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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.

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