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Flaxseed and its components differentially affect estrogen targets in pre-neoplastic hen ovaries

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Abstract

Flaxseed has been studied for decades for its health benefits that include anti-cancer, cardioprotective, anti-diabetic, anti-inflammatory properties. The biologically active components that mediate these effects are the omega-3 fatty acids and the lignan, secoisolaricirescinol diglucoside. We have previously shown that whole flaxseed supplemented diet decreases the severity and incidence of ovarian cancer while a 15% dose of flaxseed is most protective against inflammation and estrogen-induced chemical and genotoxicity. The objective of this study was to dissect the independent effects of the two flaxseed components on estrogen signaling and metabolism. Two and half year old hens were fed either a control diet, 15% whole flaxseed diet, defatted flax meal diet or 5% flax oil diet for 3 months after which the animals were sacrificed and blood and tissues were harvested. Whole flaxseed diet caused a decrease in expression of ERα. ERα target gene expression was assessed using RT^2 profiler PCR array. Some targets involved in the IGF/insulin signaling pathway (IRS1, IGFBP4, IGFBP5) were downregulated by flaxseed and its components. Flaxseed diet also downregulated AKT expression. A number of targets related to NF-κB signaling were altered by flaxseed diet including a series of targets implicated in cancer. Whole flaxseed diet also affected E2 metabolism by increasing CYP1A1 expression with a corresponding increase in the onco-protective E2 metabolite, 2-methoxyestradiol.

The weak anti-estrogens, enterolactone, enterodiol and 2-methoxyestradiol, might be working synergistically to generate a protective effect on the ovaries from hens on whole flaxseed diet by altering the estrogen signaling and metabolism.

Keywords

estrogen; flaxseed; ovarian cancer; hen

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1. INTRODUCTION

Flaxseed is mainly cultivated as an oil crop and is a rich source of the omega-3 fatty acid, alpha-linolenic acid. Other than its high oil content, flaxseed is also a source of dietary fiber, plant lignans and protein. This makes flaxseed a very rich source of nutrition [1]. For many years, scientists have been intrigued by the specific health benefits associated with the biologically active components of flaxseed. The two main biologically active components of flaxseed are the polyunsaturated omega-3 fatty acid, alpha linolenic acid, which makes up 59% of the oil content, and the lignan, [2] secoisolaricirescinol diglucoside (SDG) that comprises about 0.7%–1.9% of the whole flaxseed [3]. SDG is metabolized by the gut flora to enterolactone (EL) and enterodiol (ED).

The omega-3 fatty acids (OM-3FAs) have been reported to have chemo-preventative effects [4]. There is extensive evidence regarding the anti-inflammatory [5, 6], anti-arrhythmic [7], anti-thrombotic [8], anti-atheromatous [9] and hypolipidemic [10] effects of the OM-3FAs. The lignan, SDG, its aglycone form secoisolaricirescinol (SECO), and their metabolites, have hydroxyl radical scavenging properties which make them potent anti-oxidants [11]. The SDG metabolites, EL and ED have been shown to antagonize E2 signaling [12]. Flaxseed and its components are beneficial in reducing symptoms of benign prostate hyperplasia [13], decreasing cholesterol levels [14] and decreasing skin sensitivity while improving skin texture [15]. We have shown that flaxseed diet causes an increase in the 2-hydroxyestradiol/16-α hydroxyestradiol metabolite ratio in urine and serum [16, 17], which has been shown to decrease the risk of developing cancer in postmenopausal women [18]. Flaxseed and its components have protective effects against a myriad of cancers including breast cancer [19–21], prostate cancer [22, 23] and melanoma [24].

We have previously demonstrated that flaxseed supplemented diet reduces the incidence and severity of ovarian cancer in chickens [25, 26]. This effect corresponded with a decrease in COX-1 and COX-2 enzyme expression in parallel with decrease in prostaglandin E2 (PGE2) levels in the ovary [27]. The decrease in PGE2 levels could be a result of the high alpha linoleic acid content of flaxseed oil which is partially converted to EPA (eicosapentanoic acid) and DHA (docosahexanoic acid) in the cells. EPA and DHA can get incorporated into the cell membrane and shift the equilibrium of PG (prostaglandin) synthesis to a less inflammatory type (PGE3) [28] as previously demonstrated by our group [29]. In addition, E-cadherin, a marker characteristically upregulated in human epithelial ovarian cancers as well as in the hen ovarian tumor [30], is significantly downregulated in ovarian tumors from hens fed a flaxseed supplemented diet [31]. Flaxseed lignan metabolites, EL and ED, have been shown to decrease estrogen dependent receptor activation in breast cancer cells and flaxseed diet also results in decreased ERα expression in the chicken ovary[17]. This is significant because E2 increases tumor aggressiveness and it has been demonstrated that treatment with E2 resulted in increased rate of distant metastasis in the ovarian cancer xenograft mouse model [32].

Our group has previously shown that flaxseed diet can alter a myriad of markers implicated in ovarian cancer including estrogen receptor alpha, 2-hydroxyestradiol/16-α-

hydroxyestradiol ratio, PGE2, COX-1 and COX-2 enzymes, E-cadherin, CYP1B1, FOXA2, PAX2, EN-1, MSX2 etc. [17, 26, 30, 31, 33], but it is important to determine the precise pathways targeted by the biologically active components of flaxseed that affect the expression of a wide range of cancer targets.

The objective of this study was to understand the independent effects of the individual components of the flaxseed on estrogen signaling. This is essential in order to attribute the changes in specific E2 targets to a particular biomolecule and its downstream pathway. By unraveling the molecular targets altered by flaxseed components we can design a better preventative and therapeutic strategy against ovarian cancer.

2. MATERIALS AND METHODS

2.1 Reagents

All the reagents are as described in [17]. The AKT (9272S) and pAKT (4060S) antibodies were from Cell Signaling Technology, Beverly, MA. The ERα (sc-73479),ERα (sc-543) antibodies and CYP1A1(sc-20772) antibody were from Santa Cruz Biotechnology, Dallas, TX. The Custom Chicken RT^2 Profiler PCR array was designed by SABiosciences, Qiagen, Venlo, Netherlands. The dual luciferase reporter assay (e1910) was from Promega and lipofectamine 3000 from Invitrogen. E2 coat-a-count RIA kit from Siemens (TKE21). HEK 293 cells were obtained from ATCC (ATCC CRL-1573).

2.2 Diet composition

The diet compositions have been described in Table 1. The 4 diets included were control diet, whole flaxseed supplemented diet, defatted flax meal (DFM) supplemented diet (lignan rich) and flax oil supplemented diet (omega 3 fatty acid rich). All the diets were isocaloric. To balance the high protein and high fiber content of the flaxseed meal and whole flax diets, the flax oil and control diets were supplemented with corn gluten meal and solka floc (International Fiber Corporation), respectively. To balance for the fat content of the control and flax oil diets, the whole flax and flax meal diets were supplemented with the suitable amount of qual fat (DarPro). The diets were formulated to differentiate the effects of the flax lignan from the flax oil as illustrated in Table 1.

2.3 Animals

Two and a half year old hens (*Gallus domesticus*) were either fed control diet or diet supplemented with individual components of flaxseed for a period of 3 months (Table 2). Each diet group had 30 birds. Hens were exposed to a photoperiod of 17 h light: 7 h dark, with lights turned on at 05:00 h and turned 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 Southern Illinois University at Carbondale. Blood was collected at different time points throughout the study by wing vein puncture and tissues were harvested at the end of the study after sacrificing the hens. Tissue collection and processing was done as described previously [34].

2.4 RNA isolation and cDNA synthesis

Total RNA was extracted from ovarian tissue using Trizol reagent as described previously [34, 35]. RNA was quantified by measuring the sample absorbance at A260. RNA quality was assessed by using Experion RNA StdSens Analysis System (Biorad). RNA samples were then treated with RQ1 RNase-free DNase (Promega, Madison, WI) prior to reverse transcription reaction. cDNA was synthesized using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). This cDNA was used to analyze gene expression using real time qPCR and PCR array.

2.5 PCR array

Changes in the expression of estrogen target genes were assessed by using the SABiosciences RT^2 PCR array. The array was specifically designed for analyzing E2 targets in the chicken tissue. Ninety-six target genes were analyzed for 6 samples from each of the 4 diet groups using 6 arrays. cDNA was synthesized from the RNA samples isolated from the ovary tissues using the RT^2 First Strand kit (Qiagen). Real time qPCR was performed using the RT^2 SYBR Green qPCR Mastermix (Qiagen) and CFX384 Touch Real Time PCR detection system (Biorad). Target genes were normalized to 5 housekeeping genes (ACTB, HMBS, H6PD, RPL4 and UBC) that were pre-selected for the array. PCR array data was analyzed by using the RT^2 Profiler PCR Array Data Analysis version 3.5 on the SABiosciences website.

2.6 Real time qPCR

Target gene mRNA levels were analyzed with Real time quantitative PCR using the CFX384 Real Time System. Gene specific primers were used for target as well as reference gene amplification (Supplemental Table 2). GAPDH and 18s rRNA were used as housekeeping genes to normalize target gene expression. Amplification conditions: 95°C for 30s followed by 40 cycles of 95°C for 10s and 60°C for 15s with melt curve measured at 65 °C to 95 °C every 0.5 °C gradient for 5s. A non-template control was run for every target gene amplified.

2.7 Western blotting

Proteins were isolated from the ovary samples as described previously [34]. Twenty five micrograms of total protein was separated using a SDS-PAGE gel with 10% acrylamide/SDS separating gels and transferred to nitrocellulose membranes. Membranes were blocked using the SeaBlock blocking buffer (Pierce) followed by overnight incubation at 4° C with the target primary antibodies (CYP1A1, ER alpha, pAKT, AKT). Following the overnight incubation, blots were washed using 1X TBS with 0.01% Tween 20. Blots were further incubated with a DyLight™ 680 conjugated goat anti-mouse IgG antibody (H&L) and DyLight™ 800 conjugated goat anti-rabbit IgG antibody (H&L) for an hour at room temperature. After 3 washes with 1X TBS with 0.01% Tween 20, the blots were scanned for infrared signal using Odyssey CLx imaging system (LI-COR Biosciences). All the targets were normalized to β-Actin expression.

2.8 Immunohistochemistry

Tissues were collected, fixed and embedded as described in [34]. Five micrometer thick sections were cut and mounted on SuperFrost Plus microscope slides. Following deparaffinization, slides were rehydrated through xylene and graded ethanol solutions. Antigen retrieval was performed by using 0.9% Antigen unmasking solution (Vector Laboratories) and pressure cooked at 20 psi for 5 min. Slides were allowed to cool and sections were blocked with 5% normal horse serum. Sections were incubated with anti-estrogen receptor antibody overnight at 4° C. After washing with 1X PBS with 0.01% Tween 20, sections probed with anti-estrogen receptor antibody (sc-543) were incubated with Alexa 488 conjugated anti-mouse IgG (Jackson laboratories) for an hour at room temperature, washed with 1X PBS with 0.01% Tween 20 and mounted using Dapi Fluoromount G (Southern Biotech). Sections were visualized using a Leica DM5500Q microscope and images were captured using a Leica DFC365 FX camera. Images taken from the A4 (Dapi) and L5 (Alexa 488) channels were superimposed using the Leica Application Suite-Advanced fluorescence version 2.6.0.7266 software.

2.9 Liquid Chromatography Tandem Mass Spectrometry for Enterodiol and Enterolactone analysis

Levels of ED and EL were analyzed in the liver tissue by using LC MS/MS analysis. Extraction and analysis on the liver samples were performed as described previously [17].

2.10 High performance liquid chromatography for SDG analysis

SDG levels from the 3 different batches of diet were analyzed across all the diet groups. SDG was extracted and analyzed as described previously [17].

2.11 Gas chromatography for fatty acid analysis

Fatty acids were extracted from the ovary samples and levels of OM-3FAs and omega-6 fatty acids (OM-6FAs) were analyzed using gas chromatography as described previously [17, 29].

2.12 Statistics

All the surrogate endpoints were analyzed using 4–8 biological replicates and 2 technical replicates of each sample. Effect of the different flaxseed diets was assessed by normalizing to control diet. For experiments involving real time qPCR, western blotting, ELISA, RIA and *in vitro* reporter assay, statistical calculations were done using GraphPad Instat software by employing one-way ANOVA analysis followed by Tukey's range test. For the PCR array, data analysis was performed by the RT^2 profiler PCR array data analysis version 3.5 software available on the Qiagen website. The results that have been presented in the figures have been analyzed using the one way ANOVA. A $p < 0.05$ was considered significant while a $p < 0.01$ was considered highly significant.

3. RESULTS

3.1 Analysis of SDG and OM-3FA content in the diets using LC and GC

LCMS analysis indicated that SDG was only detected in the whole flaxseed and defatted flax meal diets while the control diet and flax oil supplemented diets did not have any detectable levels of SDG (Figure 1A). The SDG content of the defatted flax meal diet was 1.5 times more than that in the whole flaxseed supplemented diet (Figure 1A). Further calculations suggested that excess flax lignan got added while formulating the defatted flax meal diet. Levels of OM-3FAs and OM-6FAs were determined by GC analysis. Ratio of OM-3FAs to OM-6FAs was significantly higher in the whole flaxseed and flax oil supplemented diets in comparison to the defatted flax meal supplemented diet. Control diet had comparable levels of OM-3FAs and OM-6FAs (Figure 1B).

3.2 Levels of Enterolactone and Enterodiol in the liver tissue

ED and EL were only detected in the tissue of the hens that were fed either whole flaxseed or defatted flax meal diets (Figure 2). No ED or EL was detected in the hens fed flax oil supplemented diet and hens fed control diet (data not shown).

3.3 Estrogen receptor alpha expression decreases with whole flaxseed supplemented diet

Estrogen receptor alpha is predominantly expressed in the granulosa cells of the hen ovary and to some extent in the ovarian surface epithelium. Immunofluorescence staining suggested that ERα protein expression decreased in the granulosa cells of hens that were fed a diet supplemented with whole flaxseed (Figure 3C) but not in either of the other diets supplemented with its components viz. flax oil (Figure 3D) and defatted flax meal (Figure 3B). Western blot analysis corroborated the immunofluorescence results and confirmed that the hens that were given whole flaxseed supplemented diet had decreased ERα protein expression in their ovaries (Figure 3E). Real time qPCR analysis also suggested that whole flaxseed supplemented diet causes a decrease in ERα mRNA levels (Figure 3F). The decrease in ER α expression seemed to have led to a compensatory increase in ovarian CYP19 expression (Supplementary Figure 2B) along with serum E2 levels in the whole flaxseed diet hens (Supplementary Figure 2A).

3.4 Flaxseed and its components increase 2-methoxyestradiol levels in the serum with a corresponding increase in liver CYP1A1

The enzyme CYP1A1 converts E2 to 2-hydroxyestradiol, which is in turn a substrate for Catechol-O-Methyltransferase (COMT) that results in the formation of 2-methoxyestradiol. ELISA analysis for 2-methoxyestradiol on the chicken serum samples suggested that hens that were fed whole flaxseed supplemented and defatted flax meal supplemented diets had higher levels of 2-methoxyestradiol (Figure 4A). Whole flaxseed supplemented diet dependent upregulation of CYP1A1 enzyme substantiates the observed increase in the levels of the anti-angiogenic and pro-apoptotic 2-methoxyestradiol in the serum (Figure 4B).

3.5 Effect of flaxseed supplemented diet on estrogen targets involved in the IGF signaling pathway

PCR array analysis suggested that the whole flaxseed supplemented diet resulted in a decrease in the mRNA expression of IGFBP4 and IGFBP5 (Figure 5A and 5B), which are known to stabilize IGF-1. Diets supplemented with flaxseed as well its components resulted in a decrease in IRS1 mRNA expression (Figure 5C).

3.6 Flaxseed supplemented diet decreases AKT expression in the ovary

Since AKT2 is predominantly involved in glucose homeostasis we analyzed the effect of flaxseed diet on the mRNA expression of AKT2 and also examined the effect of diet on AKT activation. qPCR analysis revealed that mRNA expression of AKT2, predominantly involved in glucose homeostasis, was downregulated by diets supplemented with whole flaxseed and its components (Figure 6A). Western blotting analysis suggested that levels of total AKT protein and phosphorylated AKT protein decreased with whole flaxseed supplemented diet, when normalized to beta-actin (Figure 6B and 6C). However, the ratio of pAKT/AKT remained unaffected by flaxseed or its components (Figure 6D).

3.7 Whole flaxseed and its components may abrogate NF-κB activation

qPCR analysis on the ovary tissue indicated that hens that were fed whole flaxseed supplemented diet showed increased expression of PPAR γ mRNA, possibly due to a strong trend in NCOR2 downregulation with whole flaxseed supplemented diet (Figure 7A and 7B). Since PPAR γ can regulate NF-κB expression, we performed qPCR, which revealed that diet did not affect NF-κB mRNA expression (Figure 7D). Flax oil diet did lead to an upregulation of the NF-κB inhibitor, NFKBIA expression (Figure 7E). NFKBIA prevents nuclear translocation of NF-κB thus preventing its transcription activity. Interestingly, we observe a decrease in the expression of anti-apoptotic gene BCL-2L1, a direct target of NF-κB, in the whole flaxseed diet group (Figure 7F). NCOA1, a cofactor that regulates NF-κB's transcriptional activity was also downregulated by whole flaxseed diet (Figure 7C).

3.8 Flaxseed and its components alter ovarian expression of genes implicated in cancer

PCR array analysis of E2 targets revealed that mRNA levels PHB2 were upregulated in whole flaxseed, defatted flax meal and flax oil supplemented diets (Figure 8A) while mRNA levels of c-FOS were upregulated only with defatted flax meal (Figure 8B). mRNA levels of transcription factor, MAFF decreased with whole flaxseed, defatted flax meal and flax oil supplemented diets (Figure 8C) while mRNA levels of scaffold protein, p130cas decreased with whole flaxseed diet (Figure 8D). Other targets altered by the diets and analyzed by the PCR array are listed in Table 2.

4. DISCUSSION

The objective of the current study was to dissect the individual effects of the biologically active components of flaxseed through dietary intervention. We have already established that a diet supplemented with flaxseed is effective in reducing the severity and incidence of ovarian cancer in chickens [25]. We have further demonstrated that the effects exhibited by flaxseed are dose dependent by showing that a dose of 15% flaxseed is the most protective

against inflammation and estrogen-induced chemical and genotoxicity [17]. Based on these data, we designed a study to incorporate diets enriched with either whole flaxseed, defatted flax meal or flax oil and compared their effects to control diet (Table 1). SDG was present in the whole flaxseed and defatted flax meal diets while OM-3FAs were only present in the whole flaxseed and flax oil diets. Our data indicated that the whole flaxseed supplemented diet yielded the best results in terms of decreasing ERα expression and altering the expression of a series ERα targets genes involved in the IGF/insulin signaling pathway, cell proliferation, cancer metastasis as well as altering E2 metabolism.

LC MS/MS analysis on the liver tissue indicated that ED and EL were only detected in the tissues of the hens that were fed a diet supplemented with either whole flaxseed or defatted flax meal (Figure 2). ED and EL are metabolites of SDG, which was only present in the whole flaxseed and defatted flax meal supplemented diets (Figure 1). It has been proposed that ED and EL are ERα antagonists in the presence of E2 and weak agonists in the absence of E2 [12]. This was tested in our *in vitro* model with HEK293 cells. A high concentration of EL was able to decrease ERα activation in presence of E2 as measured by a luciferase reporter assay (Supplementary figure 1).

2-methoxyestradiol, a natural metabolite of estradiol has been shown to exhibit anti-tumorigenic and anti-angiogenic activity *in vivo* as well as *in vitro* [36]. 2-methoxyestradiol is formed by the action of COMT on 2-hydroxyestradiol, which is predominantly derived by the action of the cytochrome P450 enzyme CYP1A1. Similar to ED and EL, 2-methoxyestradiol binds weakly to the estrogen receptor and does not elicit strong estrogenic responses [37]. 2-methoxyestradiol can also induce apoptosis in cancer cells by upregulating p53 [38]. There is also evidence suggesting that 2-methoxyestradiol induces its apoptotic effects by interfering with the microtubule dynamics and by inhibition of the mitochondrial electron transport complex 1, leading to the generation of reactive oxygen species [39]. It has been suggested that the growth inhibitory effects mediated by 2-methoxyestradiol could be due to an intracellular effector or a specific receptor [40], distinct from its effects on ERα. Analysis of 2-methoxyestradiol levels suggested that the whole flaxseed supplemented diet and the defatted flax meal supplemented diet resulted in an increase in serum levels of 2-methoxyestradiol (Figure 4A). Concurrently, there was also an increase in CYP1A1 protein level in the livers of hens fed the whole flaxseed diet (Figure 4B). The whole flaxseed diet preferentially facilitates the 2-hydroxylation of E2, in turn making less E2 available for 4-hydroxylation or 16-hydroxylation. This indicates that whole flaxseed diet has a protective effect against the genotoxic 4-hydroxyestradiol and highly estrogenic 16-hydroxyestradiol [41, 42]. In addition, due to its weak affinity for the ER, 2-methoxyestradiol can act as an E2 antagonist similar to ED and EL.

The PCR array suggested that AHR mRNA levels were upregulated in defatted flax meal and whole flaxseed supplemented diets. The aryl hydrocarbon receptor (AHR) and its nuclear translocator (ARNT) are activated in presence of polyaromatic hydrocarbons (PAHs). Some plant derived compounds like resveratrol, isoflavones like diadzein, hesperetin etc. activate AHR [43]. Since flaxseed lignan metabolites ED and EL have a similar polycyclic structure, they might be stimulating AHR expression in our model. On activation, they promote the expression of cytochrome p450 enzymes like CYP1A1 and

CYP1B1, which metabolize the PAHs [44]. It has been shown that AHR can regulate CYP1A1 and CYP1B1 differentially, based on the cell type and the ligand involved in activation [45]. We did not observe any significant change in CYP1B1 expression in the ovary or liver but CYP1A1 (undetectable in chicken ovary) expression was upregulated in the liver of whole flaxseed-fed birds (Figure 4B). In addition, it has been very well established that AHR can promote ER α destabilization by targeting it for proteasomal degradation by ubiquitin ligase [46].

We observed that ERα expression was down regulated in the ovaries of the hens that were fed a diet supplemented with 15% whole flaxseed (Figure 3). The ERα gene is E2 responsive; as a result, the presence of ED and EL could be responsible for downregulating ERα gene. Effects of E2 are predominantly mediated through ERα in the ovary. E2 is critical for maintaining normal ovarian function including egg development, tissue homeostasis etc. Flaxseed diets do not impact the egg laying frequency in these hens [17, 27] indicating that the normal function of the ovary was maintained. We analyzed the E2 levels in the serum samples and found that in fact the whole flaxseed diet and the flax oil diets had higher levels of E2 (Supplementary figure 2A). Cyp19/aromatase catalyzes the conversion of testosterone to estradiol. Assessing the mRNA levels of CYP19 in the ovary revealed that it was significantly upregulated in the whole flaxseed diet (Supplementary figure 2B). Downregulation of ER could lead to a compensatory increase in E2, likely the mechanism through which E2 maintains its normal functions in the ovary.

To test the effect of ERα downregulation on the expression of its target genes, we did a PCR array for assessing the expression of 96 ERα target genes, qPCR analysis and western blotting (Table 2). Genes involved in the IGF/insulin signaling pathway and the NF-κB pathway were altered by the whole flaxseed and defatted flax meal supplemented diets. Insulin and IGF are known to regulate cell cycle progression, growth, proliferation, metabolism and over all cell survival. There is strong evidence of IGF and insulin pathway dysregulation in several cancers including breast [47, 48], prostate, colon and pancreatic cancers [49]. Several therapies have been directed towards inhibiting IGF-1R signaling with the objective of preventing cancer progression [50]. In the last two decades, there have been reports of ovarian cancer cells over-expressing IGFs, IGFBPs, IGF-1R and other components of the IGF/insulin signaling pathway [51]. We found that the mRNA levels of IGFBP4, IGFBP5 and IRS-1 were downregulated by whole flaxseed supplemented diet (Figures 5A, 5B and 5C respectively). Studies with human ovarian and breast cancer cells have established that E2 upregulates, while the anti-estrogen, ICI downregulates IGFBP4 expression [52, 53]. Since we hypothesize that flaxseed is decreasing signaling thorough ERα, these results corroborate our theory. Wang et al. have shown that IGFBP5 was upregulated specifically in high grade ovarian serous carcinomas when its expression was analyzed on a tissue microarray that included a variety of normal and cancer samples[54]. Similarly, decrease in IRS-1 mRNA levels indicate a decrease in propagation of the signal stimulated by insulin/IGF-1 as IRS-1 is an important adaptor molecule activated downstream of IGF-1R. Insulin and IGF-1 are regulated via the MAP kinase/ERK1/2 and AKT/mTOR pathways. The MAP kinase ERK1/2 pathway mainly promotes cell survival. The AKT/mTOR pathway is involved in transcription of anti-apoptotic proteins, translation of targets involved in cell cycle progression, angiogenesis and regulating glucose metabolism. AKT

can increase surface GLUT1 expression and regulate the activation of the rate limiting enzyme phosphofructokinase-1 [55]. AKT plays a role in oocyte maturation, granulosa cell development and activation of primary follicles [56]. We observed a decrease in the ovarian expression of AKT2 by supplementing the diet with whole flaxseed, defatted flax meal or flax oil (Figure 6D) but neither whole flaxseed nor its components had an effect on AKT phosphorylation in the ovary of hens (Figure 6E). We have also observed that flaxseed and its components downregulate total and phosphorylated AKT expression in hen ovarian tumors (manuscript in preparation). It is known that in endometrioid type ovarian cancer, the PI3K/AKT pathway is dysregulated due to mutation in the PTEN gene [57]. Endometrioid ovarian cancer is the most common histotype found in primary ovarian tumors of hens. As a result, alterations in the AKT pathway may be protective against cancers driven by the mutational landscape of endometrioid ovarian cancer. This suggests that flaxseed and its components regulate AKT expression at a transcriptional level and decrease carcinogenic potential of the ovary without affecting its normal function.

It has been demonstrated that E2 downregulates PPAR γ expression via the estrogen receptor [58]. We found that PPAR γ mRNA expression was upregulated by whole flaxseed supplemented diet further validating the possible decrease in signaling via ERα (Figure 7A). The Peroxisome Proliferator Activator Receptor (PPAR) family of nuclear receptors play a role in glucose metabolism and lipid homeostasis. PPAR γ is expressed in the granulosa cells of the normal ovary and plays a role in ovulation, inducing hormonal responses and tissue maintenance [59]. The anti-proliferative and pro-differentiation actions of PPAR γ render it anti-oncogenic. Transcriptional repressor cofactors like NCORs inhibit PPAR γ expression and in turn the transactivation and transrepression of PPAR γ target genes [60, 61]. As NCOR2 is an ERα target gene, flaxseed dependent downregulation of NCOR2, a PPAR γ transrepressor (Figure 7B), could be responsible for the observed increase in the expression of PPAR γ in the whole flaxseed supplemented diet. PPAR γ s also known to downregulate COX-2 expression [62] and decrease PGE2 levels in ovarian cancer cells [63], suggesting that it has anti-inflammatory effects. Analysis of PGE2 levels in the ovary revealed that supplementation with whole flaxseed and flax oil led to a decrease in PGE2 levels (data not shown).

PCR array analysis also revealed that the flax oil diet led to an upregulation of NF-κB inhibitor IkB (NFKBIA). IkBα prevents nuclear translocation of the NF-κB subunits by sequestering them in a complex in the cytoplasm [64]. Further, the array showed that the whole flaxseed diet resulted in downregulation of NCOA1 mRNA expression. NCOA1 (SRC 1) belongs to the p160 steroid receptor co-activator family and regulates NF-κB's transcription activity. We also observed that the expression of anti-apoptotic protein, BCL-2L1 was downregulated in the whole flaxseed diet. BCL-2 is a major target of NF-κB and facilitates its pro-survival responses [65]. Although its target genes were downregulated, qPCR analysis of NF-κB expression did not suggest any change in NF-κB mRNA levels with diet. Since NFkB's transcriptional activity depends on its nuclear translocation, flaxseed diet could be mediating its actions by sequestering NFkB in the cytoplasm. This definitely warrants further investigation. These data suggest that whole flaxseed and its components might decrease NF-κB mediated cellular growth, survival and inflammatory

responses by decreasing NF-κB transcriptional activation in turn affecting its target gene expression.

Several other ER targets are also modulated with flaxseed diet (Table 2). P130cas (BCAR1) was downregulated in whole flaxseed supplemented diet while AP1 transcription factor family member, MAFF was downregulated in whole flaxseed, defatted flax meal and flax oil diets. P130cas is an adhesion protein that is upregulated in breast cancer [66], promotes cell migration and induces tamoxifen resistance [67]. E2 exposure leads to upregulation of Maf transcription factors in breast cancer [68] and promotes distant metastasis [69]. C-maf is commonly upregulated in multiple myeloma[70] and promotes the tumor-stroma interaction [70]. C-FOS and estrogen repressor PHB2 were upregulated with flaxseed and its components, in our study. Downregulation of FOS is considered to be a bad prognosis in ovarian and other cancers. Since FOS is believed to have a pro-apoptotic function, loss of FOS leads to a more invasive and metastatic tumor phenotype [71]. PHB2 is a tumor suppressor that on activation has been shown to prevent the progression of ERα positive breast cancer [72]. Because of its anti-estrogenic effects, it abrogates the signaling of nuclear as well as membrane associated ERα [73]. These data indicate that flaxseed is effective in altering the expression of genes implicated in a number of cancers. Since most of these genes are involved in steroid receptor regulation, they can be targeted for therapeutic strategies in steroid dependent cancers.

Our current study suggests that surrogate end points were differentially affected by the components of flaxseed. A series of ER targets were altered by whole flaxseed and its components due to direct alterations in ERα signaling and expression. Target genes that were downregulated by flaxseed include potent oncogenes, repressors that target cytokine inhibitory receptors, important signaling mediators of growth, proliferation and anti-apoptotic factors and steroid receptors that are upregulated in cancer. Some targets that were upregulated include estrogen receptor repressor, receptors with antiinflammatory actions, etc. Besides ER target genes, flaxseed diet also increased levels of 2-methoxyestradiol in turn corroborating our previous observations where we showed that flaxseed results in upregulation of 2-hydroxyestradiol (precursor of 2-methoxyestradiol).

In conclusion, the whole flaxseed supplemented diet was more effective than the defatted flax meal and the flax oil diets, in altering molecular targets involved in inflammation, glucose metabolism and apoptosis while whole flaxseed, defatted flax meal and flax oil diets were equally potent in altering the expression of genes involved in carcinogenesis. The weak anti-estrogens; enterolactone, enterodiol and 2-methoxyestradiol might be working synergistically to generate a protective effect in the ovaries from whole flaxseed fed hens by altering estrogen signaling and metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Flaxseed and its components differentially alter estrogen signaling and metabolism
- Flaxseed was more effective than its components in altering inflammation and apoptosis targets.
- All the diets were equally potent in targeting genes involved in carcinogenesis.
- Flaxseed and its components generate a protective environment in the pre-neoplastic hen ovaries
- E2 mediated ER alpha activation was abrogated by purified Enterolactone in vitro.

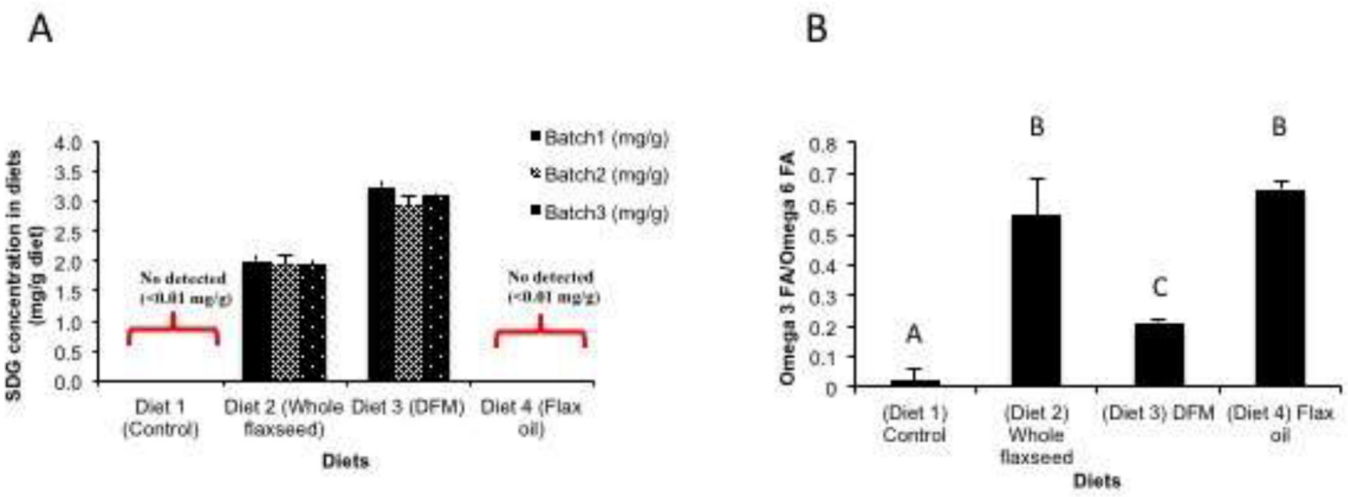

Figure 1.

Levels of SDG and omega fatty acids in the different diets. A, SDG was only detected in the whole flaxseed and defatted flax meal supplemented diet when analyzed by LC/MS. B, Ratio of omega 3 fatty acid to omega 6 fatty acid in the ovary tissue was significantly high in whole flaxseed and flax oil supplemented diets in comparison to defatted flax meal diet as analyzed by gas chromatography. Control diet had comparable levels of omega 3 and omega 6 fatty acids.

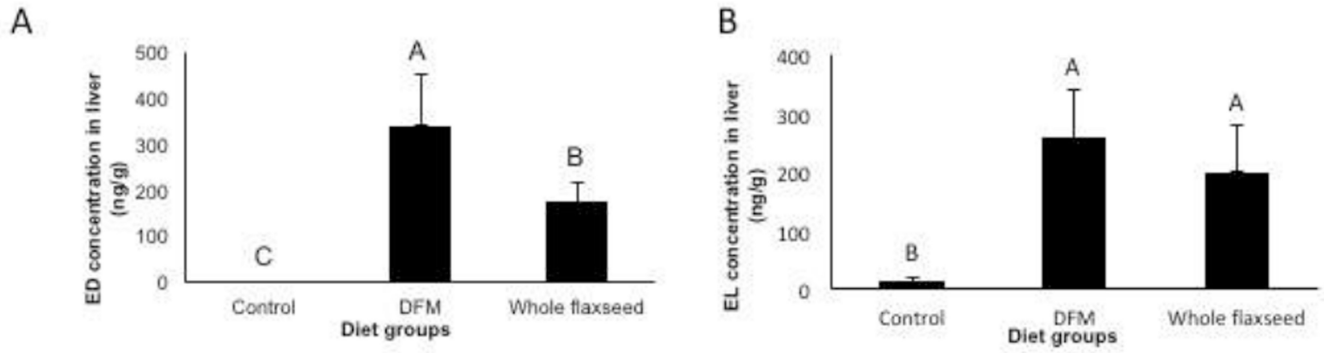

Figure 2.

Levels of ED and EL in the liver as analyzed by LCMS, n=6. A, ED was only detected in the hens that were fed defatted flax meal and whole flaxseed supplemented diets. Control vs DFM ($p<0.001$), Control vs whole flaxseed ($p<0.001$), DFM vs whole flaxseed ($p<0.01$). B, EL was only detected in the hens that were fed defatted flax meal and whole flaxseed supplemented diets. Control vs DFM ($p<0.001$), Control vs whole flaxseed ($p<0.001$). Letters on the error bar shared among groups indicate no significant difference.

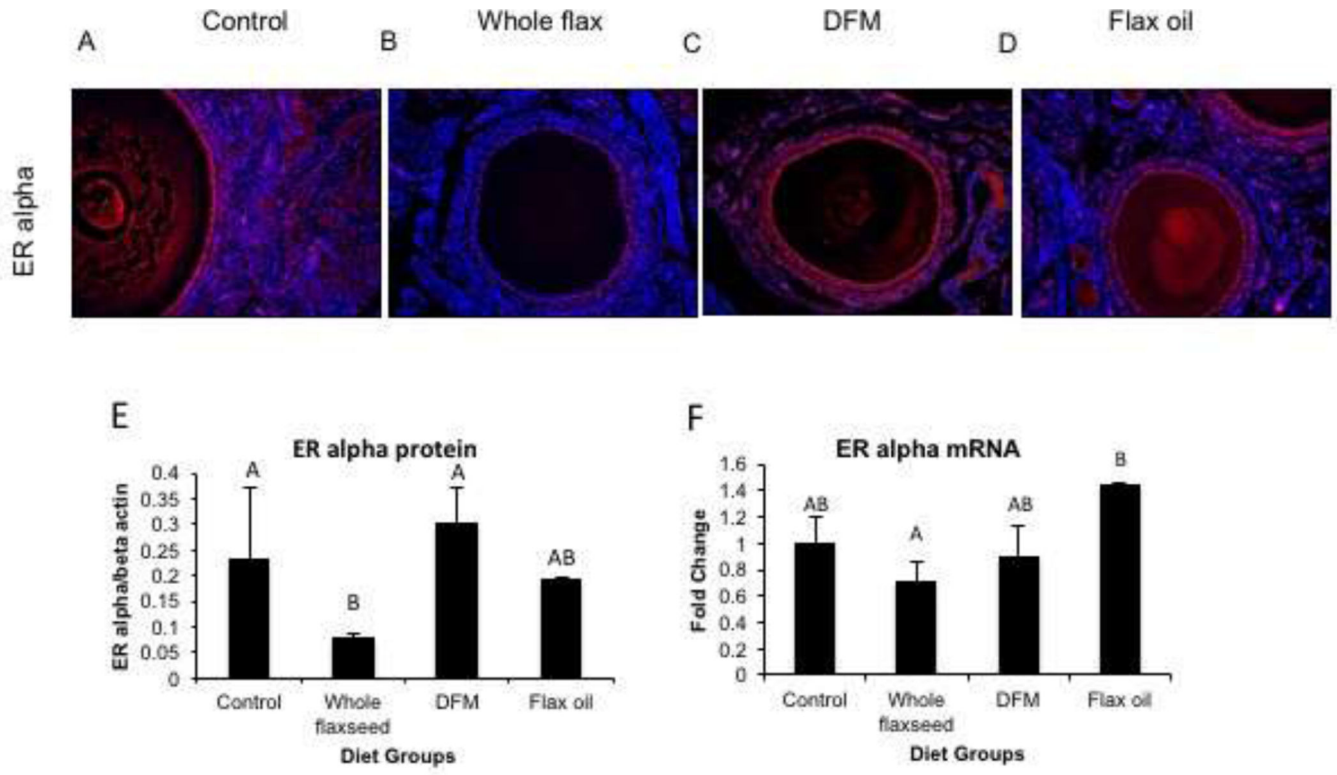

Figure 3.

Estrogen receptor alpha expression in the ovary. C, ERα expression was decreased in the granulosa cells of the ovaries of hens that were fed whole flaxseed supplemented diet in comparison to A, control fed or B, defatted flax meal supplemented or D, flax oil supplemented diets, as observed by immunofluorescence, n=3, total magnification=200x. E, ERα protein expression was decreased in the ovary tissue of whole flaxseed supplemented diet hens, assessed by western blotting, n=4. Control vs whole flaxseed ($p<0.05$), whole flaxseed vs DFM ($p<0.05$). F, mRNA levels were also downregulated in the ovaries of whole flaxseed diet fed hens in comparison to flax oil supplemented diet hens, when analyzed by qPCR n=8. Whole flaxseed vs flax oil ($p<0.05$). Letters on the error bar shared among groups indicate no significant difference.

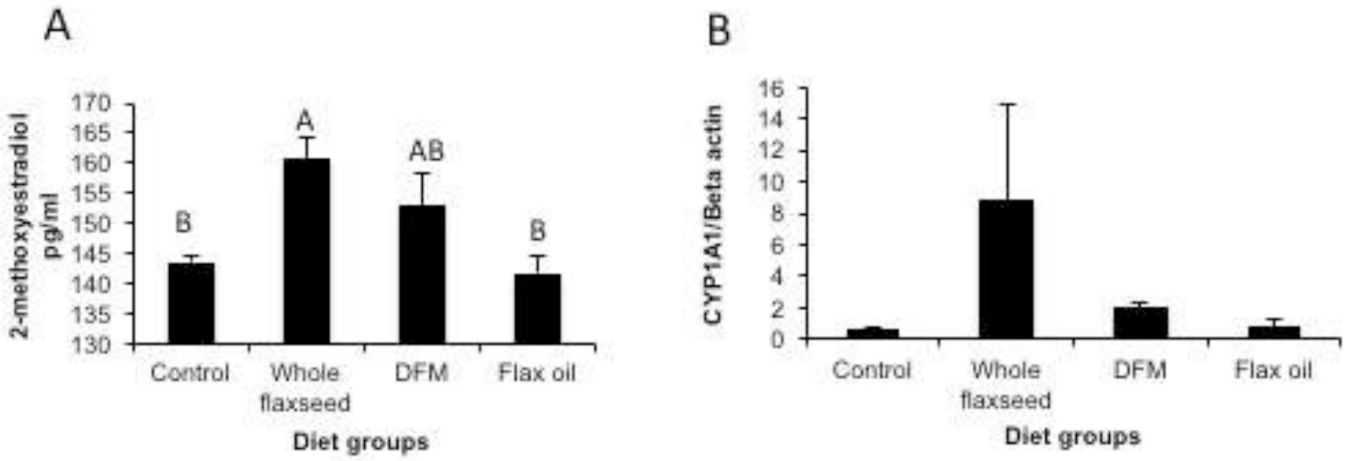

Figure 4.

Serum levels of 2-methoxyestradiol and CYP1A1 expression in the liver. A, Levels of 2-methoxyestradiol were upregulated in the serum from the hens that were fed whole flaxseed supplemented diet, when analyzed using the Cayman Chemicals EIA assay, n=6. Control vs whole flaxseed ($p<0.05$), whole flaxseed vs flax oil ($p<0.05$), B, CYP1A1 enzyme expression increased significantly in the hens that were fed whole flaxseed supplemented diet, analyzed by western blotting, n=5. Letters on the error bar shared among groups indicate no significant difference.

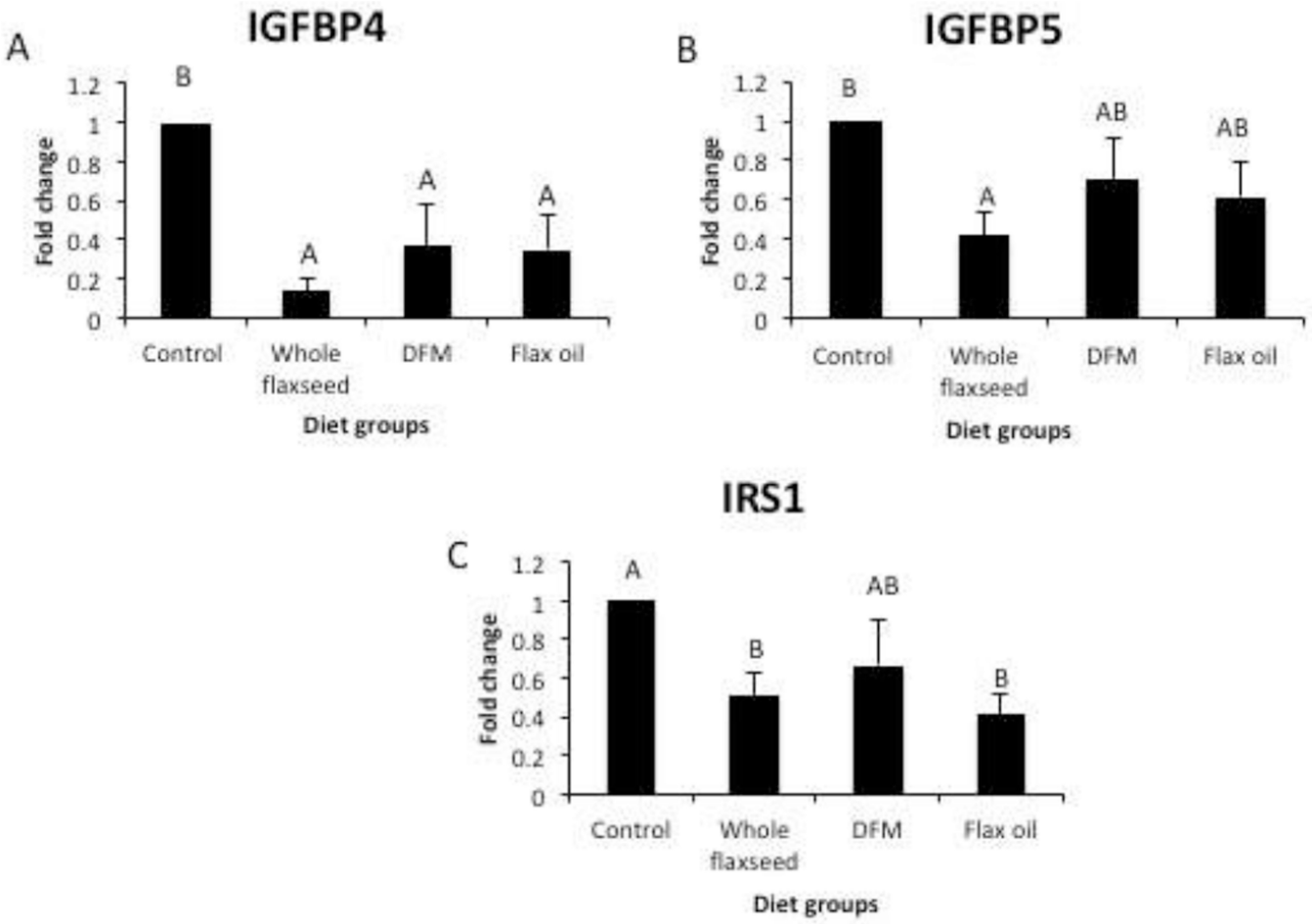

Figure 5.

Expression of ER targets involved in the IGF signaling pathway analyzed by PCR array in the ovarian tissue, n=6. A, mRNA expression of IGFBP4 decreased with whole flaxseed supplemented diet, defatted flax meal supplemented diet and flax oil supplemented diet, control vs. whole flax ($p<0.05$), control vs. whole flax ($p<0.05$), control vs. flax oil ($p<0.05$). B, mRNA expression of IGFBP5 decreased with whole flaxseed supplemented diet ($p<0.05$) C, mRNA expression of IRS1 decreased with diets supplemented with whole flaxseed and flax oil ($p<0.05$). Letters on the error bar shared among groups indicate no significant difference.

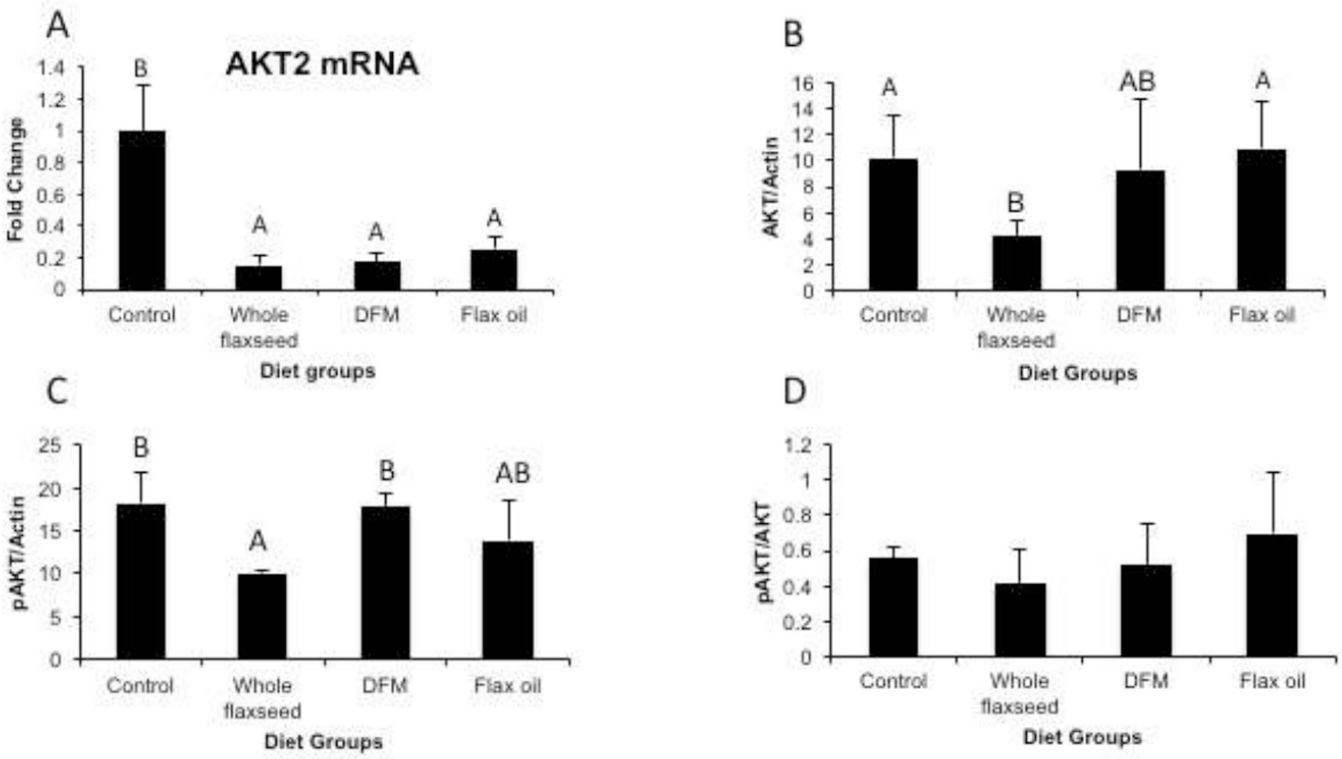

Figure 6.

Effect of flaxseed supplemented diet on AKT expression and phosphorylation. A, Real time qPCR suggested decrease in AKT2 mRNA expression by diets supplemented with whole flaxseed and its components, n=6, control vs. whole flaxseed ($p<0.01$), control vs. DFM ($p<0.01$), control vs. flax oil ($p<0.01$). B and C, western blotting revealed decreased expression of total AKT, control vs. whole flaxseed ($p<0.05$), whole flaxseed vs. flax oil ($p<0.05$) and phosphorylated AKT in the whole flaxseed supplemented diet, control vs. whole flaxseed ($p<0.05$), whole flaxseed vs. DFM ($p<0.05$). D, Ratio of phosphorylated Akt to total Akt remained unaltered with diet, n=3. Letters on the error bar shared among groups indicate no significant difference.

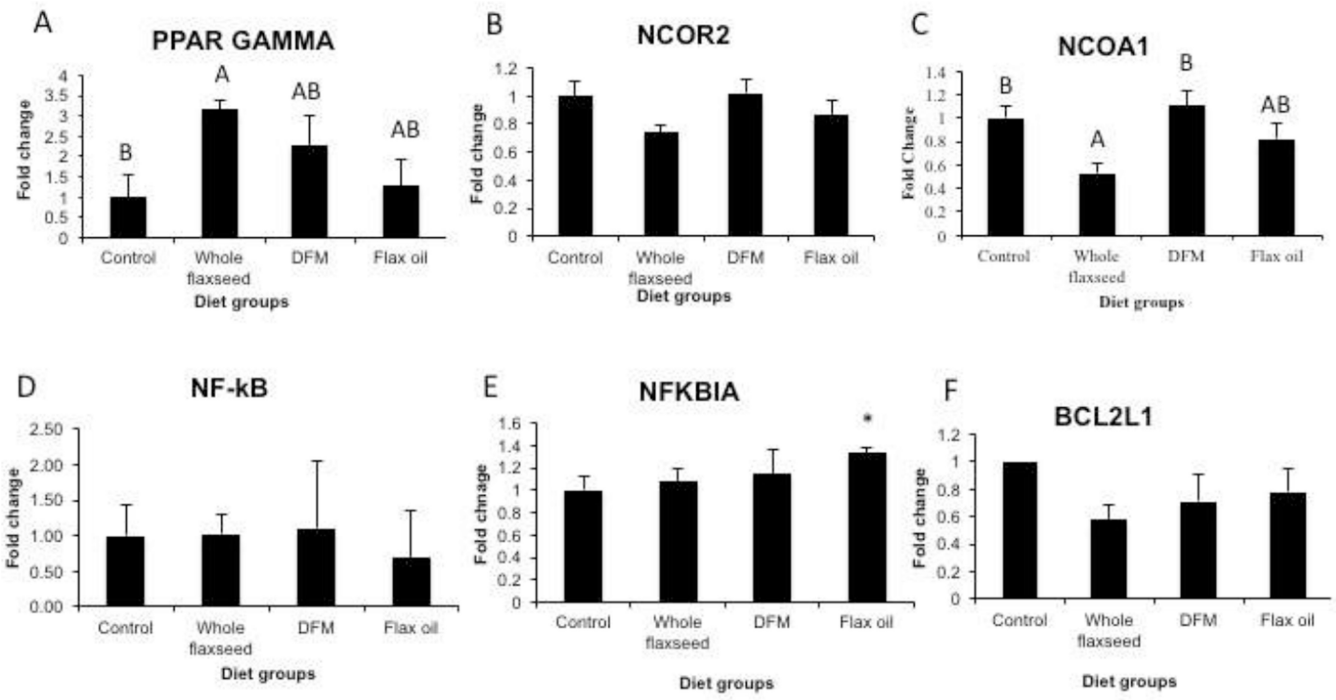

Figure 7.

Effect of flaxseed supplemented diet on targets involved in the NF-κB pathway in the ovary, n=6. A, whole flaxseed supplemented diet increased the mRNA levels of PPAR gamma in comparison to control diet when analyzed using qPCR, control vs whole flaxseed ($p<0.05$), B, expression of transcriptional repressor, NCOR2 was also downregulated with whole flaxseed diet. C, whole flaxseed supplemented diet decreased the mRNA expression of NCOA1, control vs whole flax ($p<0.05$), whole flax vs. defatted flax meal ($p<0.05$). D, NF-κB mRNA expression was not significantly altered by the any diet group, E, mRNA expression of NFkBIA was upregulated in the flax oil diet. F, expression of pro-apoptotic protein BCL2L1 was also downregulated in the whole flaxseed supplementary diet. Letters on the error bar shared among groups indicate no significant difference.

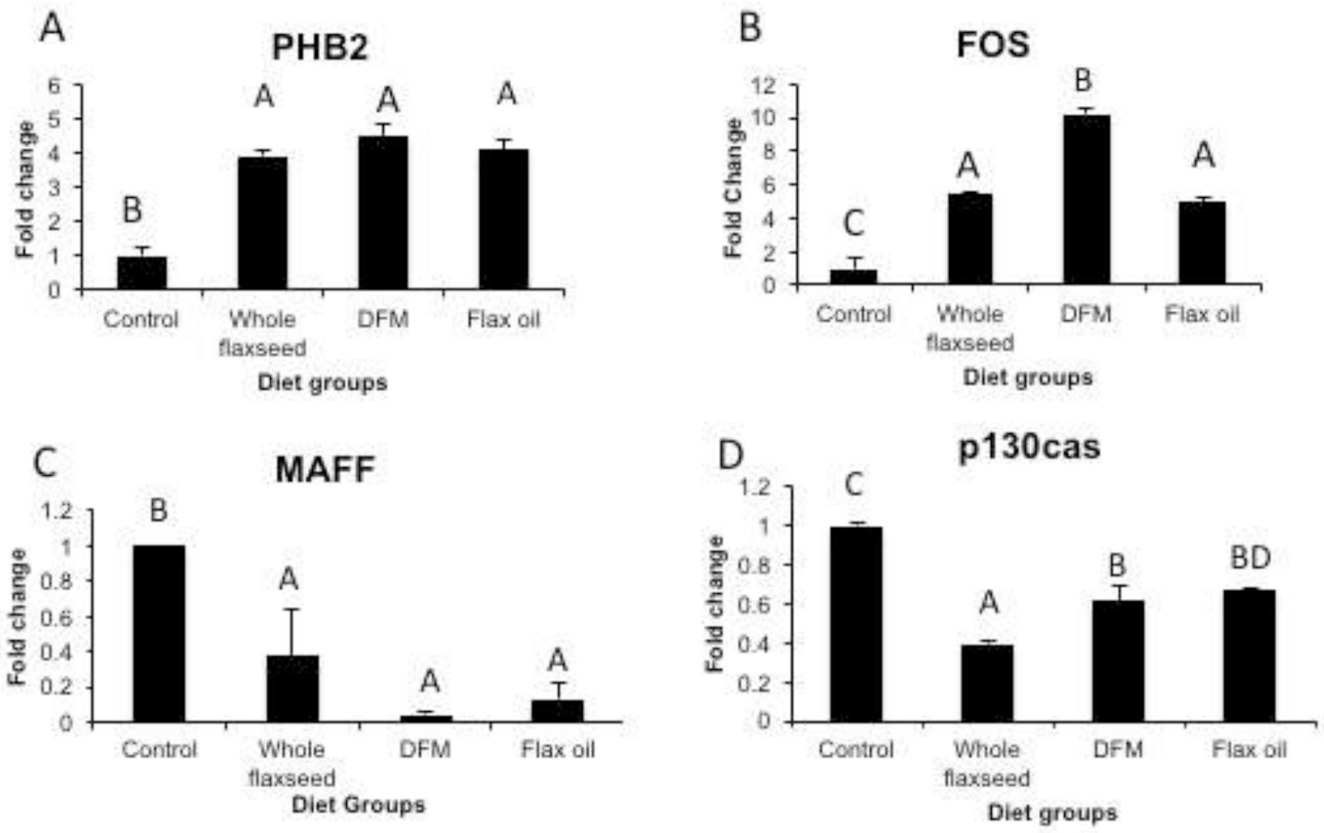

Figure 8.

Flaxseed diet and its components alter the ovarian expression of a series of genes implicated in cancer as assessed by PCR array, n=6. A mRNA expression of estrogen repressor, PHB2 increases with whole flaxseed, defatted flax meal and flax oil supplemented diets, control vs whole flaxseed ($p<0.05$), control vs DFM ($p<0.05$), control vs. flax oil ($p<0.05$). B, c-FOS mRNA expression increased with whole flaxseed, defatted flax meal and flax oil supplemented diet, control vs whole flaxseed ($p<0.001$), control vs DFM ($p<0.001$), control vs flax oil ($p<0.001$), whole flax vs. DFM ($p<0.01$), DFM vs. Flax oil ($p<0.001$). C, whole flaxseed supplemented diet, defatted flax meal supplemented diet and flax oil supplemented diet decreases mRNA expression of MAFF, control vs whole flaxseed ($p<0.05$), control vs. DFM ($p<0.05$), control vs. flax oil ($p<0.05$). E, Whole flaxseed diet, DFM diet and flax oil diet decreases the mRNA expression of p130cas, control vs whole flaxseed ($p<0.001$), control vs DFM ($p<0.001$), control vs flax oil ($p<0.001$), whole flax vs. DFM ($p<0.01$), whole flax vs. Flax oil ($p<0.001$). Letters on the error bar shared among groups indicate no significant difference.

Table 1

Formulation of diets and diet compositions

Diet	**Control**	**5% flax oil**	**15% DFM**	**15% whole flaxseed**
Enriched with:	**----**	**ALA**	**SDG**	**ALA + SDG**
Ingredient				
Corn	67.40	52.00	51.90	47.58
Flaxseed (whole)				15.00
SBM	18.30	18.30	18.30	18.30
Corn Gluten Meal	3.00	5.00		
Flax Oil		5.00		
Qual Fat			3.80	2.50
Defatted Flax Meal			15.00	
Solka Floc	0.30	8.70		5.62
Limestone	8.75	8.75	8.75	8.75
Dical	1.50	1.50	1.50	1.50
Salt	0.30	0.30	0.30	0.30
Vitamin Mix	0.20	0.20	0.20	0.20
Mineral Mix	0.15	0.15	0.15	0.15
DL-Met	0.10	0.10	0.10	0.10
Calculated Analysis				
CP, %	16.56	16.49	18.49	16.50
TME, kcal/kg	2,816	2,815	2,816	2,815
Calcium, %	3.73	3.73	3.77	3.75
Phosphorus, %	0.38	0.37	0.40	0.38
Met + Cys, %	0.67	0.67	0.72	0.64

Table 2

Genes altered in the estrogen receptor target gene PCR array

GENE NAME	FUNCTION	FOLD CHANGE	DIET GROUPS WITH ALTERED EXPRESSION
GENES UPREGULATED			
BDNF	Brain derived neurotrophic factor-involved in learning and memory; decrease in levels can increase risk of Alzheimer's.	4.8, 2.98	Whole flax and Flax oil
GPER	G-protein coupled estrogen receptor that can mediate E2 effects.	1.48	Whole flax
FOS	Generally upregulated in cancers but downregulation of Fos has been associated with decreased progression free survival in OvCa. It is proapoptotic and higher expression is associated with low grade and low malignancy potential tumors.[71, 74–77]	10.22	Defatted flax meal
LTBP1	It associates TGFβ to the extracellular matrix.	1.45	Flax oil
PHB2	Acts as an estrogen repressor and as a tumor suppressor. [72]	4.89	Whole flax
NRIP1	It interacts with nuclear receptors and promotes target gene transcription.	4.48	Defatted flax meal
NFKB1A	It is the inhibitor of the NF-κB that is constitutively active in a number of autoimmune and inflammatory diseases.	1.33	Flax oil
WISP2	This gene is downstream of WNT 1 signaling pathway is associated with malignant transformation of breast cancer cells [78].	2.27	Defatted flax meal
AHR	It is a ligand activated nuclear receptor regulates the expression of cytochrome p450 enzymes.	1.66,1.37	Defatted flax meal and whole flax
GENES DOWNREGULATED			
IGFBP4	It is known to increase the half-life of IGFs and also alter the interaction between IGFs and their cell surface receptors. Its expression is stimulated by estrogen receptor alpha.	0.13, 0.36	Whole flax and defatted flax meal
IGFBP5	It can regulate metastasis of breast cancer[79] and involved in progression of prostate cancer [80] and thyroid cancer[81].	0.42, 0.7	Whole flax and defatted flax meal
MAFF	Transcription factor involved in a number of biological processes. It is also upregulated in multiple myeloma[69] and breast cancer[68] patients.	0.37	Whole flax
RARA	Nuclear receptor, which on activation can induce targets involved in the TGF beta pathway. Also induces targets involved in cell proliferation and metastasis. Involved in promoting EMT [82]	0.5	Whole flax
NCOA1	Overexpression promotes breast cancer metastasis [83]. Acts as a transcriptional repressor for pro-apoptotic proteins in turn promoting survival. [84]	0.52	Whole flax
NCOR2	Increased expression of splice variant is associated with Tamoxifen resistance in breast cancer. [85] Over expression is associated with poor prognosis as it inhibits apoptosis and promotes cell progression. [86]	0.74	Whole flax
CTGF	It is an extracellular matrix associated protein that is involved in cell proliferation, migration. It is upregulated in a myriad of cancers [87, 88]	0.59, 0.61	Whole flax and Flax oil
BCAR1	It is a scaffold protein that controls cell motility, migration and survival [66].	0.39	Whole flax
BCL2L1	Anti-apoptotic protein belonging to the BCL-2 family of proteins.	0.57	Whole flax
APBB1	Nuclear adapter protein that interacts with amyloid precursor protein.	0.4	Whole flax
IRS1	It is a docking protein important in intracellular signaling of the IR and IGF1R. It can contribute towards tumor progression [89]	0.5, 0.41	Whole flax and Flax oil
NR3C1	Glucocorticoid receptor can act as a transcription factor and regulate inflammatory responses, proliferation and differentiation.	0.55	Whole flax

Table 3

Genes examined in the PCR array. (Qiagen RT^2 Profiler PCR array-PAGG-005Z)

Symbol	Description
ADORA1	Adenosine A1 receptor
AHR	Aryl hydrocarbon receptor
AKAP1	A kinase (PRKA) anchor protein 1
APBB1	Amyloid beta (A4) precursor protein-binding, family B, member 1 (Fe65)
ATF3	Activating transcription factor 3
BCAR1	Breast cancer anti-estrogen resistance 1
BCL2L1	BCL2-like 1
BDNF	Brain-derived neurotrophic factor
BMP4	Bone morphogenetic protein 4
BMP7	Bone morphogenetic protein 7
BRCA1	Breast cancer 1, early onset
C3	Complement component 3
CCND1	Cyclin D1
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
CKB	Creatine kinase, brain
CST3	Cystatin C
CTGF	Connective tissue growth factor
CTSD	Cathepsin D
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
EBAG9	Estrogen receptor binding site associated, antigen, 9
EFNA5	Ephrin-A5
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
ERBB3	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2 (ER beta)
FOS	FBJ murine osteosarcoma viral oncogene homolog
FOSL2	FOS-like antigen 2
FOXA1	Forkhead box A1
FST	Follistatin
GPER	G protein-coupled estrogen receptor 1
HSP90AA1	Heat shock protein 90kDa alpha (cytosolic), class A member 1
IGF1	Insulin-like growth factor 1 (somatomedin C)
IGFBP4	Insulin-like growth factor binding protein 4
IGFBP5	Insulin-like growth factor binding protein 5
IL10	Interleukin 10

Symbol	Description
IRS1	Insulin receptor substrate 1
L1CAM	Neuron-glia cell adhesion molecule (Ng-CAM)
LGALS1	Lectin, galactoside-binding, soluble, 1
LPL	Lipoprotein lipase
LTBP1	Latent transforming growth factor beta binding protein 1
MAFF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)
MED1	Mediator complex subunit 1
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
MTA1	Metastasis associated 1
MUC1	Mucin 1, cell surface associated
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
NCOA1	Nuclear receptor coactivator 1
NCOA2	Nuclear receptor coactivator 2
NCOA3	Nuclear receptor coactivator 3
NCOR1	Nuclear receptor co-repressor 1
NCOR2	Nuclear receptor co-repressor 2
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NOV	Nephroblastoma overexpressed gene
NR0B1	Nuclear receptor subfamily 0, group B, member 1
NR0B2	Nuclear receptor subfamily 0, group B, member 2
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
NR5A2	Nuclear receptor subfamily 5, group A, member 2
NRIP1	Nuclear receptor interacting protein 1
NRP1	Neuropilin 1
PDZK1	PDZ domain containing 1
PGR	Progesterone receptor
PHB2	Prohibitin 2
PTCH1	Patched 1
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
RARA	Retinoic acid receptor, alpha
S100A6	S100 calcium binding protein A6
SAFB	Scaffold attachment factor B2
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1
SLC9A3R1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1
SMAD3	SMAD family member 3
SNAI1	Snail homolog 1 (Drosophila)
SOCS3	Suppressor of cytokine signaling 3
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
TGFA	Transforming growth factor, alpha

Symbol	Description
TGFB3	Transforming growth factor, beta 3
THBS1	Thrombospondin 1
THRSP	Thyroid hormone responsive (SPOT14 homolog, rat)
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor
VEGFA	Vascular endothelial growth factor A
WISP2	WNT1 inducible signaling pathway protein 2
WNT4	Wingless-type MMTV integration site family, member 4
WNT5A	Wingless-type MMTV integration site family, member 5A
XBP1	X-box binding protein 1
ACTB	Actin, beta
H6PD	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
HMBS	Hydroxymethylbilane synthase
RPL4	Ribosomal protein L4
UBC	Ubiquitin C
GGDC	Chicken Genomic DNA Contamination
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
PPC	Positive PCR Control
PPC	Positive PCR Control
PPC	Positive PCR Control