
<td>0.34±0.02ab</td>
</tr>
<tr>
<td>Soy-IF</td>
<td>8 30.4±0.64b</td>
<td>8.94±0.52b</td>
<td>0.79±0.04b</td>
<td>0.65±0.09b</td>
<td>0.78±0.01b</td>
<td>0.40±0.01a</td>
<td>0.30±0.02b</td>
</tr>
<tr>
<td>Soy+Gen</td>
<td>9 26.7±0.60a</td>
<td>5.62±0.49a</td>
<td>0.91±0.03ab</td>
<td>1.68±0.09a</td>
<td>0.97±0.01ab</td>
<td>0.46±0.01a</td>
<td>0.38±0.02ab</td>
</tr>
<tr>
<td>Soy+E1</td>
<td>9 28.6±0.60ab</td>
<td>7.39±0.49ab</td>
<td>1.04±0.003a</td>
<td>1.92±0.09a</td>
<td>0.97±0.01a</td>
<td>0.49±0.01a</td>
<td>0.40±0.02a</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Means in a column without a common letter differ, P≤0.005 (ANOVA and Tukey)
* Difference in body weight between Soy-IF and other groups were significant only during the last week
Figure 3.1 A representative immunoblot
Figure 3.2. Body weight gain of mice fed experimental diets
Figure 3.3. Body weight of mice at 14 and 15 weeks of age
Figure 3.4 Abdominal fat expressed as a percent of body weight
Figure 3.5 Mean number of Aberrant crypts across different diet groups
Figure 3.6 Quantification of COX-2 protein expression in mice fed experimental diet

[Bar graph showing the quantification of COX-2 protein expression for different diets: Casein, Soy-IF, Soy + Gen, Soy + E1. The bars are labeled with letters a and b to indicate statistical significance.]
CHAPTER 4
DISCUSSION

It has been suggested that populations that consume soy have decreased incidence of CRC (1, 2, 4, 28). However, epidemiological and animal studies examining the relationship of soy and soy isoflavone intake and risk of CRC have produced conflicting results (28, 41, 42) (Table 1.1). Some epidemiological and case controlled studies have looked for a role for estrogen in CRC (45, 128, 128). For example, the outcome of the Women's Health Initiative (WHI) trial provided evidence for a protective role for estrogen in colon cancer risk with a 20% reduction in CRC (47, 49). However, animal studies are a means to deduce the direct role of estrogen and estrogenic compounds such as phytoestrogens (genistein) in the molecular mechanism of CRC. One possible mechanism for estrogen to affect CRC is through COX-2. NSAIDs and COX-2 inhibitors are the primary therapy for FAP patients and have been shown to decrease or regress CRC (130, 131). Although, there is evidence of estrogen influencing COX-2 in cerebral blood vessels (113), vascular system (114) and uterine tissues (138), the correlation between estrogens and COX-2 in colon has not been examined. Previously, we found diets containing soy protein with estrone decreased tumor incidence in mice (36). To further elucidate whether estrogen's influence on CRC is via suppression of COX-2 protein levels, this short term study was conducted.

Optimization of Western blot technique

In order to develop the methodology to quantify the COX-2 protein levels by western immunoblot (WIB) we ran several trials using samples obtained from mice
that had been treated with AOM for one or two weeks. As part of the method refinement, the lysis buffer used to collect the colonocytes was arrived at after several trials using lysis buffers from Singh et al (139) and Davidson et al (140).

Initial attempts to detect COX-2 protein were discouraging as we were able to detect only the positive control (Sigma, St. Louis, MO). Further trials with another positive control from Cayman Chemicals, Ann Arbor, MI and primary antibody also yielded mixed results. (Positive control – Catalog no. 160126, 10004910, primary antibody catalog no. 160106). We were able to detect only the positive control when loaded in high amounts. We also attempted to load greater than 150 μg of protein into each well. We tried different detection systems such as Super signal West Pico mouse IgG detection kit (Pierce, Rockford, IL), ECL plus western blotting detection system (Amersham Biosciences, Buckinghamshire, UK). We concluded that the amount of COX-2 expressed is too low in mouse colon samples. However, the Pierce West Femto Super sensitivity substrate yielded good detection of the COX-2 protein in the samples along with the positive control of COX-2 from Sigma, St. Louis, MO. This protocol allowed us to consistently visualize COX-2 in our samples. We relied on one mouse sample as an internal sample control that was repeated in each gel to adjust film exposure time to minimize inter-gel variance. We also optimized the protocol for β-tubulin to be used as a loading control for each sample.

**Quantification of COX-2 protein levels in samples**

In the WIB experiments each sample was repeated twice and the average of the normalized values was used for statistical analysis. To normalize, the OD ratio of COX-2 and its corresponding β-tubulin value for each sample was determined and
then a correction for inter gel variation done using the internal sample control. To generate the correction factor the ratio across all gels of the COX-2 and corresponding β-tubulin of internal sample control was averaged. Then the ratio of this average value to the individual internal sample control value for an individual gel was calculated and used as a correction factor to normalize the COX-2 OD for each sample.

We determined the coefficient of variance of repeat measures to check for the repeatability of the WIB with the same samples. To arrive at the coefficient of variance we averaged the OD of internal sample control value across all gels and determined the standard deviation. The percentage ratio of standard deviation to mean gave the coefficient of variance. The inter gel variation of COX-2 was 13% which is a very low variation. The intra gel variation was (<2%) suggesting little variance in loading as well as repeatability of the WIB. (Table 5.1) shows the mean and the standard deviation of the internal standard control across the gels and the coefficient of variance obtained in percent.

We also did a paired t-test and a one-way analysis of variance test to account for variability for the repeatability of measure values between the samples and within the samples which gave a low variance (0.045) suggesting little variability between repeatability.

Our hypothesis that dietary estrogens (estrone and genistein) suppresses the COX-2 protein levels in colon was demonstrated with a significant decrease in COX-2 levels in the mice fed estrone and genistein with soy protein in this short term study. To our knowledge this is the first study that demonstrated that oral dietary estrogens influence COX-2 protein levels in colon of mice. This observation is
supporting our earlier report of decreased tumor incidence in mice fed estrone suggesting the protective effect of estrone being mediated by suppression of COX-2. This finding is also in line with earlier reports of Singh et al (139) who reported the modulation of COX-2 protein levels in AOM-treated rat colon by high fat corn oil and high fat fish oil diet. The high fat fish oil decreased COX-2 expression in colon mucosa and tumors but a reverse effect was seen with high fat corn oil. However, they did not observe the effect of dietary estrogens on colon of mice. Another report from the same group contradicts our observation of suppression of COX-2 by dietary genistein. In AOM-treated rats fed casein based genistein diet an increase in tumor multiplicity with no effect on COX-2 activity and tumor incidence was reported (38). They also reported a significant suppressive effect on 15-PGDH (15-hydroxyprostaglandin F2α dehydrogenase) activity by genistein. Several differences in our study may provide explanation for these opposing results. Our study was done using ovariectomized female mice compared to male rats. Differences in response to AOM, the dietary treatments before and after carcinogenic treatment and response due to gender difference might have influenced the outcome. It is also important to note that they did not measure the COX-2 protein levels or the PGE2 levels, the major PG that has been demonstrated to influence tumorigenesis. They had measured 8-isoprostane that is similar to PG and is generated by nonenzymatic metabolism of AA (103)

Our observation of suppression of COX-2 by dietary estrogens is novel and has potential significance. The precise mechanisms by which estrogens mediate this effect are not clearly known. Several hypotheses to explain this observation are possible. One hypothesis is that the dietary estrogens mediate their suppressive
effects via ER, possibly by ERβ. Normal human colon expresses both ERα and ERβ but ERβ appears to be the dominant receptor in the colon that is selectively lost in malignant tissues (51-53). Ospina et al (113) observed repression of COX-2 gene by estradiol in cerebral blood vessel when stimulated with IL-β in a ER dependent manner. Also, in human uterine microvascular endothelial cells that expressed ERβ mRNA and protein abundantly, treatment of estradiol (10⁻¹⁰ to 10⁻⁶ M) increased COX-2 mRNA levels by 2.3 fold suggesting upregulation of COX-2 being mediated in an ER dependent pathway (114).

In human colon cancer DLD-1 cells genistein and other chemopreventive compounds including resveratrol and quercetin having resorcin moiety suppressed the COX-2 promoter activity with and without TGFα- stimulation suggesting modulation at the promoter level by these estrogen like compounds (115). Furthermore, Hertrampf et al (138) reported tissue specific modulation of COX-2 expression in the uterus and vena cava by estrogens and phytoestrogens. They found that administration of 17β-estradiol (3μg/mg body weight/day for 3 days) in ovariectomized female rats stimulated COX-2 mRNA expression in the uterus but downregulated in the vena cava. Also a dose-dependent downregulation of COX-2 in vena cava was observed with administration of genistein at very low dose (0.5 mg/kg of body weight) with no effect in the uterus. These studies suggest a transcriptional effect of estrogentic compounds on COX-2 (115). There is also some evidence suggesting regulation of COX-2 mRNA expression by at least two different molecular mechanisms including modulation of the transcription rate of the gene and/or by post-transcriptional regulation of mRNA stability (141). It is possible that estrogen is influencing this effect in an ER dependent pathway. Also, the two
receptors (ERα and ERβ) may function in opposite ways in modulating the effects of estrogen. The presence of different isoforms of ERβ as well as the ability of ER to form homo or heterodimers suggests a complex mechanism that involves tissue specific modulation of COX-2 by estrogen. These ligand (estrogen/phytoestrogen) bound ER form a stable dimer that then interacts with specific estrogen response elements to modulate the transcription of target genes.

Another hypothesis is that genistein and estrone alter ER-independent pathways in the colon. Weak estrogenicity of genistein and its other purported properties in inhibiting tyrosine kinases (29), DNA topoisomerasers (142) and regulation of cell growth might possibly mediate this suppressive effect. However, our data does not address this concept that genistein suppresses COX-2 via inhibition of intracellular signaling pathways related to tyrosine kinases or DNA topoisomerasers. Moreover, estrogen can also mediate its effect via nonnuclear estrogen-signaling pathway. These cell membrane receptors that are suggested to be similar to intracellular ER are located in cell membrane invaginations called caveola and are linked to the mitogen-activated protein kinase pathway resulting in rapid non nuclear effect such as short-term vasodilation of coronary arteries (143). There is also evidence suggesting a cross-talk between different signaling pathways such as wnt and ras influenced by estrogen (125). In the uterine tissue, estradiol increased the expression of wnt4 protein thereby activating the canonical wnt signaling pathway and promoted growth via modulation of downstream regulatory genes of β-catenin such as cyclin-D, c-myc, c-fos and COX-2 in an ER-independent pathway (124). Differential expression of COX-2 via the wnt signaling pathway has also been reported in colon epithelial cells that differ in APC (123). These reports
suggest a complex interplay of different signaling pathways that estrogen might possibly influence in modulating the COX-2 protein levels.

The metabolism of estrogens in the colon has not been well described, although 17βHSD activity and aromatase/cytochrome P450 activity are present in colon. Peripheral synthesis of estrogen and the interconversion of estrone and estrodiol depending on the reducing or oxidizing activity 17βHSD also might influence the estrogen mediated suppressive effect of COX-2. Inactivation of estradiol via conversion to estrone has been suggested to have a protective effect while loss of this inactivation may lead to cell proliferation via an ER-dependent pathway (59). Our study does not specifically address this question but suggested a protective effect of estrone via suppression of COX-2.

Thus, many questions remain to be answered including the role of estrogen receptors in mediating the effect of phytoestrogens on colon cells, the role of estrogen receptors and estrogen metabolizing enzymes in the colon, changes in estrogen receptor expression and/or responsiveness during the carcinogenic process and the overall role of dietary factors on estrogen receptors and estrogen related pathway in colon.

We also attempted to investigate the correlation of ACF and mucin depleted foci (MDF) with dietary factors. Our rationale to do MDF along with ACF was for the following reasons.

1. Numerous studies have used ACF as a short-term marker for predicting colon carcinogenesis especially for chemopreventive compounds (34, 35). However, conflicting results on the association between ACF and tumor development have been reported (86, 87). Zheng et al (86) reported a higher
tumor incidence in rats administered 2-carboxyphenyl retinamide (2-CPR) compared to controls, but reduced ACF incidence previously. Moreover, rats fed cholic acid had fewer ACF but more tumors than rats fed a control diet which suggest ACF may not correlate with tumor incidence (86, 87). Also, the incidence of ACF in genetically susceptible strains and resistant strains of mice were similar but differed in tumor incidence suggested a lack of correlation of number of ACF with tumorigenesis (88). These results suggested that ACF may be a preliminary biomarker but other events are involved in advancing ACF to adenoma or adenocarcinoma.

2. It has been suggested that ACF with mucin depletion and β-catenin accumulation along with other genetic alterations correlated to progression of ACF to adenoma-carcinoma (133, 144, 145). Mucin depletion of ACF has also been observed in human colon cancer specimens (133). Figure 4.1 presented from reference (133) depicts the significance of ACF with MDF (1.3% of ACF) and β-catenin accumulated crypts with their correlation to tumorigenesis.

Thus, in order to have a better preneoplastic marker and to investigate the role of estrogen and genistein in modulating ACF and MDF and their correlation, we attempted to quantify both ACF and MDF. However, we faced difficulty in clearly distinguishing the MDF. One possible reason for our inability to identify MDF could be due to the low percent of ACF with mucin depletion in our samples. Considering the incidence of ACF in our study, 5-8 per colon the probability of detecting MDF was very low (~1%) (133). Other factors such as the short duration of the study, and
the potential effect of diet and estrogenic compounds also might have influenced the outcome.

Our observation of no effect of dietary estrogens on ACF does not agree with other studies studying the effect of soy and soy isoflavones on ACF (34, 37). The use of different chemicals in inducing ACF such as AOM or dimethylhydrazine (DMH), the time of exposure to the carcinogen, species differences, as well as dietary regimens before or after carcinogen exposure might have influenced these results.

Figure 4.1 Aberrant crypt foci, Mucin depleted foci and β-catenin accumulated crypts

results. However, it is not clear if ACF are true markers of tumor development and the lack of response of ACF in the Soy+E1 fed mice would suggest that at least in the AOM-treated C57BL/J6 mouse they are not predictive of tumor development.

Mice fed the experimental diets gained weight similarly throughout the study (Figure 3.2). However, slight differences in body weight due to diet were observed at termination (Table 3.1). The differences in body weight occurred during the last 2 weeks of the study (Figure 3.2). Mice fed Soy-IF weighed slightly more than mice fed Soy+Gen. Mice fed Soy-IF had more abdominal fat than mice fed Soy+Gen which may partially explain the differences in body weight. We expressed organ weights as a percent of body weight to correct for differences in animal size. We observed that organ weights including colon, cecum, spleen and kidney of mice fed Soy-IF diets were significantly lower than from mice fed Soy+E1 ($P \leq 0.005$) (Table 3.1). The reason for this is not known.

The primary therapy for patients with HNPCC and FAP has been COX inhibition. COX-2 expression is rapidly induced by growth factors, oncogenes, and tumor promoters. Previous research has shown elevated levels of eicosanoids, particularly PGE2 during initiation and post initiation stages generated by COX-2 activity as well as the COX-2 activity increases with more advances of tumor (98, 106, 146). COX-2 is the rate limiting step in the generation of prostaglandins including PGE2 and PGF2α that may mediate cellular proliferation via the wnt or Ras signaling pathways (125). Also, it is been suggested that COX-2 generated prostaglandins mediate various physiological effects including apoptosis, immune modulation, and angiogenesis as well as modulation of second messenger signaling pathways such as elevation of intracellular cyclic AMP(102). Thus, our observation
of soy protein with estrone and soy protein with genistein in suppression of COX-2 is of potential significance. There is also a need to investigate the complex molecular mechanisms that are involved in the tissue specific regulation of the COX-gene by estrogenic compounds in the colon.

This observation is helpful in the use of plant-derived phytochemicals as chemopreventive agents which circumvent the various side effects associated with COX inhibition by NSAIDs as well as COX-2 specific inhibitors. However, genistein may not be completely without negative side effects. There is evidence for reproductive, genetic, thyroid and developmental toxicity from animal studies of genistein exposure (147, 148). There is also controversy with the exposure of genistein in infants fed soy formula and their physiological effects on the immune system as well (148). Thus, research in this area is still in its nascent stage in determining their overall affect as well as in colon carcinogenesis.
LITERATURE CITED FOR CHAPTERS 1, 2, AND 4


immunohistochemical observations of mucin-depleted foci (MDF) stained with Alcian blue, in rat colon carcinogenesis induced with 1,2-dimethylhydrazine dihydrochloride. Cancer Sci 95: 792-797.


APPENDIX
Table 5.1 Inter and intra-gel coefficient variances (CV) of western blotting

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<thead>
<tr>
<th>Inter-gel</th>
<th>COX-2</th>
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<tr>
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<table>
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<td>n=4</td>
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</table>
Example experiment data sheet

Experiment #  

**TITLE:**
Western Blot Analysis of COX-2 expression in Casein, Soy-IF, Soy+Gen, Soy+E1 fed ovariectomized C57BL/J6 mice

**PURPOSE:**
In this experiment, COX-2 protein levels will be examined in mice colon that were fed 4 diet groups viz. Casein, Soy-IF, Soy+Gen, Soy+E1 after 6 weeks of last dose of 6 weeks of AOM (10mg/kg of body weight). The colon cells were scraped using a microscope slide and collected in a lysis buffer (150mM NaCl, 50mM hepes with 1% protease inhibitors) that were flash frozen in liquid nitrogen and ultrasonicated for 3X15 secs, 15 secs apart and spun at 120,000 g called the supernatant (SN) and stored in -80°C at Food sciences building

The tubes used were the 100ul aliquots from the samples spun on Oct 17, 05  
From the earlier results that indicated thicker, stronger bands at 1 min exposure of film and 5 min exposure we decided to go for lesser concentration of the protein.

10/21/05: Quantified the protein by BCA protein assay kit.

1. Block 1.5% BSA and 1.5% milk in TBS
2. Detection system: West Femto super sensitivity substrate
3. Santa-Cruz 1° antibody (1:10,000) – overnight incubation
4. 2° antibody mouse (goat anti-mouse HRP conjugated) at 1:50,000 for anti-cox-2 and 1:5000 for b-tubulin
5. COX-2 standard (1ul (0.47mg/ml) diluted in 1400ul of 1X loading buffer)

**METHODS:**
The Western protocol is using 0.1% SDS in the running buffer and using 10% minigels made as follows:

<table>
<thead>
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<th>FOR 2 MINIGELS (1.5mm thick)</th>
<th>10% resolving</th>
<th>4% stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/0.8% Bisacrylamide</td>
<td>6ml</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>1.5M Tris, pH 8.8</td>
<td>5ml</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>0.5M Tris pH 6.8</td>
<td>8.10 ml</td>
<td>3.07 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td>200ul</td>
<td>50 ul</td>
</tr>
<tr>
<td>10% SDS</td>
<td>20ul</td>
<td>5 ul</td>
</tr>
<tr>
<td>TEMED (Biorad #161-0800)</td>
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<td>25 ul</td>
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<tr>
<td>APS (10%)</td>
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<td>Kaleidoscope prestained standards control – 99462 – Bio-Rad</td>
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<td>Cox-2 std</td>
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<td></td>
<td>Human, recombinant expressed in Sf21 cells, Sigma</td>
</tr>
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<td>Sc-19999</td>
<td>H2504</td>
<td>1:10,000</td>
</tr>
<tr>
<td>2°Cox-2</td>
<td>Sc-2005</td>
<td>B2505</td>
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<tr>
<td>□-tubulin</td>
<td>SC-5274</td>
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<tr>
<td>West femto substrate</td>
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### Gel PBCOX-2

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<th>6X SB</th>
<th>1XSB</th>
<th>Total volume/well - ul</th>
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#### Experiment Data Sheet

**Cox-2 Western Experiment #PBCox-2-03**

**Times:**

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<tbody>
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<td></td>
</tr>
<tr>
<td>Resolving gel polymerizn</td>
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<tr>
<td>Stacking gel polymerizn</td>
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<tr>
<td>Load samples</td>
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</tr>
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</tr>
<tr>
<td>volts</td>
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<td>Start/end current of run</td>
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<tr>
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