PROGRESS REPORT

North Central Region Canola Research Program
(November 2009)

Title: CANOLA OIL REDUCES BREAST CANCER RISK

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A. SUMMARY OF THE PROJECT

1. Rationale and Objective

Epidemiological and experimental studies suggesting that omega-3 fatty acid and oleic acid may exert protective effects against mammary tumors have generated a lot of interest in vegetable oils with a high level of either one of these forms of fatty acids. One vegetable oil of interest is that of the canola seed. Currently, the United States is the seventh largest canola producer and processor in the world. North Dakota produces about 90% of this canola, with smaller amounts grown in Minnesota and other states. In the last few years, consumption of canola oil has increased because of associated health benefits.

The most interesting feature of canola oil is its fatty acid profile. It has the lowest concentration of saturated fatty acids (7%) of all eight major vegetable oils, it is high in monounsaturated fatty acids (61%), and it has a favorable omega-6 to omega-3 fatty acid ratio (1.9:1). Considering the recommended 2:1 ratio of omega-6 to omega-3 fatty acids for the reduction of breast cancer risk along with the known effects of monounsaturated fatty acids, particularly oleic acid in the down-regulation (suppression) of cancer-related oncogenes, canola oil has a uniquely balanced fatty acid composition and could be beneficial in reducing breast cancer risk.

While canola oil has been linked to many health benefits, its association with cancer risk has been overlooked. We wish to develop a better understanding of a potential mechanism responsible for the inhibitory effects of canola oil on human breast cancer cell growth. The specific aims of the project were to determine the extent to which exposure to canola oil affects human breast cancer cell growth. This study entailed determining if canola oil: 1) suppresses human breast cancer cell proliferation, 2) increases anticancer caspase-3 activity, and 3) increases tumor suppressor p53 activity.

2. Experimental Approach

The basic strategies were as follows. First, we determined the optimal concentration of canola oil that causes the maximum inhibition of human breast cancer cell growth. Second, based on the level of canola oil determined from the cell proliferation study, we examined caspase-3 and p53 assays, the key enzyme and protein involved in the cancer cell death (apoptosis) pathway. A pilot experiment on the synergistic effect of canola oil with an anticancer drug, Tamoxifen, was also conducted.

Cell Culture: Three cell lines (two breast cancer cell lines, MCF-7 and T47D, and a normal mammary cell line, MCF-10A) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in a 5% CO_2-humidified atmosphere at 37°C and maintained in basal medium (Dulbecco's Modified Eagle's Medium for MCF-7, MCF-10A; Roswell Park Memorial Institute 1640 for T47D; Gibco Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Invitrogen) and 1% antibiotic-antimycotic (Gibco Invitrogen) as recommended by the supplier.

Cell Proliferation Assay: Cells were cultured in either control medium or canola oil supplemented medium (treatment). Treatment culture media (DMEM and RPMI 1640) contained 6, 10, 14, or 18 µL of canola oil (for a total volume of 100 µL). Cell proliferation was measured using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] colorimetric assay (CellTiter 96 AQueous One Solution Reagent; Promega, Madison, WI). Briefly, cells were seeded at a density of 1 X 10^5 cells/mL in flat-bottomed 96-well microtiter plates with the desired concentrations of canola oil (6, 10, 14, or 18 µL/well). After 0, 24, 48, 72, and 96 hour incubations, MTS was added to each well and the plate was incubated further for 1 to 4 hours at 37°C in a 5% CO_2 incubator. The absorbance was measured at 490 nm with a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Oil Red O Assay: Lipid contents were measured by Oil Red O assay (Sigma-Aldrich). Briefly, cells were seeded in 96-well flat-bottomed tissue culture plates (5 X 10^3 cells/well in 100 µL), and exposed to 1 mmol/L of canola oil. After an overnight incubation, medium was removed; cells were washed with phosphate-buffered saline (PBS) and fixed with 5% formaldehyde solution in PBS. The cells were washed in PBS and stained with Oil Red O solution. The plates were washed with PBS and the cell-bound Oil Red O was extracted with 100% isopropanol (VWR). The Oil Red O content was determined by measuring the absorbance at 490 nm.

Caspase-3 Apoptosis Assay: Cells were cultured at a density of 1 X 10^5 cells/mL in flat-bottomed 24-well plates in media containing the optimum canola oil concentration determined from the cell proliferation experiment. The activity of caspase-3 was measured by the colorimetric assay with CaspACE assay system (Promega, Madison, WI). Briefly, cultured cells were washed twice with PBS and resuspended in cell lysis buffer (Promega). Cell lysates were incubated with colorimetric substrate, N-acetyl-Asp-Glu-Val-Asp-amino-*p*-nitroanilide (Ac-DEVD-pNA). After a 4 hour incubation, the release

of *p*-nitroaniline from Ac-DEVD-pNA was measured at 405 nm using a microtiter plate reader (Molecular Devices).

p53 Enzyme Immunometric Assay (EIA): p53 concentration was determined by using an enzyme immunometric assay kit (TiterZyme EIA Kit, Assay Designs, Ann Arbor, MI). Briefly, cultured cells were washed twice with PBS and resuspended in cell lysis buffer (Assay Designs). The cell lysates were added to a plate preimmobilized to p53 by polyclonal antibody. After the plate was incubated overnight at 4°C, it was washed as described in the protocol provided by Assay Designs. A rabbit polyclonal antibody to p53 conjugated to Horseradish peroxidase was reconstituted in the buffer provided with the EIA kit and added to the plate. Substrate was then added to react with the labeled antibody. Stop solution was added after incubation in the dark at room temperature for 30 min. The absorbance will be measured at 450 nm using a microtiter plate reader (Molecular Devices).

Cancer Drug Treatments: The synergistic effect of canola oil with the anti-cancer drug, Tamoxifen, was examined for cell proliferation. T47D and MCF-7 cells were seeded in 96-well flat-bottomed tissue culture plates (5 X 10^4 cells/mL), and cultured simultaneously for 1-4 days with media containing chemotherapeutic drug, Tamoxifen, or Tamoxifen plus canola oil (1 mmol/L). The absorbance was measured at 490 nm with a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

3. Results

This study determined the effect of canola oil on growth and apoptosis of established human breast cancer cell lines, T47D and MCF-7. As shown in Figures 1 and 2, canola oil treatment resulted in a significant growth inhibition of T47D and MCF-7 cells in a dose- and time-dependent manner. At 24 hours after canola oil treatment, slightly increased levels of lipid contents were observed in both canola oil treated T47D and MCF-7 cells, 22.7% and 25.4% (Figure 1B) respectively. In T47D cells, 0.1 and 1 mmol/L doses of canola oil caused 7.3% and 25.2% growth inhibition (Figure 1A) respectively, and similar cell growth inhibitory effects were also evident in MCF-7 cells accounting for 13.4% and 19.4% growth inhibition (Figure 1A) respectively, following 72 hours of canola oil treatment.

In T47D and MCF-7 cells, 1 mmol/L dose of canola oil caused 31.4% (Figure 2A) and 21.8% (Figure 2B) growth inhibition respectively, following 96 hours of canola oil treatment. In T47D and MCF-7 cells, 1 mmol/L of canola oil significantly up-regulated caspase-3 and p53 activities. At 72 hours after canola oil treatment, the change in caspase-3 of T47D and MCF-7 cells was 16.2% (Figure 3A) and 8.2% (Figure 3B), and the change in p53 was 8.4% and 14.8% (Figure 3C) respectively. Significant increases were observed in the activity of apoptotic mediators in both T47D and MCF-7 cells. As shown in Figure 4, a combination of 1 mmol/L canola oil and 10 µmol/L Tamoxifen resulted in 49.0% (Figure 4A) and 5.7% (Figure 4B) growth inhibition (as compared to Tamoxifen alone) in T47D and MCF-7 cells at 96 hours after treatment.

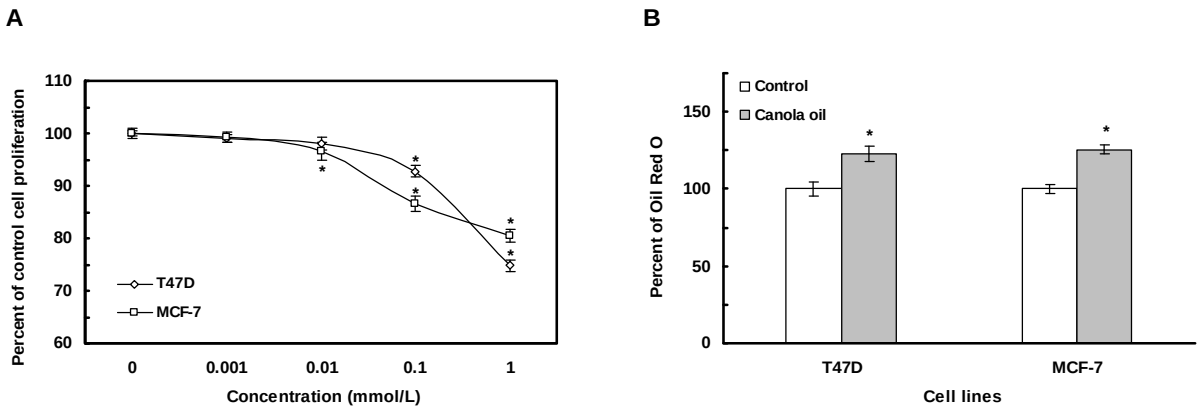

FIGURE 1. Effects of canola oil on T47D and MCF-7 cell growth and lipid content. (*a*) Cells were treated with 0.001-1 mmol/L of canola oil for 72 hours. (*b*) Cells were treated with 1 mmol/L of canola oil for 24 hours. Data in *a* are means ± SEM (*n* = 12) and expressed as percentages of control cell proliferation, (1 - [control cell absorbance - treated cell absorbance] / control cell absorbance) X 100; 100% = no effect, 0% = maximal effect. Data in *b* are means ± SEM (*n* = 9) and expressed as percentages of control cells (treated cell absorbance / control cell absorbance) X 100.

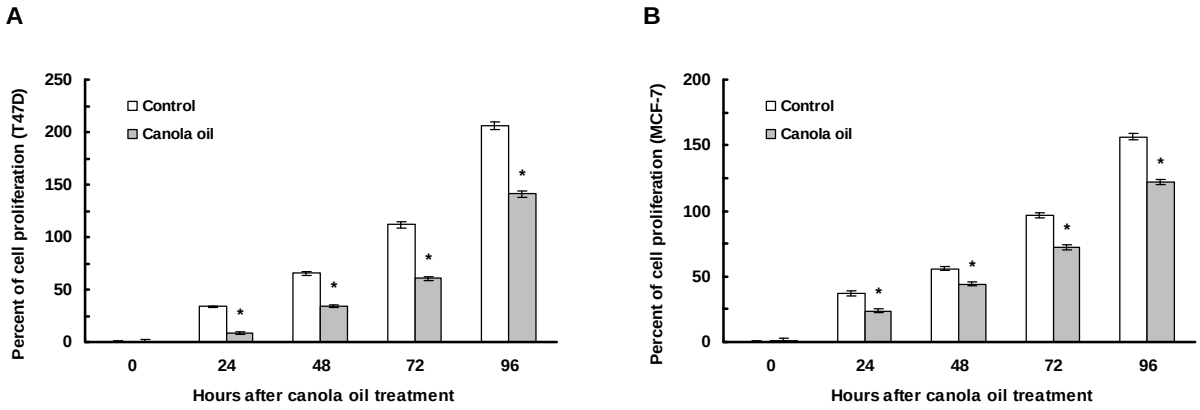

FIGURE 2. Effects of canola oil on time-dependent cell growth. (*a*) T47D cells were treated with 1 mmol/L of canola oil. (*b*) MCF-7 cells were treated with 1 mmol/L of canola oil. Data in *a* and *b* are means ± SEM (*n* = 12) and expressed as percentages of cell proliferation ([treated cell absorbance - initial (seeding) cell absorbance] / initial (seeding) cell absorbance) X 100.

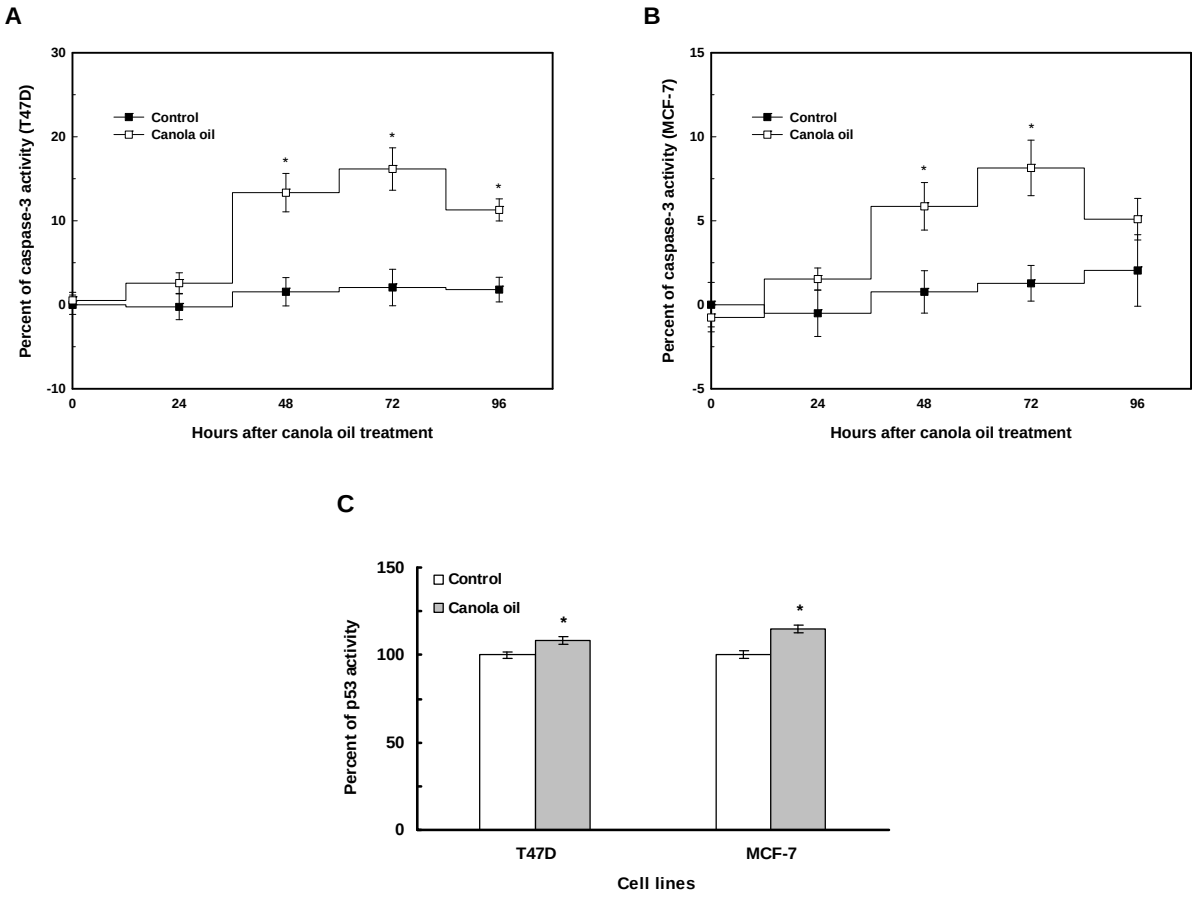

FIGURE 3. Caspase-3 and p53 activities of 1 mmol/L of canola oil treated T47D and MCF-7 cells. (*a*) Time-dependent caspase-3 activity of T47D cells. (*b*) Time-dependent caspase-3 activity of MCF-7 cells. (*c*) p53 activities of T47D and MCF-7 cells at 96 hours. Data in *a* and *b* are means ± SEM (*n* = 9) and expressed as percentages of caspase-3 activity ([treated cell absorbance - initial (seeding) cell absorbance] / initial (seeding) cell absorbance) X 100. Data in *c* are means ± SEM (*n* = 9) and expressed as percentages of control cell p53 activity (treated cell absorbance / control cell absorbance) X 100.

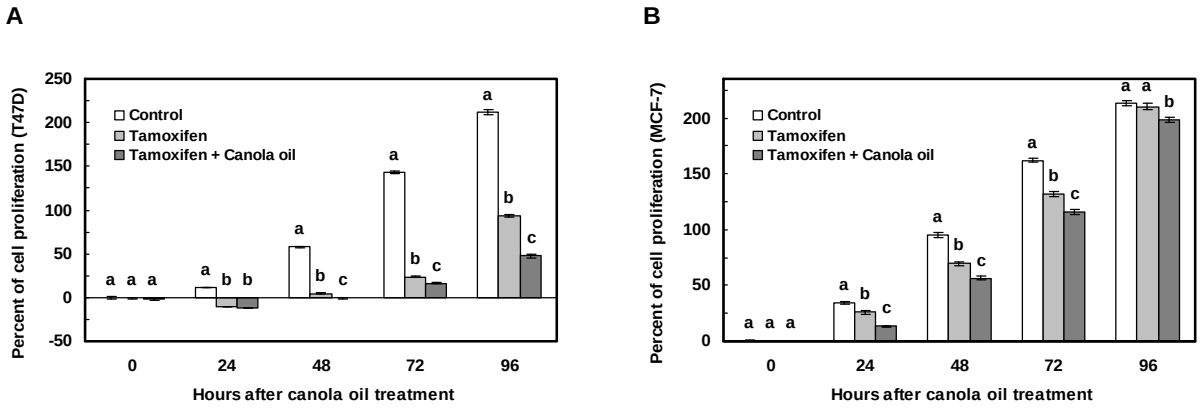

FIGURE 4. Effects of canola oil and Tamoxifen on T47D and MCF-7 cell proliferation. (*a*) Effect of 1 mmol/L canola oil and 10 µmol/L Tamoxifen on the growth of T47D cells. (*b*) Effect of 1 mmol/L canola oil and 10 µmol/L Tamoxifen on the growth of MCF-7 cells. Data in *a* and *b* are means ± SEM (*n* = 12) and expressed as percentages of cell proliferation ([treated cell absorbance - initial (seeding) cell absorbance] / initial (seeding) cell absorbance) X 100.

4. Current Research

Currently, our laboratory is investigating the effect of dietary canola oil supplementation on the growth of implanted MCF-7/adr human breast cancer cells (human breast cancer cell line with a specific degree of resistance to an anticancer drug, Doxorubicin) in athymic nude mice. We hope to show that canola oil may be useful in reversing drug resistance, thereby increasing drug uptake of resistant breast cancer cells. Due to delays beyond our control, the current study began on the 12th of November 2009, hence no data has been included on this report. This research (supported from the USDA/North Central Region Canola Research Program; FY 2009/10) is scheduled to be complete around November, 2011. Progress report on the ongoing study will be submitted as required in due course.

B. CONCLUSION

1. Accomplishments

The completed study represents the first in vitro model demonstration that canola oil, rich in oleic acid and omega-3 fatty acids, might be beneficial in reducing growth of human breast cancer cells in association to enhanced apoptosis. The research also showed synergistic effects of canola oil with a chemotherapeutic drug, Tamoxifen.

2. Impact

The prevention of cancer worldwide is one of the most pressing challenges facing scientists and public health policy-makers, among others. Although breast cancer is often considered to be one of the more chemoresponsive tumors, the duration of

response is relatively short, and most of the initially responsive breast tumors acquire a multidrug resistance phenotype. We hope that results from our *in vitro* studies will be useful in dietary counseling in breast cancer prevention and therapy. The use of canola oil, athymic nude mice, Doxorubicin, and MCF-7/adr human breast cancer cells in our current *in vivo* study is vital in extrapolating the findings to the potential clinical application of breast cancer treatment, especially in drug resistant tumors.

In addition, dissemination of findings through publication and presentation at meetings of canola producers as well as national scientific meetings may increase awareness of the health benefits of canola oil consumption. Part of the data contained herein has already been presented at the 2009 FASEB Experimental Biology Meeting in New Orleans, Louisiana. In addition to submission of a manuscript to a scientific journal, complete data will be presented at the 2009 American Society for Cell Biology Meeting in San Diego, California. We hope that our efforts and similar studies will improve the market (increased demand) for canola oil, especially in North Dakota where 90% of the U.S. canola production is based.

C. PUBLICATIONS/ABSTRACTS

1. Park, C.S., Mabasa, L., Cho, K., and Fowler A.W. 2008. Dietary Canola Oil Reduces Susceptibility to Chemically-induced Mammary Carcinogenesis. Molecular Biology of the Cell 19 (suppl 855-6).

2. Park, C. S., Cho, K., Mabasa, L., and Fowler A. W. 2009. Canola oil inhibits human breast cancer cell growth by regulating caspase-3 and p53. Faseb J. 2009 23:897.9.