Original Article

Oxidative stress pathways of flavonoid toxicity in human breast tumor cells

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Received December 4, 2016; Accepted January 15, 2017; Epub March 1, 2017; Published March 15, 2017

Abstract: African-American women have a higher breast cancer mortality rate than Caucasian women. Harmful reactive oxygen species (ROS) exert oxidative stress in cells of the human body and lead to several types of DNA damage. Recent studies have shown that flavonoids may protect against cancer through inhibition of oxidative damage. Naringenin, a universal flavonoid, can also inhibit the proliferation of cancer cells. This project studied the oxidative stress (cell death) pathways of naringenin in ER-positive (MCF-7), ER-negative (MDA-MB-468), and non-tumorigenic human breast cell (MCF-10A) lines. Cellular levels of ROS, superoxide (O2-), glutathione (GSH), and mitochondrial membrane potential (MMP) were assayed using the FACS Calibur flow cytometer and cell quest software for data collection. The results showed that naringenin induced oxidative stress that resulted in cell death in all cell lines. The highest production of ROS and O2- was observed in ER-negative cells. Naringenin did not have any effect on GSH levels in ER-negative and ER-positive but a partial increase occurred in non-tumorigenic human breast cells. The results for MMP showed that naringenin significantly increased the loss of MMP in all breast cells. In conclusion, the oxidative stress that resulted in cell death from exposure to naringenin may contribute to the cancer-preventive effects associated with an increased dietary intake of fruits containing flavonoids. We have presented evidence that naringenin is able to induce cell death in both ER-positive and ER-negative breast cancer cells; however the magnitude of the effect of naringenin is quite different. This finding suggests naringenin may have potential as a treatment for ER-negative breast cancer.

Keywords: Breast cancer, estrogen receptor, flavonoids, naringenin, oxidative stress

Introduction

African American women have lower incidence rates but higher mortality rates for breast cancer than Caucasian women [1] and are more likely to be diagnosed at the later stages of breast cancer [2-4]. The reason for mortality differences between racial groups may be cultural but also due to a different hormone receptor status [5-9]. It is increasingly recognized that breast cancer is a disease with distinct clinical behavioral and molecular properties. For example, estrogen receptor (ER) positive and negative cancers are the two most distinct breast cancer subtypes and predict the likelihood of benefits from antiestrogen therapy [10, 11]. Breast cancer tumors that are ER-positive and human epidermal growth factor receptor 2 positive (ER+/HER2+) are much more likely to respond to endocrine therapy that blocks estrogen’s effects in the body, than ER-negative (ER-/HER2+) tumors [5, 12]. According to the Surveillance, Epidemiology, and End Results [13], women with ER-positive tumors have better prognosis and need less aggressive treatment than women with ER-negative tumors.

Cancer cells have higher levels of oxidative stress than normal cells [14, 15]. Increased levels of oxidative stress result from an imbalance between the production of reactive oxygen species (ROS) and reduction of glutathione (GSH) level in the cell and high levels of ROS are known to kill cancer cells [16-22]. GSH plays a vital role in maintaining redox homeostasis and it is the main non-enzymatic component of intracellular antioxidant defense mechanisms, acting as a small scavenger molecule in living
organisms [22-24]. GSH is able to scavenge single oxygen and hydroxyl radicals directly, detoxify lipid hydroperoxides and hydrogen peroxides by the activity of glutathione peroxidase, and regenerate other antioxidant molecules [25, 26]. An early stage of oxidative stress starts with disruption of mitochondria including changes in membrane and redox potential. DNA fragmentation is preceded by disruption of mitochondria, which results in a decrease in mitochondrial membrane potential (MMP) [27-30]. This reduction in MMP is accompanied by the production of ROS contributing to cell death [17, 31-34]. There is growing awareness that oxidative stress plays an important role in various health problems and that consumption of antioxidants can prevent and even reverse these effects [24, 35-37].

Numerous studies suggest that bioflavonoids have potential beneficial effects and exert preventive effects in carcinogenesis essentially due to their antioxidant, anti-inflammatory, and anti-proliferative activities [38-40]. Naringenin, a flavonoid found in grapefruit, can alter ROS metabolism by directly lowering the intracellular pool of GSH [14, 41-44]. It was the aim of this study was to better understand the cytotoxicity of naringenin and its oxidative stress pathway(s) in human breast cancer cells and investigate the possible use of naringenin as an alternative treatment for estrogen receptor negative breast cancer.

Material and methods

Cell culture

MCF-7 (ER-positive), MDA-MB-468 (ER-negative) and MCF-10A (non-tumorigenic, control) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). For routine maintenance, Eagle’s Minimal Essential Medium (EMEM) with 2.0 mM L-glutamine, supplemented with 0.01 mg/ml bovine insulin, 10% fetal bovine serum, and 0.5% penicillin and streptomycin was used for ER-positive cells; Leibovitz’s L-15 medium with 2.0 mM L-glutamine supplemented with 10% fetal bovine serum, 0.5% penicillin and streptomycin was used for ER-negative cells; and Mammary Epithelial Growth Medium (MEGM) supplemented with 100 ng/ml cholera toxin was used for non-tumorigenic cells. All cells were cultured in 175 cm$^2$ flasks at 37°C in a humidified 95% air and 5% carbon dioxide (CO$_2$) incubator. Cells were allowed to grow and form a monolayer in the flasks. Cells were grown to 90-95% confluence, washed with phosphate buffered saline (PBS) and harvested by trypsinization (0.05% (w/v) trypsin) once a week following manufacture’s protocol (ATCC cell culture). All media were purchased from Invitrogen (Grand Island, NY) and fetal bovine serum, penicillin, and streptomycin purchased from ATCC.

Cell toxicity assay (cell death) by measuring of intracellular ROS, O$_2^-$, GSH, and MMP

All breast cells were grown in 6-well plates with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin, at 37°C in a 5% CO$_2$ incubator. Cells were allowed to grow to 90% confluence. Naringenin (Sigma Aldrich, St. Louis, MO) at different concentrations (0.0, 2.5, 10.0, or 50.0 µM) was added to the wells with 5.0 mL MEM without phenol red followed by incubation for 24 h. After 24 h of exposure to naringenin, 4.0 μL of the appropriate fluorescent indicator was added to the cells. 2’,7’-Dichlorodihydrofluorescein diacetate (H$_2$DCFDA), dihydroethidium (DHE), 5-chloromethylfluorescein diacetate (CMFDA) and rhodamine 123 (RHO) for detection of cell toxicity (cell death) using ROS, O$_2^-$, GSH and MMP, respectively using a FACS Calibur flow cytometer instrument (Becton Dickenson, San Jose, CA).

Intracellular ROS were monitored by H$_2$DCFDA dye, which is a cell permeable indicator for ROS [33, 45]. The superoxide radical (O$_2^-$) was monitored by DHE dye, which is cell permeable and reacts with O$_2^-$ to form ethidium that in turn intercalates in DNA and exhibits a red fluorescence [46]. The intracellular reduction of GSH level was monitored by CMFDA dye, which is a fluorescent chloromethyl derivative that freely spreads through the membranes of live cells and exhibits bright green fluorescence in the cytoplasm [47]. The mitochondrial membrane potential (MMP) was monitored by RHO dye, which is a cell permeable cationic dye that exhibits green fluorescence in mitochondria of living cells [48].
Flavonoid toxicity in breast tumor cells

Figure 1. Flow cytometric analysis of NT (MCF-10A Cells) naringenin concentration (µM). Flow cytometric analysis of NT (MCF-10A) following exposure to fluorescent dye for reactive oxygen species (ROS), superoxide radical (O$_2^-$), glutathione (GSH) and determining of a change in mitochondrial membrane potential (MMP). Percents indicate portion of dead cells reacting positive for each dye at 0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM naringenin.
Flow cytometric analysis of ER- (MDA-MB-468 Cells) naringenin concentration (µM). Flow cytometric analysis of ER- (MDA-MB-468) following exposure to fluorescent dye for reactive oxygen species (ROS), superoxide radical (O$_2^-$), glutathione (GSH) and determining of a change in mitochondrial membrane potential (MMP). Percents indicate portion of dead cells reacting positive for each dye at 0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM naringenin.

Figure 2. Flow cytometric analysis of ER- (MDA-MB-468 Cells) naringenin concentration (µM). Flow cytometric analysis of ER- (MDA-MB-468) following exposure to fluorescent dye for reactive oxygen species (ROS), superoxide radical (O$_2^-$), glutathione (GSH) and determining of a change in mitochondrial membrane potential (MMP). Percents indicate portion of dead cells reacting positive for each dye at 0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM naringenin.
Flavonoid toxicity in breast tumor cells

**FACS calibur flow cytometer**

After the breast cells were exposed to H$_2$DCFDA, DHE, CMFDA and RHO dyes for four hours for detection of ROS, O$_2^-$, GSH and MMP, respectively; the cells were centrifuged, the supernatant fluid with the dyes was decanted, and the cells were suspended in 1 ml PBS. The fluorescence intensity for 10,000 events for each sample was determined using a FACS Calibur flow cytometer to sort and analyze the breast cells. Results for percent dead cells showing positive for the dye were calculated with Cell Quest software (Becton Dickenson, San Jose, CA).

**Statistical analysis**

The effects of naringenin (0.0, 2.5, 10.0, and 50.0 µM) and cell type (ER+, ER-, NT) and their interactions resulting in cell death due to oxidative stress (ROS, O$_2^-$, GSH, and MMP) was assessed by two-way ANOVA using a 4 by 3 factorial design (version 6.0, Prism, GraphPad Software, San Diego, CA). Regardless of which oxidative stressor was assessed, cell death was always affected (P<0.05) by a naringenin by cell type interaction. To better understand these interactions, two means comparison tests were done. First, within a cell type, mean percent of dead cells reacting positive for given dye at 0.0 µM naringenin was compared to mean cell death for 2.5 µM, 10.0 µM, and 50.0 µM naringenin using Dunnet’s multiple comparison test at P<0.05. Second, within a dose of naringenin, mean cell death among cell types was compared using Dunnet’s multiple comparison test at P<0.05.

**Results**

**Reactive oxygen species (ROS)**

The effect of naringenin on percent of dead cells exhibiting a positive reaction to the H$_2$DCFDA dye (ROS) was significantly (P<0.05) correlated with cell type (NT, ER+, or ER+) and concentration of naringenin in the medium (Figures 1-4; Table 1). With respect to cell type ER- (MDA-MB-468) exhibited the strongest response (Figure 4; Table 1) with percent mortality of 35.4±1.28%, 81.3±1.0%, 83.3±0.74%, and 89.1±1.34% for 0.0 µM, 2.5 µM, 10.0 µM, and 50.0 µM naringenin, respectively. While NT (MCF-10A) and ER+ (MCF-7) showed significant differences (P<0.05) between naringenin concentrations of 0.0 µM and 2.5 µM. However, between 2.5 µM and 10.0 µM and 50.0 µM naringenin significant (P<0.05) differences were seen with NT showing a change of 6.4±0.24% to 9.1±0.54% between 2.5 µM and 10.0 µM and 9.1±0.54% to 16±0.45% between 10.0 µM and 50.0 µM naringenin. For ER+ a similar response occurred with a change in percent of 12±0.79% to 18±0.55% between 2.5 µM and 10.0 µM and 18±0.55% to 22±2.28% between 10.0 µM and 50.0 µM naringenin.

**Superoxide (O$_2^-$)**

For superoxide radicals (O$_2^-$) the effect of naringenin on percent of dead cells exhibiting a positive reaction to DHE dye was again significantly (P<0.05) correlated with cell type (Figures 1-3 and 5; Table 1). For ER- cells a strong and significant (P<0.05) response has been seen at all concentrations of naringenin (0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM) with percent dead cells reacting positive at 17.8±1.06%, 22.0±0.73%, 32.2±0.76% and 46.5±1.07%, respectively. For NT and ER+ cells differences were significant (P<0.05) but to a lesser degree than ER- with NT at 5.4±0.70% and ER+ at 2.3±0.44%, NT at 4.3±0.35% and ER+ 6.3±0.41%, NT at 6.1±0.49% and ER+ at 9.6±0.65%, and NT at 7.9±0.59% and ER+ at 22.2±2.23% at 0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM naringenin, respectively. Between concentrations there was a significant (P<0.05) difference with ER- cells between 0.0 µM and
Figure 3. Flow cytometric analysis of ER+ (MCF-7 Cells) naringenin concentration (µM).
Table 1. Percent positive dead NT, ER- and ER+ cells for reactive oxygen species (ROS), superoxide radical (O₂⁻), glutathione (GSH), and change in mitochondrial membrane potential (MMP) at 0.0 µM, 2.5 µM, 10.0 µM, and 50.0 µM naringenin

<table>
<thead>
<tr>
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<th>0.0 µM</th>
<th>2.5 µM</th>
<th>10.0 µM</th>
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<tr>
<td>NT (MCF-10A)</td>
<td>7.6±0.63%</td>
<td>4.5±0.7%</td>
<td>5.0±0.7%</td>
<td>0.4±0.6%</td>
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<tr>
<td>ER+ (MCF-7)</td>
<td>1.9±0.7%</td>
<td>2.3±0.5%</td>
<td>4.5±0.7%</td>
<td>12.6±0.4%</td>
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<tr>
<td>ER- (MCF-7)</td>
<td>0.6±0.7%</td>
<td>0.9±0.7%</td>
<td>0.1±0.9%</td>
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With respect to GSH response following addition of RHO to the cell medium the result was less clear. NT and ER+ cells were not significantly (P<0.05) different at 0.0 µM naringenin (5.0±0.53% and 4.5±0.61%, respectively, but ER- was significantly (P<0.05) than both at 0.74±0.19% (Figures 1-3 and 6; Table 1). At 2.5 µM naringenin all 3 cell types were significantly (P<0.05) different with NT = 5.3±0.50%, ER+ = 2.2±0.52% and ER- = 0.4±0.14%. The differences at 10.0 µM and 50.0 µM were again significant (P<0.05) with NT = 8.1±0.39% and 7.9±0.59%, respectively; ER+ = 5.7±0.42% and 6.0±0.78%, respectively; ER- = 1.3±0.36% and 1.6±0.58%, respectively. Between concentrations GSH for NT cells was not significantly (P>0.05) different between 0.0 µM and 2.5 µM naringenin with GSH positive reactions at 5.0±0.53% and 5.3±0.50%, respectively. A significant (P<0.05) difference was seen between 2.5 µM and 10.0 µM naringenin with percents positive of 5.3±0.50% and 8.1±0.39%, respectively. Between 10.0 µM and 50.0 µM naringenin no significant (P<0.05) difference was observed with percents positive of 8.1±0.39% and 7.9±0.59%, respectively. ER- cells did not show any significant (P>0.05) between concentrations with percent positives of 1.5±0.51% at 0.0 µM; 0.9±0.52% at 2.5 µM; 1.3±0.36% at 10.0 µM; and 1.6±0.58% at 50.0 µM naringenin. ER+ cells showed no significant (P<0.05) differences between 0.0 µM and 2.5 µM naringenin with percent positives of 2.2±0.52% and 4.0±0.50%, respectively; but did show significant (P<0.05) differences between 2.5 µM and 10.0 µM naringenin with percents positives of 4.0±0.50% and 5.7±0.42%, respectively. As with NT cells between 10.0 µM and 50.0 µM naringenin no significant (P<0.05) differences were seen with values at 8.1±0.39% and 7.9±0.59%, respectively.

Flavonoid toxicity in breast tumor cells

Figure 4. Effect of naringenin on generation of reactive oxygen species (ROS) and cell death in non-tumorigenic (NT), estrogen receptor negative (ER-), and estrogen receptor positive (ER+) breast cancer cell lines. ROS reported as percent dead cells exhibiting a positive reaction with the H₂DCFDA dye. Bars are means ±95% confidence intervals for six samples. Bars within a cell type with an asterisk differ (P<0.05) from the corresponding bar at 0 µM naringenin. Bars within a dose of naringenin with different superscripts differ at P<0.05.

and 2.5 µM, 2.5 µM and 10.0 µM, and 10.0 µM and 50.0 µM naringenin with percent differences of 17.8±1.06% and 22.0±0.73%, 22.0±0.73% and 32.2±0.76%, 32.2±0.76% and 46.5±1.07%, respectively. Lesser differences (P<0.05) were seen with NT and ER+ cells with no significant differences between 0.0 µM and 2.5 µM naringenin (NT = 5.4±0.70% and 4.3±0.35%, ER+ = 2.3±0.44% and 6.3±0.41%). Between 2.5 µM, 10.0 µM and 50.0 µM naringenin no significant (P<0.05) differences were seen but to a lesser degree than with ER- cells with NT cells exhibiting percents of 4.3±0.35%, 6.1±0.49%, 7.9±0.28% and ER+ cells exhibiting percents of 6.3±0.41%, 9.6±0.65%, and 22.2±2.23% at 2.5 µM, 10.0 µM and 50.0 µM naringenin, respectively.
Flavonoid toxicity in breast tumor cells

Concentrations of naringenin (0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM) NT and ER+ cells did not exhibit any significant (P<0.05) differences with NT cells showing percent positive dead cells of 0.74±0.14% at 0.0 µM, 0.4±0.12% at 2.5 µM, 0.7±0.09% at 10.0 µM and 0.9±0.06% at 50.0 µM; and ER+ cells percents of 0.74±0.19% at 0.0 µM, 0.4±0.14% at 2.5 µM, 0.7±0.19% at 10.0 µM and 0.9±0.09% at 50.0 µM. ER- cells exhibited significant (P<0.05) differences at 0.0 µM and 2.5 µM naringenin with percents of 2.2±0.52% and 2.2±0.52%; and a much more pronounced response at 10.0 µM and 50.0 µM with percents of 7.0±0.45% and 9.0±0.62%. Between concentrations of naringenin no significant (P<0.05) differences were seen in NT and ER+ cells with percents of 0.74±0.14% and 0.74±0.19% for NT and ER+ at 0.0 µM, 0.4±0.12% and 0.4±0.14% at 2.5 µM, 0.7±0.09% and 0.7±0.19% at 10.0 µM and 0.9±0.06% and 0.9±0.09% at 50.0 µM. The ER- cells did show significant (P<0.05) differences between all concentrations of naringenin with percents of 2.2±0.52% at 0.0 µM, 2.2±0.52% at 2.5 µM, 7.0±0.45% at 10.0 µM and 9.0±0.62% at 50.0 µM.

Discussion

Much interest has been directed toward the use of natural products as preventive and
remedial treatments for a variety of diseases, specifically as anticancer treatment [44, 49-51]. Thousands of flavonoids are known to occur in nature and are one of the largest groups of natural products [52]. As potential anticancer drugs flavonoids have been studied on a variety of cancer cell lines [44, 50, 53-56]. Examining different pathways to determine mode of action [41, 42, 44, 47-61]. The potential beneficial effects of flavonoids on carcinogenesis use in their antioxidative, anti-inflammatory, and antiproliferation activities [36, 62, 63]. Naringenin, a flavonoid found in high concentrations in grapefruit and other citrus fruits [63, 64-66], has been shown to exhibit antiproliferative effects and was able to cause death in various cancer cell lines [14, 49, 56, 67]. The study reported here applied flow cytometry and specific fluorescent dyes (H$_2$DCFDA, DHE, CMFDA and RHO) to evaluate intracellular ROS, O$_2^-$, GSH and MMP responses in NT, ER- and ER+ human breast cell lines. It is known that oxidative damage to mitochondrial membranes from ROS and O$_2^-$ is involved in many diseases such as Alzheimer’s disease [56] and cancer [68, 69]. In order to better understand the intracellular process involved in inducing cell death due to naringenin we determined the ROS and O$_2^-$ responses to varying concentrations of naringenin and corresponding changes in GSH and MMP.

In examining the intracellular production of ROS and O$_2^-$ at varying concentrations of naringenin in the media ROS showed a significant increase in ER- cells between 0.0 µM and 2.5 µM naringenin and while not significant between 2.5 µM and 10.0 µM, and 10.0 µM and 50.0 µM ROS response remained high at all three naringenin concentrations (Figure 4). This corresponds to O$_2^-$ values (Figure 5) where there was a direct coordination with recent dead cells showing positive increasing as naringenin concentrations increased. This suggests that both ROS and O$_2^-$ in ER- cells are profoundly affected by naringenin. However, this was not the case with NT and ER+ cells where ROS percents (Figure 4) remained the same between 0.0 µM and 2.5 µM naringenin and between 2.5 µM and 10.0 µM, and 10.0 µM and 50.0 µM naringenin showing significance for both NT and ER+ cells between 2.5 µM and 10.0 µM naringenin but not significant between 10.0 µM and 50.0 µM naringenin. These results are similar to O$_2^-$ percents with significant differences between 10.0 µM and 50.0 µM naringenin for NT and ER+ cells. These results support the assumption that bioflavonoid compounds can induce growth inhibition in breast tumor cells through increasing rates of apoptosis [69-71] and support the finding of McLean [72] where amino-flavonones induce ROS formation and oxidative DNA damage in ER- breast cell lines. In a review by Sac [53] there is substantial evidence that flavonoids through induction of oxidative stress inhibit tumor growth in ER- cells. The O$_2^-$ radical can rapidly change into other reactive oxygen species such as hydrogen peroxide and hydroxyl radicals [21, 73-76] thus it is clear that naringenin has significant effect in increase ROS and O$_2^-$ in estrogen negative breast tumor cell lines which is supported by other studies [70, 71, 73, 77].

Protective mechanisms such as GSH serve to break down free radicals and minimize oxidative damage [19, 23]. In this study GSH response appeared to be minimal (Figure 6) with the highest percent of cells positive for GSH at 8.1±0.39% in NT cells. Significant increases in GSH did not occur until 10.0 µM naringenin was added to the medium for NT and ER+ cells. ER- cells did not respond to increasing naringenin; which when compared with ROS and O$_2^-$ responses showing similarly low percents confirming studies [26] that ROS, O$_2^-$ and GSH production are much lower in non-malignant cell line [26, 78-80]. In general with GSH levels remaining low in spite of increase in ROS and O$_2^-$ as naringenin concentration were increased, it would appear that naringenin may, as suggested by others [12, 41, 78, 81, 82] that flavonoids can cause GSH to be depleted and increase ROS. Whether this is due to GSH being converted to GSSH (Glutathione disulfide) at a rate that far exceeds regeneration or if some other mechanism is involved cannot be answered by this study. None the less, GSH is important in preventing cell death [20, 22, 23, 83, 84].

Cell death is usually proceeded by mitochondrial degeneration [28] which was determined in this study by the uptake of the RHO dye by mitochondria in dead cells indicating a change in the MMP [28, 30, 31, 37, 85-87]. In this study MMP did not show any significant change in percent cell positive in ER- cells between 0.0
μM and 2.5 μM naringenin, but did exhibit significant differences between 2.5 μM and 10.0 μM, and 10.0 μM and 50.0 μM naringenin showing a near linear response (Figure 7). This is consistent with finding by Suski [88] who found that during apoptosis MMP and activity of the respiratory chain decreased coincident with an increase in ROS production. However, for NT and ER+ no significant changes in MMP were detected (Figure 7). Overall the percent of dead cells positive for RHO dye was low with the highest percent at 9.0±0.62% for ER- cells. This suggests that other mechanisms may be at play affecting mitochondrial integrity including deficiency in oxidizable substances for mitochondria, blockage of respiration, and withdrawal of growth factors and lack of extra cellular glucose supply [27, 89-92]. In addition, pathways leading to cell death do not necessarily involve mitochondria but rather can directly affect nuclear DNA leading to cell death.

Take in it is entirety there was a clear difference in cell susceptibility to naringenin between NT, ER- and ER+ cells with respect to oxidative stress. Of significant ROS and O\textsuperscript{2-} percents (Figures 4 and 5) were highest in ER- cells which is in agreement with other studies [26, 79] were flavonoids exhibited low cytotoxicity in normal human cells. GSH while not showing high percent positive cells was more significant in NT and ER+ cells, and very low in ER- cells (Figure 6). As noted previously this may be due to high ROS and O\textsuperscript{2-} which overwhelms the rate at which GSH is produced or regenerated from GSSH [23, 35, 93]. The change in MMP was clear in ER- cells, but low; which suggests that naringenin did increase oxidative stress on mitochondria as shown by several researches [85-87, 91]. It is probably not the major pathway leading to cell death as discussed by several authors [59]. Since anti-cancer therapeutic strategies are focusing on oxidative stress [20, 94-97] early studies have suggested that cancer cells have high metabolic activities and therefore produce high levels of ROS as compared with normal cells which in this study only ER- showed high ROS and O\textsuperscript{2-} activity and is consistent with previous studies [68, 70-72, 74].

Acknowledgements

This research was supported by a U56 grant from National Institute of Health (NIH). Thanks to the Toxicology Laboratory at the Department of Natural Sciences at the University of Maryland Eastern Shore for equipment and facility support. Special thanks go to Dr. Jeanine Harter-Dennis for her invaluable assistance in statistical analysis of the data and to Dr. Thomas P. Oscar for his review and helpful suggestions during preparation of this work for publication.

Disclosure of conflict of interest

None.

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