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RESEARCH ARTICLE

Flaxseed oil reduces the growth of human breast tumors (MCF-7) at high levels of circulating estrogen

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Flaxseed (FS) has been shown to attenuate mammary tumorigenesis, possibly due to its high α-linolenic acid (ALA)-rich oil (FSO) content. This study determined the effect of FSO on the growth of estrogen receptor-positive human breast tumors (MCF-7) in ovariectomized athymic mice at high premenopausal-like estrogen (E2) levels. Mice with established MCF-7 tumors were fed basal diet (control) or basal diet supplemented with FSO (40 g/kg) for 8 wks. Compared with control, FSO reduced tumor size (33%, $p<0.05$) and tumor cell proliferation (38%, $p<0.05$) and increased apoptosis (110%, $p<0.001$). FSO also reduced human epidermal growth factor receptor-2 (79%, $p<0.05$) and epidermal growth factor receptor (57%, $p=0.057$) expression, which then may have led to a reduction in Akt (54%, $p<0.05$) and phosphorylation of mitogen-activated protein kinase (MAPK) to phosphorylated MAPK (pMAPK, 28%, $p<0.05$). Insulin-like growth factor-1 receptor, vascular endothelial growth factor receptor, MAPK and phosphorylated Akt were not affected. FSO increased ($p<0.001$) serum ALA, eicosapentaenoic acid and docosahexaenoic acid and, *in vitro*, ALA reduced MCF-7 cell proliferation (33%, $p<0.001$). Thus, FSO regressed estrogen receptor-positive human breast tumorigenesis at high E2 levels *via* downregulation of the growth factor mediated pathway, likely through its ALA content, and may explain the anti-tumorigenicity of FS.

Keywords:
α-linolenic acid / Athymic nude mice / Breast cancer / Flaxseed oil / MCF-7 xenografts

1 Introduction

Flaxseed (FS) has approximately 40% oil, of which about 60% is the n-3 PUFA α-linolenic acid (ALA) [1]. FS is also rich in the lignan secoisolariciresinol diglucoside (SDG) with metabolites which possess estrogenic or anti-estrogenic properties due to their structural similarity to estrogen (17β-estradiol, E2) [2–4].

Numerous studies have consistently shown that, at low circulating E2 simulating postmenopause, FS and its oil and lignan components can reduce the growth of established estrogen receptor-positive (ER+) human breast tumors (MCF-7), alone [5–8] or combined with the breast cancer drug tamoxifen (TAM) [7, 9, 10]. This has been shown to occur *via* modulation of the estrogen receptor (ER)- and growth factor-mediated signaling pathways [5, 9–13]. Without TAM treatment, the effect of SDG was stronger than FS oil (FSO) at reducing tumor growth, but with TAM treatment, FSO had a stronger effect than SDG. A clinical study supported the animal studies in showing the effectiveness of FS in reducing tumor cell proliferation and human epidermal growth factor receptor-2 (HER2) expression and increasing apoptosis in tumors of postmenopausal breast cancer patients [13].

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Abbreviations: ALA, α-linolenic acid; **BD,** basal diet; **DHA,** docosahexaenoic acid; **E2,** 17β-estradiol; **EGFR,** epidermal growth factor receptor; **EPA,** eicosapentaenoic acid; **ER,** estrogen receptor; **ER+,** estrogen receptor-positive; **FAS,** fatty acid synthase; **FS,** flaxseed; **FSO,** flaxseed oil; **HER2,** human epidermal growth factor receptor-2; **IGF-1R,** insulin-like growth factor-1 receptor; **MAPK,** mitogen-activated protein kinase; **NEG,** negative control; **OVX,** ovariectomized; **pAkt,** phosphorylated Akt; **pMAPK,** phosphorylated mitogen-activated protein kinase; **POS,** positive control; **SDG,** secoisolariciresinol diglucoside; **TAM,** tamoxifen; **VEGF,** vascular endothelial growth factor; **VEGFR,** vascular endothelial growth factor receptor

Relatively less work has been conducted on the effect of FS at high circulating E2 levels simulating premenopause. At high E2 levels, FS also reduced MCF-7 tumor growth through decreased cell proliferation, increased apoptosis [7] and reduced angiogenesis through lowering of vascular endothelial growth factor (VEGF) expression [14]. However, it is unclear whether this effect is in part due to the FSO and the mechanisms of its action. The objective of this study was to investigate whether FSO is a component contributing to the anti-cancer action of FS at high circulating levels of E2 by determining the effect of FSO on established MCF-7 tumor growth and its potential mechanisms of action, with greater focus on the growth factor-mediated signaling pathway. The results may provide guidance to premenopausal breast cancer patients regarding the potential use of ALA-rich foods such as FS in the management of their disease and whether FSO may be used as an alternative when intake of large amounts of FS cannot be tolerated. This is important considering many breast cancer patients consume a variety of dietary supplements, with FS as the third-most common, only behind green tea and Vitamin E [15].

2 Materials and methods

2.1 Cell line and cell culture

The ER+ human breast cancer cell line, MCF-7 (The American Type Culture Collection, Manassas, VA), was maintained in DMEM/F-12 medium supplemented with 10% fetal bovine serum and 1% antibiotics. Cells were grown to 70–90% confluence in T-150 flasks and cultivated in fresh medium a day before harvest. The experimental medium used was identical to the above medium; however, it was phenol red-free (Gibco, Carlsbad, CA) and fetal bovine serum was replaced by 10% charcoal-stripped calf bovine serum (Gibco).

MCF-7 cells were freshly prepared for injection by trypsinization and resuspension in serum-free medium with 1:1 Matrigel (4×10^7 cells/mL) on ice. Cell viability (>95%) was confirmed by Trypan blue exclusion assay after each day of injection.

For the *in vitro* study, MCF-7 cells were cultured as previously mentioned; however, the experimental medium used for cell proliferation analysis consisted of phenol red-free DMEM/F-12 1× (1:1, Gibco) supplemented with 5% charcoal-stripped calf bovine serum (Gibco) and 1% antibiotics, including penicillin, streptomycin and amphotericin (Sigma, St. Louis, MO).

2.2 Animals and diet

Ovariectomized (OVX) athymic mice (BALB/c nu/nu, 4–5 wk old) (Charles River Canada, St-Constant, PQ, Canada) were housed and maintained in the manner described in Chen *et al.* [7]. Animal care and study were approved by the University of Toronto Animal Ethics Committee (permission ♯ 2000-6856) and were performed according to the *Guide to the Care and Use of Experimental Animals* (Canadian Council on Animal Care, 1993). The basal diet (BD) was based on the AIN-93G formulation [16], modified with 20% fat from corn oil rather than soybean oil. It contains casein (200 g/kg), L-cystine (3 g/kg), sucrose (100 g/kg), cornstarch (301.06 g/kg), dextrose (99.5 g/kg), corn oil (200 g/kg), t-butylhydroquinone (0.04 g/kg), cellulose (50 g/kg), AIN-93G mineral mix (35 g/kg), AIN-93G vitamin mix (10 g/kg) and choline chloride (1.4 mg/kg). The FSO diet consisted of BD with 40 g/kg of pure FSO (Pizzey's Milling, Angusville, MB, Canada) and 160 g/kg corn oil so that all diets were isocaloric. The FSO level was equivalent to the amount present in 10% dietary FS, *i.e.* 40 g/kg diet, which was found to be effective in previous studies [6–8, 10–13, 17, 18]. Diets were prepared by Dyets (Bethlehem, PA) and sterilized by Co^{60} radiation by Isomedix Corporation (Whitby, ON, Canada). All diets were double-bagged in sealed bags and stored at 4°C in the dark until use to ensure diet stability.

2.3 Experimental design

The study design was similar to our previous study [7]. After 7-day acclimatization to BD, mice were placed under ketamine–xylazine anesthesia and subcutaneously implanted with a sterilized E2 pellet (0.36 mg, 60-day release; Innovative Research of America, Sarasota, FL) in the interscapular region, followed by sealing of the incision with tissue adhesive Vetbond (3 M Animal Care Products, St. Paul, MN). A 100-μL cell suspension (4×10^6 MCF-7 cells) was injected into the right and left thoracic and abdominal mammary fat pads, producing four mammary tumors *per* mouse. The palpable tumor area, measured weekly, was calculated with the formula (length/$2 \times$ width/2) $\times \pi$.

When the tumor area reached 20 mm^2 (at week 4), the negative control (NEG) mice had their E2 implants removed, while positive control (POS) and FSO mice had their E2 pellets replaced to simulate a premenopausal situation. Mice were assigned to three groups ($n = 14$/group) such that the mean tumor size and body weight were similar: (i) POS fed BD, (ii) NEG fed BD and (iii) FSO group fed the FSO diet. Food intake, body weight and tumor size were monitored weekly until sacrifice at week 12 (week 8 after treatment) by CO_2 asphyxiation and cervical dislocation. A part of each tumor was fixed in 10% buffered formalin solution for immunohistochemical analysis and another part was fast frozen in liquid nitrogen and stored at −80°C for Western blot analysis. During the study, several mice died unrelated to the diet treatments, leaving 10–11 mice/group. Mice that died were not included in the data analyses.

2.4 Immunohistochemistry: Cell proliferation and apoptosis

Immunohistochemistry assay to measure tumor cell proliferation as Ki-67 Labeling Index was as described previously [7]. The rabbit anti-human Ki-67 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted (1:200) in diluent buffer. All slides were coded and read without knowledge of treatment groups under a light microscope at 400 × magnification. Over 1000 cells from different fields were counted for Ki-67 Labeling Index that was calculated as a percentage of positively stained cells over total cells counted.

In situ TUNEL assay was used to show DNA fragmentation by ApopTag detection kit (Chemicon, Temecula, CA), according to the manufacturer's protocol, also as described previously [7]. The number of apoptotic cancer cells was counted without knowledge of treatment groups and expressed as apoptotic cell number *per* mm^2 at 400 × magnification.

2.5 Western blot

Western blotting was performed on the tumors as described in Ealey *et al.* [19]. Briefly, tumor proteins solubilized in Laemmli buffer containing β-mercaptoethanol were heated for 5 min at 95°C, separated by electrophoresis on 10–12% polyacrylamide gels under denaturing conditions, and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C in blocking buffer (5% non-fat dry milk in Tris-buffered saline/0.1% Tween-20). The rabbit anti-human antibodies used included Akt, phosphorylated Akt (pAkt), p44/42 mitogen-activated protein kinase (MAPK), phosphorylated MAPK (pMAPK), HER2, epidermal growth factor receptor (EGFR), VEGF receptor (VEGFR) (all 1:750), as well as insulin-like growth factor-1 receptor (IGF-1R) and actin (both 1:1000) (Cell Signaling Technology, Beverley, MA). After extensive washing in Tris-buffered saline/0.1% Tween-20, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (BioShop Canada, Burlington, ON, Canada) and developed with a standard enhanced chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescence was detected on X-ray film (Clonex Corporation, Markham, ON, Canada), and the intensity of bands was quantified using FluorChem Imaging System (Alpha Innotech, San Leandro, CA). Consistent protein levels were ensured through protein determination immediately prior to sample preparation, as well as Coomassie Blue staining of membranes. The "relative intensity unit" of each tumor biomarker was calculated by dividing the tumor biomarker band intensity by the average intensity of all actin bands.

2.6 Serum lipid analysis

Total lipids were extracted from 25 μL of serum according to the method of Folch *et al.* [20]. Extracts were collected into a test tube containing a known amount of heptadecanoic acid (17:0) and analyzed by gas chromatography as described in Chen *et al.* [21]. Peaks were identified by retention times of fatty acid methyl ester standards (Nu-Chek Prep, Elysian, MN). Fatty acid concentrations were estimated by proportional comparison of gas chromatography peak areas with that of the heptadecanoic acid internal standard and expressed as percent of total fatty acids.

2.7 *In vitro* study

MCF-7 cells were counted and plated at 3×10^3 cells *per* well on a 96-well plate and incubated for 3 days at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Working treatment solutions were prepared by dissolving pure ALA initially with 100% ethanol solution then serially diluted with experimental medium on day 1 of the experiment. Treatments included POS and ALA (50 μM), both with 1 nM of E2. ALA at 50 μM was used, as this was similar to the serum ALA concentration found *in vivo* in the present study (Section 3).

After removing existing media, treatment solutions were added to wells on day 1 (4 wells *per* treatment *per* 96-well plate), and changed with fresh treatment solution on day 3. On day 5, cell proliferation was measured using a BrdU assay kit, as *per* manufacturer's instructions (Calbiochem, San Diego, CA). The experiment was repeated three times in quadruplicate.

2.8 Statistical analysis

Data are presented as mean ± SEM. Analysis of variance with general linear model-repeated measures procedure was used to determine palpable tumor growth difference among treatment groups over treatment time, followed by *post-hoc* Tukey test using SPSS (Statistical Package for Social Sciences, version 17.0, Chicago, IL). Palpable tumor area was analyzed with mouse as the unit, *i.e.* mean area of all tumors in each mouse. Differences between groups in all other data were analyzed by unpaired *t*-test when variance was equal or by Mann–Whitney rank sum test when variance was unequal (Sigma Stat, version 3.5, Chicago, IL). Significance was set at $p<0.05$ for all statistical analyses. The NEG group was compared with the POS and FSO groups only for palpable tumor growth, as it was added in the study only to determine whether the model system was working, *i.e.* tumors grow with an E2 implant and regress without an E2 implant.

3 Results

3.1 Food intake and body weight

Food intake was significantly higher ($p<0.05$) in the FSO group (2.66 g/day) than the POS (2.42 g/day); however, body

weight did not differ significantly between groups at start or end of treatment.

3.2 Palpable tumor area

Over the study period, palpable tumors in the POS group (with E2) continued to grow to a significantly larger size than those in NEG group (no E2) (Fig. 1). The tumors in POS group grew 162% ($p<0.001$), while those in NEG group regressed 58% ($p<0.001$) from pre-treatment size (Fig. 1). This indicates that the MCF-7 tumors were E2-sensitive and that our experimental model was working. FSO increased palpable tumor area by 78.5% ($p<0.01$) compared with pre-treatment, resulting in a 33% ($p<0.05$) smaller palpable tumor area than POS at week 12 (post-treatment week 8).

3.3 Tumor biomarkers

Compared with POS, FSO reduced tumor cell proliferation (Ki-67 Labeling Index) by 38% ($p<0.05$) (Fig. 2A) and increased tumor cell apoptosis by 110% ($p<0.01$) (Fig. 2B). FSO diet did not affect IGF-1R and VEGFR expressions, but significantly reduced HER2 expression by 79% ($p<0.05$) and tended to reduce EGFR by 57% ($p = 0.057$) (Fig. 3). FSO

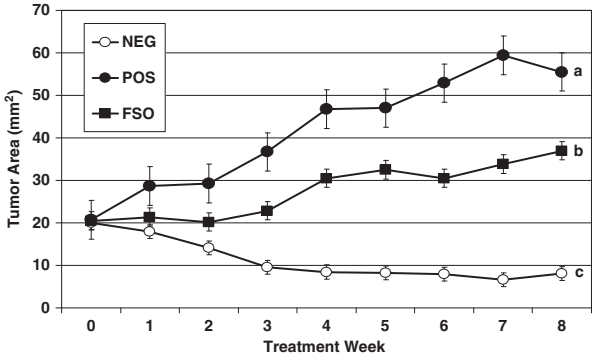

Figure 1. Effect of FSO on MCF-7 tumor growth in OVX athymic mice. Lines with different letters are significantly different ($p<0.05$). $n = 10$–11/group.

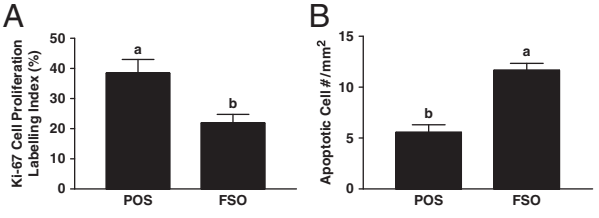

Figure 2. Effect of FSO on MCF-7 tumor cell proliferation (A) and apoptosis (B) in OVX athymic mice. Bars with different letters are significantly different for cell proliferation ($n = 7$–10/group; $p<0.05$) and apoptosis ($n = 10$/group; $p<0.01$).

reduced Akt expression (54%, $p<0.05$) and levels of pMAPK (28%, $p<0.05$), but not pAkt or MAPK expression.

3.4 Serum fatty acids

The ALA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) serum concentrations in the POS group were 4.5 μM, not detectable and 50.4 μM, respectively, and 61.0, 22.2 and 193.2 μM in the FSO group, respectively. Figure 4 provides the fatty acids as percent of total fatty acids. FSO diet significantly increased serum ALA ($p<0.001$), EPA and DHA ($p<0.001$) levels and decreased the serum n-6:n-3 PUFA ratio from 12:1 to 5:1, ($p<0.001$) compared with POS (Fig. 4).

3.5 *In vitro* cell proliferation

Compared with POS, ALA (50 μM) reduced MCF-7 cell proliferation *in vitro* by 33% ($p<0.001$) (Fig. 5).

4 Discussion

This study has shown for the first time that in OVX athymic mice with high circulating E2 concentration simulating premenopause, the ALA-rich FSO is effective in attenuating the growth of ER+ MCF-7 tumors and this is due to a significant reduction in cell proliferation and increase in apoptosis.

To understand the potential mechanisms of these effects on tumor growth, various signaling proteins of the growth factor-mediated and angiogenesis pathways were analyzed. EGFR, HER2 and IGF-1R are tyrosine kinase receptors that can be activated by binding with their ligands (growth factors epidermal growth factor and IGF-1) or by dimerization in the case of HER2, which has no known ligand. They can indirectly activate growth-inducing signal transduction molecules such as anti-apoptotic Akt and proliferatory MAPK, both of which can be phosphorylated and activated to form pAkt and pMAPK, respectively [22, 23]. Together, Akt and MAPK promote cell survival through downstream activation of transcription factors, and also by regulating anti-apoptotic Bcl-2 expression [24, 25]. E2 can also influence the growth factor-mediated pathway by activating Akt and MAPK *via* membrane-associated receptors [22, 26]. In the present study, FSO reduced HER2 and EGFR but not IGF-1R protein expression, which then led to downregulation of Akt and pMAPK protein expressions downstream. These results indicate that the tumor-reducing effect of FSO may in part be due to reduced growth factor signaling. Although FSO did not affect pAkt, there may be other molecules downstream of the Akt signaling pathway, *e.g.* anti-apoptotic Bcl-2 [27], which was not measured, that may have been downregulated by FSO.

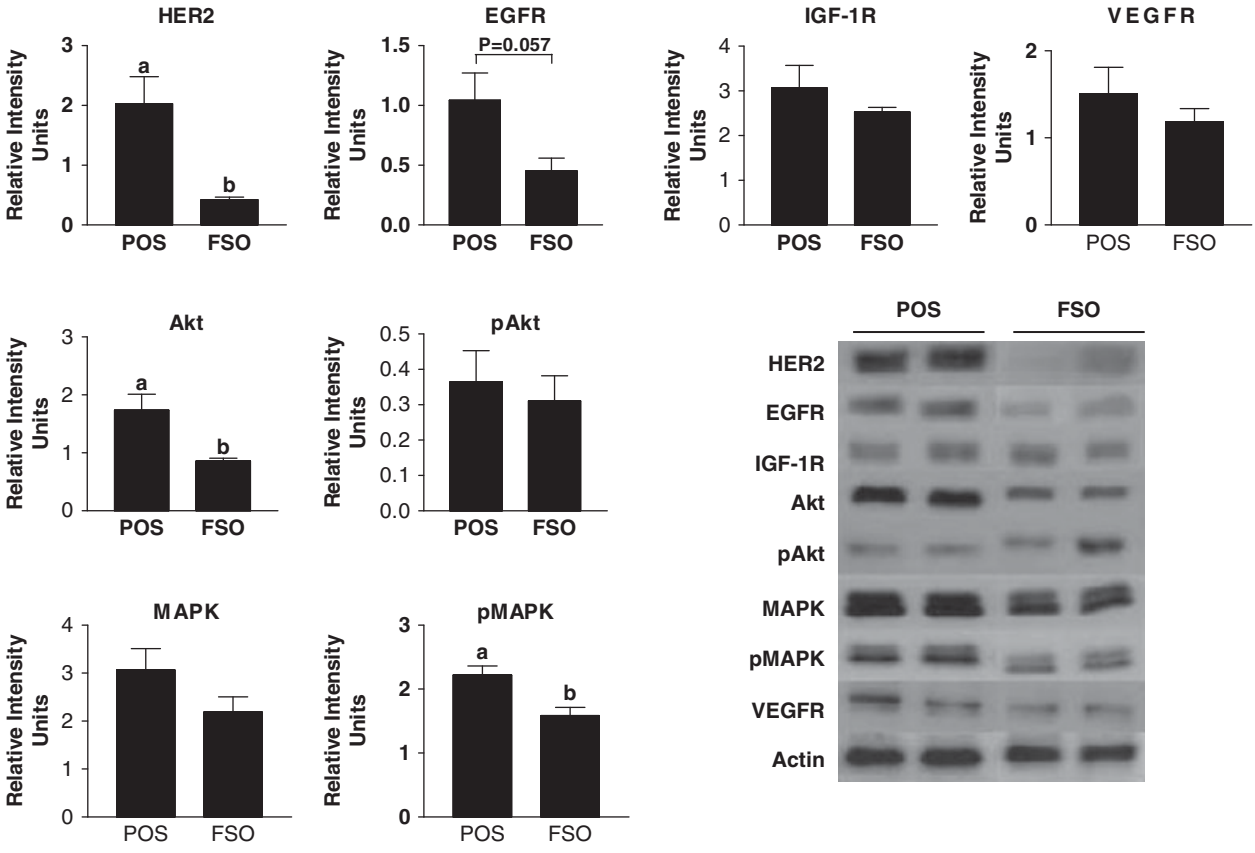

Figure 3. Effect of FSO on the expression of MCF-7 tumor biomarkers in athymic mice. Bars with different letters are significantly different ($p<0.05$). $n=4$/group, except POS of VEGFR ($n=3$) and IGF-1R ($n=5$), in duplicate.

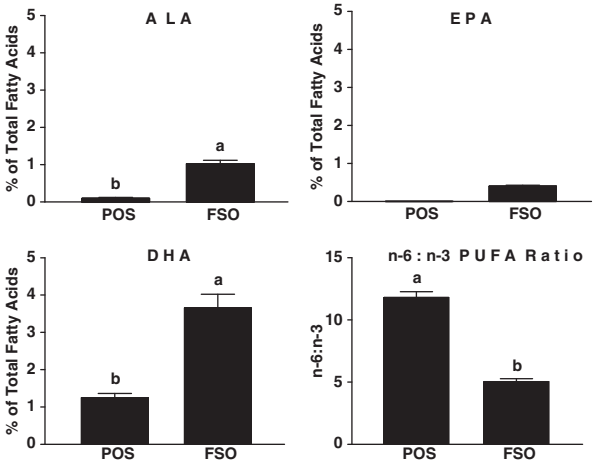

Figure 4. Effect of FSO on the serum fatty acid distribution and *n*-6:*n*-3 PUFA ratio in OVX athymic mice with MCF-7 tumors. Bars with different letters are significantly different ($p<0.001$). $n=10$/group.

Angiogenesis is the development of capillaries which supply nutrient-rich blood to tumors, thus, fueling their growth, progression and possibly metastasis [28]. This involves the action of VEGF, a very potent and specific anti-apoptotic mitogen of newly formed vascular endothelial cells upon binding to the receptor [28]. FSO did not affect VEGFR protein expression, suggesting that the angiogenesis pathway was less involved in its tumor-reducing effect. This is in contrast to the results of Dabrosin *et al.* [18] in ER-negative human tumors (MDA-MB-435) and of Jungestrom *et al.* [14] in ER+ human tumors (MCF-7) at high circulating E2 levels, who both showed that 10% FS can reduce extracellular VEGF. However, tumor VEGFR expression may not be positively correlated with extracellular or tumor VEGF levels. It is possible that the VEGF ligand, which was not measured in this study, was downregulated, but not the receptor (VEGFR), potentially explaining this discrepancy.

The effects of FSO are in agreement with the study in postmenopausal breast cancer patients [13], and in athymic mice with established MCF-7 tumors at low E2 levels [10], which showed a reduction in tumor cell proliferation and HER2 expression, and increased apoptosis following FS treatment; with the study in mice with established MCF-7 tumors at low E2 levels, which showed a reduction in tumor pMAPK protein after FSO treatment with or without TAM treatment [5, 9]; and with the *in vitro* study, which showed that ALA can downregulate HER2 expression in BT-474

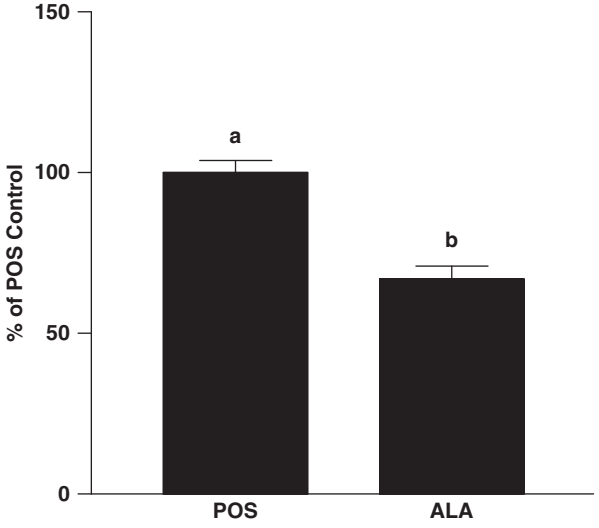

Figure 5. Effect of ALA on MCF-7 cell proliferation *in vitro*. Bars with different letters are significantly different ($p<0.001$). $n=3$ experiments/group, in quadruplicate.

cancer cells overexpressing HER2 [29]. All these studies indicate that FSO is one component of FS that can exert a tumor-reducing effect both at high and low circulating E2 levels and the mechanisms include the downregulation of the growth factor-mediated signaling pathway.

ALA can be metabolized to the longer chain n-3 fatty acids EPA and DHA by the action of desaturase and elongase enzymes, which are also responsible for metabolizing linoleic acid to its longer chain fatty acids [30]. Serum fatty acid levels were analyzed in this study to determine the availability and metabolism of ALA in FSO, and thus, its contribution to the observed effects. ALA-rich FSO diet increased the serum ALA, EPA and DHA concentrations and decreased the n-6:n-3 PUFA ratio compared with POS in OVX athymic mice, indicating that ALA is absorbed and metabolized and likely contributed to the observed FSO effect. Ideally, tumor tissue levels of fatty acids should have been analyzed, as serum and tumor fatty acid levels may differ. However, the limited available tumor tissues made it difficult to do tumor fatty acid analysis as well. Nevertheless, it has previously been shown that tumor ALA, EPA and DHA are increased with the intake of 5% FS by rodents with established carcinogen-induced tumors [31, 32]. Thus, dietary supplementation with FSO at levels found in a 10% FS diet may also be expected to affect fatty acid composition in established MCF-7 tumors in this study.

To further determine whether the results seen *in vivo* were due to the ALA component of FSO, an *in vitro* study examined the effect of ALA on the proliferation of MCF-7 cancer cells. The serum ALA concentration in mice fed FSO was approximately 61 μM (Section 3); hence, a similar concentration of 50 μM was tested in the *in vitro* study. ALA reduced MCF-7 cell proliferation *in vitro* by 33%, while reduction in palpable tumor growth in mice was also 33%. This indicates that ALA itself exerts an effect and need not be converted to EPA and DHA to exert an anti-tumorigenic effect.

Previous studies have shown that EPA and DHA treatment of breast cancer cells *in vitro* caused an increase in their concentrations in membrane lipid rafts, where growth factors tend to co-localize, that then led to a decrease in cancer cell EGFR levels and growth compared with control [33]. Similarly, ALA may have been incorporated in the membrane lipid rafts and reduced the expression of growth factors, particularly HER2 and EGFR, that then led to the downregulation of signaling biomarkers and reduced tumor cell growth.

Other mechanisms by which ALA may affect tumor growth include increased lipid peroxidation and reduced fatty acid synthase (FAS). The highly unsaturated nature of n-3 PUFAs makes them highly susceptible to lipid peroxidation, which generates apoptosis-inducing free radicals [34], which eventually leads to decreased tumor growth. FAS, an enzyme that is overexpressed in aggressive breast cancers, may also potentially be involved, as ALA has been shown to inhibit FAS activity in FAS-overexpressing SK-Br3 breast cancer cells [35].

This study provides further support for the effectiveness of FSO in reducing tumor growth, although there are several limitations. First, although athymic mice are commonly used as a model in human cancer studies [36, 37], they are immunodeficient (lack T-cell function) [38]; therefore, results using this mouse model must be confirmed in human trials. However, results on the development of breast cancer drugs, such as TAM [39] and aromatase inhibitors [40], using this model have translated well to humans. In addition, results of our athymic mouse studies on the anti-tumorigenic effect of FS [7, 8, 10–12, 17, 18] have been demonstrated as well in a clinical trial with breast cancer patients [13]. Secondly, serum fatty acids were analyzed, but not tumor fatty acids because of a limited amount of tumor tissue. Although a previous study has shown that dietary profiles reflect serum and mammary tumor fatty acid profiles [41], and other studies have shown that tumor ALA, EPA and DHA were increased in rats fed FS [31, 32], tumor fatty acid analysis will provide information regarding actual levels that may have caused an effect on the tumors in this study. Finally, only select protein biomarkers related to growth factor signaling were analyzed. Other mechanisms of action are likely involved as well, which should be further explored in the future.

In conclusion, at high levels of E2, FSO and its high ALA content reduced MCF-7 breast tumor growth by reducing cell proliferation and increasing apoptosis through modulation of the growth factor-mediated signaling pathway, and may in part account for the anti-cancer effect of FS. The results also suggest that ALA-rich FSO may potentially be consumed as an alternative to FS by those who are not able to tolerate the large dose of FS. The amount of FSO used in this study is based on 10% FS in animal diet (25–50 g of FS

in human diet [11]), which is equivalent to the consumption of 10–20 g of FSO in human diet. However, although the results support the use of FSO as a potentially safe, cost-effective and accessible complementary breast cancer treatment, clinical trials still need to be conducted before a more definitive recommendation can be given regarding its use.

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The authors have declared no conflict of interest.

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