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Whole flaxseed diet alters estrogen metabolism to promote 2-methoxtestradiol-induced apoptosis in hen ovarian cancer☆,☆☆

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Abstract

The study reported here demonstrates that a flaxseed-supplemented diet causes ovarian tumors in the laying hen to undergo apoptosis, resulting in a reduction of tumor burden, reducing the frequency and severity of ovarian cancer. We have previously shown in normal ovaries that flaxseed and its components down-regulate ERalpha and alter the expression of enzymes that metabolize estrogen. In this study, we analyzed the effects of the two main components of whole flaxseed, ligan and omega 3 fatty acids on estrogen metabolism and the estrogen receptor in ovarian tumors. ER alpha expression was up-regulated in the ovarian tumors and was not affected by diet. Liver CYP1A1 expression was significantly increased by the whole flaxseed diet with a corresponding increase in 2-methoxyestradiol plasma levels. We also observed increased p38 and ERK 1/2 MAPK activation in the ovary as well as an increase in apoptosis in the tumor epithelium. SMAD 7, a factor involved in the 2-methoxyestradiol-mediated apoptosis pathway was also up-regulated in tumors from the whole flaxseed diet group. 2-methoxyestradiol-induced antitumor effects were further validated by in human ovarian cancer cells. This study details the effect of flaxseed diet on estrogen metabolism and demonstrates the antiovarian cancer effects of 2-methoxyestradiol.
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Keywords: 2-Methoxtestradiol; Ovarian cancer; Flaxseed; Apoptosis; Estrogen metabolism

1. Introduction

Ovarian cancer is aptly referred to as the silent killer because patients are not diagnosed until the disease is advanced and when the prognosis is poor and treatment options are limited. The 5-year relative survival rate for ovarian cancer patients in the United States is 45%. The SEER statistics database of the National Institute of Health predicted that there will an estimated 21,290 new cases of ovarian cancer in 2015, and an estimated 14,180 women will succumb to ovarian cancer in 2015 in the United States alone [1].

In women, as well as in the laying hen, the risk of ovarian cancer increases with age [2].

The risk of ovarian cancer is correlated to lifetime number of ovulations, in both women and hens. Hens start developing ovarian cancer by 2.5 years of age after ovulating daily. The laying hen develops ovarian cancer spontaneously and is therefore an excellent natural model to study initiation of the disease and test prevention strategies [3].

Whole flaxseed is the richest plant source of both the omega-3 polyunsaturated fatty acid, alpha linolenic acid (ALA) and the lignan, secoisolaricirescinol diglucoside (SDG). Flaxseed also contains a significant amount of other macronutrients, fiber and minerals [4]. ALA is converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are antiinflammatory and cardioprotective [5], while SDG is converted to enterolactone (EL) and enterodiol (ED) which have antiestrogenic and antioxidant properties [6]. Both DHA and EL have been shown to decrease endothelial cell proliferation and migration thereby protecting against tumor progression [7–10].

Estradiol is hydroxylated by different cytochrome p450 (CYP) enzymes in the C-2 position to form 2-hydroxyestradiol/2-hydroxyestrone (CYP1A1), in the C-4 position to form 4-hydroxyestradiol/4-hydroxyestrone (CYP1B1) or in the C-16 position to form estriol (16-hydroxyestradiol/16-hydroxyestrone) (CYP3A4) [11]. We have previously established that 15% whole flaxseed-supplemented diet increases the serum 2-hydroxyestradiol/16-hydroxyestradiol ratio, in turn suggesting a reduced risk of cancer [12]. The 2-hydroxy and 4-hydroxy metabolites are oxidized by catechol-o-methyl transferase (COMT) to methoxy-metabolites [13]. 2-hydroxyestradiol is preferentially converted to 2-methoxyestradiol [14], whereas 4-hydroxyestradiol is readily oxidized to the 3,4 quinone, a genotoxic metabolite.

Two-methoxyestradiol has antiangiogenic properties that were demonstrated both *in vivo* as well as *in vitro* [15,16]. Two-methoxyestradiol has also been shown to exhibit proapoptotic and

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Table 1
Table of abbreviations

Abbreviation	Full form
16-OHE1	16-hydroxyestrone
2-MeE2	2-methoxyestradiol
2-OHE1	2-hydroxyestrone
4-OHE1	4-hydroxyestrone
ARNT	Aryl hydrocarbon receptor nuclear translocator
AHR	Aryl hydrocarbon receptor
ALA	Alpha linolenic acid
COMT	Catechol-O-methyl transferase
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
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
E2	Estradiol
ED	Enterodiol
EL	Enterolactone
EIA	Enzyme immunosorbent assay
EPA	Eicosapentaenoic acid
ERK1/2 kinase	Extracellular signal regulated kinase 1/2
ERα	Estrogen receptor alpha
FIGO	International Federation of Gynecologic Oncologists
JNK	Jun amino-terminal kinase
MAPK	Mitogen-activated protein kinase
OM3 FA	Omega-3 fatty acid
OM6 FA	Omega-6 fatty acid
OSE	Ovarian surface epithelium
OvCa	Ovarian cancer
p38 kinase	p38 mitogen activated kinase
PBS	Phosphate-buffered saline
PGE_2	Prostaglandin E2
ROS	Reactive oxygen species
SDG	Secoisolariciresinol diglucoside
SECO	Secoisolariciresinol
SMAD	SMA/Mothers against decepentaplegic homolog
TGF-beta	Transforming growth factor beta
TUNEL	TdT mediated dUTP nick end labeling

antiproliferative properties in a variety of cells *in vitro* [17]. Studies also showed that breast cancer cells are sensitive to 2-methoxyestradiol and undergo apoptosis, but normal mammary cells do not, suggesting that its proapoptotic effects might be tumor cell specific [18,19]. 2-methoxyestradiol induces apoptosis by destabilizing tubulin dimers [20]. More recently, 2-methoxyestradiol has been shown to increase activation (phosphorylation) of the MAP kinase p38 and up-regulation of SMAD7 to promote its proapoptotic, antitumorigenic effects [21].

Previously we have shown that flaxseed diet decreases the incidence and severity of ovarian cancer in old laying hens [22–24].

Table 2
Antibody table

Protein target	Manufacturer, catalog #	Species raised in	Antibody dilution
CYP1A1	Santa Cruz, 20,772	Rabbit polyclonal	1:400 (WB)
CYP1B1	Abcam, 33,586	Rabbit polyclonal	1:500 (WB)
CYP3A4	My-Biosource, 18,227–1-ap	Rabbit polyclonal	1:500 (WB)
ER alpha	Santa Cruz, 543	Rabbit polyclonal	1:200 (IHC)
Caspase-3	Abcam, 115,183	Rabbit polyclonal	1:700 (WB)
Cleaved caspase-3	Cell Signaling Technology, 9664S	Rabbit polyclonal	1:500 (WB) 1:200 (IHC)
ERK1/2	Cell Signaling Technology, 4696	Rabbit polyclonal	1:1000 (WB)
pERK1/2	Cell Signaling Technology, 4370	Mouse monoclonal	1:1000 (WB)
p38	Cell Signaling Technology, 9212S	Rabbit polyclonal	1:700 (WB)
p-p38	Cell Signaling Technology, 4511S	Rabbit polyclonal	1:700 (WB)
SMAD7	Santa Cruz, 11,392	Rabbit polyclonal	1:200 (WB)
Beta actin	Novus, 600–505	Rabbit polyclonal	1: 2000 (WB)

Table 3
Diet composition

Diet	Control	15% Whole flaxseed	10% Defatted flax meal	5% Flax oil
Enriched with:	–	ALA+ SDG	SDG	ALA
Ingredient				
Corn	67.40	47.58	54.90	52.00
Flaxseed (whole)		15.00		
SBM (soy bean meal)	18.30	18.30	18.30	18.30
Corn gluten meal	3.00			5.00
Flax oil				5.00
Defatted flax meal			10.00	
Qual Fat		2.50	3.80	
Solka Floc	0.30	5.62	1.99	8.70
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	16.50	17.04	16.49
TME, kcal/kg	2816	2815	2816	2815
Calcium, %	3.73	3.75	3.77	3.73
aPhosphorus, %	0.38	0.38	0.40	0.37
Met + Cys, %	0.67	0.64	0.72	0.67

This study was designed to tease apart the effects of the bioactive components of flaxseed on targets associated with ovarian health. Our main objective was to understand the effects of flaxseed-induced increase in 2-methoxyestradiol on molecular pathways promoting apoptosis in tumors and normal hen ovarian tissue. We demonstrated that whole flaxseed diet increased the levels of 2-methoxyestradiol in the hens but only promoted significant apoptosis in the tumors tissues and not the normal ovary *via* increased phosphor-p38 (pp38) and SMAD7. We corroborated these results *in vitro* by demonstrating that 2-methoxyestradiol increased expression of cleaved caspase-3, pp38 and SMAD7 in human ovarian cancer cells. (See Table 1 for abbreviations.)

2. Material and methods

2.1. Materials

Biotinylated antirabbit IgG, Vector laboratories. AffiniPure Alexa 488 conjugated Donkey Anti-Mouse IgG, Jackson ImmunoResearch. Streptavidin, Alexa Fluor® 488 conjugate, Life technologies; DeadEnd TUNEL Detection System from Promega (Madison, WI, USA); BG1FR cells were procured from Dr. Ken Korach's lab at NIEHS; Cayman Chemicals 2-methoxyestradiol EIA kit (582261); 2-methoxyestradiol was obtained from Sigma Aldrich, (M6383), HyClone DMEM culture medium (SH30243.03), HyClone DMEM culture medium w/o Phenol red (SH30604.02). DyLight™680 conjugated goat antimouse IgG antibody (H&L) (35518) and DyLight™800 conjugated goat antirabbit IgG antibody (H&L) (35571) from ThermoFisher. 100X Halt™ Protease and Phosphatase Inhibitor Cocktail from ThermoFisher (78440).

2.2. Animals care and study description

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.

Two and a half-year-old hens (*Gallus domesticus*) were either fed control diet, diet supplemented with flaxseed, diet supplemented with defatted flax meal or diet supplemented with flax oil for a period of 11 months. The control group had 175 birds, while all the other groups had 160 birds. Blood was collected at different time points throughout the study by wing vein puncture. At the end of each study, hens were euthanized using CO_2 asphyxiation, and tissues (ovary and liver) were harvested upon dissection. Ovaries were removed from hens, and small yellow follicles (6–8 mm) and preovulatory follicles (9–35 mm) were excluded. Ovaries that were suspected of having abnormalities were assessed to confirm cancer status by histology. Tumors were classified by stage based on the tumor dissemination, oviductal involvement and presence or absence of ascites, similar to the FIGO guidelines for women with ovarian cancer [25]. The ovaries were dissected into several pieces and either flash frozen in

liquid nitrogen and later stored at −80 °C or fixed in NBF fixative solution and later embedded in paraffin for histological staining. (See Tables 2 and 3.)

2.3. Protein isolation and Western blotting

Western blotting was performed and described previously [26]. Snap-frozen tissue was homogenized in protein lysis buffer (0.1% SDS in PBS+1X Halt™ Protease and Phosphatase Inhibitor Cocktail in water) to isolate protein. Twenty-five micrograms of total protein was separated using an 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. 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 antimouse IgG antibody (H&L) and DyLight™800-conjugated goat antirabbit 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.

2.4. Hematoxylin and eosin staining

Formalin fixed ovarian tissue was embedded in paraffin and 5-μm thick sections were cut and mounted on microscope slides. Slides were deparaffinized in xylene and rehydrated by using graded ethanol solutions. Hematoxylin and eosin staining was performed on the slides as described by Sheehan and Haarpchak [27].

2.5. Immunohistochemistry

Immunofluorescence was performed as described previously [26]. Formalin fixed ovarian tissue was embedded in paraffin, and 5-μm thick sections were cut and mounted on SuperFrost Plus microscope slides. Followed by deparaffinization, slides were rehydrated by running them 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 antiestrogen receptor antibody overnight at 4 ° C. After washing with 1X PBS with 0.01% Tween 20, sections probed with antiestrogen receptor antibody (sc-543) were incubated with Alexa 488-conjugated antimouse 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). Control sections were incubated with ER alpha antibody preabsorbed with the ER alpha blocking peptide. 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.

2.6. 2-methoxyestradiol ELISA assay

Plasma samples were analyzed for levels of 2-methoxyestradiol. 2-methoxyestradiol was extracted from the plasma samples using methylene chloride and analyzed using the Cayman Chemicals 2-methoxyestradiol ELISA kit [26].

2.7. Cell culture

BG1FR human ovarian adenocarcinoma cell lines were obtained from the lab of Dr. Ken Korach at NIEHS and cultured in DMEM complete medium (DMEM+10% FBS+0.5% Pen/Strep/Fungizone) by incubating at 37 °C under 5% CO_2.

2.8. 2-methoxyestradiol treatment on BG1FR cells

Cells were seeded at a density of 2×10^6 in 100-mm dishes for 24 h in phenol red negative DMEM medium. Next day, medium was replaced with phenol red negative DMEM medium containing different concentrations of 2-methoxyestradiol (2uM, 5uM, 10uM, 20uM) for 6 h and 24 h. Cells were harvested, and proteins were isolated from at the aforementioned time points.

2.9. TUNEL assay for detection of apoptotic cells

Cells in their late-stage apoptosis were detected with the DeadEnd TUNEL system from Promega (Madison, WI) according to manufacturer's protocol. Formalin-fixed paraffin-embedded (FFPE) sections were deparaffinized using xylene and rehydrated in decreasing concentrations of ethanol. Sections were rinsed in 0.85% NaCl followed by washes with phosphate buffered saline (PBS). Sections were then fixed using neutral buffered formalin in PBS for 15 min followed by washes with PBS. Sections were then permeabilized using 0.02-mg/ml proteinase K at room temperature for 10 min. Sections were washed with PBS and refixed in neutral buffered formalin for 5 min. After washing with PBS, sections were incubated with a TUNEL labeling mixture of terminal deoxytransferase (TdT) and biotinylated dUTP at 37° C for 1 h, followed by two washes in PBS for 5 min each. Sections were incubated with Streptavidin Alexa 488 fluorophore (Life Technologies, Carlsbad, CA, USA; Alexa Fluor 488) for 1 h at room temperature followed by washes in PBS. Sections were coverslipped using DAPI Fluoromount G mounting media. For control staining, the positive control section was treated for 10 min at room temperature, with DNase I (8 U) before incubation with TUNEL labeling mix, while the negative control section was incubated in the TUNEL labeling mix without TdT. Sections from three hens per treatment group were stained, and pictures of four different fields were taken for each section. TUNEL Positive cells as well as DAPI positive cells per field per section were counted using Image J. Percentage TUNEL positive cells per section was calculated and averaged to obtain percentage TUNEL positive cells for each group.

2.10. Statistics

All the surrogate endpoints were analyzed using four to six biological replicates and two technical replicates of each sample. Effect of the different flaxseed diets was assessed by normalizing to control diet. Statistical calculations were done using GraphPad Instat software by employing two-way ANOVA followed by Tukey's range test. A $P<.05$ was considered significant, while a $P<.01$ was considered highly significant.

3. Results

3.1. Expression of estrogen receptor alpha protein was up-regulated in ovarian tumors

The normal ovarian tissue histology showed presence of an OSE lining the outside of the ovary and the stromal cells interspersed between the follicles (Fig. 1A–D). The histology of the tumor samples, however, appeared to have very few or no follicles, and the tumor glands formed by the epithelial cells occupied most of the tissue (Fig. 1E–H). Diet did not appear to have any significant effect on tumor or normal tissue histology. In the normal tissue, ERα was only expressed in the granulosa cells and the ovarian surface epithelium, but in the ovarian tumors (Fig. 1I–L), ERα was observed to be strongly expressed in the tumor epithelium as demonstrated by immunofluorescence staining. Diet did not alter the expression of ERα in the ovarian tumors (Fig. 1M–P).

3.2. Effect of flaxseed and its components on estrogen-metabolizing enzymes in ovary

CYP3A4 and CYP1B1 are E2 metabolizing enzymes that convert E2 to 16-hydroxyestradiol and 4-hydroxyestradiol, respectively. Our study demonstrates that flaxseed and its components decreased expression of CYP3A4. There was a significant decrease in CYP3A4 protein expression in whole flaxseed-supplemented, defatted flax meal-supplemented and flax oil-supplemented diet-fed hen ovarian tumors compared to control hen ovarian tumors. In the control diet group, birds with tumors had significantly higher expression of CYP3A4 than normal ovaries. In the DFM diet, birds with tumors had significantly lower expression of CYP3A4 than normal ovaries. In addition, normal ovary tissues from the flax oil diet group had considerably lower expression of CYP3A4 protein than control normal tissue (Fig. 2A). Assessment of CYP1B1 protein expression revealed that it was significantly increased in ovarian tumors from flax oil-supplemented hens compared to normal tissue from flax oil-supplemented hens as well as ovarian tumors from control diet group (Fig. 2B).

3.3. Whole flaxseed diet increases expression of CYP1A1 and levels of 2-methoxyestradiol

CYP1A1 can metabolize E2 to 2-hydroxyestradiol, a weak estrogen. CYP1A1 is not expressed at a detectable level in the hen ovary but is present in the liver. Analyzing CYP1A1 expression by Western blotting suggested that whole flaxseed diet led to an up-regulation of CYP1A1 expression compared to the control diet. DFM and flax oil diets did not significantly alter CYP1A1 expression in the liver (Fig. 3A). Analyzing plasma levels of 2-methoxyestradiol by EIA suggested that plasma of

hens that were fed whole flaxseed diet had significantly higher levels of 2-methoxyestradiol, in parallel to the observed CYP1A1 expression pattern (Fig. 3B). 2-methoxyestradiol levels in plasma samples from DFM and flax oil-supplemented diet groups were not altered significantly.

3.4. Whole flaxseed diet increases the activation of p38 and ERK1/2 MAP kinases in hen ovaries

p38 MAP kinases are activated by environmental stress and inflammatory stimuli. Phosphorylation (activation) of p38 MAP kinase was significantly increased in the ovary tissue from hens that were fed a whole flaxseed-supplemented diet irrespective of the pathology (Fig. 4A). ERK1/2 MAP kinase is a vital signaling molecule downstream from other MAP kinases that are activated by a series of growth factors and cytokines. Supplementation with whole flaxseed, defatted flax meal or flax oil in the diet did not impact its expression in the normal tissue. However, whole flaxseed diet significantly increased the activation of ERK1/2 in the tumor tissue as compared to the normal tissue (Fig. 4B).

3.5. Whole flaxseed diet increases the expression of SMAD7 in the hen ovary

SMAD7 is an inhibitory SMAD belonging to the SMAD family of proteins that are predominantly involved in the TGF beta signaling pathway and is believed to be a key mediator in promoting the activation of p38 in presence of 2-methoxyestradiol. SMAD7 expression was significantly increased in the tumors from hens on the whole flaxseed-supplemented diet compared to tumors from hens on control and flax oil-supplemented diets (Fig. 5).

3.6. Whole flaxseed diet induces apoptosis in hen ovarian tumors

TUNEL staining is indicative of DNA fragmentation in late-stage apoptotic cells. TUNEL staining revealed a significantly higher percentage of positive cells in the tumors from whole flaxseed fed hens compared to normal ovaries from whole flaxseed-fed hens or in tumors from control diet-fed hens (Fig. 6A and B). Whole flaxseed diet increases cancer cell apoptosis in hen ovarian tumors.

3.7. 2-methoxyestradiol induces activation of MAP kinases in BG1FR cells

P38 MAP kinase is known to induce apoptosis under oxidative stress. We observe that 5 uM, 10 uM and 20 uM of 2-methoxyestradiol treatment can increase phosphorylation (activation) of p38 after 24 h but not at 6 h of treatment. Expression of total p38 protein remained consistent across all treatment groups (Fig. 7A). 2-methoxyestradiol treatment also induced the activation ERK1/2 at all concentrations at 6 h as well as 24-h time points (Fig. 7B).

3.8. 2-methoxyestradiol induces apoptosis in BG1FR cells by up-regulating cleaved caspase 3 expression

One of the early events of apoptosis is cleavage of caspase-3 protein in the caspase cascade. Treating cells with 2-methoxyestradiol for 24 h increased apoptosis along with detectable expression of cleaved caspase-3 in the cell lysates. All 2-methoxyestradiol treatment doses induced cell death (Fig. 8A and B). The expression pattern of cleaved caspase 3 parallels the expression pattern of active p38 kinase.

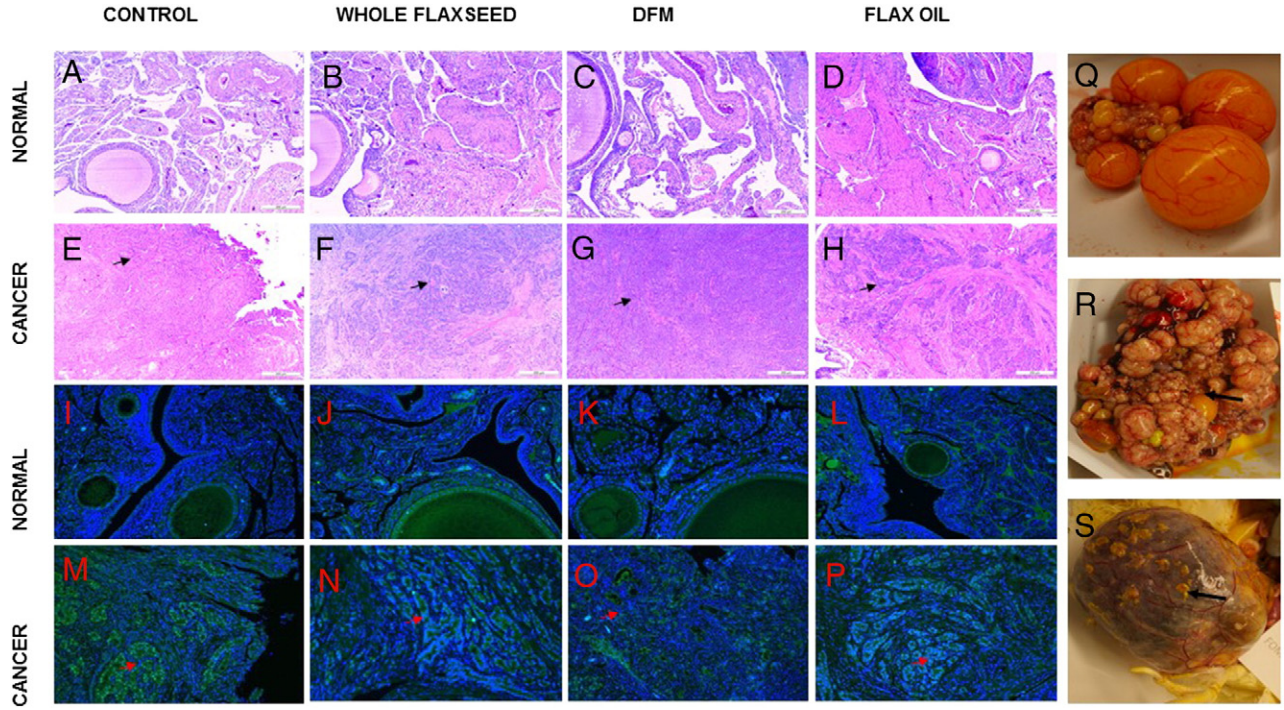

Fig. 1. H and E staining depicting normal and cancerous ovarian tissue histology and immunofluorescence demonstrating ER alpha protein expression from the different diet groups, A, B, C and D indicate normal hen ovarian histology with presence of follicles. E, F, G and H show ovarian tumors appearing as glandular structures throughout the ovarian sections from control, whole flaxseed, defatted flax meal and flax oil diet groups (indicated by black arrow). (I, J, K and L) Faint green fluorescence indicated low expression of ER alpha in the OSE and the granulosa cells of the normal ovary tissue from control, whole flaxseed, DFM and flax oil diets, while M, N, O and P indicate that ER expression was highly up-regulated in the ovarian tumors for all the diet groups (indicated by red arrow). Total magnification ×200, Q and R, closeup pictures of a healthy hen ovary and an ovary with a solid tumor (indicated by black arrow), respectively. (S) Picture of a hen abdomen in an advanced stage of ovarian cancer suggested by presence of ascites and peritoneal metastasis (indicated by black arrow).

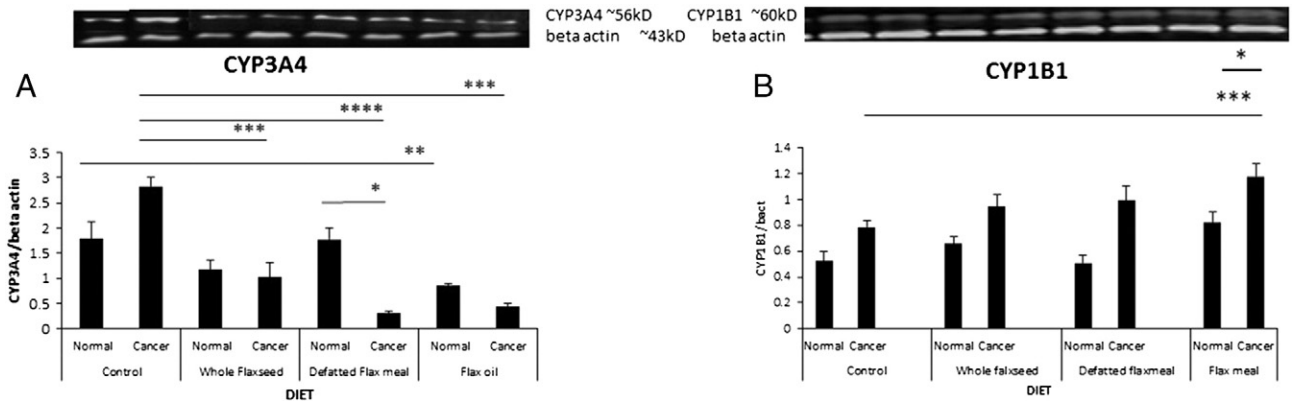

Fig. 2. Expression of cytochrome p450 enzymes in the ovary by Western blotting. Western blots representing the expression of CYP3A4 plus actin, or CYP1B1 plus actin are shown. The gel images have been cropped to show the region of the blots with either CYP3A4 or CYP1B1 to improve the clarity and conciseness of the figure. (A) whole flaxseed, defatted flax meal and flax oil-supplemented diets decreased CYP3A4 protein expression in tumor tissues. Control diet-fed hen tumors had significantly higher expression of CYP3A4 compared to control normal tissues. DFM-fed hen tumors had significantly lower expression of CYP3A4 compared to control normal tissues while normal tissue from flax oil-supplemented diet group had significantly lower CYP3A4 expression compared to control normal group. (B) tumors from flax oil fed diet group showed higher expression of CYP3A4 compared to flax oil fed normal hen ovaries and control diet-fed normal hen ovaries. (* indicates $P<.05$, # indicates $P<.05$, ** indicates $P<.01$, *** indicates $P<.001$, ### indicates $P<.0101$, **** indicates $P<.0001$), $n=6$.

4. Discussion

Previously, we have shown that supplementing the diets of cancer-prone old laying hens with flaxseed significantly reduces the incidence and severity of ovarian cancer. The purpose of this study was to determine the mechanism through which flaxseed reduces cancer severity (causes apoptosis) and to determine the contribution of the individual components to this outcome. The two most biologically active components of flaxseed are the omega-3 fatty acids from the germ and the phytoestrogen lignan from the hull. Herein, we show that whole flaxseed causes apoptosis in ovarian tumors but not normal ovarian tissue, and control diets do not cause significant increase in apoptosis. We demonstrated that whole flaxseed and defatted flaxmeal (the lignan component) cause an induction of CYP1A1, resulting in an increase in 2-methoxyestradiol, which is a potent apoptosis-inducing agent. Increased 2-methoxyestradiol is correlated with increased phospho-p38 and SMAD7, resulting in increased cleavage of caspase 3 and apoptosis. *In vitro*, we demonstrated that 2-methoxyestradiol induced the phosphorylation of p38, up-regulated SMAD7 and increased cleavage of caspase 3. Together, these results indicated that flaxseed diets cause the production of an endogenous, tumor-specific apoptosis causing molecule (2-methoxyestradiol), likely involved in the mechanism through which flaxseed reduces the tumor burden in old laying hens.

When the current feeding study culminated, the hens were almost 4 years old. Overall, 30–40% of the hens developed ovarian cancer with no significant difference among the different diet groups (data not shown). Most of the ovarian tumors observed in the hens were late stage and appeared to be of the endometrioid subtype. The tumor morphology was not altered significantly by diet. Immunofluorescence staining for ERα suggested that the receptor was moderately expressed in the granulosa cells and the OSE in the normal ovary but very highly expressed in the tumor glands. Clinically, it has been observed that the majority of ovarian cancer patients have moderate to high expression of estrogen receptor, and these tumors are responsive to estrogen [28].

Estrogen metabolites can lead to mutations in DNA and drive cell proliferation. E2 can induce CYP1B1 which results in the formation of 4-hydroxyestradiol that can form quinones on oxidation and covalently bind to DNA leading to mutations [29,30] [11,13]. The action of CYP3A4 generates 16 hydroxyestradiol (estriol), a metabolite that can potentiate cell proliferation by increasing ER activation. The levels of

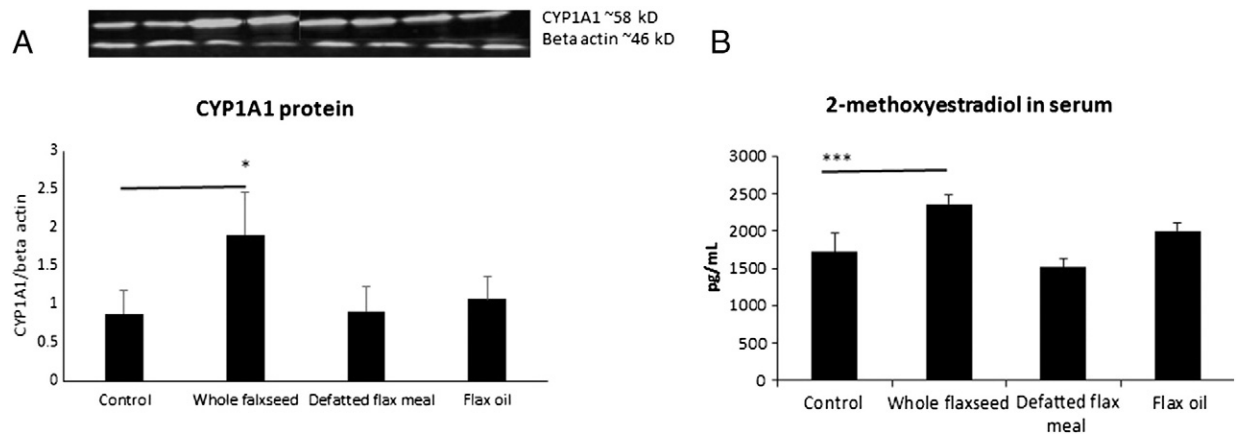

Fig. 3. Expression of CYP1A1 in the liver and levels of 2-methoxyestradiol in serum. Western blots representing the expression of CYP1A1 plus actin are shown. The gel image has been cropped to show the region of the blot with CYP1A1 plus actin to improve the clarity and conciseness of the figure. (A) whole flaxseed-supplemented diet led to a significant increase in CYP1A1 protein expression in the liver. $n=6$, Control *versus* Whole flaxseed, $P<.05$, B, 2-methoxyestradiol levels were significantly increased in the serum from whole flaxseed-fed hens as analyzed by enzyme immunoassay. $n=10$, $P<.001$.

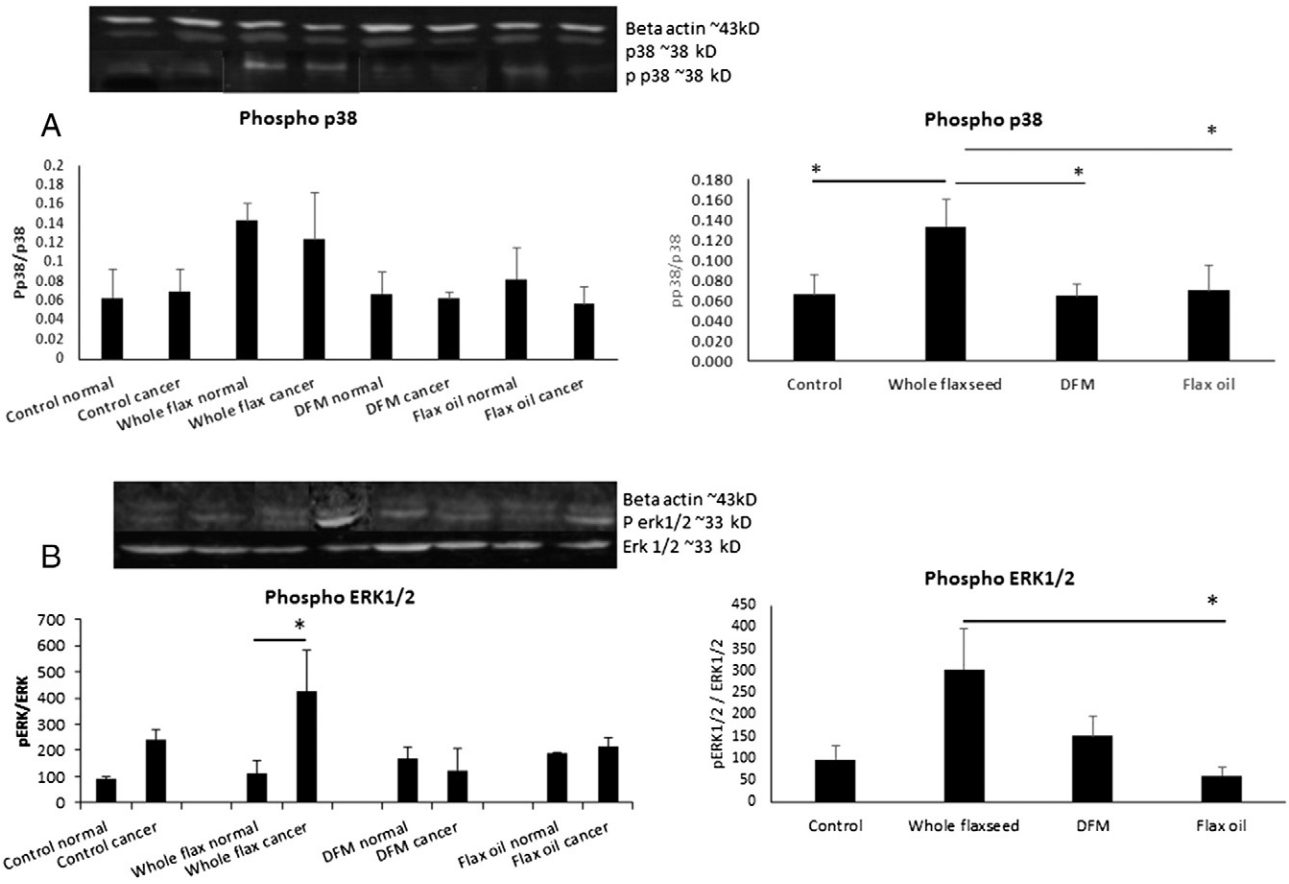

Fig. 4. Activation of MAP kinases in normal ovary and ovarian tumors by Western blotting. Western blots representing the expression of total and activated p38 MAP kinase along with total and activated ERK1/2 MAP kinase plus actin are shown. The gel images have been cropped to show the region of the blot with p38 or phosphor-p38 plus actin or the region of the blot with total ERK 1/2 or phosphor-ERK 1/2 plus actin to improve the clarity and conciseness of the figure. (A) Whole flaxseed diet significantly increased the activation of p38 MAP kinase in the normal as well as the cancerous ovaries. (B) ERK1/2 activation was increased significantly in the whole flaxseed-fed hen ovarian tumors compared to normal ovaries. Whole flaxseed normal *versus* Whole flaxseed cancer, $P<.05$, whole flaxseed *versus* flax oil, $P<.05$, $n=6$.

CYP3A4 protein were significantly decreased in ovarian tumors from whole flax, DFM and flax oil diet groups. This is in keeping with our previous observations that showed a dose-dependent decrease in CYP3A4 expression with flax seed diet [12]. CYP3A4 and CYP1B1 are overexpressed in some cancers [12] [31]. CYP3A4 can also metabolize docetaxel, a commonly used chemotherapy drug for treating ovarian and breast cancer, into a less active and less effective metabolite [32]. Therefore, decreasing CYP3A4 expression with flaxseed prior to

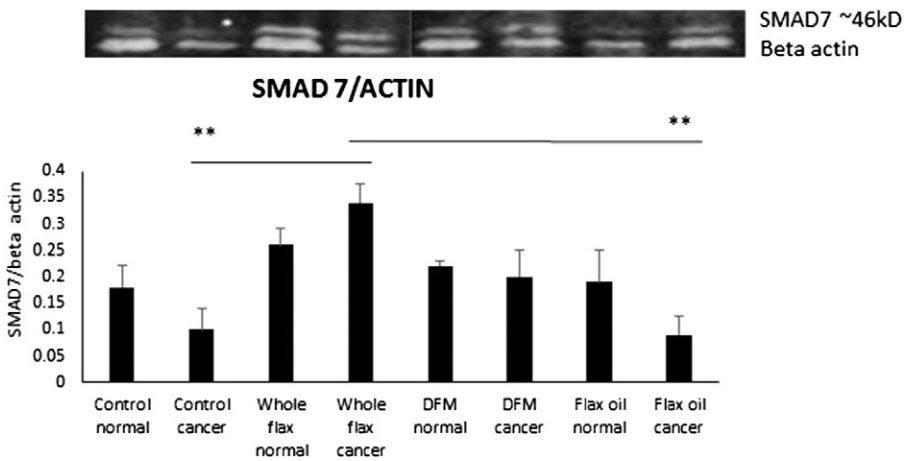

Fig. 5. Expression of SMAD7 in the ovary by Western blotting. Western blots representing the expression of SMAD7 are shown. The gel image has been cropped to show the region of the blot with SMAD7 plus actin to improve the clarity and conciseness of the figure. SMAD7 protein expression was significantly higher in the cancerous ovaries of hens supplemented with whole flaxseed diet compared to control diet and flax oil-supplemented diet. The expression of SMAD7 appeared to be higher in the normal ovaries of the whole flaxseed-fed hens but not statistically significant. $n=6$, control cancer *versus* whole flax cancer, $P<.01$, whole flax cancer *versus* flax oil cancer, $P<.01$.

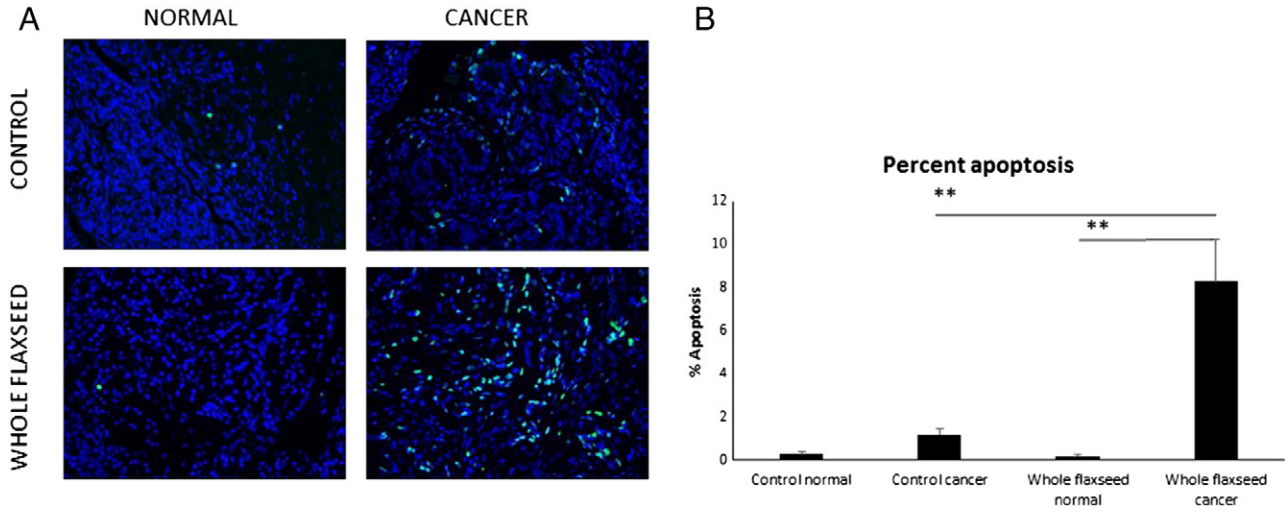

Fig. 6. TUNEL staining on normal and cancer ovaries from control and whole flaxseed-supplemented diet groups. Terminal deoxynucleotidyl transferase (TUNEL) staining was performed on sections of normal and cancerous ovaries from whole flaxseed-fed and control diet-fed hens. (A) TUNEL staining indicated that hens with ovarian cancer that were on whole flaxseed-supplemented diet had a significantly higher number of late-stage apoptotic cells compared to the normal hens on whole flaxseed diet as well as hens with ovarian cancer on control diet. (magnification ×400). (B) Graphical representation of percentage of apoptotic cells on an average from control and whole flaxseed diet groups in normal and cancerous hens, $n=3$. Control cancer *versus* whole flaxseed cancer $P<.01$, whole flaxseed normal *versus* whole flaxseed cancer, $P<.01$.

chemotherapy might enhance the therapeutic effect. In the liver, we observed that CYP1A1 expression was up-regulated by the whole flaxseed diet with a corresponding increase in the plasma levels of 2-methoxyestradiol (Fig. 3A and B). Naturally occurring polycyclic compounds like isoflavones, found in soybeans, can induce activation of AHR (Aryl hydrocarbon receptor) [33]. Our previous study also indicated that the flaxseed diet induced AHR expression [26]. CYP1A1 is one of the primary targets of AHR and its partner, ARNT (Aryl hydrocarbon receptor nuclear translocator). Flaxseed lignan metabolites ED and EL are polyphenols and might be responsible for flaxseed-induced activation of CYP1A1. Two-hydroxyestradiol is further methylated by catechol-o methyltransferase (COMT) to form 2-methoxyestradiol [34].

Fukui *et al.* demonstrated that 2-methoxyestradiol treatment can increase activation of JNK, ERK and p38, followed by induction of apoptosis in breast cancer cells [35]. *In vitro* studies with prostate cancer cells suggested that 2-methoxyestradiol stabilizes SMAD7 and promotes p38-mediated apoptosis [21]. Current study showed that phospho-ERK1/2 was significantly increased in the cancer samples from the whole flaxseed diet group while p38 activation was increased with whole flax diet irrespective of the pathology. The up-regulation of ERK1/2 with the whole flax diet was a surprising observation and

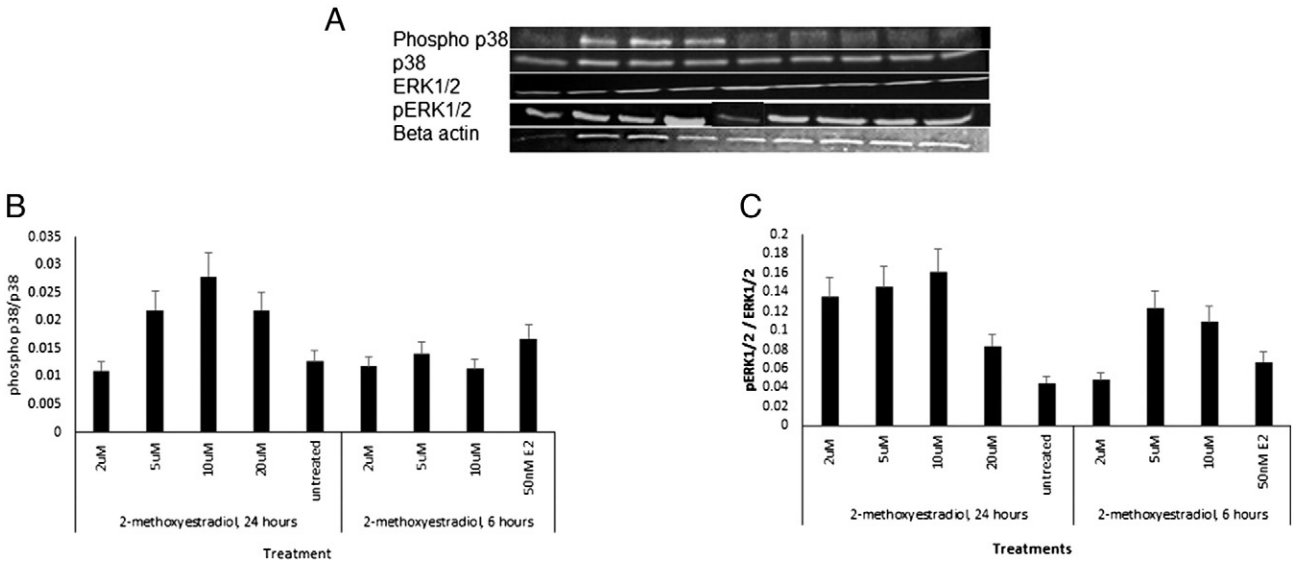

Fig. 7. Effect of 2-methoxyestradiol on activation of MAP kinases on BG1FR cells. (A) Western blots representing the expression of total and activated p38 MAP kinase along with total and activated ERK1/2 MAP kinase, normalized to actin. The gel image has been cropped to show the region of the blot with p38 or phosphor-p38 plus actin or the region of the blot with total ERK 1/2 or phosphor-ERK 1/2 plus actin to improve the clarity and conciseness of the figure. (B) Treatment with 2-methoxyestradiol drastically up-regulated the activation of p38 MAP kinase at 5°uM, 10°uM and 20°uM concentrations upon 24 h of treatment but not at 6 h of treatment. (C) treatment with 2-methoxyestradiol up-regulated the activation of ERK1/2 MAP kinase at all the concentrations after treatment for 6 h and 24 h.

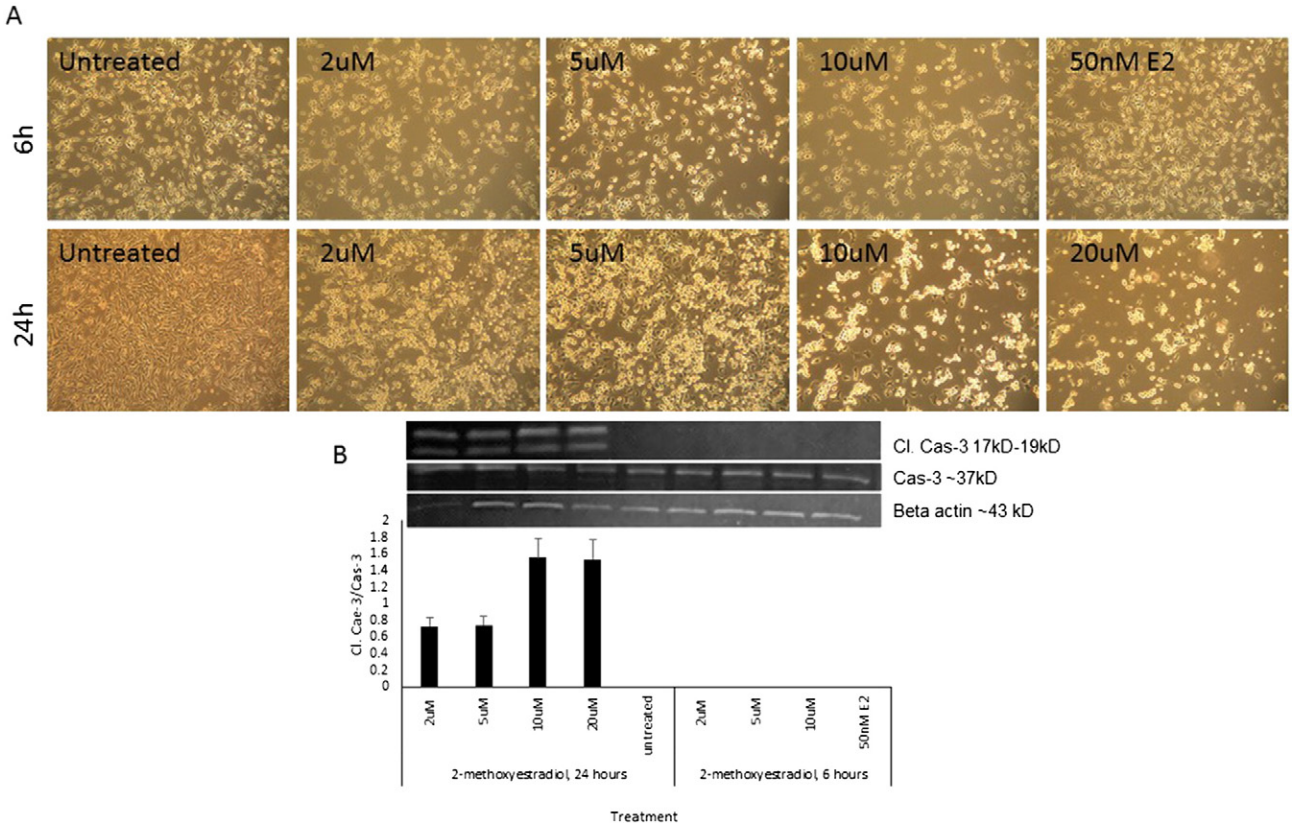

Fig. 8. Treatment of BG1FR ovarian cancer cells with 2-methoxyestradiol induces apoptosis. (A) BG1FR cells were treated with different concentrations of 2-methoxyestradiol, and cells were harvested at 6 and 24 h, respectively. Higher concentrations of 2-methoxyestradiol appeared to induce apoptosis at a much higher rate, similarly cells treated with 2-methoxyestradiol for 24 h seemed to have far fewer cells that were adherent. (B) cleaved caspase-3 expression was markedly increased in the cells that were treated with 2u°M, 5u°M, 10°uM and 20°uM 2-methoxyestradiol for 24 h, but the expression was undetectable after 6 h of treatment.

needs further investigation since it traditionally promotes cell survival. Recently, there has been evidence suggesting the involvement of ERK in apoptosis induced by ROS and FADD-dependent caspase-8 activation [36].

In prostate cancer cells, SMAD7 protein increases the interaction of MKK3 and p38, thus promoting p38 activation [37]. SMAD7 was significantly increased in the ovarian tumors from hens that were fed the whole flaxseed diet. This suggests that 2-methoxyestradiol might be specifically regulating SMAD7 expression and action in the tumors of the whole flaxseed-diet fed hen and not the normal ovary. SMAD7 has also been shown to increase beta-catenin stabilization at the membrane, thus preventing its nuclear translocation, and thereby promoting apoptosis [37,38]. We observed a dramatic increase in TUNEL-positive cells in the tumors from hens on the whole flaxseed diet compared to tumors from hens on the control diet as well as normal ovary from hens on the whole flaxseed diet. This suggests that one of the two MAP kinases (p38 or ERK1/2) might be promoting 2-methoxyestadiol-induced apoptosis in the tumors from whole flaxseed diet-fed hens. To further validate this, we used an *in vitro* system to demonstrate the effect of 2-methoxyestradiol treatment on human ovarian adenocarcinoma cell line, BG1FR. Activation of p38 peaked after 24 h of 2-methoxyestradiol treatment while ERK 1/2 was activated at both time points. p38 activation coincided with the expression of cleaved caspase 3 and the appearance of dying cells in the culture, suggesting that 2-methoxyestradiol signals through p38 and not ERK1/2.

This study was crucial in dissecting the independent effects of flaxseed components and studying their effects in normal and tumor tissues. Hen tumors, like human tumors, show increased expression of ER. Only the diet supplemented with whole flaxseed led to an increase in levels of 2-methoxyestradiol and increased apoptosis specifically in tumor cells. Our data suggests that flaxseed has several possible mechanisms to exert its negative effects on cancer cells. In addition to the actions of the omega-3 fatty acid component of flaxseed, which we have shown that reduces the production of inflammatory prostaglandins [24,39], we now show that flaxseed may promote tumor cell death through 2-methoxyestradiol, increase therapeutic success by decreasing CYP3A4 activity and decrease the aggressiveness and invasiveness of these tumors through decreased levels of 16-hydroxyestradiol.

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