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Decreased severity of ovarian cancer and increased survival in hens fed a flaxseed enriched diet for one year

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Abstract

Objective—With the exception of the laying hen, no other animal model of spontaneous ovarian surface epithelial cancer replicates the human disease. Flaxseed is the richest vegetable source of omega-3 fatty acids, which are chemopreventative in breast cancer and may be important in other cancers. The objective of this study was to determine if a flaxseed enriched diet had a chemopreventative effect on ovarian cancer in the laying hen.

Methods—White leghorn hens were fed a 10% flaxseed enriched or standard diet for one year. The incidence and severity of ovarian cancer were determined by gross pathology and histology in the two groups. General health markers were also measured. Eggs were collected and analyzed by gas chromatography to determine omega-3 fatty acid levels.

Results—A significant reduction in late stage ovarian tumors was detected in the flaxseed fed hens. Incidence rates of ovarian cancer were not significantly different between the two groups. The results indicate that a flaxseed diet increases overall survival in the laying hen. Flaxseed fed hens' eggs incorporated significantly more omega-3 fatty acids compared to control hens.

Conclusions—These findings show that 10% flaxseed supplementation for one year in the laying hen results in a significant reduction in the severity of ovarian cancer, but no change in the incidence of the disease. Hens fed flaxseed had overall better health and reduced mortality. These findings may provide the basis for a clinical trial that evaluates the efficacy of flaxseed as a chemosuppressant of ovarian cancer in women.

Keywords

Ovarian cancer; Flaxseed; Omega-3 Fatty Acids; Laying Hen; Dietary Intervention

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Introduction

A woman's risk of developing invasive ovarian cancer during her lifetime is about 1 in 71 and her lifetime chance of dying from this disease is about 1 in 95. Research into the discovery of early detection markers and therapeutics continues, but there are still no effective treatments for ovarian cancer today. This makes the search for effective ovarian cancer chemopreventative agents ever more important [1]. As research into the effect of nutrition on cancer development continues, it is increasingly evident that nutrition plays a major role [2]. The American Institute for Cancer Research estimates that 30–40 percent of all cancers can be prevented by appropriate diets, physical activity and maintenance of appropriate body weight [3]. Obesity, nutrient sparse foods such as concentrated sugars, refined flour products, low fiber intake, consumption of red meat, and an imbalance of omega-3 (OM-3FA) and omega-6 (OM-6FA) fatty acids all contribute to excess cancer risk [4]. OM-3FA and OM-6FA are polyunsaturated fats that are essential fatty acids because they cannot be synthesized by the body and must be obtained from the diet [5]. OM-3FA have been shown to reduce inflammation and help prevent risk factors associated with chronic diseases such as heart disease, cancer, and arthritis [4]. OM-3FA also appear to be important for proper brain and behavioral functions [4]. Epidemiologic studies indicate that populations that consume high amounts of OM-3FA have lower incidences of breast, prostate and colon cancers than do those that consume lower amounts of OM-3FA [6–8].

Dietary intervention with flaxseed to prevent cancer was first proposed by Budwig in the 1950s [9]. Flaxseed is the richest plant source of one kind of OM-3FA, alpha-linolenic acid (ALA). In several laboratory studies, flaxseed inhibited the formation of colon, breast, skin and lung tumors [10]. Flaxseed supplementation reduces epithelial cell proliferation in female rats, suggestive of a protective effect against breast (and possibly ovarian) cancer [11]. Flaxseed supplementation significantly reduces the incidence of breast tumors in the dimethyl benzanthracene (DMBA) rat model, as well as protects against colon carcinogenesis [12,13]. The addition of 10% flaxseed to the diet decreases metastatic spread in breast cancer xenograft models [14]. These data indicate that flaxseed supplementation may act to suppress metastatic cancer spread as well as prevent initial tumor formation.

Animal models that can be used for large scale dietary intervention studies for human ovarian cancer are lacking. We, and others, have characterized the laying hen (*Gallus domesticus*) as an animal model for the study of ovarian cancer [15–17]. Several studies have demonstrated the utility of the laying hen for pre-clinical trials and dietary intervention studies [18–20]. One of the most prevalent theories about the etiology of ovarian cancer is the “incessant ovulation hypothesis” which was proposed by Fathalla in 1971 [21]. He hypothesized that the inflammation associated with continuous ovulation leaves ovarian surface epithelial (OSE) cells susceptible to malignant transformation. The observation that egg-laying domestic hens frequently develop ovarian cancer supports this hypothesis [22]. Hens spontaneously develop ovarian adenocarcinomas that are similar in histological appearance to human ovarian carcinomas and share similar symptoms of the disease, such as perfuse ascites fluid and peritoneal metastatic dissemination [15,23,24].

The laying hen provides a model for conducting large scale dietary intervention studies with statistically significant numbers of animals. The purpose of this study was to determine if flaxseed slowed the development, or progression, of ovarian cancer in hens fed a diet enriched with omega-3 fatty acids compared to hens fed a control diet. The results indicate that a diet enriched with flaxseed increases overall health in the laying hen. No significant differences in the incidence rates of ovarian cancer were found between the two groups; however, a significant decrease in late stage ovarian tumors was observed in hens fed flaxseed. New insight into the ability of flaxseed and OM-3FA to decrease ovarian cancer may establish the foundation for

clinical studies to test the efficacy of dietary intervention with flaxseed for the prevention and suppression of ovarian cancer in women.

Materials and Methods

Animal Care

387 single-comb 2.5 year old White Leghorn hens were used for this study. Hens were maintained three per cage, provided with measured feed and water ad libitum and exposed to a photoperiod of 17 h light: 7 h dark, with lights on at 05:00 h and lights off at 22:00 h. Animal management and procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Illinois at Urbana-Champaign and University of Illinois at Chicago.

Composition of meals

2.5 year old hens were randomly divided into control and flaxseed groups with 194 and 193 hens respectively. The composition of the test diets is shown in Table S1 (Supplementary Material). A percentage of both corn and soy were removed from the experimental diet to keep the diet isocaloric with the addition of the flaxseed. The hens were fed 110 grams of food a day and were provided water ad libitum. Hens on the flaxseed diet consumed around 11 grams of flaxseed per day, or 6.2g/kg body weight.

Tissue Collection

Hens were euthanized by CO₂ asphyxiation. Upon necropsy, ovaries were removed and small yellow follicles (6–8mm) and pre-ovulatory follicles (9–35mm) were removed from ovaries of the normal hens. Suspected abnormal ovarian tissues were noted and confirmed as cancerous or normal ovarian tissue by histology. Tumors were classified by stage as shown in Table 1. Tumors were characterized based on the size of the ovarian tumor, oviductal involvement, and if there was any ascites fluid and peritoneal metastases present by gross observation. Basic histology was performed on early stage tumors to determine the extent of tumor growth [25].

Experimental Design

The hens were fed either a 10% flaxseed enriched diet or standard (control) diet for one year. Ovulation frequency was measured by counting the total number of eggs laid each week by the whole group (flax or control), then dividing by the total number of hens in each group. At the six and nine month time points, 20 hens from each group were randomly selected, euthanized, and necropsied, to ensure that the OM-3FA from the flaxseed were incorporating into the ovary. Eggs were collected both at these time points and at the end of the study from both groups of hens and measured for total omega-3 fatty acid content by gas chromatography.

Gas Chromatography

Measuring OM-3FA in egg yolks is a common practice in the poultry industry as a way to measure total body OM-3FA incorporation [26,27]. The yolk was separated from the rest of the egg and diluted 1:25 with a phosphate buffered saline solution. A solution containing 30ug 17:0 standard (Sigma-Aldrich), 30ug 22:2 standard (Sigma-Aldrich), and 200ug butylated hydroxy toluene (Sigma-Aldrich) in methanol was then added to the diluted egg yolks. Lipid extraction was performed using HPLC grade chloroform, water and HPLC grade hexane (Sigma-Aldrich). The lipids were then dried under a stream of nitrogen and methylated using the Instant Metanolic HCl kit (Alltech). After methylation, the lipids were extracted twice with hexane. After drying under nitrogen, the lipids were re-dissolved in hexane and injected into the Shimadzu 17A gas chromatograph equipped with a flame ionization detector. Hydrogen was used as the carrier gas and nitrogen as the make up gas with an Omegawax column. Fatty

acids were determined from their retention time and quantified by integration of area under peak after normalization to 17:0 and 22:2 standards.

Statistical analysis

Statistical analysis was performed with GraphPad InStat by using One-way ANOVA with Student-Newman-Keuls comparison and also 2-way Contingency Table and Chi-Square Tests. A value of ($P<0.05$) was considered significant whereas a value of ($P<0.01$) was considered highly significant.

Results

Incorporation of OM-3FA into Egg Yolk

The incorporation of OM-3FA into tissue was measured by gas chromatography as shown in Figure 1. Total OM-3FA content was determined in egg yolks collected from control and flaxseed fed hens at the twelve month time point. Six egg yolks from 6 hens in each group were used to measure total OM-3FA. Flax eggs incorporated 78.20ug OM-3FA/mg yolk while control eggs only incorporated 32.05ug OM-3FA/mg yolk. There was significantly more Om-3FA incorporated into the eggs from flaxseed fed hens when compared to the control group ($P<0.007$).

Tumor Incidence and Severity

After feeding the hens flaxseed for one year, we found no significant difference in the incidence of total ovarian cancers between the flaxseed fed hens (24%) and control fed hens (22%) ($P<0.1$) (Fig. 2A). Tumors from both groups were analyzed and characterized according to Table 1. Hens fed flaxseed had more early stage tumors that were confined to the ovary and oviduct compared to the control diet hens, indicative of the chemosuppressive actions of flaxseed (Fig. 2B). In contrast, hens on the control diet had significantly more late stage tumors (T3/T4) that presented with ascites fluid and metastases compared to the flaxseed group (61% vs. 47%)($P<0.05$).

Effect of Flaxseed on Egg Laying Frequency

No significant differences in egg laying frequency were seen between the two groups after feeding for one year (Figure 3), indicating that diet had no effect on ovulation. There appears to be a decrease in egg laying frequency in the last quarter of the study in both diet groups, but these data were not statistically significant.

Effect of Flaxseed on Body Weight

The graph in Figure 4 represents the average weights of hens on the control diet and flaxseed enriched diet necropsied at the six and twelve month time points. At six months, the control fed hens' average weight was 1.83kg while the flaxseed fed hens weighed significantly less at 1.67kg ($P<0.05$). By twelve months, the control diet hens weight had significantly decreased to 1.58kg, while the flaxseed fed hens weight remained stable at 1.71kg ($P<0.001$). There was no difference in food intake in hens fed flaxseed compared to the control diet.

Effect of Flaxseed on Mortality

Flaxseed increased overall survival of the hens over the course of the one year study (Fig 5). Initially, 193 hens were in the control group and 194 hens in the flax group. Twenty hens from each group were euthanized at the six and nine month time points. Of the remaining 153 hens in the control diet group, only 78 hens survived to the twelve month time point (51.6%), compared to 111 flaxseed hens out of the remaining 154 (72.1%) ($P<0.0001$). Mortality rates include all natural causes of death over the total study.

Discussion

The goal of this study was to determine if a diet enriched with flaxseed for one year prevented ovarian cancer development and progression in the laying hen. Flaxseed fed hens had fewer late stage tumors, but no statistically significant reduction of total ovarian cancers was found between the two diet groups. Hens fed a flaxseed rich diet over one year had better overall health and reduced mortality compared to hens fed the control diet.

A significant reduction in severity of ovarian tumors was observed in hens fed flaxseed, with more tumors confined to the ovary and decreased metastatic spread. This is an important finding as the metastases that accompany late stage ovarian cancer are the main cause of death from this disease [28]. Over 60% of all women diagnosed with ovarian cancer present at stage 3 or 4 when metastases has already occurred [29]. If the cancer is found at an early stage, when the tumor is still confined to the ovary, women have a much better prognosis and much better treatment options compared to a late stage diagnosis when it has already metastasized. Flaxseed supplementation for one year is able to reduce the severity of ovarian cancer in hens. This finding may have therapeutic potential as women at high risk for developing ovarian cancer could supplement their diet with flaxseed to prevent tumors from forming metastases.

Feeding hens a flaxseed supplemented diet for one year did not prevent ovarian cancer. No statistical difference in incidence rates was detected between the control group and flax group. This may be because the diet was not administered until the hens were 2.5 years of age. By this age, a hen has ovulated approximately 500 times, similar to the number of ovulations of a woman entering menopause [22]. At 2.5–3.5 years of age, the incidence rate of ovarian cancer in hens is between 4–20%. A chemoprevention with flaxseed may not have been seen because the damage caused by ovulation had already occurred. A one year feeding study is not adequate to test if flaxseed supplementation prevents ovarian cancer. A new four year study is currently being conducted in order to determine if long-term dietary intervention with flaxseed may reduce the incidence of ovarian cancer development. In this new study, a 10% flaxseed-supplemented diet has been provided to the hens as soon as they commenced egg laying (approximately 22 weeks of age), before the damage from ovulation accumulates. The findings from this study will provide more definitive results indicating the value of flaxseed as a chemopreventative agent.

The importance of diet in the development of cancer has only been studied for 30 years, while the chemotherapeutic efficacy of certain foods in the treatment and prevention of cancer is an age old idea [30]. The increasing awareness that certain compounds, such as lycopene or selenium, have anti-cancer activities has lead to an increase in dietary intervention studies [31,32]. However, many of these compounds that showed promise *in vitro*, show little or no positive effects once tested *in vivo* [33,34]. Dietary intervention with individual compounds can be difficult to study since many individual compounds can work together synergistically. Thus, given alone as treatment, effects may be minimal or not seen at all.

The hens were fed a diet enriched in flaxseed as it contains many nutrients that have been shown to have anti-cancer effects, including OM-3FA and phytoestrogen lignans. Flaxseed contains the highest level of plant lignans that are metabolized in the digestive tract to enterodiol (END) and enterolactone (ENL), which have antioxidant activities [35]. Lignans have also been show to alter estrogen metabolism which may decrease ovarian cancer risk and improve health [36,37]. During the course of the one year trial study, the overall health of the hens fed flaxseed was improved based on survival compared to the hens fed the control diet. Hens were able to incorporate high amounts of OM-3FA into their eggs from the flaxseed as shown by gas chromatography, yet had no differences in egg laying frequencies.

Hens fed flaxseed maintained a low steady weight compared to the control fed hens whose weight precipitously declined over the course of the study, indicative of their failing health. The overall reduced weight in the flaxseed fed hens may have played a role in the decrease in late stage ovarian cancers as obesity and increased body mass index have been linked to an increased risk for developing ovarian cancer in women [38]. Hens fed a low-calorie diet also have a reduced risk of developing the disease [38,39]. Overall mortality rates were also significantly decreased in hens fed flaxseed compared to control. A diet high in flaxseed has anti-cancer activity as well as important overall health benefits. As OM-3FA reduce inflammation and phytoestrogens can act as antioxidants, the combination of OM-3FA and phytoestrogens may create a synergistic effect that results in an overall health benefit. *In vitro* studies are currently underway to elucidate the mechanisms by which these compounds act to protect against tumor progression and improve survival.

The laying hen is an excellent model to study the effects of dietary intervention on ovarian cancer. The findings here show that dietary intervention with flaxseed in the laying hen increases overall health and reduces mortality. A chemosuppressive effect of flaxseed on ovarian cancer progression was reported after feeding for one year. These findings have important clinical relevance and may provide the basis for a large-scale clinical trial testing the efficacy of flaxseed as a chemopreventative agent of ovarian cancer in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Flaxseed fed hens lay eggs with higher OM-3FA content. Eggs were collected from control and flaxseed fed hens at time of necropsy after 12 months on the diet. There was no difference in the number of eggs laid between the groups. 6 egg yolks from each group were measured for total OM-3FA content by gas chromatography. Yolk was separated from the rest of the egg and diluted with a phosphate buffered saline solution. Lipids were extracted with hexane and measured by a gas chromatograph. Fatty acids were determined from their retention time and quantified by integration of area under peak after normalization to 17:0 and 22:2 standards. Eggs from flaxseed fed hens contained significantly more OM-3FA compared to control fed hens ($P<0.007$).

Figure 2.

Flaxseed suppresses the severity of ovarian cancer in the laying hen. Hens were fed a diet enriched with 10% flaxseed or a control diet for 1 year. After 1 year, the hens were euthanized, necropsied, and examined for the presence of ovarian cancer. There was no significant difference in incidence rates between the two groups (A). There was a reduction in the severity of the disease in hens fed flaxseed (B). Hens fed flaxseed had more ovarian tumors in stage T1/T2, when the tumor was still confined to the ovary ($P<0.05$). Hens on the control diet had more late stage tumors (T3/T4), where the cancer had spread to other organs ($P<0.05$).

Figure 3.

Egg laying frequency is unaffected by the addition of flaxseed to the diet. Eggs were collected and counted weekly over the 1 year study from the two groups. There were no significant differences in the numbers of eggs laid in the flaxseed fed group compared to the control group. Egg laying frequency in both groups appeared to slow in the last quarter of the study, but this change was not significant.

Figure 4.

Flaxseed fed hens maintain a steady weight compared to control hens over a 12 month study. At 6 months, 20 hens from each group were randomly selected to be euthanized and necropsied. All hens were weighed before necropsy. At the 12 month time point, the remaining hens were weighed and necropsied. At the 6 month time point, the flaxseed fed hens weighed significantly less than the control diet hens ($P < 0.05$). By 12 months, the control fed hens weight had significantly decreased ($P < 0.001$), while the weight of the flaxseed fed hens remained stable.

Figure 5.

Flaxseed increased survival rates over a year-long study. At the beginning of the study there were 194 hens in the control diet group and 193 hens in the flaxseed diet group. At the 6 and 9 month time points, 20 hens from each group were randomly selected to ensure that the treatment was effective. By the end of the study, only 79 hens from the control group were alive out of the 154 remaining compared to 111 hens out of 153 hens from the flaxseed group ($P<0.0001$), indicating that flaxseed reduces mortality. Mortality rates include all natural causes of death over the total study.

Table 1

Histological Staging of Ovarian Cancer in the Laying Hen. T0- Stage 0. The ovary is normal and presents with large preovulatory follicles and small peri-ovulatory follicles. The tissue is organized into follicular structures around the edge of the ovary and the internal stromal tissue in the medullary region of the ovary. T1-Stage 1. The ovary is mostly normal, except for a small area of early stage cancerous tissue, seen here surrounding an otherwise normal pre-ovulatory follicle. The earliest lesions seen in the hen are acini created by a single layer of cells surrounding a lumen (black arrows) (22). The tumor has not broken through the basement membrane to invade the rest of the ovary. There is no tumor on the surface. T2- Stage 2. The majority of the ovary is abnormal and there may be oviductal involvement. The tumor has broken through the basement membrane (black arrows) and transformed the normal ovary. Tumor may be on the surface of the ovary. T3- Stage 3. The tumor has taken over the whole ovary and may have metastasized to other organs within the peritoneum. Ascites may be present. T4- Stage 4. The ovary is completely cancerous. Extensive peritoneal metastases and ascites fluid are seen by gross observation. (20X bar=50um, 40X bar=100um).

	20X	
T0- Normal ovary, follicles present by histology

	20X	
T1-Tumor confined to the ovary, some areas may be normal

	20X	
T2-Most of the ovary is abnormal, may have oviductal involvement

	20X	
T3-Tumor has spread to the whole ovary, other organs may be involved, ascites may be present

	20X	
T4- Extensive peritoneal metastases and ascites fluid