Extra virgin olive oil potentiates the effects of aromatase inhibitors via glutathione depletion in estrogen receptor-positive human breast cancer (MCF-7) cells

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Abstract

There have been numerous evidences supporting the relationship between olive oil and cancer, with most of the attention being directed toward its fat and phenolic content. The aims of this study were to investigate whether EVOO and OA could enhance the effects of aromatase inhibitors (letrozole and anastrozole) in ER-positive MCF-7 cells, as well as to investigate its influence on cytochrome c release and GSH levels. It was observed that upon combination treatment, anti-proliferation effects and apoptosis induction were augmented. Apoptosis was triggered via the intrinsic pathway in accordance with cytochrome c release into the cytosol based on IF–IC and ELISA observations. Intracellular GSH levels were also reduced upon EVOO/OA treatment in combination with aromatase inhibitors, and were found to be inversely correlated to cytosolic cytochrome c levels. Additionally, the estrogenic suppressive effects of letrozole and anastrozole were amplified when used in combination with EVOO/OA. Therefore, the employment of aromatase inhibitors in combination with EVOO/OA could orchestrate a reduction in intracellular estrone biosynthesis which feeds ER-positive cells, while simultaneously depleting GSH levels and increasing ROS generation, thus releasing cytochrome c and subsequent induction of apoptosis in MCF-7 cells.

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1. Introduction

Epidemiological studies have implicated that the incidence of breast cancers were found to be the lowest in Mediterranean women (La Vecchia and Bosetti, 2006). It has been suggested that this was largely due to high olive oil (OO) consumption, which contains a rich source of oleic omega-9 monounsaturated fatty acids (OA:ω-9 MUFA) (Colomer and Menendez, 2006) ranging from 56% to 84% of total fatty acids (FA), and ω-6 linoleic polyunsaturated fatty acids (LA PUFA:18:2n-6) ranging from 7% to 10% of total FA in the Mediterranean diet. Collectively, these fatty acids provide OO with unique characteristics such as resistance to acidification and oxidation (Perez-Jimenez et al., 2005). Beside FAs, the main phenolic component present in olive leaves, drupes and extra virgin olive oil (EVOO) is oleuropein which discriminates the characteristic differences between various types of OOs in terms of taste and stability (Andrewes et al., 2003). Previous studies have shown that olive tree (Olea europaea) products possess several pharmacological properties including anti-oxidant, hypocholesterolemia (Bogani et al., 2007; El and Karakaya, 2009), anti-inflammatory (Bogani et al., 2007; Fabiani et al., 2008), anti-microbial (Omar, 2010), anti-viral (Lee-Huang et al., 2007) and anti-cancer properties (Fu et al., 2010). In spite of these claims, the mechanism by which specific FA components in OO exert its potential protective

Abbreviations: Bcl-2, B-Cell Lymphoma 2; ER, estrogen receptor; E1, estrone; EVOO, extra virgin olive oil; FA, fatty acids; GSH, glutathione; MUFA, monounsaturated fatty acid; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; OA, oleic acid; OO, olive oil; PUFA, polyunsaturated fatty acid.

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and anti-cancer effects on estrogen receptor (ER) positive human breast cancers remain poorly understood.

Past studies have begun to address the specific biological and molecular mechanisms by which FAs such as OA exert its effects on cancer (Simopoulos, 2004; Trichopoulou et al., 2000). It has been reported that OA, which is the main MUFA of OO specifically suppresses Her-2/neu overexpression, which in turn interacts synergistically with immunotherapies involving Herceptin in breast cancers expressing the Her-2/neu oncogene (Menendez et al., 2005). It was also reported that in non-malignant cells, OA promoted an additive growth effect when used together with insulin-like growth factor 1 (IGF1). In contrast, OA had no growth inducing effects and inhibited IGF1-induced proliferation in malignant cells. This was reported in rat models where a rich diet of OO resulted in a slower progression of induced mammary tumors (Zeng et al., 2010).

Apocytochrome c is the heme-free precursor of cytochrome c, which is synthesized in the cytosol and post-translationally imported into the mitochondria (Wang et al., 2003). Once it enters the intermembrane space, apocytochrome c is converted into holocytochrome c and is attached to the inner mitochondrial membrane where it plays a role in the production of energy (Stuart et al., 1990). However, when cytochrome c detaches and escapes into the cytosol, a cascade of events commences, which ultimately results in the triggering of apoptosis via the intrinsic mitochondria-mediated pathway (Fulda and Debatin, 2006; Kromer and Reed, 2000). In this pathway, Bcl-2 proteins regulate apoptosis by altering the mitochondrial membrane potential leading to the release of cytochrome c (Newmeyer and Ferguson-Miller, 2003). Bcl-2 overexpression was also shown to increase cellular glutathione (GSH) levels, which is associated with increased resistance to apoptotic-inducing drugs (Meredith et al., 1998; Voehringer, 1999), while GSH depletion enhances apoptosis induced by anti-cancer drugs such as cisplatin in Bcl-2 expressing cells (Rudin et al., 2003). Depletion of GSH levels have also been reported to cause elevations in lipid peroxidation and protein carbonylation in MCF-7 and MDA-MB-231 cells (Parihar et al., 2010). Studies have also shown that cellular levels of GSH are linked to NADPH levels, a cofactor of glutathione reductase which catalyzes the reduction of glutathione disulfide to GSH. The production of GSH then reduces hypoxides to water, maintaining a low level of oxidative stress within cells. All these indicate the importance of GSH during oxidative stress and apoptosis in human mammary adenocarcinoma cells (Parihar et al., 2010; Spitz et al., 2000).

Aromatase, a member of the cytochrome P450 (CYP) enzyme family, catalyzes the rate-limiting conversion of androstenedione to estrone and of testosterone to estradiol, using NADPH as a cofactor (Ghosh et al., 2009). Increase in aromatase activity has been correlated to alcohol consumption, old age, obesity, insulin and gonadotropins levels (Smith and Dowsett, 2003). In post-menopausal women, ER-positive breast cancers often fail to respond, or develops resistance during treatment leading to disease recurrence (Ali and Coombes, 2002). Third generation non-steroidal aromatase inhibitors such as letrozole, vorozole and anastrozole potentially prevent estrogen biosynthesis rather than blocking ER activation (Santen and Harvey, 1999) and are currently being used as adjuvant therapies for early and late stage breast cancer (Cruciotta et al., 2001).

The present study was undertaken to investigate the effects of EVOO and its major FA component (omega-9 OA) in combination with aromatase inhibitors such as anastrozole and letrozole in ER-positive mammary adenocarcinoma cells. Here we hypothesized that by depleting GSH levels with EVOO and OA, the efficacy of aromatase inhibitors will be enhanced in MCF-7 cells.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO) (Cat. No. 317275), 3-(4,5-dimethylthiazol-2-4)-5-2.5-diphenyl tetrazolium bromide (MTT) (Cat. No. 474989) and annexin V-FITC apoptosis detection kit (Cat. No. PP032) were purchased from EMD Millipore (Billerica, MA, USA). Rooswell Park Memorial Institute (RPMI-1640) media (Cat. No. 17-702F), fetal bovine serum (FBS) (Cat. No. 14-502F), trypsin (Cat. No. 17-160E), and penicillin/streptomycin (Cat. No. 17-602F) were purchased from Lonza Inc. (Basel, Switzerland). Letrozole (Cat. No. L6545), Anastrozole (Cat. No. A2736) and oleic acid (Cat. No. O1383) were purchased from Sigma Aldrich (St. Louis, MO, USA). Commercial EVOO was purchased from Monte Verde Pte. Ltd. (KL, Malaysia).

2.2. Cell lines and culture conditions

ER-positive human breast adenocarcinoma cell lines (MCF-7) were kindly donated by Prof. Datuk Dr. Rohana Yusof from the Department of Molecular Medicine, Faculty of Medicine, University of Malaya, while normal human mammary epithelial cells (HMEC) were purchased from Lonza Inc. (Basel, Switzerland). MCF-7 cells were cultured as monolayers in RPMI-1640 media, supplemented with l-glutamine, 10.0% /C176 (v/v) FBS, 100.0 U/ml penicillin and 100.0 mg/ml streptomycin. HMEC were maintained in mammary epithelial growth media (MEGM) with growth supplements provided by the manufacturer, Lonza Inc. (Basel, Switzerland). All running cultures were maintained at 10 passages or fewer in a humidified incubator at 37°C in 5.0% CO2 and 95.0% air. All treatments in this study were conducted in FBS-free media.

2.3. GC and GC–MS analysis

Analysis of EVOO constituents were carried out by GC and GC–MS. EVOO samples were dissolved in peptane (C2H8O), followed by GC analysis on the Agilent 7890A gas chromatograph instrument (Agilent Technologies, Santa Clara, CA, USA) with HP-5 capillary column (30 m × 0.32 mm × 0.25 μm). The carrier gas was nitrogen at a 1.0 ml/min constant flow and with an injection volume of 1 μl. Injector and inlet temperatures were set at 250°C and a splitting ratio of 1:20. The oven temperature program was set as: initial temperature 90°C hold for 1 min, 90–190°C (5°C/min) hold for 10 min, 190–250°C (3°C/min) and hold for 10 min, with a total run time of 61 min. GC–MS analysis was performed using the Shimadzu GCMS-QP 2010 PLUS (Shimadzu Corporation, Kyoto, Japan) with DB-5 capillary column (30 m × 0.25 mm × 0.25 μm) and helium as the carrier gas.

The oven temperature program was set similar to the GC parameters. Mass spectra were performed at 70 eV over a scan range of 40–600 amu. Retention indices were determined with C7 to C40 alkane standards as references. Relative amounts of individual compounds were based on peak areas obtained without FID response correction factor. Identification of compounds was based on the GC–MS library (NIST08) while comparing Kovats retention index (RI) with literature.

2.4. MTT cell viability assay

cytotoxic effects of EVOO, OA, letrozole and anastrozole on MCF-7 cells were determined using the MTT assay. Briefly, 2.0 × 104 cells were treated in triplicates with various concentrations of letrozole (up to 50 μM) or anastrozole (up to 100 μM) and/or in combination of EVOO (up to 125 μg/ml) or OA (up to 80 μg/ml) for 24 h. All EVOO and OA dosages used in this study were within or less than physiologically relevant concentrations. Solvent controls were conducted simultaneously using either DMSO (for EVOO) or ethanol (for OA) with appropriate concentrations corresponding to each treatment. MTT reagent (5.0 mg/ml) was added and cells were incubated in the dark at 37°C. DMSO was then added to dissolve purple formazan precipitates and a Tecan Sunrise microplate well reader (Tecan, Mannef, Switzerland) was used to detect absorbance/reference at 570/650 nm.

2.5. Annexin V-FITC/PI apoptosis assay

Detection of apoptosis was conducted using the annexin V–FITC/PI apoptosis detection kit according to manufacturer’s protocol. Briefly, MCF-7 cells were seeded in RPMI-1640 with 10% (v/v) FBS for 24 h and replaced with FBS-free media during treatments. Cells were then treated for 24 h with various combinations of OA (20 and 40 μg/ml) and EVOO (100 and 125 μg/ml) in combination with anastrozole (40 μM) or letrozole (20 μM). Cell pellets were washed in 1x PBS and resuspended in 0.5 ml of media binding reagent. Annexin V-FITC (1.25 μl) was added and cells were incubated for 15 min in the dark. After incubation, 10 μl of PI was added and cells were analyzed immediately using a BD FACSCANTO™ II flow cytometer and the CellQuest Pro IVD software (Becton Dickinson, Mountain View, CA, USA).
2.6. Cytochrome c immunofluorescence–immunocytochemistry (IF–IC)

Determination of cytochrome c localization was done using the ApoTrack™ cytochrome c apoptosis IF–IC antibody kit (Cat. No. MSA07) (NittoSciences Inc., Eugene, OR, USA) according to manufacturer’s protocol on MCF-7 cells treated with EVOO, OA and aromatase inhibitors. Briefly, monolayer cells were grown and treated on coverslips in 6-well plates, using untreated cells containing treatment-equivalent DMSO/ethanol volumes as a solvent control for 24 h. Cells were then fixed with 4% (v/v) paraformaldehyde dissolved in 1x PBS for 20 min at room temperature. Coverslips were placed in antigen retrieval buffer (100 mM Tris, 5% w/v urea, pH 9.5), at 95°C for 10 min to improve antigen detection. Permeabilization buffer (0.5 ml of 1% Triton® X-100) was added and specimens were blocked with 10% (v/v) normal goat serum for 1 h. Cells were incubated with primary anti-cytochrome c IgG and anti-complex (Vv) IgG for 2 h. Unbound antibodies were washed away, followed by incubation with secondary goat anti-mouse IgG2a–FITC and goat anti-mouse IgG2b–Texas Red (TXRD) for 1 h. Imaging was processed using an inverted fluorescence microscope, Nikon ECLIPSE TS100 with excitation/emission wavelength at 590/620 nm for TXRD and 488/520 nm for FITC.

2.7. Cytochrome c and glutathione ELISA

In order to quantify the intracellular levels of cytochrome c and GSH, the human cytochrome c sandwich ELISA kit (Cat. No. K0160) (Abnova, Taipei City, Taiwan) and the GSH 96-wells sandwich ELISA kit (Cat. No. 703002-96) (Cayman Chemical, Ann Arbor, MI, USA) were used according to manufacturer’s protocol. Briefly, treated MCF-7 cells were collected and lysed with 500 µl of cell lysis buffer. Protein samples were collected and normalized using the Bradford assay to a final concentration of 10 µg/ml. A total of 100 µl of protein samples were added into each pre-coated well followed by the addition of biotinylated antibodies against cytochrome c and GSH. Detection was done using streptavidin-HRP conjugates and TMB substrate and H2SO4. The absorbance of developed color was inversely proportional to the amount of antigen in samples.

2.8. Quantification of estrone (E1) levels

A solid phase competitive inhibition enzyme immunoassay was used to determine estrone levels in MCF-7 lysates using the Estrone ELISA kit (Cat. No. DB52051) (IBL International, Hamburg, Germany) according to manufacturer’s protocol. Briefly, rabbit anti-E1 polyclonal IgGs were pre-coated onto microplate wells followed by competitive binding between 100 µl of avidin-HRP conjugated human E1 and human E1 from standard or samples. Unbound E1 were washed off followed by the addition of TMB substrate and H2SO4 stop solution. A microtiter plate reader, Tecan Sunrise® was used to detect absorbance/reference at 450/650 nm and mean concentrations from triplicates were calculated using a standard curve. The intensity of developed color was inversely proportional to the amount of antigen in the sample.

2.9. Statistical analysis

All cell proliferation, flow cytometry, IF–IC and ELISA experiments were carried out in triplicates. Data from all experiments were presented as mean ± SD. Differences between treated and untreated groups were considered statistically significant with a p-value threshold of <0.05 using a two-tailed paired Student’s t-test. Significant correlation (R) between cytochrome c and GSH levels were calculated using the Pearson’s linear correlation test.

3. Results

3.1. EVOO and OA enhances the cytotoxicity of aromatase inhibitors in MCF-7 cells

 Determination of cytotoxicity using different concentrations of EVOO up to 125 µg/ml and OA up to 80 µg/ml indicated that OA did not affect the viability of MCF-7 cells after 24 h of exposure (Fig. 1A). However, when OA was combined with the use of aromatase inhibitors such as letrozole and anastrozole, increased efficacies were observed after 24 h where cell viability levels dropped from 76.4 ± 5.1% (anastrozole treated) to 54.7 ± 4.5% (anastrozole + OA treated), and from 70.5 ± 6.4% (letrozole treated) to 46.0 ± 4.8% (letrozole + OA treated) (Fig. 1B and C). Preliminary concentrations of EVOO and OA used were fixed at 50 µg/ml and 15 µg/ml respectively, while concentrations of both anastrozole and letrozole were allowed to vary. Increase in cytotoxicity levels were also found to be weaker in aromatase inhibitor combination regimes involving EVOO in comparison to purified OA, which was the major MUFA constituent of EVOO (Table 1). Even though...
3.2. Induction of apoptosis by EVOO and OA in combination with aromatase inhibitors

In order to justify the optimum combination ratio between EVOO/OA with letrozole and anastrozole, a series of combination treatments were performed using the MTT cell viability assay. Since EVOO and OA were non-cytotoxic components in a dual drug combination regime, combination index values as well as the presence of synergistic relationships cannot be determined. Therefore, optimal combinations were justified based on normalized cell viability fold reduction values against untreated groups. This describes the level of enhancement by a single cytotoxic drug component on a second non-cytotoxic component (Chou, 2006). Optimum combination concentrations were obtained and used for subsequent assays (Fig. 2D). Annexin V-FITC/PI flow cytometry results indicated that the population of cells shifted from viable cells to early and late stage apoptosis, followed by secondary necrosis after 24 h of treatment (Fig. 2A and B). As with previous MTT cytotoxicity analysis, the extent of apoptosis was greatest in letrozole + OA and anastrozole + OA combination treatment groups at 11.32% and 12.48% respectively, followed by combined treatment groups containing EVOO and lastly standalone aromatase inhibitors (Fig. 2C). This indicated that both EVOO and OA played a role towards aromatase inhibitor’s augmentation of apoptosis, and that OA was indeed the major FA constituent in EVOO responsible for this observation.

3.3. EVOO/OA in combination with aromatase inhibitors modulates the release of cytochrome c from the mitochondria

Since a combined treatment of EVOO and/or OA with aromatase inhibitors (letrozole and anastrozole) successfully induced apoptosis in MCF-7 cells, we then investigated the facilitation of cytochrome c release from inner mitochondria membrane to the cytosol. IF–IC results showed that untreated cells indicated an overlapping presence of cytochrome c within the mitochondrial membrane with minimal signs of cytochrome c release. However, when both aromatase inhibitors were used in combination with OA, a release of cytochrome c into the cytosol was observed (Fig. 3A). ELISA on cytosolic cytochrome c levels confirmed our IF–IC observations, where both standalone letrozole and anastrozole treated cells did not show any significant difference in cytosolic cytochrome c levels compared to untreated cells (Fig. 3B). In contrast, combination treatment involving EVOO or OA showed a 1.5–2.5 fold increase in cytosolic cytochrome c levels, thus affirming the involvement of the intrinsic pathway. The effect of OA–combined with letrozole in comparison to anastrozole was also shown to be superior in terms of cytochrome c release by 19.2 ± 2.1% (Fig. 3B). In reference to annexin V-FITC data demonstrated opposing observations on the superiority of letrozole over anastrozole compared to cytochrome c release and initial MTT data, its findings were statistically insignificant (1.16% variation between anastrozole and letrozole with a p-value of 0.28) and was therefore disregarded.

3.4. EVOO/OA nullifies the increase of GSH levels by aromatase inhibitors

Cellular levels of GSH are positively correlated to NADPH levels, which are in turn increased through the inhibition of aromatization reactions by aromatase inhibitors. Therefore, we investigated whether EVOO and OA could counteract the increasing levels of GSH following exposure to letrozole and anastrozole in MCF-7 cells through ELISA. Our observations show that upon letrozole and anastrozole treatment, levels of GSH were increased to 10.5 ± 1.3 μM and 11.2 ± 1.6 μM respectively, compared to untreated cells (7.5 ± 1.2 μM) presumably from an increase in NADPH levels (Fig. 3C). However, when both aromatase inhibitors were used in combination with EVOO or OA, this increase in GSH levels were nullified and reduced to 6.0 ± 1.0 μM by EVOO and 4.9 ± 1.3 μM by OA, which was below untreated control levels (Fig. 3C). These findings revealed that treatment with aromatase inhibitors alone increases GSH levels, while combination of aromatase inhibitors with EVOO or OA depletes cellular GSH levels.

3.5. Cytochrome c release is inversely correlated to GSH levels

In order to investigate whether the occurrence of apoptosis through the intrinsic pathway was associated to GSH levels, a Pearson correlation analysis was performed. The correlation dot plot indicated that the release of cytochrome c from the mitochondria inner membrane was negatively correlated towards the levels of GSH with a correlation coefficient, R of −0.82 (Fig. 4A). The strength of the relationship as represented by the coefficient of determination, R², indicates that at least 68% (R² = 0.68) of the total variation in cytosolic cytochrome c release is inversely related to GSH levels, which is in turn hypothetically affected by levels of excess NADPH within the cell.

3.6. EVOO/OA enhances the estrogenic suppressive effects of aromatase inhibitors

To determine whether EVOO/OA disrupted the efficacy of aromatase inhibitors owing to reduced GSH levels and the under utilization of NADPH, an ELISA was performed on estrone (E1) levels, which is the predominant estrogen in post-menopausal women. It was observed that intracellular E1 levels were reduced as expected when exposed to standalone letrozole (10.3 pg/ml) and standalone anastrozole (15.1 pg/ml) from untreated cells (24.8 pg/ml). E1 levels were further significantly reduced when cells were exposed to both aromatase inhibitors in combination with EVOO.
(let: 2.6 pg/ml + anas: 1.0 pg/ml) or OA (let: 0.95 pg/ml + anas: 0.56 pg/ml) (Fig. 4B). These results indicated that the combination between aromatase inhibitors and EVOO/OA did not disrupt the effects of letrozole and anastrozole, but instead, augmented its estrogenic suppressive effects.

4. Discussion

Epidemiological studies have shown that populations predominantly consuming an OO based Mediterranean diet exhibited lower incidences of breast cancer and other chronic diseases (Colomer and Menendez, 2006; Visioli et al., 2002). A diet rich in OO also showed a slower progression of DMBA-induced rat mammary carcinogenesis (Costa et al., 2004), and the ability to suppress the lipogenic enzyme FAS which may provide a well-tolerated novel anti-cancer therapy through metabolic changes (Notarnicola et al., 2011). This study has provided evidence that treatment of ER-positive MCF-7 cells with OA or EVOO enhances the efficacy of two commercially available aromatase inhibitors (letrozole and anastrozole) without affecting non-transformed breast cancer cells. We also demonstrated that the combination of aromatase inhibitors with OA is superior to EVOO, thus piling on the benefits of OAs, which have also been previously reported to result in the down-regulation of overexpressed Her-2/neu oncogene in breast carcinomas (Menendez et al., 2005).

To date, several third generation non-steroidal aromatase inhibitors have been developed such as Anastrozole (Arimidex®), Letrozole (Femara®), Vorozole and Exemestane (Aromasin®), which prevents the aromatase cytochrome P450 complex from catalyzing a series of reactions leading to the aromatization of androgens to estrogens (Brodie et al., 1986; de Jong et al., 1997). In this study, treatment with letrozole displayed a slightly greater growth inhibition effect compared with anastrozole in MCF-7 cells. The superiority of letrozole over anastrozole was further extended to combined treatment regimes comprising of both EVOO and OA, thus confirming previous findings that letrozole possessed a stronger binding affinity and functional association than anastrozole.
towards aromatases (Kijima et al., 2005). In addition to that, past reports also showed that there was no median change from baseline in total serum cholesterol for letrozole, but a slight increase was observed for anastrozole where cholesterol was used as a substrate for estrogen synthesis (Markopoulos et al., 2009).

Previous studies have shown that mitochondria-targeted pro-apoptotic proteins enable the formation of membrane channels or pores which loosen the mitochondrial permeability barrier. Based on a past report, we hypothesized that this event together with exposure to EVOO allowed the occurrence of lipid peroxidation which disrupts the ability of cardiolipin to interact with cytochrome c, hence initiating a sequence of events that ultimately lead to apoptosis (Ragione et al., 2000). Based on this model of cell death induction, IF–IC and ELISA was used to measure the release of cytochrome c from the mitochondria, where it was demonstrated that both EVOO and OA can enhance cytochrome c release in MCF-7 cells.

We also observed that GSH content in MCF-7 cells was elevated after treatment with letrozole and anastrozole, suggesting that aromatase inhibitors interfere with pro-apoptotic signals. Since the reducing equivalent of NADPH is required to regenerate GSH from the oxidized disulfide form, GSSG (Voehringer, 1999), an increase in cytosolic GSH level after aromatase inhibitor treatments may be hypothetically attributed to a build up in reduced forms of NADPH which are required as coenzymes for aromatization, GSH synthesis and FA synthesis. Interestingly, the depletion of cytosolic GSH upon exposure to combined regimes consisting of EVOO and OA was not only shown to boost the percentage of

Fig. 3. EVOO and OA in combination with aromatase inhibitors (letrozole and anastrozole) induces the release of cytochrome c from mitochondria, and reduces intracellular GSH levels. (A) Representative IF–IC photomicrographs of MCF-7 cells where cytochrome c (green fluorescence) was seen leaking out (white arrows) from the inner mitochondrial membrane (red fluorescence). (B) ELISA quantification on cytosolic cytochrome c levels between various treatment groups on MCF-7 cells. (C) ELISA quantification of intracellular GSH levels in MCF-7 cells. All data were presented as mean ± SD of three independent replicates, where (*) indicates p-value ≤ 0.05, while (**) indicates p-value ≤ 0.01 in comparison to untreated samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. EVOO and OA reduces the level of estrone (E1) production when used in combination with aromatase inhibitors (A) Dot plot indicating a negative correlation between cytoplasmic cytochrome c and GSH levels upon treatment with various combinations of letrozole and/or anastrozole with EVOO and OA, where R is the correlation coefficient; and R² is the coefficient of determination. (B) ELISA on cellular E1 levels demonstrating the estrogenic biosynthesis suppressive effect of EVOO and OA in combination with aromatase inhibitors. Data was shown as mean ± SD of three independent replicates, where (*) indicates p-value ≤ 0.05, while (**) indicates p-value ≤ 0.01 in comparison to untreated samples.
apoptotic cells, but also enhanced the efficiency of aromatase inhibitors as shown by reduced E1 biosynthetic levels. Through mechanisms that have yet to be elucidated, we concluded that OAs in EVOO play a significant role in GSH synthesis, lipid peroxidation and ROS generation, which have been shown to be involved in the induction of apoptosis as well as other cell death-mediated factors originating from severe oxidative stress (Buttke and Sandstrom, 1994; Toborek et al., 2002).

A negative correlation between GSH levels and cytosolic cytochrome c also reinforced the hypothesis that depletion in GSH is connected to lipid peroxidation, the release of cytochrome c and subsequently, apoptosis. GSH requires constant replenishment from GSSG, and NADPH is known to be essential for its regeneration in cellular defense against oxidative stress to promote cancer cell growth and survival (Kil et al., 2010). The fact that NADPH utilization occurs as a result of FA oxidation inhibition even in the presence of glutamine and glucose suggests that NADPH production powered by these nutrients may either be insufficient or imposes a minimal impact on the antioxidant system. These suggest that there is a link between FA, GSH, ROS and aromatase inhibitors, where NADPH is shared and provided for regeneration of the GSH anti-oxidant system to protect against oxidative damage (Yuneva et al., 2007). It is also proposed that the inhibition of aromatization by letrozole or anastrozole on its own increases cellular GSH levels, resulting in higher drug resistance occurrence. The administration of FA, specifically natural OA from EVOO can therefore be employed in combination with aromatase inhibitors to reduce GSH levels, which has previously been shown to be a major culprit for drug resistance (Chen and Kuo, 2010).

This study shows that treatment of MCF-7 cells with aromatase inhibitors presumably increases reduced forms of NADPH, leading to the provision of reducing equivalents to regenerate intracellular GSH. The decline of GSH levels in the presence of aromatase inhibitors and EVOO/OA provides direct evidence that EVOO plays a role in GSH depletion through disruption of membrane potential and lipid peroxidation which enhances the release of cytochrome c from the mitochondria, inducing apoptosis in MCF-7 cells (Fig. 5). Even though EVOO contains a wide variety of other metabolites including polyphenols, iridoids and triterpenes which could possibly influence the overall activity of EVOO, the consistent parallel effects observed between EVOO and purified OA in all experiments served as a strong indicator that OA remains the major constituent and key player responsible in augmenting the anti-cancer effects of aromatase inhibitors.

5. Conclusion

In conclusion, the employment of aromatase inhibitors in combination with EVOO/OA could orchestrate a reduction in intracellular estrogen biosynthesis which feeds ER-positive cells, while simultaneously depleting GSH levels and the subsequent induction of apoptosis.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.10.024.

References