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Flaxseed sprouts induce apoptosis and inhibit growth in MCF-7 and MDA-MB-231 human breast cancer cells

Jaehee Lee · Kyongshin Cho

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Abstract Flaxseeds have been shown to play a role in the prevention of cancer and heart disease, and it is believed that their more favorable fatty acid composition is responsible. Sprouting is a natural method to modify nutritional components and to decrease cyanide poisoning of raw flaxseeds. Here, we investigated the in vitro effects of flaxseed sprouts on cell growth and apoptosis of human breast cancer cells. In a series of in vitro experiments, estrogen-receptor-positive (MCF-7) and estrogen-receptor-negative (MDA-MB-231) cells were cultured and treated with flaxseed sprouts, and then cell proliferation, apoptosis, and gene expression were measured. Flaxseed sprouts significantly reduced the growth of both of MCF-7 and MDA-MB-231 cells and also increased apoptosis. However, flaxseed sprouts did not affect the growth of MCF-10A mammary epithelial cells. In gene transcription analysis using quantitative real-time polymerase chain reaction, flaxseed sprout treatment significantly upregulated *p53* mRNA in both cell cancer lines. These results suggest that flaxseed sprouts induce apoptosis and inhibit cancer cell growth, thereby demonstrating their anti-proliferative effects in breast cancer cells. This study may provide important information for devising dietary strategies to reduce breast cancer risk.

Keywords Flaxseed sprouts · Breast cancer · Apoptosis · p53

J. Lee
Department of Chemistry and Biochemistry,
North Dakota State University,
Fargo, ND 58108, USA

K. Cho (✉)
Department of Animal Sciences, North Dakota State University,
1300 Albrecht Avenue,
Fargo, ND 58108, USA
e-mail: kyongshin.cho@ndsu.edu

Introduction

Flaxseeds are rich sources of plant lignans and omega-3 fatty acids, the latter of which has tumor-suppressive effects (Tou et al. 1998). Previous studies have shown that consuming flaxseeds may have beneficial effects not only in reducing the risk of cancer and cardiovascular disease but also in improving immune responses (Kelley 2001). Dietary flaxseeds have demonstrated inhibitory effects on the growth of breast cancer cells both in vitro and in vivo (Bergman Jungeström et al. 2007; Chen et al. 2009) and are also associated with a decreased risk of developing breast cancer, but little is known about the implications of flaxseed sprouts in the growth and death of breast cancer cells (Chen et al. 2009).

Although flaxseeds are an excellent source of nutrients with anti-cancer properties, raw flaxseeds have limitations of their use because they can cause indigestion and are not readily absorbed during digestion (Chadha et al. 1995). Sprouting is a natural method to increase bioavailability and to decrease cyanide poisoning of raw flaxseeds, and flaxseed sprouts are easily digestible and contain bioactive nutrients (Wanasundra et al. 1999). Flaxseeds have been extensively studied in several decades, and anti-cancer mechanisms were discovered including the induction of apoptosis and arrest of cell cycle progression (Tou et al. 1998). However, it is not clear that flaxseed sprouts do indeed reduce breast cancer risk through apoptotic mechanisms.

Apoptosis plays critical roles in cancer pathogenesis, and its regulation is commonly impaired in cancer cells (Call et al. 2008). The *p53* tumor suppressor gene is important in suppressing cancer cell growth, and the MDM2 protein binds specifically to the p53 protein to negatively regulate its transcriptional activity through multiple mechanisms in cancer cells (Kussie et al. 1996; Eischen and Lozano 2009).

Increasing apoptosis in tumor cells is one of the main strategies in cancer therapy (Call et al. 2008), but the interruption of p53 activity by MDM2 is one of the main mechanisms by which cancer cells evade apoptotic cell death (Eischen and Lozano 2009). While there is a growing body of literature linking the intake of flaxseeds to reduced breast cancer risk, less is known about flaxseed sprouts and their potential to decrease breast cancer risk. In this study, we investigated the in vitro effects of flaxseed sprouts on cell growth and apoptosis in two different human breast cancer cell lines.

Materials and Methods

Cell lines and cell culture. The estrogen-receptor-positive MCF-7 (HTB-22, adenocarcinoma) and estrogen-receptor-negative MDA-MB-231 (HTB-26, adenocarcinoma) human breast cancer cell lines and non-cancerous MCF-10A (CRL-10317, fibrocystic disease) human mammary epithelial cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA) in June 2011 and March 2005, respectively, and serially passaged and stored in liquid nitrogen until assay. The cells were grown in Dulbecco's modified Eagle's medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 10 % (*v/v*) heat-inactivated fetal bovine serum (Gibco Invitrogen) and 1 % (*v/v*) antibiotic–antimycotic (Gibco Invitrogen) solution as recommended by the supplier. Cells were maintained as monolayer cultures in 25 and 75 cm^2 tissue culture flasks (BD Biosciences, Franklin Lakes, NJ) at 37°C in a 5 % CO_2-humidified atmosphere during experimentation.

Materials. The commercially available and commonly consumed flaxseed sprouts (Natural Alpha Omega, Santa Fe Springs, CA) were used for this study. After lyophilization for 72 h, flaxseed sprouts were powdered by a ZM1 grinder (Retsch, Haan, Germany) equipped with 0.08 mm sieve. Then, flaxseed sprouts were added to absolute ethanol and vortex-mixed. The appropriate or optimal dose of flaxseed sprouts was determined from dose–response studies, and controls always included ethanol at the appropriate concentration (0.5 % *v/v*).

Cell proliferation assay. The colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96 Aqueous One Solution Reagent, Promega, Madison, WI) was used to measure cell proliferation. Briefly, cells were seeded in 96-well flat-bottomed tissue culture plates (5×10^3 cells/well in 100 μl) and incubated with various concentrations of flaxseed sprouts. After 0, 24, 48, 72, or 96 h in cultures, 10 μl of MTS solution was added, and cells were incubated at 37°C for 1–4 h. The degree of cell proliferation was evaluated numerically by measuring the absorbance at 490 nm with a Spectra-Max Microplate Reader (Molecular Devices, Sunnyvale, CA). The cell proliferation was calculated and expressed based on the following formula: [(treated cell absorbance − seeding cell absorbance)/seeding cell absorbance] × 100.

Flow cytometric apoptosis assay. Apoptosis was determined by double-staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) as described previously (Cho et al. 2010). Briefly, cells were cultured in six-well flat-bottomed tissue culture plates (5×10^4 cells/ml) and treated with flaxseed sprouts (100 μg/ml) for 72 h. After harvesting, cells were washed with phosphate buffered saline and resuspended in assay buffer (Sigma-Aldrich). Cells were stained with FITC-conjugated Annexin V and PI, and then analyzed using an Accuri C6 cytometer and Cflow software (Accuri Cytometers, Ann Arbor, MI).

Quantitative real-time RT-PCR. The cells were cultured in six-well flat-bottomed tissue culture plates (5×10^4 cells/ml) and treated with flaxseed sprouts (100 μg/ml) for 72 h. The cultured cells were harvested and placed in RNAlater (Ambion, Austin, TX) prior to freezing and then disrupted into small pieces. RNA was purified by the standard method. Briefly, cells were homogenized in TRI-Reagent (Molecular Research Center, Cincinnati, OH). Total RNA was isolated using 1-bromo-3-chloropropane phase separation reagent (Molecular Research Center). RNA was precipitated by isopropanol and washed with 75 % ethanol. The RNA pellet was dried and resuspended in RNase-free water. The RNA concentration was quantified using a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA). A total of 1 μg RNA of each sample was reverse-transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) and a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) at 42°C for 15 min and 95°C for 3 min, in accordance with the manufacture's recommended protocol. Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a 7500 Fast Real-Time PCR System (Applied Biosystems) with QuantiTect Primers (Qiagen, product reference is in brackets); tumor protein p53 [*p53*, QT00060235] and murine double minute oncogene [*MDM2*, QT00056378]. The relative amounts of gene expression were standardized and calculated by the expression of house-keeping control gene, beta-actin [*ACTB*, QT01680476] as an internal standard, using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis. Cell culture experiments were conducted at least two times. For the comparison of two groups with similar variance, a paired *t* test was used. Statistical data analyses were performed using Minitab Release 14.1

(Minitab Inc., State College, PA). Differences were considered significant at $P<0.05$.

Results

Flaxseed sprouts significantly reduced MCF-7 and MDA-MB-231 cancer cell growth. The cell growth inhibitory effects of flaxseed sprouts in MCF-7 and MDA-MB-231 cells were examined over a wide range of doses and times. Flaxseed sprouts reduced the cell proliferation of MCF-7 and MDA-MB-231 cells at 100 μg/ml concentration and 24, 48, 76, and 96 h after treatment (Fig. 1). At a 10 μg/ml concentration, flaxseed sprouts showed a mild growth inhibitory effect on MCF-7 and MDA-MB-231 (Fig. 1). As shown in Fig. 1*A*, flaxseed sprouts (100 μg/ml) resulted in a significant growth reduction of MCF-7 and MDA-MB-231 cells in dose- and time-dependent manners. In MCF-7 cells, flaxseed sprouts caused 15.8 % growth reduction at 72 h after treatment. In MDA-MB-231 cells, flaxseed sprouts

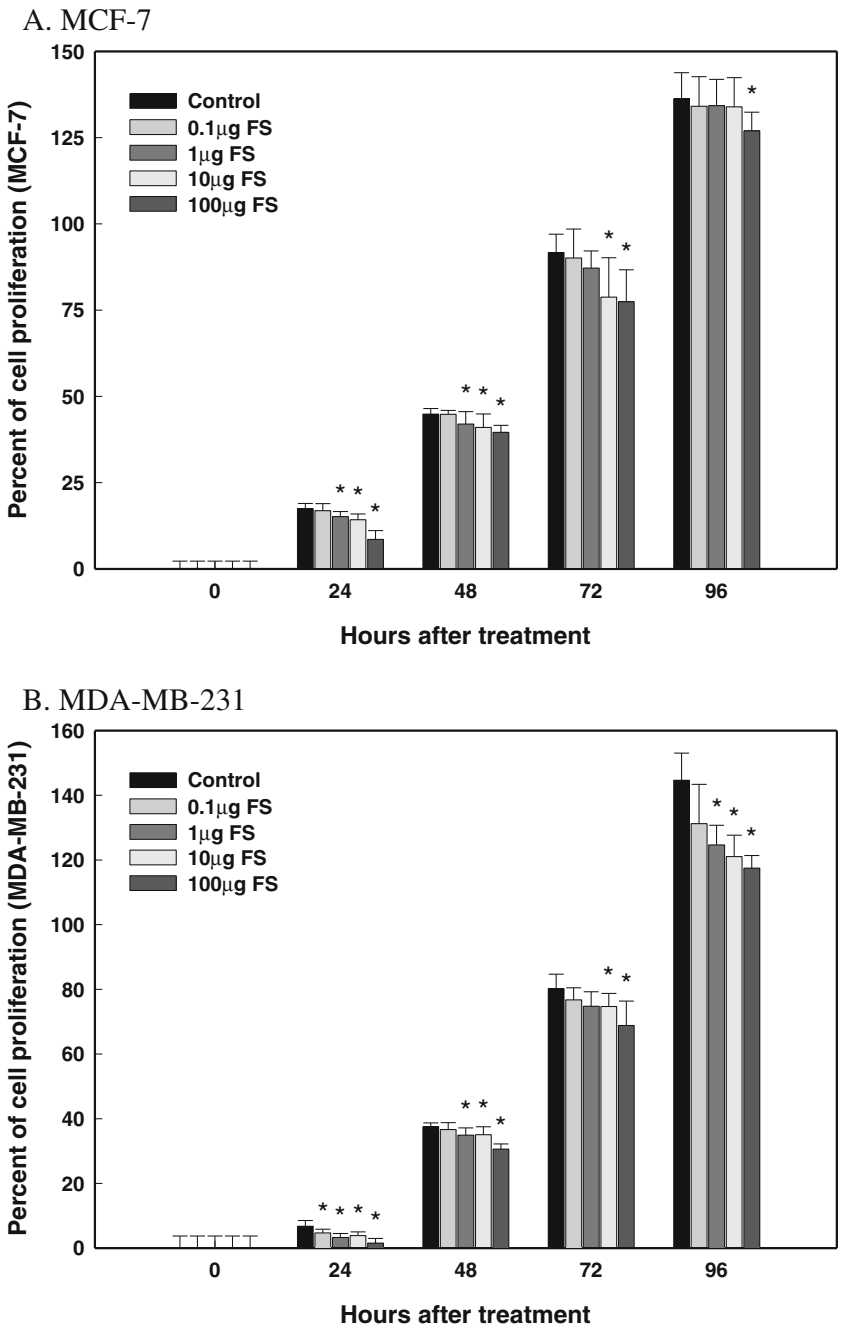

Figure 1. Effects of flaxseed sprouts (*FS*) on cell proliferation of (*A*) MCF-7 and (*B*) MDA-MB-231 cells. Cells were treated with flaxseed sprouts (0.1, 1, 10, and 100 μg/ml) for 0, 24, 48, 72, or 96 h, and cell growth was determined by MTS assay. Data are expressed as means±SD ($n=8$). *Asterisk* indicates a statistically significant difference compared with control cells ($P< 0.05$).

showed 11.4 % growth reduction at 72 h after treatment. The dose of 100 μg/ml of flaxseed sprouts was used for subsequent experiments.

Flaxseed sprouts induced apoptosis in MCF-7 and MDA-MB-231 cells. Apoptosis was measured 72 h after treatment using a flow cytometric assay with Annexin V and PI at

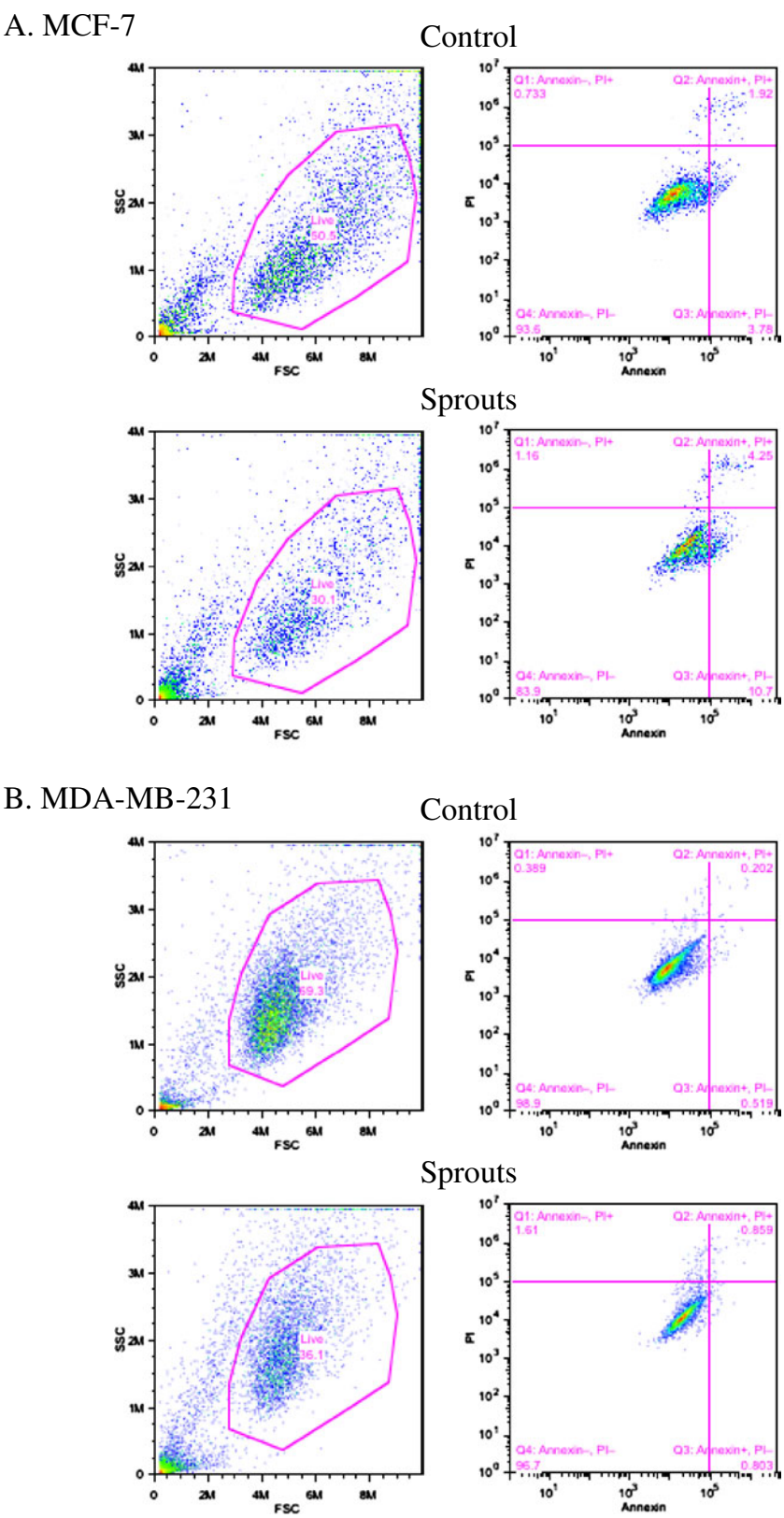

Figure 2. Apoptosis of MCF-7 and MDA-MB-231cells treated with flaxseed sprouts (100 μg/ml) for 72 h. Harvested cells were stained with Annexin V-FITC (FL1, *X* axis) and PI (FL2, *Y* axis). Cells were measured by forward scattering (FSC, size) and side scattering (SSC, granularity) of flow cytometer. Results represent *lower-left quadrant* (viable cells), *upper-left quadrant* (necrotic cells), *lower-right quadrant* (early apoptotic cells), and *upper-right quadrant* (late apoptotic cells).

Table 1. Expression of apoptosis-related genes in MCF-7 and MDA-MB-231 cells

Gene symbol	Treatment		Fold difference	*P* values
	Control	Sprouts		
MCF-7				
p53	0.87±0.22	1.33±0.29	1.53	0.01*
MDM2	1.11±0.29	0.96±0.34	0.86	0.07
MDA-MB-231				
p53	0.84±0.30	1.65±0.41	1.96	0.04*
MDM2	1.19±0.24	0.78±0.32	0.66	0.16

Data represent mRNA levels (n=5) and expressed as means±SD. The relative amounts of gene expression were standardized and calculated by the expression of house-keeping gene, beta-actin

Tumor protein 53 (*p53*) and murine double minute 2 oncogene (*MDM2*) were used as apoptosis markers

*P< 0.05, indicating statistically significant difference between two groups of cells

100 μg/ml. Flaxseed sprouts increased apoptosis (upper- and lower-right quadrants) of MCF-7 (5.70 % vs 14.95 %) and MDA-MB-231(0.72 % vs 1.66 %) cells as shown in Fig. 2. Cell death (upper-left quadrants) was also elevated in MCF-7 and MDA-MB-231 cells treated with flaxseed sprouts (Fig. 2). Moreover, flaxseed sprouts showed a decreased percentage of live cells in MCF-7 (50.5 % vs 30.1 %) and MDA-MB-231 (69.3 % vs 36.1 %) cells. These results indicate that flaxseed sprouts induce apoptotic cell death in MCF-7 and MDA-MB-231 cells.

Expression of genes associated with apoptosis in MCF-7 and MDA-MB-231 cells. The mRNA levels of apoptosis genes in MCF-7 and MDA-MB-231 cells were analyzed by quantitative real-time PCR. As shown in Table 1, flaxseed sprouts did not affect the transcription of *MDM2* in MCF-7 and MDA-MB-231 cells treated with 100 μg/ml of flaxseed sprouts for 72 h. However, flaxseed sprouts significantly increased *p53* gene transcription (53 % in MCF-7 and 96 % in MDA-MB-231 compared with the control) in both cell lines. These data support the results of the flow cytometric apoptosis assay and imply that flaxseed sprouts reduce breast cancer cell growth by increasing p53-mediated apoptosis.

Flaxseed sprouts did not affect MCF-10A cell growth. To determine a possible toxic effect of flaxseed sprouts on cell proliferation, non-cancerous MCF-10A human mammary epithelial cells were examined using the MTS assay (Fig. 3). While flaxseed sprouts showed a decreasing trend (P=0.08) in cell growth at 100 μg/ml and 72 h, there was no statistically significant difference between control and treated cells. The growth of MCF-10A was not influenced by dose or treatment duration of flaxseed sprouts. This result suggests that the cell growth inhibitory effects of flaxseed sprouts are specific to mammary cells that are cancerous.

Discussion

Flaxseeds (*Linum usitatissimum*) are well known for their mammalian lignan precursor secoisolariciresinol diglucoside and essential fatty acid precursor alpha-linolenic acid (ALA), both of which have protective effects against many types of cancer (Chen et al. 2009). Flaxseeds contain cyanogenic glycosides which are toxic because they yield

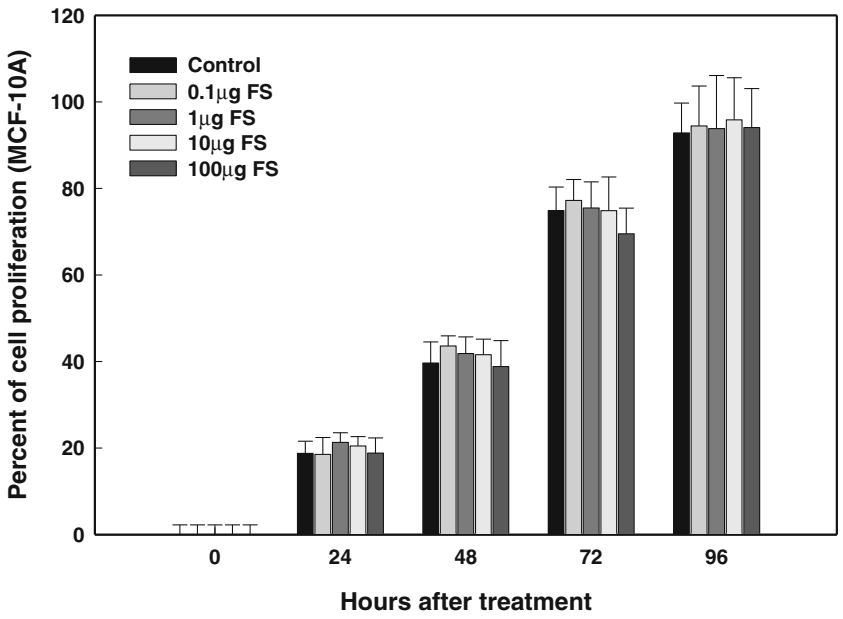

Figure 3. Effects of flaxseed sprouts (*FS*) on cell proliferation of MCF-10A cells. Cells were treated with flaxseed sprouts (0.1, 1, 10, and 100 μg/ml) for 0, 24, 48, 72, or 96 h, and cell growth was determined by MTS assay. Data are expressed as means±SD (n=8). *Asterisk* indicates a statistically significant difference compared with control cells (P< 0.05).

hydrogen cyanide in the presence of water (autohydrolysis) (Shahidi and Wanasundara 1997). Given that consuming raw flaxseeds results in cyanide poisoning, a certain amount of raw flaxseeds can only be digested and absorbed safely in humans (Shahidi and Wanasundara 1997). To reduce cyanogenic glycosides from flaxseeds, a number of artificial detoxification methods such as roasting, autoclaving, and boiling can be used (Yang et al. 2008). However, these methods using heat shocks may destroy not only cyanogenic glycosides but also certain nutrients (Garcia-Gonzalez et al. 2007). Germination is a natural method to increase diverse bioactive components and to decrease toxicity, and the sprouting of seeds has been a considerable topic of interest in cancer prevention research. Some studies have shown that cyanogenic glycosides are significantly reduced during sprouting (Wanasundra et al. 1999).

We investigated the anti-cancer effects of flaxseed sprouts using two different human breast cancer cell lines, estrogen-receptor(ER)-positive MCF-7 and estrogen-receptor-negative MDA-MB-231 cells (Fig. 1). The ER plays important roles in mammary development and differentiation (Lewis and Jordan 2005), and estrogens, which are essential hormones in both males and females, are intimately associated with breast cancer (Lewis and Jordan 2005). ER alpha and beta have distinct roles in mammary development, and they mediate the breast cancer-promoting (ER alpha) or breast cancer-inhibiting (ER beta) effects of estrogens (Paruthiyil et al. 2004). In our investigation, flaxseed sprouts significantly reduced the growth of MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) cells in an ER-independent manner (Fig. 1).

Several in vitro and in vivo studies have also revealed that ALA has inhibitory effects on the initiation, promotion, and progression stages of breast cancer (Chajès et al. 1995). Epidemiological studies report that groups of people who consume diets high in omega-3 fatty acids could have lower breast cancer incidence (Rose and Connolly 1999), and omega-3 fatty acids have been found to inhibit mammary tumor cell growth and to also enhance immune responses against breast cancer (Rose and Connolly 1999). Flaxseed sprouts can be used as an alternative dietary source, and consuming adequate amounts of them may be helpful for decreasing breast cancer risk.

The flow cytometric assay was used to quantify the relative amount of live or dead cells. Treatment with flaxseed sprouts decreased the percentage of live cells and also induced apoptosis in both ER-positive and ER-negative breast cancer cells (Fig. 2). Considering the higher percentage of live cells in the MDA-MB-231 cells, the lower level of apoptosis may be due to early apoptosis. However, mRNA data showed a significant increase in the transcription of *p53* in both cell lines (Table 1). The essential nutrients of flaxseed sprouts may be responsible for the induction of apoptosis, and *p53* may play a role as one of the mechanisms leading to cancer cell growth inhibition.

Apoptosis is a complex biological process and is involved in tumor growth and sustenance (Ridel and Shi 2004). The functions of the p53 protein are important for normal cell development to program cell death, and the p53 protein is inhibited or degraded by MDM2 protein (Haupt et al. 1997; Kubbutat et al. 1997). While *p53* mRNA was significantly increased by flaxseed sprout treatment, there was no significant difference in *MDM2* mRNA.

We observed that flaxseed sprouts reduce the growth of ER-positive and ER-negative human breast cancer cells in vitro by increasing apoptotic cell death. These novel findings suggest that dietary intervention using flaxseed sprouts may potentially reduce breast cancer risk, and further investigation in vivo will be required to determine if flaxseed sprouts can become a critical tool in the overall campaign to reduce breast cancer.

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Conflict of interest statement None declared.

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