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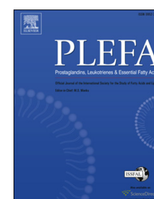

Flaxseed enriched diet-mediated reduction in ovarian cancer severity is correlated to the reduction of prostaglandin E_2 in laying hen ovaries ☆

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ABSTRACT

Prevention of ovarian cancer is the best approach for reducing the impact of this deadly disease. The laying hen is a robust model of spontaneous ovarian cancer that recapitulates the human disease. Dietary intervention with flaxseed, the richest vegetable source of omega-3 fatty acids (OM-3FAs) and phytoestrogen lignans, demonstrate the potential for effective prevention and amelioration of ovarian cancer by targeting inflammatory prostaglandin pathways. Prostaglandin E_2 (PGE_2) is the most pro-inflammatory ecoisanoid and one of the downstream products of two isoforms of cyclooxygenase (COX) enzymes: COX-1 and COX-2. Our objective was to investigate the effect of flaxseed supplementation for one year on ovarian cancer and correlate its effects to expression of COX enzymes and concentrations of prostaglandins. White Leghorn hens were fed 10% flaxseed-enriched or standard diet for one year. The severity of ovarian cancer was determined by gross pathology and histology. COX-1 and COX-2 localization and protein and mRNA expression and PGE_2 and PGE_3 concentrations in ovaries were measured by IHC, western blot, quantitative real-time PCR and LC–MS–MS, respectively. The results demonstrated a significant reduction in late stage ovarian tumors in the flaxseed-fed hens compared with the control diet-fed hens. In correlation with decreased ovarian cancer severity, concentrations of PGE_2 and expression of COX-2 were diminished in ovaries of flaxseed-fed hens. PGE_3 concentrations were below the level of detection. The results demonstrated that in normal ovaries, COX-1 was localized to the granulosa cell layer surrounding the follicles and ovarian surface epithelium (OSE) whereas COX-2 protein was localized to the granulosa cell layer in the follicle. Extensive COX-1 and COX-2 protein expression was found throughout the ovarian carcinoma. Our findings suggest that the flaxseed-mediated reduction in the severity of ovarian cancer in hens is correlated to the reduction in PGE_2 in the ovaries of flaxseed-fed hens. These findings may provide the basis for clinical trials of dietary intervention targeting prostaglandin biosynthesis for the prevention and treatment of ovarian cancer.
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1. Introduction

Dietary intervention with flaxseed, the richest vegetable source of $n-3$ polyunsaturated fatty acids ($n-3$ PUFAs) and phytoestrogen lignans, demonstrate the potential for effective amelioration and prevention of ovarian cancer by targeting inflammatory prostaglandin pathways.

Cyclooxygenase (COX) is the rate limiting enzyme in the production of prostaglandins (PG) and thromboxanes. COX has two isozymes, COX-1 and COX-2, which have similar structure but are encoded with different genes (PTGS1 and PTGS2) and show distinct expression patterns. COX-1 is known to be constitutively expressed in many tissues and cell types and is involved in normal cellular physiological functions, whereas COX-2 is pro-inflammatory in nature and can be induced by mitogens, cytokines, tumor promoters and growth factors [1]. Aberrantly high expression of COX-2 has been implicated in the etiology of many forms of cancer [2]. However, we and others have shown elevated expression of COX-1 in ovarian cancer [3–5]. PGE_2 is the most common prostaglandin found in different human cancers including colon, lung, breast, and head and neck cancers [6] and exerts its autocrine/paracrine effects on target

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cells by coupling to four subtypes of G-protein-coupled receptors, which have been classified as EP1-4 [7]. EP4 is over-expressed in many cancers such as castrate-resistant prostate cancer [8] and colorectal cancer [9] and using EP4 antagonist inhibits breast cancer metastasis [10].

Ovarian carcinoma is the most lethal gynecologic malignancy and fifth leading cause of cancer death in women [11]. The high mortality rate is due to the late stage of detection when approximately 75% of ovarian cancers are diagnosed. The genetic and molecular mechanisms underlying ovarian cancer remain largely unknown and treatment options for patients with advanced disease are limited. Research on ovarian cancer and development of new therapies has been hampered due to lack of appropriate animal models. Laying hens spontaneously develop ovarian adenocarcinomas that are similar to human ovarian cancer in both histopathology [12] and expression of some molecular markers [5,13,14] and share similar symptoms of the disease, such as perfuse ascites fluid and peritoneal metastatic dissemination [12,15]. Thus, the laying hen provides a tractable model for in vivo testing of therapeutic modalities [16].

Epidemiological and preclinical studies indicate that increased dietary intake of $n-3$ PUFAs reduces the incidence and growth of various cancers [17–21]. Thus, increasing the consumption of $n-3$ PUFAs may be a nontoxic way to prevent or suppress ovarian cancer. The $n-3$ PUFAs include alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). EPA and DHA are both found in oily cold-water fish such as tuna and salmon. ALA is found primarily in dark green leafy vegetables and flaxseed which is the richest plant source of $n-3$ PUFAs. ALA can be elongated by Δ^5 and Δ^6 desaturase enzymes to form EPA and DHA in digestive tract. EPA competes with arachidonic acid for COX enzymes and limits the production of PGE_2.

Flaxseed is an excellent source of $n-3$ PUFAs but its potential mechanism of action on ovarian cancer is unknown. The objective of this study was to investigate the effect of flaxseed supplementation for one year on ovarian cancer and correlate its effects to expression of COX enzymes and concentrations of prostaglandins. Our data showed that flaxseed decreases the severity of ovarian cancer by reducing the expression of COX-2 mRNA and concentration of PGE_2 in hen ovarian tissue. Therefore, our findings provide important evidence for the use of flaxseed in the chemo-suppression and treatment of human ovarian cancer.

2. Materials and methods

2.1. Animal care

Three hundred eighty seven single comb 2.5 year old White Leghorn hens were randomly divided into 2 groups and were fed either a 10% flaxseed-enriched diet ($n=193$) or control diet ($n=194$) for one year. To ensure that incorporation of OM3-FAs into ovaries of hens fed whole flaxseed was not different than hens fed ground flaxseed, 6 hens ($n=6$) were fed ground flaxseed for 3 months. Ovulation frequency was measured by counting the total number of eggs laid each week by the whole group (flaxseed or control), then dividing by the total number of hens in each group. 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.

2.2. Composition of meals

The composition of the diets has been shown previously as supplementary data [12]. 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 g of food per day and were provided water ad libitum. Hens on the flaxseed diet consumed around 11 g of flaxseed per day, or 6.2 g/kg body weight.

2.3. Tissue collection

Hens were euthanized by CO_2 asphyxiation. Upon necropsy, ovaries were removed and small yellow follicles (6–8 mm) and pre-ovulatory follicles (9–35 mm) were removed from normal ovaries of the hens. Basic histology was performed [22] on suspected abnormal ovarian tissues to confirm the presence of cancer. Tumors were classified by stage as previously reported [12] and 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.

2.4. Gas chromatography

The yolk was separated from the rest of the egg and diluted 1:25 with a phosphate buffered saline solution. A solution containing 30 μg C17:0 standard (Sigma-Aldrich), 30 μg C22:2 standard (Sigma-Aldrich), and 200 μg butylated hydroxyl 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). Then 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 C17:0 and C22:2 standards.

2.5. Histology and immunohistochemistry

Ovarian tissues fixed in neutral buffered formalin solution were processed and paraffin embedded. 5 μm sections were cut and mounted on SuperFrost Plus microscope slides (Fisher Scientific, IL). Slides were deparaffinized and rehydrated through xylene and graded ethanol solutions (Fisher Scientific). Hematoxylin and eosin (H&E) staining was done as described [23]. Immunohistochemistry was performed by using the Vectastain Elite ABC kit (Vector Laboratories, CA). Antigen retrieval was done using Antigen Unmasking Solution (Vector Laboratories) and pressure cooked at 20 psi for 5 min in a Decloaking Chamber electric pressure cooker (Biocare Medical, Walnut Creek, CA). Slides were cooled and quenched in 0.3% H_2O_2 (Sigma-Aldrich) in methanol for 15 min. Slides were blocked with normal serum and incubated in anti-human COX-1 (1:50), and anti-human COX-2 (1:50) monoclonal antibodies (Abcam Inc., MA) overnight at 4°C. Non-immune IgG was used for negative control. After rinsing in Tris-buffered saline (TBS), sections were incubated with biotinylated secondary antibody and avidin-biotin complex (Vector Laboratories). Specific binding was visualized using DAB (Vector Laboratories) in the presence of H_2O_2 and sections were counterstained with Gils hematoxylin (Sigma-Aldrich), mounted with Histomount (Fisher Scientific), examined on a Nikon ECLIPSE E400 microscope and

were documented using SPOT Advanced version 4.0.1 software (Diagnostic Instruments, Inc., Sterling Heights, MI).

2.6. Western blot

Hen ovarian tissue homogenates were prepared from snap frozen samples, pulverized on dry ice, re-suspended in ice-cold lysis buffer (PBS/0.1% sodium dodecyl sulfate (SDS), supplemented with protease inhibitor cocktail HALT), and homogenized using an Ultra-Turrax (Jenke and Kunkel, Staufen, Germany). Protein concentrations were determined by BCA protein assay (Pierce). Twenty micrograms of total protein were separated by SDS-PAGE using 12.5% acrylamide/SDS separating gels and transferred to nitrocellulose membranes as described previously [24]. Monoclonal anti-human COX-1 and COX-2 (Abcam Inc., MA) and anti human EP4 Cayman Chemical (Ann Arbor, MI, USA) were used for detection of COX-1, COX-2 and EP4, respectively. Data were normalized to monoclonal anti-chicken ß-Actin (Santa Cruz, CA). Detection of bound antibody on the blot was assessed with a horseradish peroxidase-conjugated, goat anti-mouse IgG antibody (OuthernBiotech, Birmingham), visualized by chemiluminescent detection (SuperSignal West Pico Chemiluminescent Substrate, ThermoScientific, IL), and quantified after densitometry using Imagequant software (Molecular Dynamics, CA). Data for protein are represented as integrated OD.

2.7. RNA extraction and analysis

Ovaries preserved in RNAlater were used for RNA isolation. Total RNA was extracted from ovaries using Trizol reagent (Invitrogen, CA) and was quantified by determination of absorbance at A260. RNA samples were then treated with RQ1 RNase-free DNase (Promega, WI) prior to reverse transcription reaction. Synthesis of first strand of cDNA was performed using the high capacity cDNA Archive Kit (Applied Biosystems) and cDNA was quantified by Quant-iT fluorescent reagent (Invitrogen). Equal amounts of cDNA from all samples were subjected to quantitative real-time PCR.

2.8. Quantitative real-time PCR (qRT-PCR)

Hen specific primers were designed to recognize target genes using Primer Express (ABI). The primer pairs were designed so that at least one spanned an intron. Primer sequences for COX-1 (Prostaglandin G/H synthase 1, PTGS1, XM_425326): forward: 5′ TCAGGTGGTTCTGGGACATCA 3′; reverse: 5′ TGTAGCCGTACTGGGAGTTGAA 3′; for COX-2 (Prostaglandin G/H synthase 2, PTGS2, XM_422297): forward: 5′ CTGCTCCCTCCCATGTCAGA 3′; reverse: 5′ CACGTGAAGAATTCCGGTGTT 3;′ for EP4: forward 5′ GGTGTTTCATAGACTGGCGA 3′; reverse: 5′ GCAGATCACCGTAACCATGA 3′; for internal control gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase, GAPDH, NM_204305): forward: 5′ GATGGGTGTCAACCATGAGAAA 3′; reverse: 5′ CAATGCCAAAGTTGTCATGGA 3′. Plasmids for standards of each target of interest and internal control GAPDH were cloned as described previously [13,25]. qRT-PCR was conducted by amplifying cDNA with SYBR® Green (Applied Biosystems) on ABI 7900HT using a 384 well plate format and analyzed with AB1 Prism software. Control reactions lacking template were run for each gene. Reactions were 10 μL in total volume and 200 nM of each primer. The plasmid standards and cDNA were simultaneously assayed in duplicate reactions. The amplification conditions were as follows: 50 °C 2 min, 95 °C 10 min, 40 cycles for 95 °C 15S, 60 °C 1 min.

2.9. Prostaglandin analysis

Hen ovarian tissue samples were homogenized and weighed before storage in glass tubes at−80 °C. For analysis, tissue samples were thawed, and a 300 mg sample of each was spiked with 10 μL of d_4-PGE_2 in methanol/water (1:1, v/v) (100 ng/mL) as a surrogate standard. Next, 40 μL of 1 M citric acid in water and 5 μL of 10% BHT in hexane were added to prevent free radical-catalyzed peroxidation. Prostaglandins were extracted by adding 6 mL of hexane/ethyl acetate (1:1, v/v) followed by vortex-mixing for 2 min. After centrifugation at $3500 \times g$ for 10 min, the upper organic phase was collected. The extraction procedure was repeated, and the organic phases were combined and evaporated to dryness under a stream of nitrogen gas. Immediately before analysis using LC–MS–MS, each extract was reconstituted in 100 μL methanol/water (1:1, v/v) and centrifuged at $12000 \times g$ for 10 min [26]. Normal hen ovarian tissue was used to prepare a standard curve, and the preparation process was identical to that of the samples. Recovery tests indicated 104 ± 12 % recovery for two standards, PGE_2 and PGE_3.

2.10. Liquid chromatography–tandem mass spectrometry (LC–MS–MS)

For the quantitative analysis of PGE_2 and PGE_3, HPLC separations were carried out using a Shimadzu (Columbia, MD) Prominence UFLC system with a Waters (Milford, MA) XTerra MS C18 (2.1×50 mm, 3.5 μm) analytical column and a 5-min isocratic mobile phase consisting of acetonitrile/ aqueous 0.1% formic acid (37:63, v/v) at a flow rate of 200 μL/min. PGE_2 and PGE_3 were resolved to baseline in less than 4 min using these chromatographic conditions. The HPLC system was interfaced to an Applied Biosystems (Foster City, CA, USA) API 4000 triple quadrupole mass spectrometer, which was operated using negative ion electrospray [27]. The deprotonated molecules of m/z 351 and m/z 355 corresponding to PGE_2 and the surrogate standard d_4-PGE_2, respectively, were selected for collision-induced dissociation at a collision energy of −23 eV. The abundant product ions of m/z 271 and m/z 275, corresponding to the $[M–H–2H_2O–CO_2]–$ product ions of PGE_2 and d_4-PGE_2, respectively, were measured using selected reaction monitoring [28]. Likewise, selected reaction monitoring of the transition of m/z 349 to m/z 269 was used for quantitative analysis of PGE_3. The retention times of PGE_3, PGE_2 and d_4-PGE_2, were 2.33, 3.09, and 3.01 min, respectively. For quantitative analysis using LC–MS–MS with SRM, the injection volume was 10 μL. The standard curves for PGE_2 and PGE_3 were linear ($r^2 > 0.999$) over the concentration range of 0.1–100 ng.

2.11. 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 as highly significant.

3. Results

3.1. Ovarian cancer severity

More late stage tumors with ascites fluid and metastasis were presented in hens fed the control diet compared with hens fed the flaxseed enriched diet (Fig. 1A and B; 61% vs. 47%; $P < 0.05$). In contrast, the hens fed the flaxseed diet had more early stage

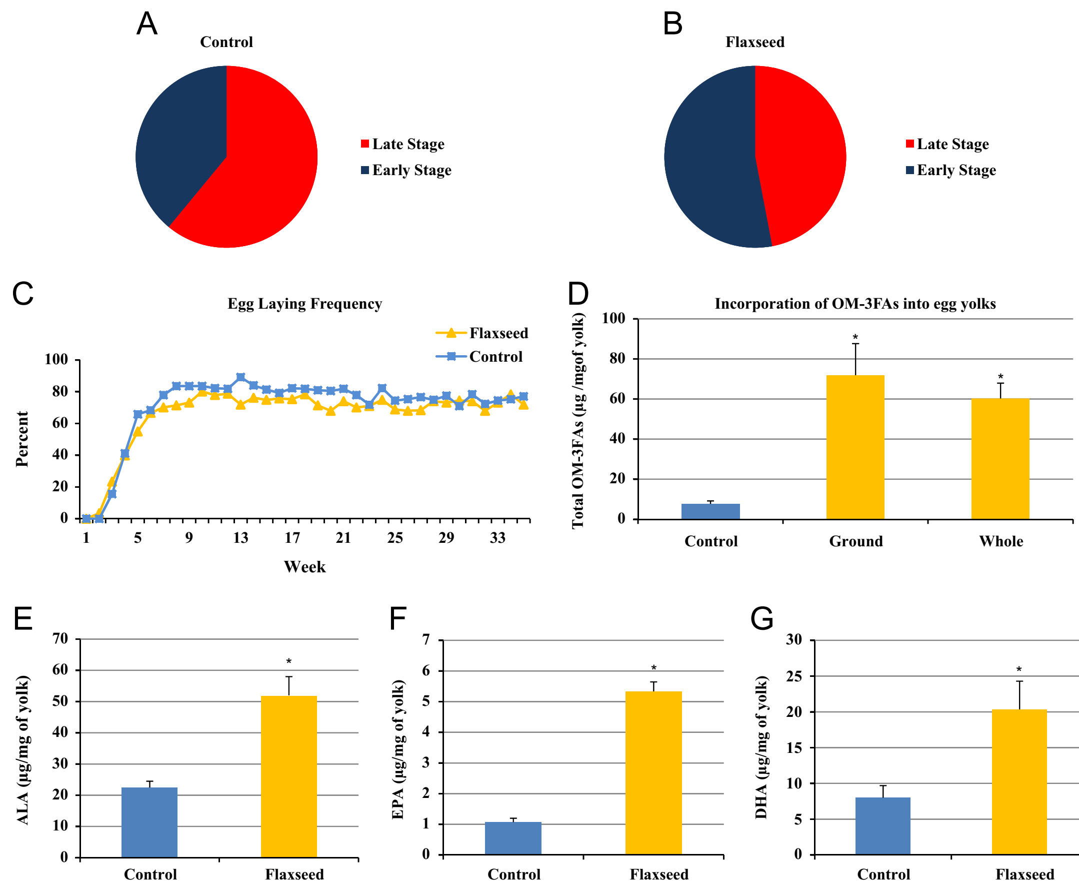

Fig. 1. (A) and (B) There was a reduction in the severity of the disease in flaxseed-fed hens. Hens fed the flaxseed enriched diet had more early stage ovarian tumors which were still confined to the ovary ($P<0.05$). Hens on the control diet had more late stage tumors and the cancer had spread to other organs ($P<0.05$). (C) Egg laying frequency is unaffected by the addition of flaxseed to the diet. There were no significant differences in the numbers of eggs laid in the flaxseed-fed group compared to the control group. (D) There was a significant increase in total $n-3$ PUFAs in egg yolks collected from the flaxseed-fed hens (both ground and whole) for 3 month compared to hens fed control diet for 3 month ($n=6$; $P<0.05$). There was no statistically significant difference between total $n-3$ PUFAs in egg yolks collected from hens fed ground flaxseed versus whole flaxseed. (E)–(G) The yolks from the flaxseed-fed hens for one year had higher ALA, EPA and DHA compared to yolks collected from the control diet fed hens ($P<0.05$). $^{*}P<0.05$.

tumors that were confined to the ovary and oviduct indicative of the chemo-suppressive effect of flaxseed.

3.2. Egg laying frequency

The ovulation rate (egg laying frequency) was not changed under the effect of the flaxseed enriched diet (Fig. 1C) indicating that the reduction in ovarian cancer severity in hens fed the flaxseed diet is not due to lower number of laid eggs.

3.3. Fatty acid analysis

The incorporation of $n-3$ PUFAs into tissue was measured using gas chromatography. Six egg yolks at the three and twelve month time points from hens in both flaxseed and control diet groups were used. The total amount of $n-3$ PUFAs in yolks collected from the whole flaxseed-fed hens for 3 months was not significantly different than total amount of $n-3$ PUFAs in yolks collected from the ground flaxseed-fed hens for 3 months (Fig. 1D); however, yolks collected from the hens fed both whole flaxseed and ground flaxseed had higher incorporation of $n-3$ PUFAs compared with hens fed the control diet ($P<0.05$). The egg yolks collected from the whole flaxseed-fed hens for one year had higher amounts of ALA, EPA and DHA compared with yolks from the control diet-fed hens for a year (Fig. 1E–G; $P<0.05$).

3.4. Localization of COX-1 and COX-2 in the hen ovary

The distribution of COX-1 and COX-2 proteins in the hen ovary was examined by immunohistochemistry. Increased expression of COX-1 protein was observed in cancerous ovaries (Fig. 2C and D) compared to normal ovaries (Fig. 2A and B) of hens in both control and flaxseed groups. In the normal ovaries, COX-1 was seen mostly in the granulosa cell layer (arrow) surrounding the follicles and in ovarian surface epithelial cell layer (Fig. 2A and B); however extensive COX-1 protein expression was found throughout the ovarian carcinoma (Fig. 2C and D). COX-2 protein was expressed in a similar pattern to COX-1. In normal ovaries (Fig. 3A and B), COX-2 expression was mostly seen in the granulosa cell layer of the follicles; whereas in cancerous ovaries, COX-2 was widely distributed over the tissue (arrow, Fig. 3C and D).

3.5. COX protein expression

COX-1 and COX-2 protein expression in ovaries of hens from both control and flaxseed groups were quantified by western blot (Fig.4A–D). COX-1 protein expression was significantly increased in cancerous ovaries compared to normal ovaries, independent of diet ($P<0.05$). There was no statistical difference in COX-2 protein expression between cancerous ovaries of the control diet-fed hens and cancerous ovaries of the flaxseed diet-fed hens (Fig. 4B and D). However, there was a significant decrease in COX-2 protein expression in normal ovaries of the flaxseed-fed hens compared

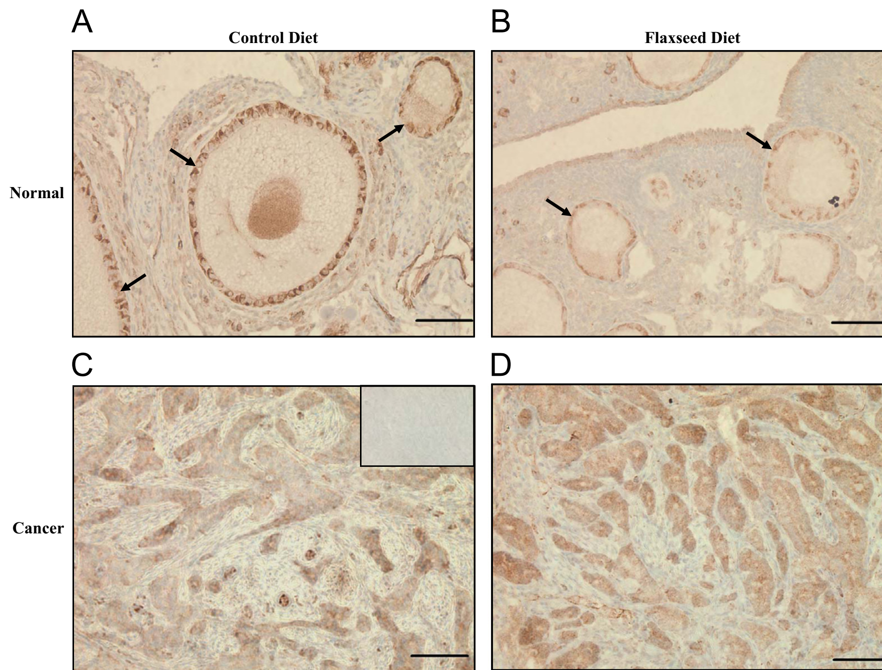

Fig. 2. COX-1 immunohistochemistry; (A) normal ovary of the control-fed hens; (B) normal ovary of the flaxseed-fed hens, arrows in A and B point to granulosa cell layer stained by COX-1; (C) ovarian tumor of the control-fed hens (inset: non-immune IgG, 20X); (D) ovarian tumor of the flaxseed-fed hens. Calibration bar, 50 μm.

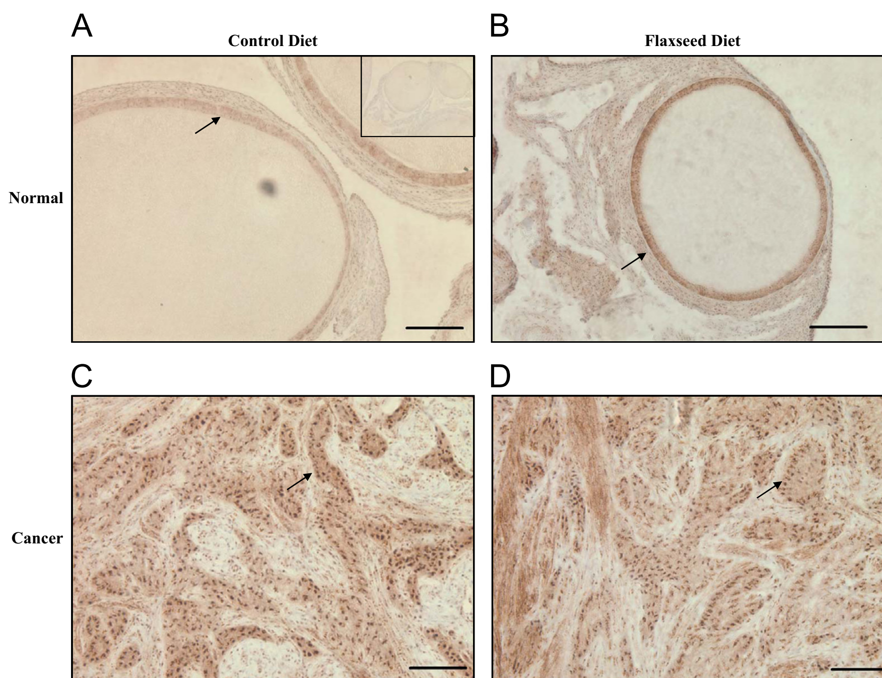

Fig. 3. COX-2 immunohistochemistry: (A) normal ovary of the control-fed hens, (inset: non-immune IgG, 20X); (B) normal ovary of the flaxseed-fed hens, arrows in A and B point to granulosa cell layer stained by COX-2; (C) ovarian tumor of the control-fed hens; (D) ovarian tumor of the flaxseed-fed hens, arrows in C and D point to punctate-located COX-2. Calibration bar for A & B: 100 μm, Calibration bar for C and D: 50 μm.

with the normal ovaries of the control-fed hens indicating that flaxseed decreases COX-2 protein expression in normal ovaries of hens ($P<0.05$; Fig. 4A and D).

3.6. COX mRNA expression

To determine if the observed changes in COX protein expression in ovaries are transcriptionally regulated, COX-1 and COX-2 mRNA expression were measured in normal and cancerous ovaries of hens fed the flaxseed enriched and control diet by qRT-PCR. COX-1 mRNA expression was significantly higher in cancerous ovaries compared to normal ovaries, independent of the diet ($P<0.01$; Fig. 4E). There was no significant difference in COX-1 mRNA expression between cancerous ovaries of hens fed the flaxseed enriched diet and cancerous ovaries of hens fed the control diet. In contrast, we observed a significant inhibition of COX-2 mRNA expression in both normal and cancerous ovaries of hens fed the flaxseed enriched diet compared to normal and

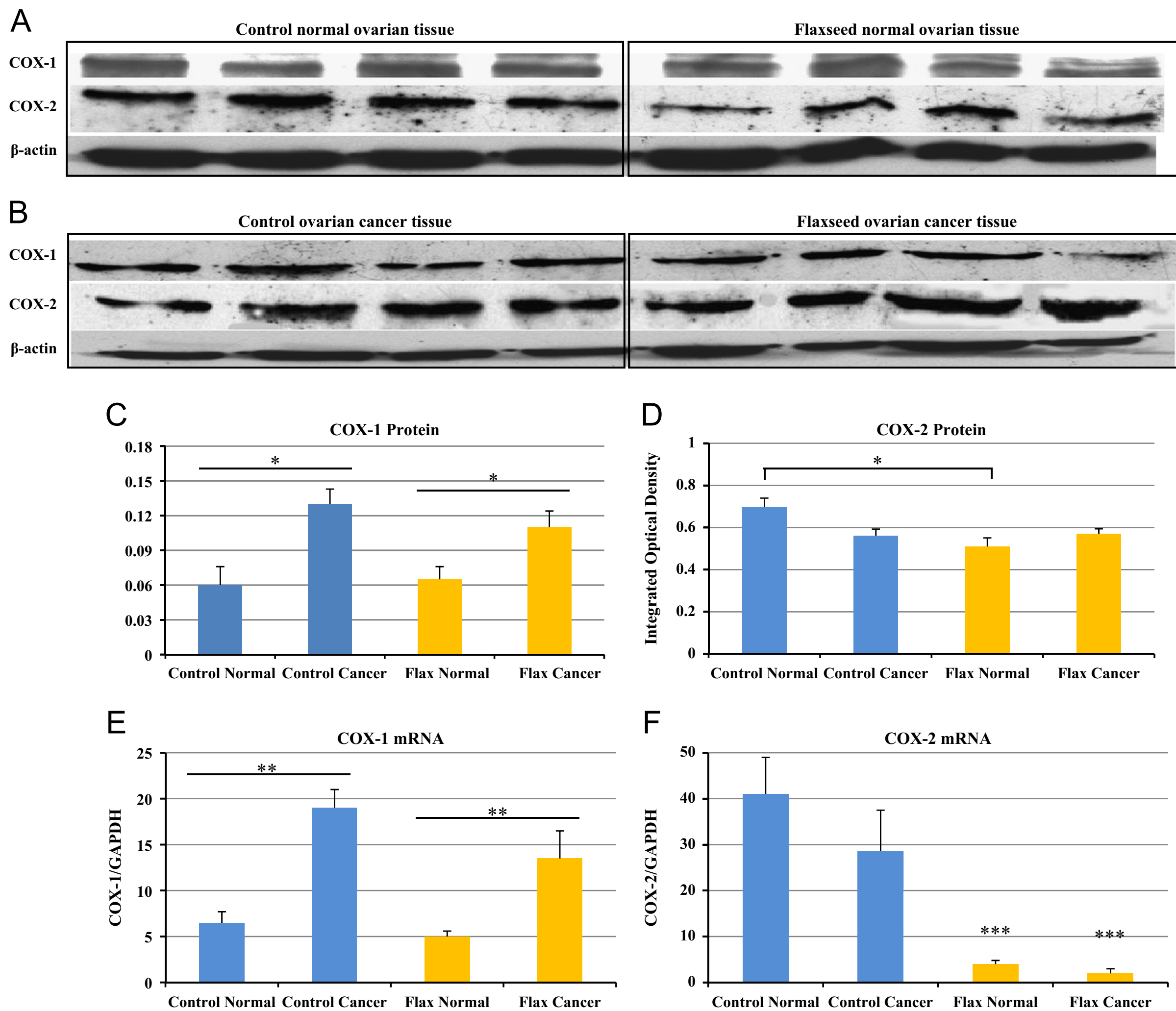

Fig. 4. Western blot and qPCR for COX-1 and COX-2 in ovaries of hens from both control and flaxseed groups. (A)–(C) COX-1 protein expression was significantly higher in cancerous ovaries compared to normal ovaries, independent of diet ($n=8$; $P<0.05$). (A), (B) and (D) There was a significant decrease in COX-2 protein expression in normal ovaries of the flaxseed-fed hens compared with the normal ovaries of control-fed hens ($n=8$; $P<0.05$). (E) COX-1 mRNA expression was measured in ovaries from hens in control cancer ($n=19$), control normal ($n=11$), flaxseed cancer ($n=15$) and flaxseed normal ($n=17$) groups. COX-1 mRNA expression was significantly decreased in all normal ovaries compared to cancerous ovaries ($P<0.01$). (F) COX-2 mRNA expression was quantified in ovaries of hens from control cancer ($n=16$), control normal ($n=10$), flax cancer ($n=14$) and flax normal ($n=16$) groups. There was a significant decrease in COX-2 expression in the flaxseed feeding group as compared to control ($P<0.001$). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

cancerous ovaries of hens fed the control diet, respectively ($P<0.001$; Fig. 4F). COX-2 mRNA expression was similar between cancerous and normal ovaries of hens fed the flaxseed diet for one year indicating that the flaxseed diet is targeting COX-2 at the transcriptional level.

3.7. *Prostaglandins*

The levels of PGE_2 and PGE_3 were measured by LC–MS–MS in normal and cancerous ovaries of hens fed the flaxseed and control. The cancerous ovaries of hens had higher concentrations of PGE_2 compared with normal ovaries, regardless of diet (Fig. 5). The results indicated that consumption of a flaxseed enriched diet for one year significantly reduces the concentrations of PGE_2 in both normal and cancerous ovaries of hens compared to normal and cancerous ovaries of hens fed the control diet, respectively. PGE_3 concentrations were below the level of detection (data not shown).

3.8. *Effect of flaxseed on EP4 expression in ovaries*

There were no significant differences in expression of EP4 mRNA and protein among groups indicating that existence of cancer or flaxseed supplementation does not affect the expression of EP4 in ovaries of hens (Fig. 6).

4. Discussion

PGE_2 is the most pro-inflammatory lipid and plays an important role in cancer therefore suppressing its pathway may be a suitable target to prevent/suppress ovarian cancer. We fed hens a 10% flaxseed enriched diet for one year and investigated its effect on ovarian cancer. Our results indicated that feeding the laying hens with a flaxseed enriched diet for one year decreased the severity of ovarian cancer but the mechanism by which flaxseed supplementation suppresses ovarian cancer severity was unknown. Thus, we postulate that feeding the hens with flaxseed reduces the expression of COX enzymes and PGE_2 concentrations

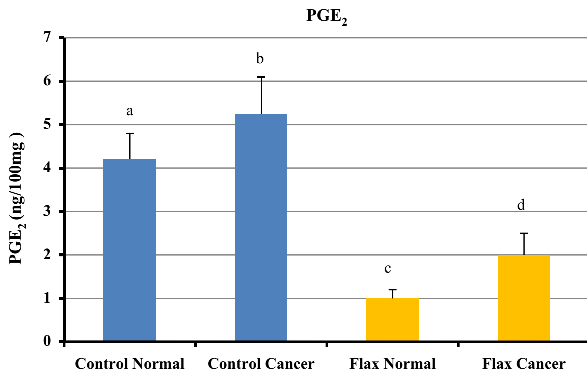

Fig. 5. Prostaglandin E_2 (PGE_2) concentrations in hen ovarian tissues were measured by LC–MS–MS. The cancerous ovaries of hens had higher concentrations of PGE_2 compared with normal ovaries, regardless of diet. Flaxseed reduced the concentrations of PGE_2 in normal and cancerous ovaries of the flaxseed-fed hens compared with normal and cancerous ovaries of the control-fed hens, respectively. a vs b and c vs d, $P<0.05$; a vs c and b vs d $P<0.01$.

in ovaries and thereby decreases the severity of ovarian cancer. Here we report that the reduced ovarian cancer severity in hens fed the flaxseed enriched diet for one year is correlated to decreased concentration of prostaglandin E_2 and COX-2 in the ovaries of hens.

Reduction in ovulation results in a decrease in ovarian cancer [29]. This raises the question: is the reduced severity that we observed in this study due to a decrease in ovulation rate? Therefore, we monitored the egg laying (ovulation) rate and the data showed that the flaxseed enriched diet does not change the ovulation rate compared with the control diet indicating that the reduced ovarian cancer severity in hens fed the flaxseed diet is not due to reduction in ovulation.

Incorporation of $n-3$ PUFAs into egg yolks of flaxseed-fed hens provides a non-invasive measure of the incorporation of $n-3$ PUFAs into ovaries of the hens. Our data indicated that incorporation of $n-3$ PUFAs into egg yolks of hens fed ground flaxseed is not statistically different than incorporation of $n-3$ PUFAs into egg yolks of hens fed whole flaxseed. Thus, hens in this study were fed a diet supplemented with 10% whole flaxseed. Higher amounts of ALA, EPA and DHA in egg yolks of the hens fed flaxseed were detected compared to yolks collected from the control diet-fed hens. Our data indicated that the hens were able to incorporate high amounts of $n-3$ PUFAs from flaxseed into their eggs. This was in agreement with a study published by Scheideler et al. that showed 10% flaxseed diet increased the incorporation of $n-3$ PUFAs into the eggs [30].

We detected higher expression of COX-1 protein and mRNA in cancerous ovaries compared with normal ovaries, regardless of diet. Hales et al. reported that COX-1 is over-expressed in ovarian cancer in hens [5] and Urick et al. reported that increased COX-1 expression in hen ovaries with tumors was correlated to increased PGE_2 levels [4]. In present study, the expression of COX-1 was localized to the granulosa cell layers and confined to the OSE and cortical stroma adjacent to the follicle in normal ovaries. This was in agreement with previous studies conducted in rats [31] and hens [5] that have shown COX-1 is constitutively expressed in the ovary and confined to interstitial thecal cells.

There are many reports on the role of COX-2 enzyme and its over-expression in cancer. In the mammalian ovary, COX-2 is localized to the granulosa cells and induced by luteinizing hormone at the time of the preovulatory gonadotropin surge [32]. In the hen ovary, COX-2 is expressed in the granulosa cells, but distinct from the reported distribution in the mammalian ovary, COX-2 is also highly expressed in the interstitial tissue of the ovary [4,5]. Because chickens have a high ovulation frequency, high expression and accumulation of COX-2 in hen ovaries are possible. Our data indicated that COX-2 expression in ovarian carcinoma becomes more localized to discrete foci, trapped within the glandular like structures of the ovarian tumor. Arico et al. reported that COX-2 can induce angiogenesis via vascular endothelial growth factor (VEGF) and prostaglandin production and can also inhibit apoptosis [33]. Therefore, COX-2 may be a plausible target for the prevention and treatment of ovarian cancer. One feasible way to blunt the cancer-promoting activity of COX-2 is to limit its access to arachidonic acid, the precursor for the prostaglandins which mediate this activity. Flaxseed contains high amounts of ALA which can be converted to EPA and DHA. EPA can act as a competitive inhibitor of arachidonic acid binding to COX-2 [34]. There was a significant decrease in COX-2 mRNA expression in both normal and cancerous ovaries of flaxseed-fed hens compared to hens fed the control diet. However, there was no similar reduction observed in the expression of COX-2 protein in ovaries of hens in any of the groups. The lack of correlation between COX-2 mRNA and protein expression has been previously reported. Kim et al. has shown that HuR knockdown colon cancer cells have reduced COX-2 mRNA levels which did not lead to a reduction in COX-2 protein levels [35]. Furthermore, the semi-quantitative identification of COX-2 protein by IHC or western blot does not necessarily correlate with enzymatic activity [36]. The data suggest that flaxseed is a specific COX-2 inhibitor at the transcriptional level and may also inhibit COX-2 activity by substrate level modulation. The flaxseed-mediated inhibition in COX-2 is likely important to the mechanisms of action of flaxseed in the reduction of ovarian cancer in our study. Our data are in agreement with previous studies which have shown the inhibition of COX-2 by $n-3$ PUFAs [37–39].

High concentrations of PGE_2 are believed to be immunosuppressive [40], increase angiogenesis [41], stimulate cell proliferation and inhibit apoptosis in ovarian cancer cell lines [42]. PGE_2 mediated immune suppression may lead to increased susceptibility to tumor formation and impaired defense against previously formed tumors [4,43]. The influence of PGE_2 pathway on contributing factors in tumor formation and progression is clear; however, the mechanisms by which the $n-3$ PUFAs inhibit PGE_2 pathway remains to be fully elucidated. Our results indicated that a flaxseed enriched diet significantly reduces the levels of PGE_2 in ovaries of hens compared to ovaries of hens fed the control diet. This might be due to down-regulation of COX-2 at the transcription level, and to the substrate-level modulation of the enzymatic activity of both COX enzymes. The arachidonic acid (AA) is the predominant substrate for both COX enzymes. Dietary $n-3$ PUFA may modulate substrate pools available to COXs and lipoxygenases (LOX), thereby controlling the formation of downstream eicosanoids such as PGE_2 [44]. The EPA can compete with AA and act as an alternative substrate for COX enzymes [45] and decrease PGE_2.

The ability of flaxseed to suppress ovarian cancer growth might be due to reducing proliferation and increasing apoptosis in ovarian tumors via diminishing PGE_2 concentrations. Furthermore, ovarian tumors with high tissue contents of PGE_2 have a weak response to chemotherapy [46], therefore, a flaxseed enriched diet not only may prevent progression of cancer, but it may increase the sensitivity of tumors to chemotherapy. Moreover, it is likely that anti-estrogenic properties of lignans play role in anti-cancer effects of flaxseed.

EP4 receptor promotes tumor progression by increasing pro-angiogenic factor and tumor cell invasiveness in ovarian carcinoma cell lines. Spinella et al. reported that EP2–EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion [47]. However, we did not detect a high expression of EP4 in ovaries with high concentration of PGE_2. It might be due to use of in vivo model instead of ovarian cell lines.

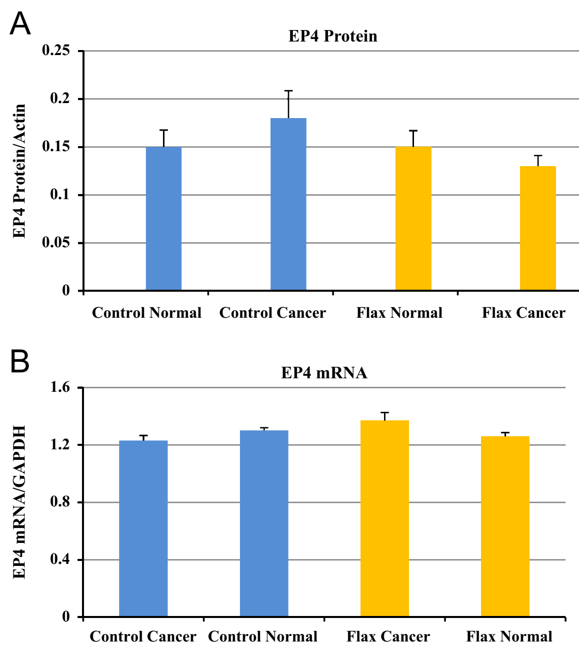

Fig. 6. Expression of EP4 protein and mRNA was measured in ovaries of hens. No significant differences were found among the groups.

Our study demonstrated that consumption of 10% flaxseed enriched diet for one year does not affect expression of EP4 neither at the level of transcription nor translation.

Taken together, the present results indicate that the flaxseed mediated reduction in the severity of ovarian cancer in hens [12] is correlated to the flaxseed-mediated reduction in PGE_2. The reduction in PGE_2 appears to be primarily due to inhibition of COX-2, and it is likely that substrate modulation of both COX enzymes further contributes to the reduction in PGE_2 levels. Concomitant flaxseed mediated reduction in PGE_2 and ovarian cancer severity suggests that inflammatory prostaglandins drive the progression of the disease; therefore PGE_2 may be an effective target for the reduction of ovarian cancer. We have previously shown that exactly at the age that ovarian cancer is first detected in chickens, PGE_2 levels increase indicating cumulative inflammation may also contribute to initiation of ovarian cancer [48]. We have recently shown that life-long consumption of flaxseed reduces not only the severity but also the incidence of ovarian cancer in hens [49]. In conclusion, our study provides new insight into the mechanism of action of flaxseed in the reduction of ovarian cancer severity and will establish the foundation for clinical trials to test the efficacy of dietary intervention with flaxseed for the prevention and suppression of ovarian cancer in women.

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