EFFECTS OF ESTROGEN AND PHYTOESTROGENS ON THE DEVELOPMENT OF COLONIC INFLAMMATION

A Senior Scholars Thesis

by

KARI M'LYNN GALIPP

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Majors: Nutritional Sciences and Biology

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Approved by:

Research Advisor: Clinton Allred
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Effects of Estrogen and Phytoestrogens on the Development of Colonic Inflammation.
(May 2012)

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Colon cancer exhibits the third highest cancer mortality rate of the US. Patients with inflammatory bowel disease (IBD) display a much higher risk for development of the disease. Women experience protective effects against colon cancer due to naturally increased levels of estrogen compared to men. Preliminary studies were performed to determine methods of producing an IBD mouse model displaying suitable levels of inflammation for future studies regarding the effects of estrogen in a precancerous state. By inducing colon inflammation to create an IBD model, appropriate levels of estrogen supplementation will be obtained to enhance the results of future investigations. In phase one, male mice were exposed intrarectally to varying amounts of trinitrobenzene sulfonic acid (TNBS), and a relevant IBD state was achieved by the 3% TNBS dose, which was then used in phase two. For this phase, colitis was induced with TNBS in ovariectomized (OVX) female mice supplemented with varied levels of estrogen. TNBS induced inflammation and was not detrimental to mice at the 3% TNBS solution exposure level. OVX mice supplemented with estrogen achieved the colitis state without

additional health implications. Mice supplemented with .5 mg implants displayed estrogen blood levels half the value of those with 1 mg estrogen implants. Results obtained in this pilot study represent the first step toward determining how estrogen may decrease the risk of inflammation-associated development of colon cancer.

ACKNOWLEDGMENTS

The completion of this program and the following research would not have been possible without the expert guidance and direction provided by all the members of the Allred laboratory. I cannot express enough gratitude for all the opportunities I have been given and the doors that have been opened for me as the result of their support. I am extremely thankful for Dr. Allred's investment in my academic career and the privilege to work with such dedicated, intelligent scientists as Kim, Cameron, Charles, Carlos, and other students and faculty involved in my experience. I have developed a vast appreciation for research and active science through my participation in the lab and the numerous procedures and studies I have been able to assist.

I would like to extend a special thank you to Cameron Armstrong for diligently coordinating our projects with my less than accommodating schedule and helping me gain so much knowledge that cannot be taught in the classroom. I am very appreciative of Kim Allred for recognizing my genuine interest and rough talent in the lab, and polishing those skills to increase my fascination even further. Finally, I am very thankful for Dr. Allred's guidance and support of my future medical career, despite our frequent conversations to persuade my further interest in graduate school.

Additional acknowledgments are extended to those responsible for the funding of this research, including the American Institute of Cancer Research and the International Life Sciences Institute.

NOMENCLATURE

EIA	Enzyme Immunoassay
ER	Estrogen Receptor
ERα	Estrogen Receptor-α
ERβ	Estrogen Receptor-β
IBD	Inflammatory Bowel Disease
OVX	Ovariectomized
TNBS	Trinitrobenzene Sulfonic Acid
WT	Wild Type

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CHAPTER I

INTRODUCTION

Colon cancer

Colon cancer is the third most prevalent type of cancer diagnosis within the US population, and also is the third highest cause of cancer mortality. An estimate of 141,210 people will be diagnosed with the disease and 49,380 deaths will be caused by colon cancer in 2011, according to predictions of the American Cancer Society (1).

Colon cancer affects both men and women, especially in developed nations, and is considered a hormone-dependent cancer, largely influenced by estrogen. The influence of estrogen is evident due to the low number of women inflicted with the disease compared to men, as well as the protection exhibited by hormone supplementation. The risk factors associated with colon cancer range from age, gender, family history, previous infliction with colonic diseases, and exposure to procarcinogens in the environment and the diet. Current treatments consist of surgery, chemotherapy, and radiotherapy. The desire to shift toward chemopreventive actions focuses on the examination of dietary modifications and hormone supplementation for disease intervention and control (2). Diet is a factor that can easily be adjusted and offers a unique opportunity to target the colon through exposure to digested compounds (3).

This thesis follows the style of The Journal of Nutrition.

The development of colon cancer is promoted by oxidative stress, inflammation, genetic damage, and changes to normal molecular processes (2). The progression can begin as a result of chronic inflammation, which is a characteristic of inflammatory bowel disease (IBD). Patients with IBD display an increased risk of developing colon cancer, and insight to this correlation can be gained through an animal model of the disease.

Estrogen

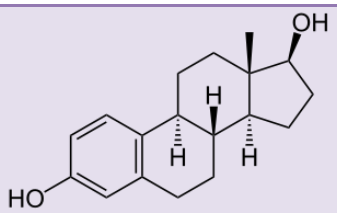
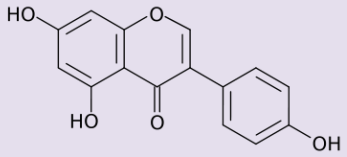
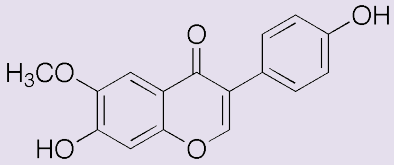
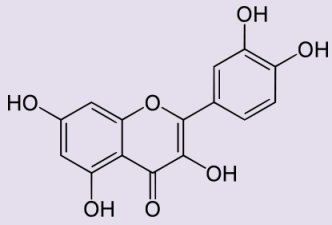
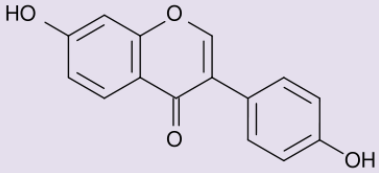
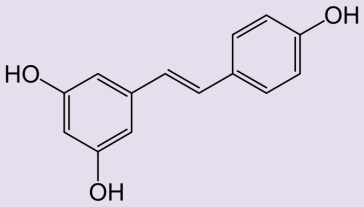
Estrogen exhibits a protective role against the development of colon cancer as the hormone substrate of estrogen receptor beta (ERβ), the predominant estrogen receptor of the digestive tract epithelium. As the disease state of colon cancer advances, the expression of ERβ declines, influencing many aspects of the progression pathway, such as the inhibition of cell cycle arrest and decrease of apoptosis (4). The correlation of estrogen and colon cancer prevention is evident through the larger risk of colon cancer for men compared to women, along with the additional decreased risk for women who use hormone replacement therapy and oral contraceptives compared to those who do not receive supplementary hormones (5).

Phytoestrogens

Phytoestrogens are natural plant derivatives present in the diet that structurally resemble estrogen (**Table 1**) and have the ability to imitate its function by binding to estrogen receptors (4). Phytoestrogens exhibit a broad range of potential health benefits including antioxidant, anti-inflammatory, and free radical reduction (2).

There are three major classes of phytoestrogens, consisting of isoflavones, lignans, and coumestans. Isoflavones, especially genistein, have received the most attention for their potential biological activities and are found in soybeans, legumes, and peanuts (6). The absorption of phytoestrogens utilizes the intestinal microflora, and is an important process for the proper metabolism of these compounds and their eventual ability to affect the pathways leading to colon cancer (4).

Table 1 – *Common Phytoestrogen Structures:* A comparison of phytoestrogens to the endogenous form of estrogen, estradiol (7).

Estradiol		Genistein	
Glycitein		Quercetin	
Daidzein		Resveratrol	

Phytoestrogens possess many possible mechanisms that could account for their effects on colon cancer pathways. The most obvious method of phytoestrogen action is through its interaction with estrogen receptors, and its high binding affinity for ERβ to produce the effects of hormonal mechanisms. However, phytoestrogens also display ER-independent effects on colon cancer pathways, including the regulation of gene expression that influences signaling cascades and reduces proliferation, inflammation, metastasis, and angiogenesis (2). The chemopreventive effects of phytoestrogens can infuence both the initiation and promotion of colon cancer tumors. The mechanisms include many different targets that can be studied through cell-culture models, the use of animal experiments, and clinical trials.

To begin to study the roles of estrogen and phytoestrogens in inflammation-associated colon cancer, we aimed to develop an IBD mouse model for use in future investigations. The inflammatory agent trinitrobenzene sulfonic acid (TNBS) was selected for its potential to induce inflammation in the colon emulating that observed in ulcerative colitis. We sought to determine the levels of TNBS necessary to achieve the amount of inflammation relative to that detected in the colons of human IBD patients. Another parameter of the study tested the coordination of estrogen supplementation levels with TNBS exposure and the relationship between the two compounds within the animal model.

CHAPTER II

MATERIALS AND METHODS

The materials and methods utilized to conduct the study were carried out as a pilot study for future investigations. A sample group of mice was induced with colon inflammation using variable amounts of TNBS. Following the stimulation of colitis, the animals were sacrificed to collect data to determine the optimum level of TNBS exposure that resulted in the most accurate representation of IBD symptoms and the disease state, while causing fewer unwarranted ailments. The second phase observed the effects of estrogen supplementation and its suitable combination with the TNBS exposure. The findings will be used in future studies to induce inflammation in mouse models to observe additional parameters of estrogen influence on colon cancer.

Mouse model

Sexually mature C57BL/6 WT mice were utilized in both phases of the study. The first phase used male mice that received no estrogen supplementation. During the second phase of the trial, female mice were ovariectomized and implanted with supplemental pellets of estradiol (E_2) two weeks prior to initial TNBS treatments. The females received sylastic implant pellets in doses of .5 mg E_2 + 19.5 mg of cholesterol, or 1 mg E_2 + 19 mg cholesterol. All mice were weighed prior to treatment.

TNBS

TNBS induction of colitis is an important parameter of the study, with the purpose to mimic inflammation indicative of ulcerative colitis. TNBS was administered to the animals via intrarectal injection. The TNBS solutions were prepared according to the guidelines of Nature Protocols (8).

The first phase of the study consisted of 5 experimental groups of 4 male mice apiece. Each group was assigned a specific concentration of TNBS, including a control group (0%), 1, 2, 3, and 4% TNBS solutions. The animals were presensitized to the TNBS solutions to promote increased colonic response one week prior to intrarectal treatment, as indicated in **Figure 1**. Dermal presensitization to TNBS is thought to increase subsequent immune response to the chemical (8). Animals were treated with TNBS 5 days prior to sacrifice, and the optimum TNBS concentration found in this stage was utilized in Phase 2 of the study.

Phase 2 utilized 16 female OVX mice divided into 4 trial groups. Two groups were supplemented with .5 mg E_2, and two received 1 mg E_2 pellets. Presensitization occurred one week prior to TNBS treatment, which was administered 5 days prior to sacrifice. One group of each supplementation level was treated with 3% TNBS solution 3 weeks after E_2 implantation (Group A), while the remaining two groups were treated 7 weeks after E_2 exposure (Group B).

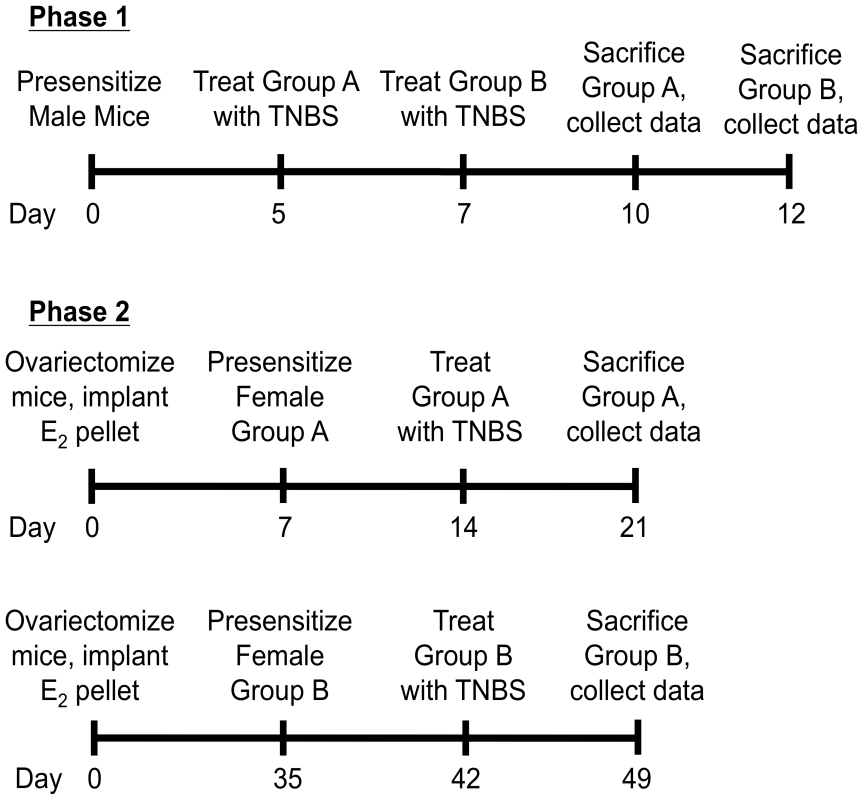


Figure 1 – *Study design.* Both Phases of the study utilized sexually mature C57BL/6 WT mice. The first Phase used male mice to set the control levels of TNBS exposure. Phase 2 used the optimum TNBS levels found in Phase 1 on female mice to observe the interactions of estrogen supplementation and TNBS-induced inflammation.

Tissue collection

Mice were weighed immediately before sacrifice, and any abnormalities in health were noted. The animals were humanely euthanized in order to collect appropriate tissues and data related to the effects of the chemical administered. The colons were collected and measured in length (cm) and weight (g). The inflammatory damage caused by TNBS exposure was assessed macroscopically. Trends in animal weight loss were investigated, as well as the ratio of colon length to colon weight as a measure of induced inflammation, which increases the ratio as length decreases and weight increases.

Enzyme immunoassay

At the time of sacrifice, the blood of the phase 2 animals was also collected for plasma analysis of E_2 levels. The Enzyme Immunoassay (EIA) was performed using the Estradiol EIA Kit (Cayman Chemicals) and following the outlined procedures.

CHAPTER III

RESULTS

Phase 1

The male mice in the first phase of the study showed a general decrease in weight across each group from presensitization to treatment, then a general increase in weight, as evident from **Figure 2**. The 4% TNBS group showed a large weight decrease, however, indicating the toxicity of the higher levels of the inflammatory agent. This group also displayed visible signs of poor health, including lethargy and dehydration.

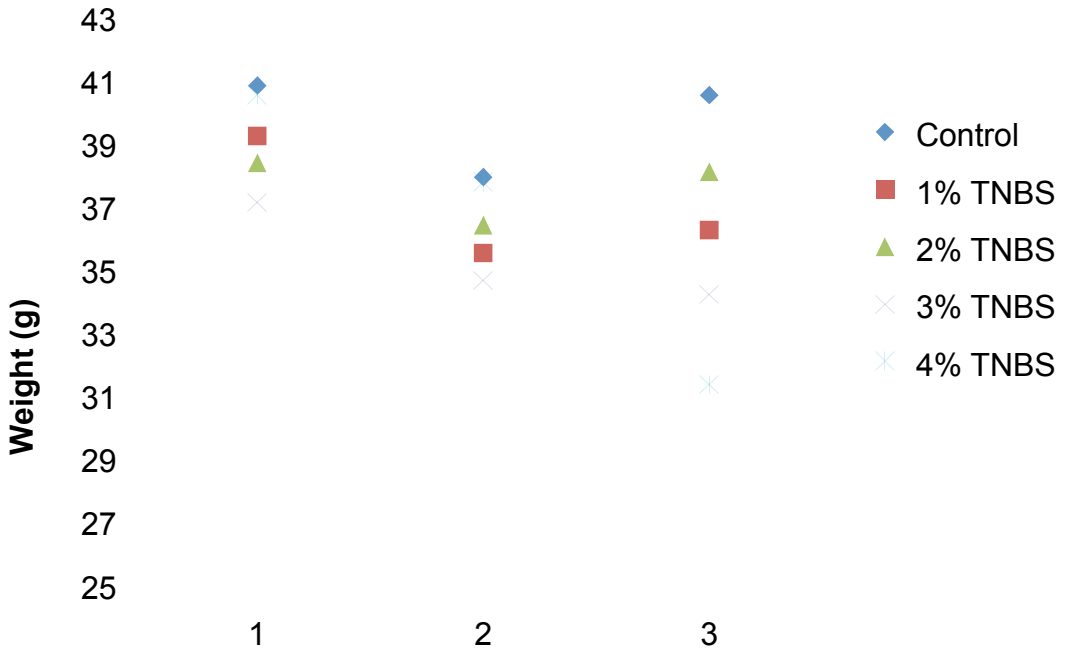


Figure 2 –*Weight of Phase 1 male mice measured at 1) presensitization, 2) treatment, and 3) sacrifice*. The data shows a general decrease in weight across all treatment groups in the first period. The 4% TNBS group displayed the only weight decrease of all groups after treatment, with an average weight loss of almost 9g per animal. Poor health and extreme colon damage were visibly evident for this treatment group, indicating the toxicity of the 4% exposure level.

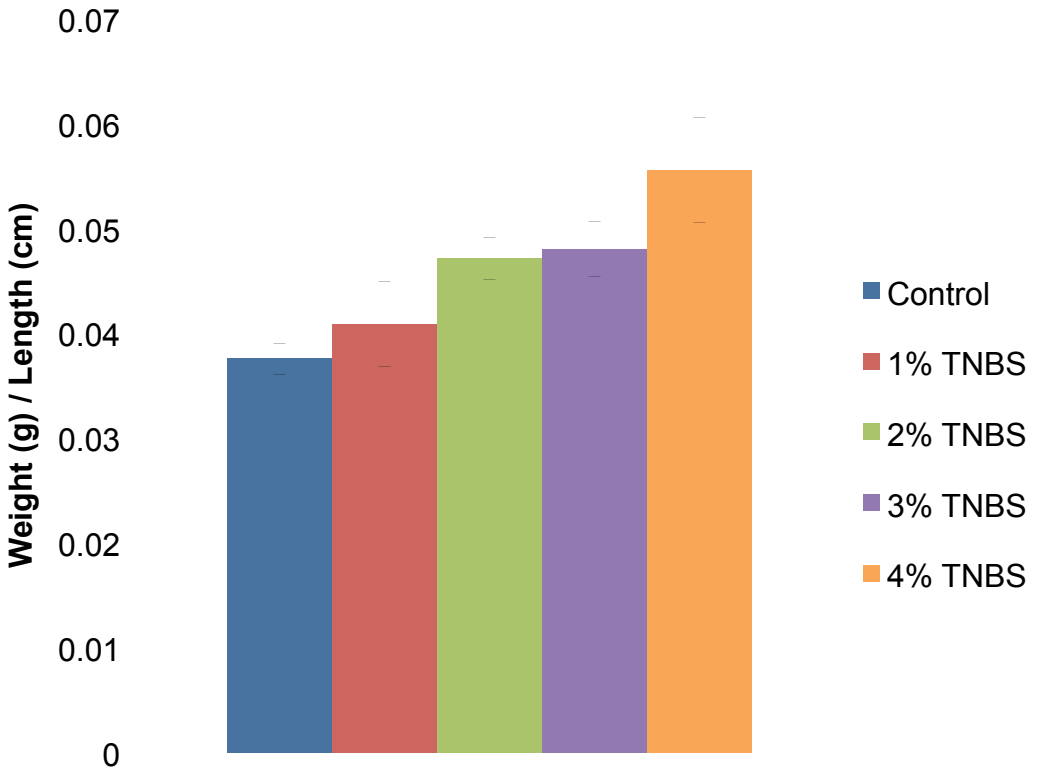


Figure 3 – *Weight to length ratio of colons per Phase 1 treatment group*. Colons of animals that received lower TNBS exposure displayed lower weight to length ratios. Groups displayed ratios of 0.0376, 0.0409, 0.0472, 0.0481, and 0.0556, respectively.

Following sacrifice, the colon of each animal was weighed and measured. In a weight to length ratio, the colons of animals that received lower TNBS doses displayed lower ratios, with a general upward trend, indicating higher levels of inflammation. The 4% TNBS group displayed a ratio of .0556, compared to the .0376 ratio of the control group. The general trend can be seen in **Figure 3**. Along with a high colon weight to length ratio, the 4% group displayed visible damage and inflammation to the colon upon removal, leading to fragile colons difficult to harvest and cleanse of excretory debris.

From the results of the first leg of the study, it was decided that the 3% TNBS exposure level provided a desirable IBD model without excess toxicity to the animal. The female mice of the second phase were treated with a 3% TNBS solution in order to monitor the estrogen variable and its relationship to the inflammation model.

Phase 2

In the short estrogen exposure group of female mice, the colon weight to length ratio exceeded those of both the male control group and standard 3% TNBS group. **Figure 4** shows the comparison of these ratios. Data could be slightly skewed, as 3 of the 4 high-level estrogen mice were sacrificed 4 days early due to adverse response to the experimental treatment.

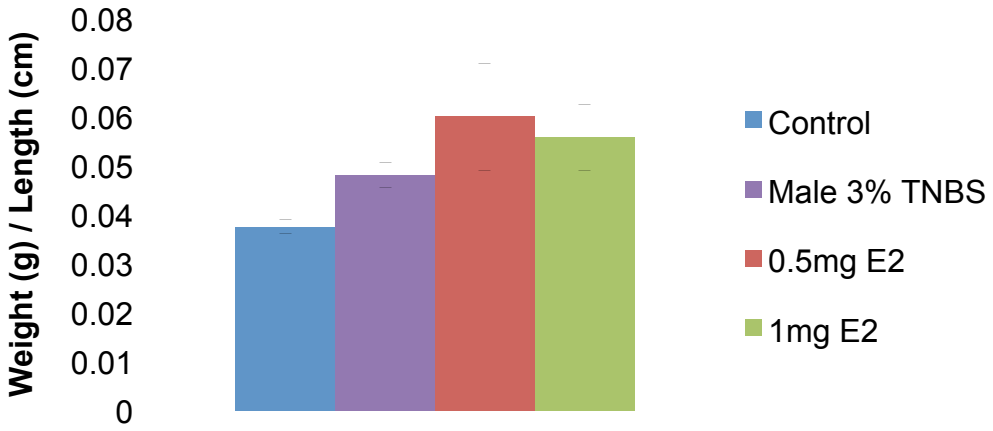


Figure 4 – *Weight to length ratio of colons in Phase 2 Group A short-term estrogen exposure*. Females receiving 0.5mg E_2 pellets displayed a ratio of 0.0600, and those with 1.0mg implants showed an average 0.0558 ratio. These ratios are compared to the 0.0376 and 0.0481 ratios of the Phase 1 male control and 3% TNBS groups, respectively.

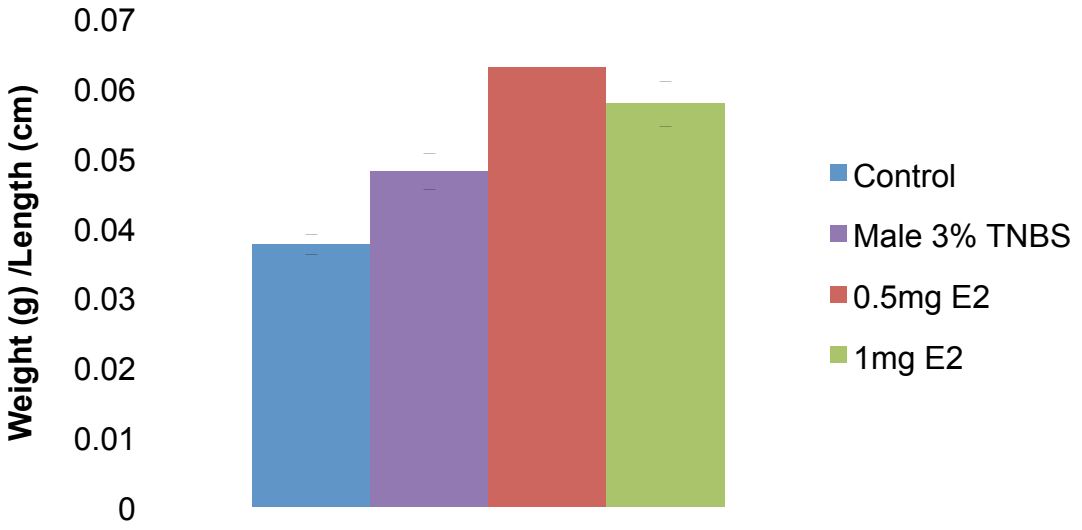


Figure 5 – *Weight to length ratio of colons in Phase 2 Group B long-term estrogen exposure*. Females receiving 0.5mg E_2 pellets displayed a ratio of 0.0630, and those with 1.0mg implants showed an average 0.0578 ratio. These ratios are compared to the 0.0376 and 0.0481 ratios of the Phase 1 male control and 3% TNBS groups, respectively.

Mice that received a longer exposure period to estrogen followed a similar trend, evident in **Figure 5**. Again, several mice did not reach the end of the study, but data from both groups were available to compare.

Estrogen levels

Finally, the estrogen levels present in the blood of 1 mg estrogen implant mice were twice those of mice that received a .5 mg implant for both the short and long exposure groups. **Figure 6** also shows that the long exposure group displayed levels of blood estrogen that were roughly half of the shorter exposure group, indicating that the levels decrease over time according to the amount of estrogen remaining in the pellet.

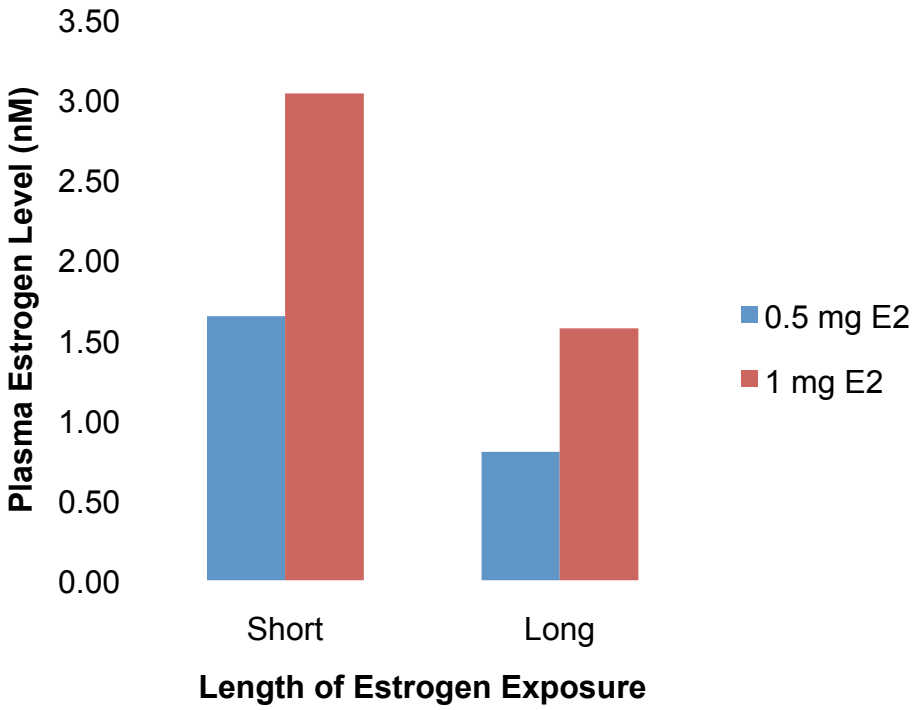


Figure 6 – *Plasma levels of estrogen measured in Phase 2 female mice*. The Enzyme Immunoassay (EIA) was performed using the Estradiol EIA Kit (Cayman Chemicals) and followed the outlined procedures. Estrogen levels present in the blood of 1mg estrogen implant mice were twice those of mice that received a 0.5mg implant for both Group A (short) and Group B (long) exposure groups. The long exposure group displayed levels of blood estrogen that were roughly one half of the shorter exposure group, indicating that the blood levels decrease over time according to the amount of estrogen remaining in the pellet.

The blood levels of estrogen were investigated to determine the proper supplementation to imitate levels displayed by a human female throughout the hormonal cycle. These levels provide a good background for future studies and suggest that a plasma estrogen level near 1.50 nM at the time of TNBS exposure should be targeted.

CHAPTER IV

SUMMARY AND CONCLUSIONS

According to the results obtained, the administration of TNBS successfully induced inflammation and was not detrimental to mice at the 3% TNBS exposure level. Although there were several premature mortalities during the study, it is not thought to correlate with TNBS exposure, but another unknown error that may be ruled out in future studies with larger sample sizes. The mortalities observed were generally confined to single cages and could be the result of cage-specific disease. In the future, mice should be caged in smaller groups to lessen the impact of the proposed cage effect.

Mice that were OVXed and supplemented with estrogen achieved the colitis state without additional health implications for other organ systems. This finding is especially important for future studies that will utilize the TNBS-induced inflammation model in place of previous problematic models. This model will be used to study the effects of phytoestrogens, particularly genistein, on the development and treatment of the inflamed colitis state.

The blood estrogen levels achieved by supplementation reflect those of a cycling human female. Mice supplemented with 0.5 mg E_2 implants displayed estrogen blood levels approximately half the value of those that received 1.0 mg E_2 implants. The ratio observed indicates that levels of estrogen circulating in the bloodstream are predictable

and can be controlled according to the concentration of the supplement. The level is therefore dose-dependent and also decreases with time as the pellet is distributed and diminished. In future studies, it would be interesting to obtain blood data periodically in order to produce a curve denoting the blood levels over time. The resulting blood levels indicate that future studies with a long-term estrogen exposure should utilize a higher concentration of estrogen, while those with shorter exposure should make use of the smaller dose of estrogen. The plasma estrogen concentration of 1.50 nM demonstrated an effective combination with TNBS administration.

Results obtained in this pilot study represent the first step toward determining how estrogen and phytoestrogens may decrease the risk of inflammation-associated development of colon cancer. The knowledge gained about the TNBS model will be utilized in future studies involving dietary phytoestrogens and their effects on inflammation in the colon. The subsequent study is currently planned and funded, and will begin once proper sample sizes are available. Future studies will also further investigate the development of colon cancer as a consequence of IBD once additional information has been obtained regarding the inflammation pathways phytoestrogens affect.

The outcomes of this study and its future counterparts will provide a strong foundation for the advancement of colon cancer treatment and prevention in humans. Because the model closely resembles the IBD state of a human colon, future findings on the effects

of estrogen and phytoestrogens will be applied to the battle against colon cancer and could lessen its devastating affects on high-risk populations. The increasing knowledge of pathways involved in colon cancer and precursor diseases may also be helpful in the study of other disease states and cancers and will have a large impact on the increased success of future medical efforts.

LITERATURE CITED

1. American Cancer Society. Colorectal cancer facts & figures 2011-2013. Atlanta, GA: American Cancer Society; 2011.

2. Pan MH, Lai CS, Wu JC, Ho CT. Molecular mechanisms for chemoprevention of colorectal cancer by natural dietary compounds. Mol Nutr Food Res. 2011;55:32.

3. Pin AL, Houle F, Huot J. Recent advances in colorectal cancer research: The microenvironment impact. Cancer Microenvironment. 2011;4:127.

4. Barone M, Tanzi S, Lofano K, Scavo MP, Guido R, Demarinis L, Principi MB, Bucci A, Di Leo A. Estrogens, phytoestrogens, and colorectal neoproliferative lesions. Genes Nutr. 2008;3:7.

5. Hartman J, Gustafsson JA. Estrogen receptors in colorectal cancer: Goalkeepers, strikers, or bystanders? Cancer Prev Res. 2010;3(8):897.

6. Lechner D, Kallay E, Cross HS. Phytoestrogens and colorectal cancer prevention. Vit and Hormones. 2005;70:169.

7. ChemSpider: The free chemical database [homepage on the Internet]. RSC. 2011 [accessed 11/10/11]. Available from: http://www.chemspider.com.

8. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. Nat Protocols. 2005;2(3):541.

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