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# Flaxseed reduces the pro-carcinogenic micro-environment in the ovaries of normal hens by altering the PG and oestrogen pathways in a dose-dependent manner

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## Abstract

The objective of the present study was to find the optimum dose of flaxseed that would decrease PG and alter oestrogen pathway endpoints implicated in ovarian cancer. In the study, four groups of fifty 1·5-year-old chickens were fed different amounts of flaxseed (0, 5, 10 or 15 % of their total diet) for 4 months and were then killed to collect blood and tissues. Levels of flaxseed lignan metabolites, Enterolactone (EL) and Enterodiol (ED) were measured in the serum, liver and ovaries by liquid chromatography–MS/MS, and *n*-3 and *n*-6 fatty acid (FA) levels were measured by GC. The effects of the varied flaxseed doses were assessed by measuring levels of $PGE_2$ and oestrogen metabolites (16-hydroxyestrone (16-OHE1) and 2-hydroxyestrone (2-OHE1)) as well as by analysing the expression of the oestradiol metabolising enzymes CYP3A4 (cytochrome p450, family 3, subfamily A, polypeptide 4), CYP1B1 (cytochrome p450, family 1, subfamily B, polypeptide 1) and CYP1A1 (cytochrome p450, family 1, subfamily A, polypeptide 1) and that of oestrogen receptor α (ERα) in the ovaries. The ratio of *n*-3:*n*-FA increased with an increase in flaxseed supplementation and corresponded to a dose-dependent decrease in cyclo-oxygenase-2 protein and $PGE_2$ levels. EL and ED increased in the serum, liver and ovaries with increased concentrations of flaxseed. Flaxseed decreased the expression of ERα in the ovaries. The ratio of 2-OHE1:16-OHE1 in the serum increased significantly in the 15 % flaxseed diet, and there was a corresponding increase in CYP1A1 in the liver and decrease in CYP3A4 in the ovaries. *CYP1B1* mRNA also decreased with flaxseed diet in the ovaries. The 15 % flaxseed-supplemented diet significantly decreased inflammatory $PGE_2$, ERα, CYP3A4, CYP1B1 and 16-OHE1, but it increased CYP1A1 and 2-OHE1, which thus reduced the inflammatory and pro-carcinogenic micro-environment of the ovaries.

**Key words:** **Oestrogen: PG: Flaxseed: Ovarian cancer prevention**

Flaxseed (*Linum usitatissimum)* is an excellent source of α-linoleic acid, a polyunsaturated *n*-3 fatty acid (FA), and dietary fibre; it is also the richest source of plant lignan[(1)]. Secoisolariciresinol diglucoside (SDG) is the predominant lignan present in flaxseed[(2)]. Flaxseed is known to exhibit protective effects against a multitude of chronic ailments, including CVD, stroke and cancer. Investigations have shown that flaxseed reduces the risk of breast and colon cancers, atherosclerosis, insulin-dependent diabetes mellitus and hyperlipoproteinaemia[(3–5)].

Using the chicken model, we have demonstrated in an earlier study that flaxseed is beneficial in mitigating the severity and reducing the incidence of ovarian cancer[(6)]. The anti-oncogenic properties attributed to flaxseed are mainly a result of the *n*-3 FA and the lignan component. *n*-3 FA inhibit the synthesis of certain arachidonic acid-derived PG, such as $PGE_2$, which are known to be elevated in many cancers. $PGE_2$ can initiate a tumour's growth by binding to its receptors on target tissues and activating signalling pathways which control processes such as cell proliferation, migration, apoptosis and angiogenesis[(7)]. Cyclo-oxygenase (COX) enzymes catalyse the synthesis of PG from arachidonic acid. The COX-1 enzyme is expressed constitutively and imparts its homeostatic effects by synthesising PG that maintain the

**Abbreviations:** 2-OHE1, 2-hydroxyestrone; 16-OHE1, 16-hydroxyestrone; 2-OHE2, 2-hydroxyestradiol; 4-OHE2, 4-hydroxyestradiol; 16-OHE2, 16-hydroxyestradiol; COX, cyclo-oxygenase; CYP1A1, cytochrome p450, family 1, subfamily A, polypeptide 1; CYP1B1, cytochrome p450, family 1, subfamily B, polypeptide 1; CYP3A4, cytochrome p450, family 3, subfamily A, polypeptide 4; $E_2$, oestradiol; ED, enterodiol; EL, enterolactone; FA, fatty acid; ERα, oestrogen receptor α; SDG, secoisolariciresinol diglucoside.

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integrity of the stomach lining. COX-1 is elevated with ovarian cancer in humans[8] as well as laying hens[9–11]. COX-2 is an inducible enzyme and is mainly stimulated under inflammatory responses, commonly by inflammatory cytokines[12]. COX-2 is known to be up-regulated in a myriad of cancers, including gastric adenocarcinoma[13], colorectal cancer[14], breast cancer[15], prostate carcinoma[16], cervical cancer[17], pancreatic cancer[18] and lung cancer[19].

SDG is a precursor to the phytoestrogens enterolactone (EL) and enterodiol (ED). The initial step includes the conversion of SDG to secoisolariciresinol by hydrolysis. Gut flora demethylate and dehydroxylate secoisolariciresinol form ED, which can be oxidised further to EL[20]. ED and EL are known to have phytoestrogenic and antioxidant effects. Recent studies[21,22] have shown that dietary lignan reduces the risk of post-menopausal breast cancer. In other control studies[23,24], women who consumed a lignan-rich diet also showed a decreased risk of breast cancer.

There is evidence to suggest that in response to oestradiol ($E_2$), oestrogen receptor α (ERα) stimulates growth and invasion in ovarian cancer cells[25]. The anti-oestrogenic property of the phytoestrogen lignans, which can be attributed to their weak antagonism to ER[26], might mitigate the oestrogen-dependent aggressiveness in ovarian cancer cells.

Ovarian cancer is one of the deadliest gynaecological malignancies: it has a 5-year survival rate of 44% in the United States[27]. This is a result of its late stage of detection, when the prognosis is poor and treatment options are limited. Although identifying early detection markers is the need of the day, developing a preventative approach is equally essential. Ovarian cancer in the laying hen (*Gallus domesticus*) develops spontaneously and significantly resembles the human disease with respect to histopathology as well as gross pathology[28–31]. We have previously shown that a 10% flaxseed diet decreases the incidence and severity of ovarian cancer in chickens and decreases COX-2 and $PGE_2$ levels in the ovaries[6,32].

The objective of the present study was to determine the effects of different doses of a flaxseed-supplemented diet on the levels of $PGE_2$, ER and the $E_2$ metabolising enzymes involved in the oestrogen pathway. An earlier study has shown that a 10% flaxseed diet reduced the aggressiveness of ovarian cancer and was correlated with a decrease in $PGE_2$[6]. The present study was therefore designed to determine the optimum dose of flaxseed in the diet to maximise the beneficial effects without being toxic. A maximum dose of 15% was selected for the study, because an earlier study indicated that a 20% dose of flaxseed could be hepatotoxic for chickens (DB Hales, K Ansenberger and JM Bahr, unpublished results).

## Materials and methods

### Reagents

The reagents were obtained from the following: Biotinylated Anti-Rabbit IgG (Vector Laboratories); AffiniPure Alexa 488 Conjugated Donkey Anti-Mouse IgG (Jackson ImmunoResearch); Streptavidin, Alexa Fluor® 488 Conjugate (Life Technologies); the iScript Complementary DNA Synthesis Kit and Ssofast EvaGreen Supermix (SYBR Green; Bio-Rad Laboratories, Inc.); the TUNEL Apoptosis Detection Kit (GenScript); Estramet ELISA Kits for 2-hydroxyestrone (2-OHE1) and 16-hydroxyestrone (16-OHE1) analysis (Immuna Care Corporation); ED and EL standards (Sigma-Aldrich); Genistein–*d*4 (4-hydroxyphenyl-2,3′,5′,6–*d*4, 98% atom% D; C/D/N Isotopes, Inc.); and β-glucuronidase enzyme, ≥30 000 units/g solid and SDG standard (Sigma-Aldrich). The HPLC analysis was done on a Shimadzu LC-20A HPLC system (Shimadzu Company). The GC analysis was performed on a GC-2010 (Shimadzu Company). All of the other reagents were those used in previous studies[9].

### Animals

A total of 200 single comb White Leghorn hens (*G. domesticus*) aged 1·5 years were used for the present study. The hens were exposed to a photoperiod of 17 h light–7 h dark, with lights being turned on at 05.00 hours and turned off at 22.00 hours. Animal management 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.

Each group of fifty chickens was fed a different percentage (0, 5, 10 or 15%) of a flaxseed-supplemented diet for a period of 4 months. At the end of 4 months, the birds were euthanised, and their tissues were harvested.

### Diet composition

The composition of the test diets is shown in Table 1. The hens consumed 110 g of food per day and were provided water *ad libitum*. Their levels of SDG and α-linoleic acid were routinely analysed to ensure that the diets were consistent.

### Tissue collection

All of the birds included in the study were euthanised and their tissues were collected on necropsy as described in our earlier study[6]. Histology was performed to confirm that all of their ovaries were normal[33].

### Histology and immunofluorescence

Formalin-fixed ovarian tissue was embedded in paraffin, and 5 μm thick sections were cut and mounted on SuperFrost Plus microscope slides. Following deparaffinisation, the slides were rehydrated by being run through xylene and graded ethanol solutions. Hematoxylin and eosin staining was performed on the slides as described by Sheehan & Hrapchak[34]. The slides were also used for assessing COX-1 and COX-2 tissue expression as well as localisation by immunofluorescence. Antigen retrieval was performed by using a 0·9% Antigen unmasking solution (Vector Laboratories) and pressure cooking the slides for 5 min at 20 psi. The slides were allowed to cool, and sections were blocked

**Table 1.** Composition (%) of diets* containing different levels of flaxseed

| Ingredient | Control | 5 % Flaxseed | 10 % Flaxseed | 15 % Flaxseed |
|---|---|---|---|---|
| Maize | 67·40 | 63·20 | 58·70 | 53·60 |
| Soyabean meal | 18·30 | 18·30 | 18·30 | 18·30 |
| Maize gluten meal | 3·00 | 1·80 | 0·60 | 0·00 |
| Flaxseed | 0·00 | 5·00 | 10·00 | 15·00 |
| Calculated analysis | | | | |
| CP (%) | 16·5 | 16·5 | 16·5 | 16·5 |
| TME (kcal/kg) | 2915 | 2915 | 2915 | 2915 |
| Ca (%) | 3·75 | 3·75 | 3·75 | 3·75 |
| Available P (%) | 0·38 | 0·38 | 0·38 | 0·38 |
| Met+Cys (%) | 0·67 | 0·67 | 0·67 | 0·67 |

CP, crude protein; TME, true metabolisable energy.

* Each diet contains: limestone (8·75%); dicalcium phosphate (1·50%); iodised salt (0·30%); DL-methionine (0·10%); vitamin premix (0·20%); trace mineral premix (0·15%); and Solka Floc (cellulose; International Fiber Corporation) (0·30%). Vitamin premix provided per kg of diet: retinyl acetate, 4400 IU; cholecalciferol, 25 μg; DL-α-tocopheryl acetate, 11 IU; vitamin $B_{12}$, 0·01 mg; riboflavin, 4·41 mg; D-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite, 2·33 mg. Trace mineral premix provided as mg/kg of diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0·75 from ethylene diamine dihydroiodide; and Se, 0·1 from $Na_2SeO_3$.

with a 5 % normal goat serum (COX-1 and COX-2) in 1× Tris-buffered saline for 1 h at room temperature. Sections were incubated with either an anti-human COX-1 (1:200) or an anti-human COX-2 (1:200) antibody at 4°C overnight. For COX-1 and COX-2 staining, sections were washed with 1× Tris-buffered saline, incubated with decanted antibody and then incubated with Streptavidin Alexa Fluor 488 conjugate for 30 m at room temperature. Sections were rinsed in 1× Tris-buffered saline with 0·5 % Tween 20. Slides were mounted using Dapi Fluoromount G (Southern Biotech). All antibody dilutions were made in blocking solution. Control sections were incubated with non-immune anti-rabbit IgG (COX-1 and COX-2).

### *RNA extraction and analysis*

Total RNA was extracted from the ovarian tissue using a Trizol reagent as described previously[9,35]. Quantification was performed by determining absorbance at $A_{260}$, and RNA quality was assessed with the Experion RNA StdSens Analysis Kit. RNA samples were then treated with RQ1 Rnase-free Dnase before a reverse transcription reaction was performed. Complementary DNA was synthesised using the Bio-Rad iScript Kit.

### *Real-time PCR*

Target gene mRNA levels were analysed with real-time quantitative PCR using the CFX384 Real-Time System. Gene-specific primers were used for target as well as reference gene amplification (Table 2). To normalise target gene expression, three housekeeping genes were used. The amplification conditions were: 95°C for 30 s; forty cycles for 10 s at 95°C and 15 s at 58°C with a melt curve measured at 65 to 95°C for 5 s every 0·5°C gradient. A non-template control was run for every target gene that was amplified.

### *Cell apoptosis and proliferation staining*

We assessed 5 μm paraffin-embedded histological sections of chicken ovarian tissue from the control and all of the dosage groups for apoptotic and proliferating cells using TdT-mediated dUTP nick end labelling and proliferating cell nuclear antigen staining, respectively, as described previously[36].

### *$PGE_2$ enzyme immunoassay*

A total of six ovary tissue samples from each dietary group were analysed for $PGE_2$ levels using the $PGE_2$ Enzyme

**Table 2.** Primers used for real-time quantitative PCR

| Gene symbol | Common name | Gene ID | Forward primer | Reverse primer |
|---|---|---|---|---|
| Target | | | | |
| *PTGS1* | Cyclo-oxygenase 1 | 427752 | 5′-TCAGGTGGTTTCTGGGACATCA-3′ | 5′-TGTAGCCGTACTGGGAGTT GA-3′ |
| *PTGS2* | Cyclo-oxygenase 2 | 396451 | 5′-CTGCTCCCTCCCATGTCAGA-3′ | 5′-CACGTGAAGAATTCCGG TGTT-3′ |
| *ESR1* | Oestrogen receptor 1 | 396099 | 5′-CCACAGCATTCGTGAGAGG-3′ | 5′-GCATAGTCGTTGCACAC AGC-3′ |
| *CYP1B1* | Cytochrome p450, family 1, subfamily B, polypeptide 1 | 421466 | 5′-CAAGATTCCTGGATGAGAACG-3′ | 5′-GCTGCACCTTGGA TAATTCC-3′ |
| *CYP1A1* | Cytochrome p450, family 1, subfamily A, polypeptide 1 | 396051 | 5′-TGGATACCCTCTGCCTCTCT-3′ | 5′-GGAACTAAGGGGAAG CGTG-3′ |
| *CYP3A4* | Cytochrome p450, family 3, subfamily A, polypeptide 4 | 416477 | 5′-CCATCTTGGTGAAGGAGTGC-3′ | 5′-TTCCACTGGTC ATCCATAGC-3′ |
| Housekeeping | | | | |
| *TBP* | TATA box binding protein | 395995 | 5′-CGTGAGGGAAATAGGCA-3′ | 5′-GACTGGCAGCAAGGAAG-3′ |
| *SDHA* | Succinate dehydrogenase complex, subunit A | 395758 | 5′-CAGGGATGTAGTGTCTCGT-3′ | 5′-GGGAATAGGCTCCTTAGTG-3′ |
| *RPL4* | Ribosomal protein L4 | 415551 | 5′-TTATGCCATCTGTTCTGCC-3′ | 5′-GCGATTCCTCATCTTACCCT-3′ |

Immunoassay Kit (Cayman Chemicals). Snap-frozen ovarian tissues maintained at −80°C were pulverised on dry ice, resuspended in homogenising buffer and transferred to clean tubes in preparation for solid-phase extraction. Extraction and analysis were carried out as described previously[6].

### 2-Hydroxyestrone and 16-hydroxyestrone ELISA assay

Blood samples were collected from all of the chickens during necropsy. Serum samples from the control and each of the dosage groups were analysed for their levels of both the 2-OHE1 and the 16-OHE1 metabolites using the 2-hydroxyestrone and 16-hydroxyestrone Estramet Double ELISA Kit (Immuna Care Corporation). Measurement of the oestrone metabolites is proportional to the concentrations of the oestrone and $E_2$ metabolites combined.

### Western blot analysis

Snap-frozen ovary tissue samples (25 μg) from each of the groups were analysed for *COX-1/COX-2* expression and normalised to β-actin, as described previously[6].

### GC for fatty acid analysis

The *n*-3 FA and *n*-6 FA levels in the chicken ovaries were analysed using a Shimadzu GC-2010 (Shimadzu Company). A solution containing 12·5 μg/ml 17 : 0 standard (Sigma-Aldrich) in methanol was added to the tissue sample (25 μg) as a recovery standard. Extraction and analysis was performed as described previously[36].

### HPLC for secoisolariciresinol diglucoside analysis

*Secoisolariciresinol diglucoside extraction*. We hydrolysed 1 g of flaxseed with 50 ml of 0·5 M-NaOH at 135 W, microwaving intermittently (30 s on–30 s off) for 3 min. Acidified hydrolysate (pH 3, 2·5 M-$H_2SO_4$) was added to 100 ml of methanol to precipitate the carbohydrates and proteins. After centrifugation for 10 min at 3000 rpm, the supernatants were filtered through a 0·22 μm filter and analysed by HPLC.

*HPLC analysis*. All extracts were analysed by a Shimadzu LC-20A HPLC system (Shimadzu Company) by measuring $A_{280\ nm}$ using a variable wavelength detector. SDG was separated on a Kinetex 2.6u C18 column (2·6 μm, 50 × 3·0 mm; Phenomenex) using a slightly modified version of the gradient elution method[37]. The column thermostat was set to 40°C, and the injection volume was set to 10 μl. The mobile phase consisted of 1 % acetic acid (solvent A) and methanol (solvent B) mixed A/B (v/v) for 0 min (95:5), 12 min (40:60), 18 min (60:40) and 18·2 min (95:5) and used at a flow rate of 0·4 ml/min. The SDG peaks were identified and quantified by comparison to the SDG standard.

### Liquid chromatography tandem MS for enterodiol and enterolactone analysis

*Extraction*. Tissues from all of the different diet groups were finely homogenised in citrate buffer (25 mM, pH 5·0) while the serum samples were being centrifuged before extraction. We then added four times the volume of methanol to the samples and treated them with ninety units of β-glucuronidase enzyme. The majority of the lipid was removed using *n*-hexane. A 1:4 solution of the EL and ED standards were prepared in methanol. Internal standard genistein–$d_4$ was added to all of the samples and standards. The supernatant was applied to a preconditioned solid phase extraction (SPE) cartridge, and bound compounds were then eluted in methanol. The eluted sample was concentrated, and the residue was then reconstituted with the loading solvent and acetonitrile–water (1:3).

*Liquid chromatography MS/MS analysis*. All of the extracts were analysed by a Shimadzu Prominence UFLC-8080 system (Shimadzu Company). The ED and EL were separated from the other compounds in the extract by chromatography on a Water XTerra MS C18 column (3·0 μm, 2·1 × 50 mm). The mobile phase consisted of 0·2 % formic acid in water (solvent A) and acetonitrile (solvent B) mixed A/B (v/v) for 0·3 min (80:20), 1·8 min (10:90), 1·81 min (80:20) and 2·50 min (end) and used at a flow rate of 0·6 ml/min and an injection volume of 5 μl. The compound peaks were identified and quantified by comparison with those of standard ED, EL and genistein–$d_4$. The molecules with an $m/z$ ratio of 301, 296·9 and 273·1 corresponded to ED, EL and genistein–$d_4$, respectively.

### Statistics

All of the experiments were performed in duplicate, and the target values were normalised to control. Statistical calculations were done using GraphPad Instat software by employing one-way ANOVA analysis. A $P<0·05$ was considered significant, whereas a $P<0·01$ was considered highly significant.

## Results

### ELISA analysis for $PGE_2$ in ovaries

$PGE_2$ levels in the ovarian tissue decreased with an increase in the percentage of flaxseed in the diet. $PGE_2$ levels were significantly lower in the 15 % diet group as compared to the control and 5 % groups (Fig. 1(A)).

### Hematoxylin and eosin staining on ovary sections

Ovary tissues from all of the diet groups were assessed using hematoxylin and eosin staining. Ovaries from all of the diet groups appeared normal histologically (Fig. 2(A)).

### Cyclo-oxygenase-1 and cyclo-oxygenase-2 enzyme expression

COX-1 expression was observed in the granulosa cells and also in some stromal cells, and COX-2 was predominantly expressed in the granulosa cells and the ovarian surface epithelium (Fig. 2(B) and (C)). COX-1 protein was expressed consistently across all of the groups (Fig. 2(B)). COX-2 protein was deceased in the 15 % flaxseed diet group (Fig. 2(C)

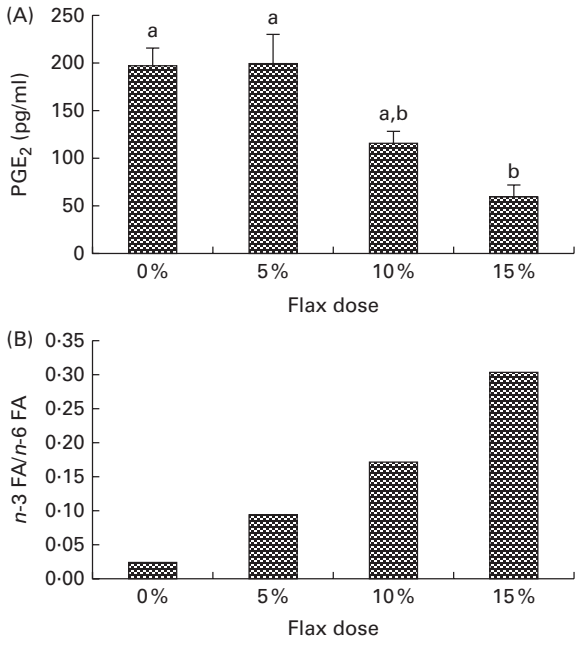


**Fig. 1.** Flaxseed dose-dependent alteration in the levels of *n*-3 fatty acids (FA) and PG. (A) $PGE_2$ levels were assessed in the ovary tissue of the control and flaxseed-fed hens using an ELISA assay kit. Values are means (*n* 6), with standard deviations represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0·01$; one-way ANOVA). (B) Ovary tissue levels of FA and *n*-6 FA measured using GC. Values are means (*n* 6).

and (D)). *COX-1* mRNA and protein levels in the ovaries were not affected by the amount of flaxseed (Fig. 3(A) and 3(B)). *COX-2* mRNA levels appeared to be consistent across all of the different diet groups (Fig. 3(C)), whereas COX-2 protein expression was significantly reduced in the ovaries of the chickens that were fed a 15% flaxseed diet (Fig. 3(D)).

## n-3 Fatty acid and n-6 fatty acid levels in ovaries

*n*-3 FA and *n*-6 FA analysis revealed that the higher the dose of flaxseed in the diet was, the higher the level of *n*-3 FA incorporation in the chicken ovaries. The *n*-3 FA:*n*-6 FA ratio increased with higher doses of flaxseed (Fig. 1(B)).

## Cell proliferation and apoptosis assay

TdT-mediated dUTP nick end labelling staining for the detection of apoptotic cells indicated that there was no significant difference with respect to the number of apoptotic cells in the ovarian tissue between the birds in the different dietary groups (Fig. 4(B) and (D)). Similarly, proliferating cell nuclear antigen expression was not affected by the flaxseed diet (Fig. 4(A) and (C)). As a result, the ratio of proliferating to apoptotic cells in the ovaries was comparable across all of the diet groups (Fig. 4(E).

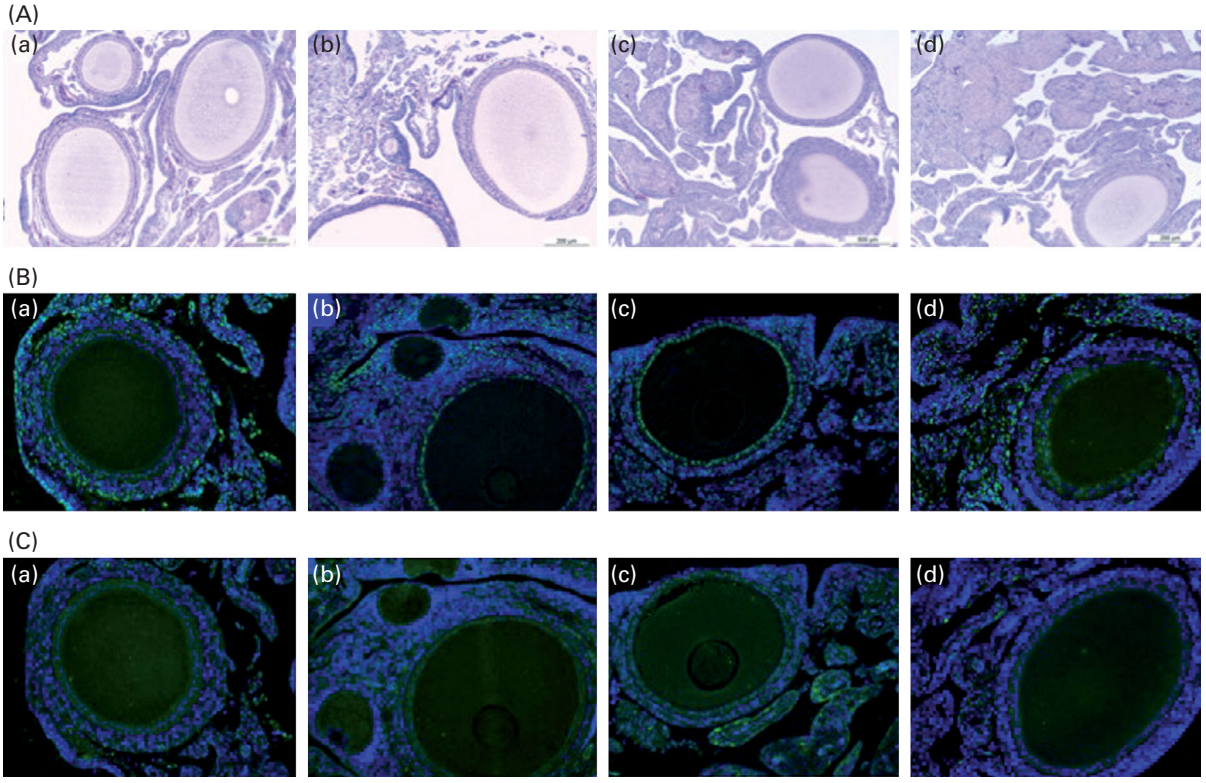


**Fig. 2.** Hematoxylin and eosin (H&E) staining on ovarian section and immunofluorescence staining for cyclo-oxygenase (COX)-1 and COX-2 proteins. (A) Ovary tissue sections from the control (a), 5% flaxseed (b), 10% flaxseed (c) and 15% flaxseed (d) groups were stained using H&E and assessed histologically under 100× magnification, *n* 3. (B) and (C) Ovary sections from the control (a), 5% flaxseed (b), 10% flaxseed (c) and 15% flaxseed (d) groups were stained for COX-1 and COX-2 proteins, respectively, and observed at 200× magnification. (Insert: non-immune IgG, 200×), *n* 3.

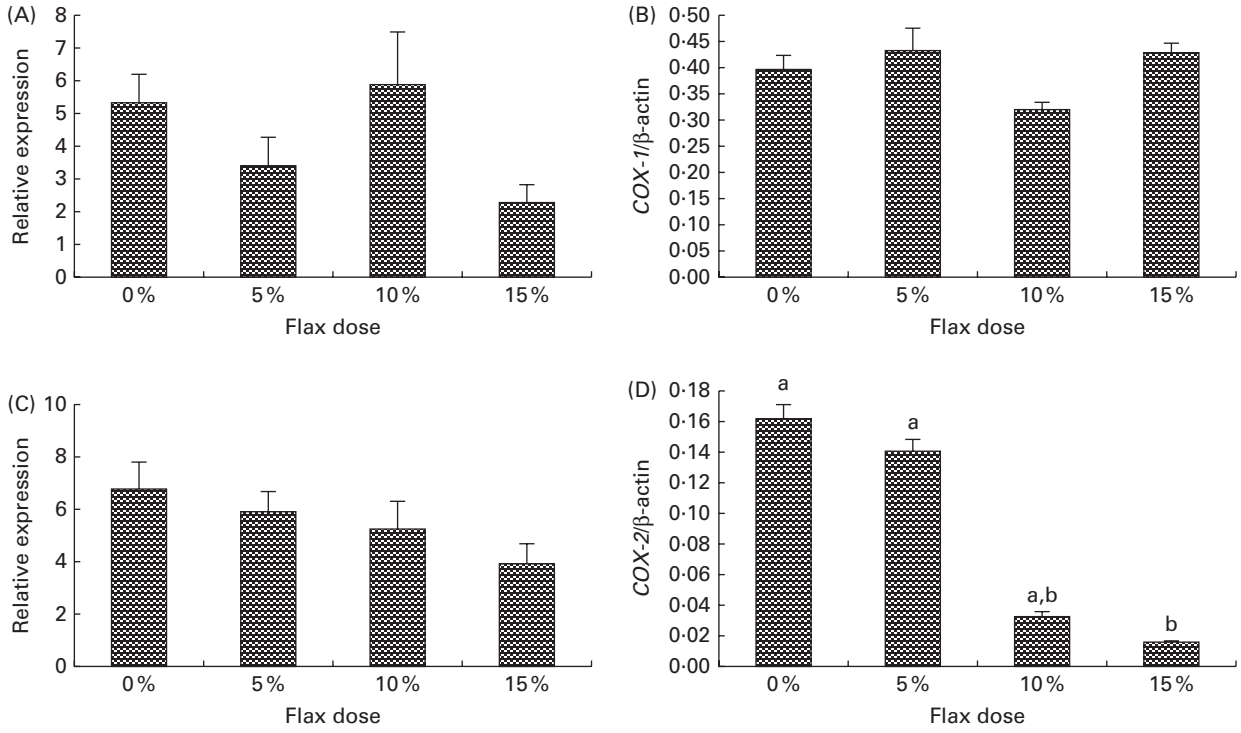


**Fig. 3.** Cyclo-oxygenase (COX)-1 and COX-2 protein and mRNA expression in the ovaries. (A) and (B) *COX-1* enzyme mRNA expression was measured using real-time PCR, and protein levels were analysed using Western blotting in control and flaxseed-fed chickens, respectively. Values are means ($n$ 6), with standard deviations represented by vertical bars. (C) and (D) *COX-2* enzyme mRNA expression was measured using real-time PCR, and protein levels were analysed using Western blotting in control and flaxseed-fed chickens, respectively. Values are means ($n$ 6), with standard deviations represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0{\cdot}05$; one-way ANOVA).

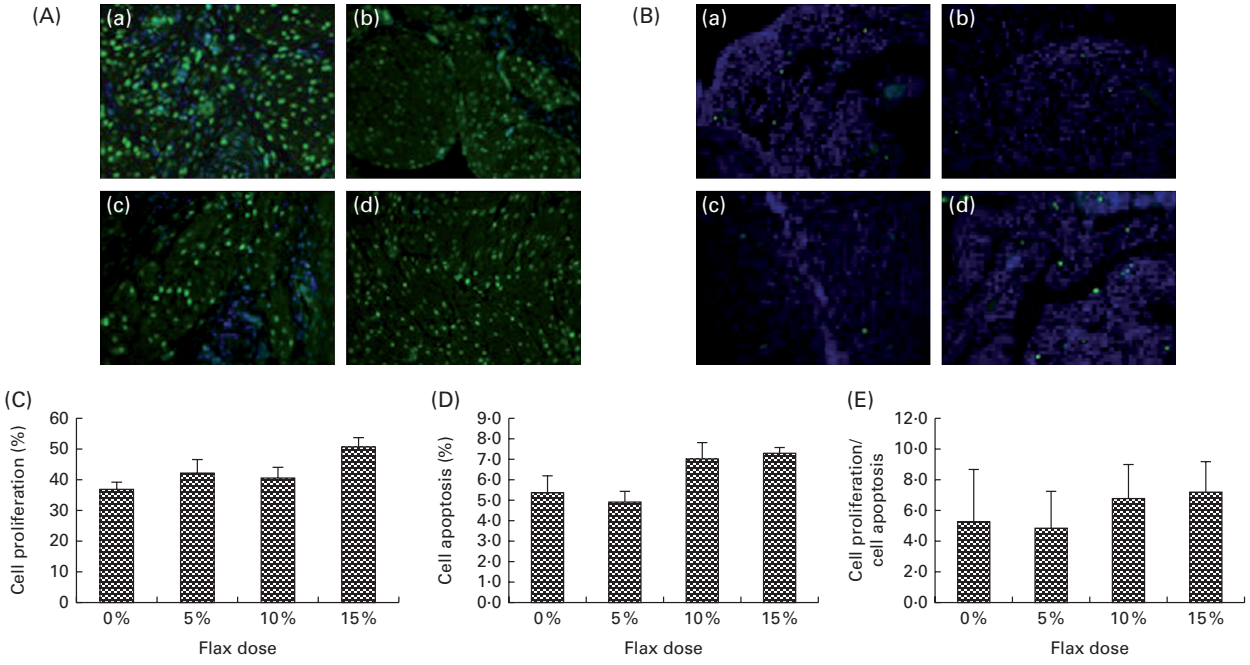


**Fig. 4.** Effect of 5, 10 and 15 % flaxseed-supplemented diet on cell proliferation and apoptosis in the ovaries. (A) Green fluorescent staining indicated proliferating cellular nuclear antigen (PCNA)-positive proliferating cells for ovary sections from the control (a), 5 % flaxseed (b), 10 % flaxseed (c) and 15 % flaxseed (d) groups. (B) Green fluorescent staining indicated TdT-mediated dUTP nick end labeling (TUNEL)-positive apoptotic cells for ovary sections from the control (a), 5 % flaxseed (b), 10 % flaxseed (c) and 15 % flaxseed (d) groups observed at 200× magnification. (Insert: no primary control, 200× magnification). (C) Graph indicating percentage of cell proliferation. (D) Graph indicating percentage of cell apoptosis. (E) Graph indicating ratio of cell proliferation to cell apoptosis in normal ovary tissue from chickens ($n$ 3).

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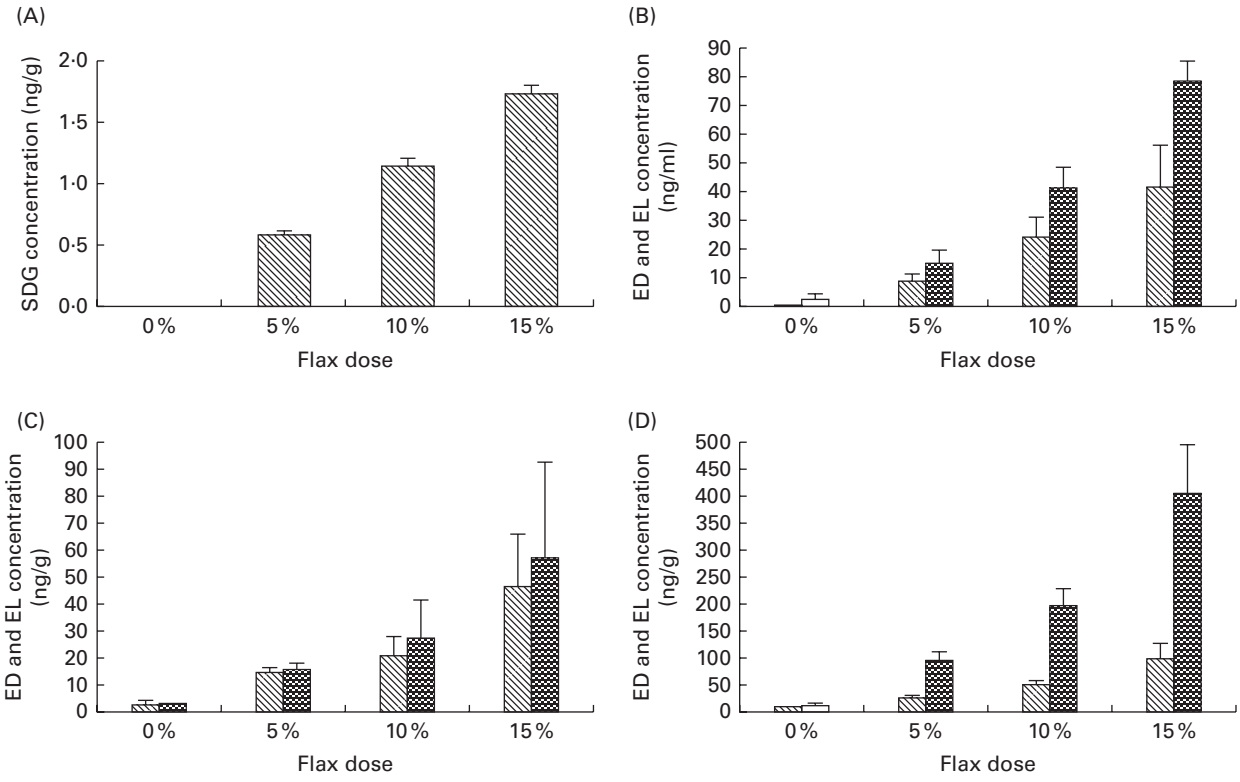


**Fig. 5.** Levels of secoisolariciresinol diglucoside (SDG), enterodiol (ED) and enterolactone (EL) in the diets and tissues, respectively. SDG is hydrolysed and ultimately metabolised by the gut flora to EL and ED. (A) Levels of SDG were measured in the varying percentage of flaxseed-supplemented diets using liquid chromatography (LC). (B)–(D) Levels of ED (▨) and EL (▩) were analysed in the different flaxseed-supplemented diet groups using LC, MS and MS analysis in the serum, ovary and liver tissues, respectively (*n* 6).

### *Secoisolariciresinol diglucoside concentration in different diets*

The level of SDG was measured in the 0, 5, 10 and 15% flaxseed diet groups using liquid chromatography. As expected, the level of SDG increased proportionally with the percentage of flaxseed (Fig. 5(A)). The SDG concentrations were 0·56, 1·12 and 1·65 mg/g in the 5, 10 and 15% flaxseed diets, respectively.

### *Enterolactone and enterodiol levels in the tissue were proportional to the flaxseed content of the diet*

The concentrations of the phytoestrogen lignans EL and ED were measured in the serum, ovary and liver samples from birds that were fed different doses of flaxseed in their diets. The levels of EL and ED in serum (Fig. 5(B)), ovary (Fig. 5(C)) and liver (Fig. 5(D)) tissues increased proportionally to the increase in flaxseed dose. EL levels were considerably higher than those of ED in the liver tissue for all of the dosage groups, whereas in the serum, EL levels were lower than ED for all of the doses. ED and EL levels were comparable in the ovarian tissues.

### *Flax diet influences levels of oestrogen metabolites*

Serum samples from the different diet groups were analysed for their levels of certain oestrogen metabolites. The level of 16-OHE1 was considerably reduced in the flax-fed birds as compared to those in the control group, whereas the level of 2-OHE1 was substantially increased in the 15% flax diet group (Fig. 6(A)). The clinically relevant 2-OHE1:16-OHE1 ratio was seen to increase significantly in the serum of the birds in the 15% dosage group (Fig. 6(B)).

### *Cytochrome p450 enzymes expression*

CYP1B1 (cytochrome p450, family 1, subfamily B, polypeptide 1), CYP1A1 (cytochrome p450, family 1, subfamily A, polypeptide 1) and CYP3A4 (cytochrome p450, family 3, subfamily A, polypeptide 4) enzymes metabolise $E_2$ to 4-hydroxyestradiol (4-OHE2)[38], 2-hydroxyestradiol (2-OHE2) and 16-hydroxyestradiol (16-OHE2; Estriol), respectively[39]. Ovarian tissue expression of *CYP1B1* and *CYP3A4* mRNA decreased proportionally with the dose of flaxseed in the diet (Fig. 7(A) and (B)). *CYP1A1* mRNA expression in the liver increased with an increase in the amount of flaxseed in the diet (Fig. 8(A)), but its expression was negligible in the ovary. The mRNA expression of *CYP3A4* and *CYP1B1* in the liver remained unchanged between the groups (Fig. 8(B) and (C), respectively).

### *Oestrogen receptor expression decreases with flax diet*

An increase in the dosage of flax in the diet was observed to parallel a decrease in *ERα* mRNA expression (Fig. 9(A)).

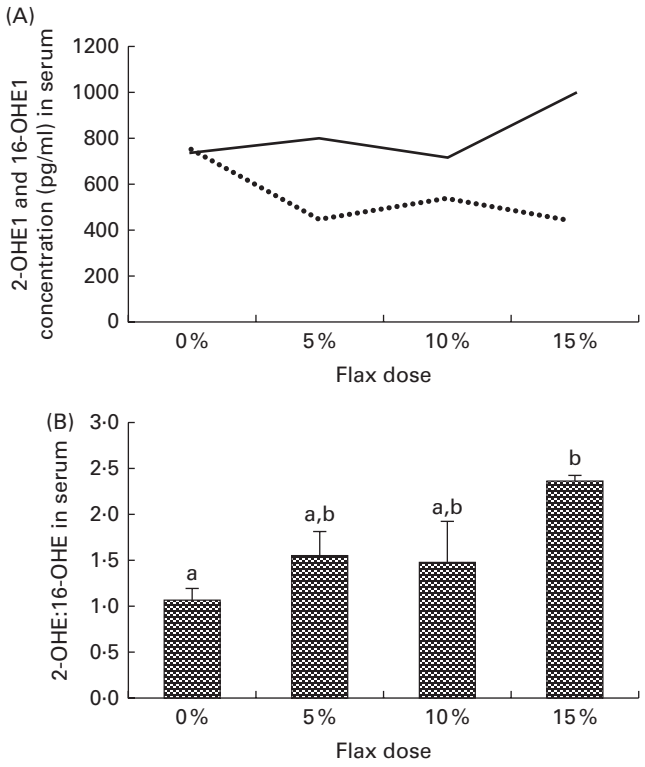


**Fig. 6.** Serum levels of 2-hydroxyestrone (2-OHE1) and 16-hydroxyestrone (16-OHE1). (A) 2-OHE1 (—) and 16-OHE1 (••••) were analysed in the serum samples from control and flaxseed-supplemented diet groups using the 2-hydroxyestrone and 16-hydroxyestrone Estramet Double ELISA Kit (*n* 3 for the 5, 10 and 15% groups; *n* 9 for the control group). (B) 2-OHE1: 16-OHE1 ratio in the serum samples of chickens fed varying doses of flaxseed-supplemented diets. Values are means, with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA).

mRNA levels were significantly decreased in the 10 and 15% flaxseed diet groups as compared to the control group. ERα protein was predominantly expressed in the granulosa cells of the chicken ovarian follicle and appeared to decrease in the 15% flaxseed diet group (data not shown).

## Discussion

In our previous studies, we demonstrated that feeding 2·5-year-old chickens a 10% flaxseed diet for 1 year reduced the severity but not the incidence of ovarian cancer[32]. When younger (22-week-old) chickens in their first lay were fed a flaxseed diet for 4 years, the severity as well as incidence of ovarian cancer was found to be reduced[6]. These findings suggest that flaxseed diet is most effective in combating ovulation-induced inflammation when it is consumed at the start of ovulation and continued throughout the reproductive life. The objective of the present study was to determine the optimum dose of flaxseed in the diet by analysing the levels of surrogate end points that are representative of its effect on ovarian cancer initiation and progression.

We previously established that COX-2 enzyme expression increases with age, and the consumption of flaxseed decreases COX-2 expression[35]. COX enzymes act on *n*-6 FA to generate series-2 pro-inflammatory PG such as $PGE_2$, and their action on *n*-3 FA generates series-3 anti-inflammatory PG[40]. Flaxseed is a rich source of *n*-3 FA, which competes as a substrate with *n*-6 FA for COX enzymes. This in turn leads to a possible decrease in the synthesis of pro-inflammatory PG, which are known to stimulate the release of inflammatory cytokines[41] that are associated with cancer. In the present study, we observed that an increase in the percentage of flaxseed in the diet led to an increase in the *n*-3 FA:*n*-6 FA ratio and a concurrent statistically significant reduction in the ovarian $PGE_2$ levels in hens that were fed a 15% flaxseed diet (Fig. 1(B)). *COX-1* mRNA and protein expression was not altered significantly with a flaxseed diet, which was consistent with our previous finding[6] that suggested a flax diet did not have any effect on *COX-1* expression in normal young chickens (Figs. 2(B), 3(A) and (B)). Also consistent with our previous results, we observed that COX-2 protein expression decreased significantly with a 10 and 15% flax diet (Fig. 3(D)). With the present study, we confirm our previous results[6,35] and take a step further by demonstrating that not only does a flax diet decrease the levels of inflammatory $PGE_2$[35], but it does so in a dose-dependent fashion. This further suggests that the anti-apoptotic and pro-angiogenic[42] COX-2 enzyme can be targeted by a flaxseed diet.

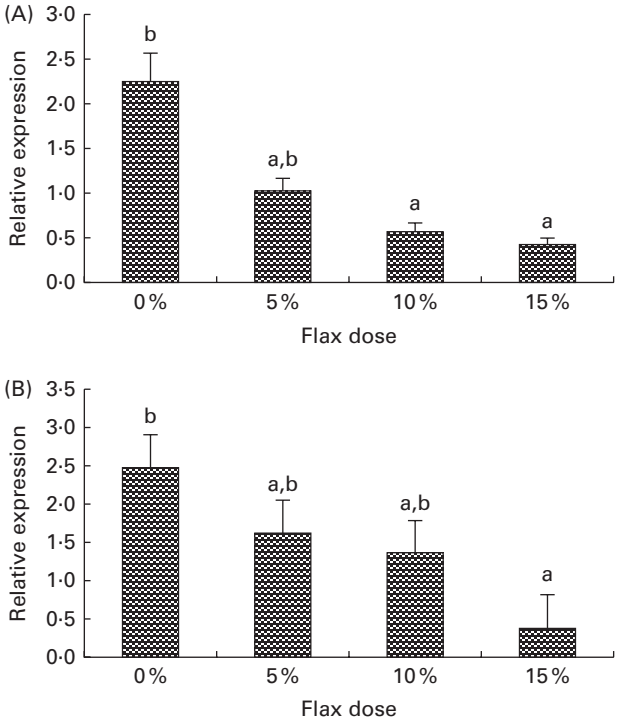


**Fig. 7.** Cytochrome p450, family 1, subfamily B (*CYP1B1*) and cytochrome p450, family 3, subfamily A (*CYP3A4*) enzyme mRNA expression in the ovary tissue. (A) *CYP1B1* mRNA expression was measured using real-time quantitative PCR (qPCR). Values are means (*n* 6), with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA). (B) *CYP3A4* mRNA expression was measured using real-time qPCR. Values are means (*n* 6), with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different ($P<0·01$; one-way ANOVA).

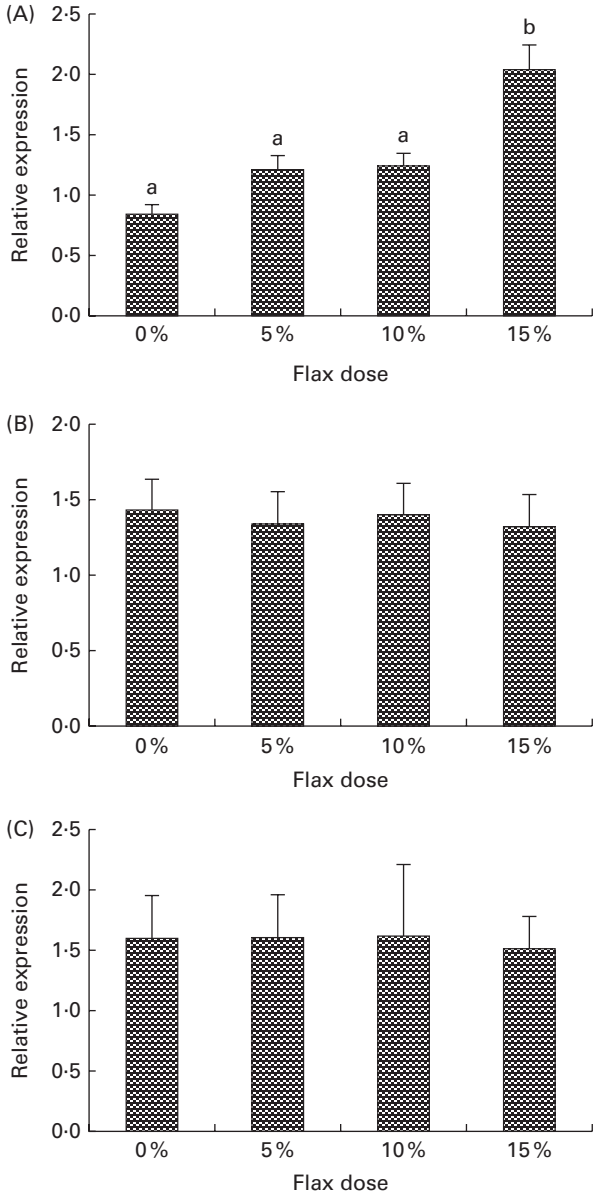


**Fig. 8.** Cytochrome p450, family 1, subfamily A (*CYP1A1*) (A), cytochrome p450, family 1, subfamily B (*CYP1B1*) (B) and cytochrome p450, family 3, subfamily A (*CYP3A4*) (C) mRNA expression in the liver. mRNA levels were quantified using real-time quantitative PCR. Values are means (*n* 6), with standard deviations represented by vertical bars. [a,b]Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA).

Phase I of oestrogen metabolism begins with hydroxylation, which is initiated by the CYP (cytochrome p450 enzymes) CYP1B1, CYP1A1 and CYP3A4 acting at different positions on $E_2$ to form 4-OHE2, 2-OHE2 and 16-OHE2, respectively. Phase II of oestrogen metabolism includes conjugation by glucuronidation, sulfation or O-methylation by catechol-*O*-methyl transferase[43]. The 4-OHE2, 2-OHE2 and 16-OHE2 metabolites are known to have varying degrees of oestrogenicity themselves[39]. The corticle follicles and small follicles in the chicken ovary produce $E_2$[44], which thus provides a substrate for these metabolising enzymes. The 4-OHE2 is further oxidised to oestrogen-3, 4-quinones, which can form depurinating DNA adducts that can cause mutations that result in the initiation of cancer[45]. The 16-OHE2 is known to be pro-carcinogenic[46], and evidence suggests that the 16-OHE2 increases the risk of breast cancer because it increases the rate of cell proliferation by binding covalently to the ER[47]. The 2-OHE2 has a relatively low binding affinity for ER and has no carcinogenicity, unlike some other $E_2$ catechols. For example, Liehr *et al.* demonstrated that the 2-OHE2 could not induce kidney tumours in hamsters, whereas the 4-OHE2 could[48]. The 2-OHE2 is also known to protect against hormone-dependent cancers by interfering with the binding of sex hormones to the sex steroid binding protein[49]. It is also converted to 2-methoxyestradiol at a much faster rate by catechol-*O*-methyl transferase, as compared to the other catechol oestrogens. The 2-methoxyestradiol has anti-proliferative and anti-angiogenic properties[50].

The serum analysis in the present study suggested that the 2-OHE1 increased considerably in the 15 % flax diet, but the 16-OHE1 decreased in all of the flax diet samples and significantly in the 15 % group. A decrease in the 2-OHE1:16-OHE1 ratio can be correlated with an increased risk for a number of cancers, including breast, endometrium and cervical[51–54]. In the present study, we observed that the 2-OHE1:16-OHE1 ratio increased significantly in the 15 % flax diet as compared to the control group. Consistent with the 16-OHE1 levels, we observed that a flaxseed diet led to a down-regulation of *CYP3A4* mRNA expression in the ovaries of chickens (Fig. 7(B)). *CYP1A1* expression could not be detected in the ovaries of either control or flax-fed birds, but its expression in the liver increased with a flax diet (Fig. 8(A)). The mechanism through which flaxseed regulates the expression of these CYP enzymes is currently being investigated.

$E_2$ induces *CYP1B1* expression in the female reproductive tract as well as in ER-positive breast cancer cells and ER-over-expressing endometrial cancer cells[38]. We have previously shown that *CYP1B1* expression is up-regulated in cancerous chicken ovaries and is highest in the post-ovulatory follicle 3, which is usually buried inside the ovary, where the $E_2$ concentration is high[45]. $E_2$ levels in the ovary are about 100-fold higher than circulating levels[55]. The high oestrogen levels in

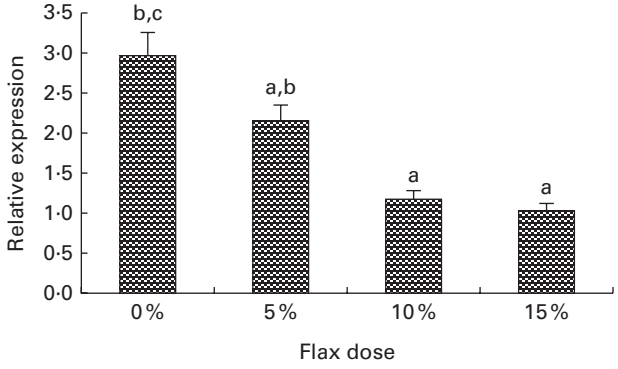


**Fig. 9.** Oestrogen receptor α (*ERα*) expression in the ovaries. *ERα* mRNA levels were analysed using real-time quantitative PCR in the different diet groups. Values are means (*n* 6), with standard deviations represented by vertical bars. [a,b,c]Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA).

the ovary could not only facilitate increased *CYP1B1* expression, but they could also provide a large substrate pool for CYP1B1, which would result in an increase in genotoxic 4-OHE2 catechol levels. Different doses of flaxseed in the diet did not alter $E_2$ concentrations in the chicken sera (data not shown); however, concentrations in the ovary were not measured. In the present study, *CYP1B1* mRNA expression was seen to decrease in the ovary with a flax diet in a dose-dependent manner (Fig. 7(A)). A decrease in *CYP1B1* expression in the ovary with a flax diet demonstrates the anti-oestrogenic and possibly anti-oncogenic properties of flaxseed.

Oestrogen stimulates cell proliferation and can promote lymph node metastasis in ER-positive ovarian cancer tumours but not in ER-negative cancers[56], which demonstrates the significance of ER in promoting tumour growth and mobilisation in ovarian cancer. CYP1B1 is primarily regulated by the aryl hydrocarbon receptor and its dimerising partner, the aryl hydrocarbon receptor nuclear translocator[57]. Tsuchiya *et al.*[38] have shown that $E_2$ can up-regulate *CYP1B1* through ER independent of the aryl hydrocarbon receptor, which further suggests that ER plays a role in inducing oestrogen-mediated toxicity. We observed that feeding hens a flaxseed-supplemented diet led to a dose-dependent decrease in the expression of *ERα* mRNA (Fig. 9). A decrease in *ERα* expression did not alter the egg-laying frequency of these birds, which indicates that flaxseed does not impair the normal functioning of the ovary. We have previously published that hens that were fed a diet of 10% flaxseed had an average egg-laying frequency of about 71% per week[35]. From one of our recent studies, we know that hens that were fed a 15% flaxseed diet produced eggs at a frequency of 69·4% per week, whereas the control group produced eggs at 72·23% per week. These data clearly indicate that the different doses of flaxseed diet do not affect the egg-laying frequency of the birds. The effect of a flaxseed diet on ERα could be a result of the flaxseed lignan SDG, which is ultimately converted to the weakly oestrogenic/anti-oestrogenic EL and ED. The partial agonist/antagonist property of these compounds could be responsible for decreasing ER activity and eventually its expression, given that ER transactivates its own expression[10].

In our previous long-term study, we showed that a 10% flaxseed-supplemented diet decreased the levels of inflammatory $PGE_2$, which also correlated with a reduced incidence and severity of ovarian cancer[6]. We observed that $PGE_2$ levels were also higher in older chickens regardless of pathology, which suggests that there are other factors that contribute to ovarian cancer initiation and dissemination. The present findings indicate that a 15% flaxseed dose is most effective in decreasing the levels of the highly potent inflammatory PG $PGE_2$, carcinogenic oestrogen metabolites and ER in the ovaries of normal young chickens without having any toxic effects. The findings suggest that a 15% flaxseed diet can beneficially affect a number of clinically relevant surrogate endpoints that are implicated in cancer. This is especially significant because it was recently demonstrated that ER-positive ovarian cancer is more chemo-resistant than ER-negative ovarian cancer, which makes it extremely challenging to treat[58]. The ER-positive status of these tumours possibly plays an important role in the progression of the disease.

Taken together, the chemo-preventative properties of flaxseed alleviate the expression of inflammatory $PGE_2$, ER, toxic oestrogen metabolites and enzymes which might contribute to the development of ovarian cancer. As a result, the present data strongly supports early dietary intervention using flaxseed as a preventative approach for ovarian cancer.

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The study was conceptualised by D. B. H. in order to understand the effect of different doses of flaxseed on inflammation/oxidative stress, PG-associated pathways and oestrogen-associated pathways. It was included as a specific aim in the NIH RO1 AT00408 grant.

The immunohistochemistry (COX-1, COX-2) and quantitative PCR (CYP1A1 (liver), CYP1B1 (liver and ovary), CYP3A4 (liver and ovary) and ERα) experiments were performed by A. D. In addition, A. D. processed the serum samples and wrote and formatted the manuscript. M. A. G. F. performed the quantitative PCR and Western blotting for COX-1 and COX-2. E. E. measured the $PGE_2$ levels in the ovary tissue. S. M. performed the proliferating cell nuclear antigen and TdT-mediated dUTP nick end labelling staining on the ovary tissue sections. C. G. processed the samples for the enterolactone and enterodiol analysis and also measured the SDG levels in the diets. C. S. analysed the *n*-3 FA and *n*-6 FA levels in the ovaries. T. K. analysed the 2-OHE1 and 16-OHE1 in the serum samples.

The authors have no known conflicts of interest.

The authors have nothing to disclose.

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