The effects of Nobiletin, Hesperetin, and Letrozole in a combination on the activity and expression of aromatase in breast cancer cells

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Abstract: Nobiletin (NOB) and hesperetin (HES) are the citrus polymethoxyflavone and flavonone. Aromatase or cytochrome P450 (CYP19) enzyme is a key enzyme in estrogen biosynthesis. The objective of this study was to investigate the combinational effects of HES, NOB and letrozole (LET) as aromatase inhibitors on the activity and expression of aromatase in MCF-7 cells. In this study, aromatase enzyme activity based on the conversion of androgen substrate testosterone to 17β-Estradiol was determined. Estradiol concentrations were measured using an electrochemiluminescence immunoassay. CYP19 gene expression was determined by quantitative real-time PCR. Our findings demonstrated that none of combinations including LET+NOB, LET+HES, LET+NOB+HES, and NOB+HES had no significant effects on aromatase activity and expression. The present study showed for the first time that the combination of HES, NOB, and LET had no effects on activity and expression of aromatase in MCF-7 breast cancer cells.

Key words: Nobiletin; Hesperetin; Letrozole; Aromatase; Breast cancer; MCF-7 cells.

Introduction

According to WHO reports, cancer is currently the leading cause of death and prevails coronary heart disease or stroke (1). Among Iranian women, breast cancer is a commonly diagnosed malignancy and the most frequent cause of cancer death, accounting for 23% of all cancers and 14% of the cancer deaths (2, 3).

Approximately 80% of breast cancer cases are estrogen receptor positive; thus endocrine treatment is of one major therapeutic value in these patients along with surgery (4). 17β-Estradiol (E2) plays an important role in the pathogenesis and progression of breast cancer (5, 6). Estrogen biosynthesis requires the key enzyme aromatase (7). Aromatase is a member of the cytochrome P450 (CYP 19) enzyme family, which converts androgens such as testosterone and androstenedione to estrogens such as 17β-estradiol and estrone (8). Activity and expression of aromatase as well as plasma estrogen concentrations increase in breast cancer tissue (6, 9, 10). Hence, aromatase inhibitors (AIs) are being used clinically in the estrogen-responsive breast cancer treatment (4, 8). Letrozole (LET) is a useful non-steroidal AIs used as first-line therapy in the treatment of estrogen receptor positive breast cancer in postmenopausal women (4, 6, 7). Several studies have demonstrated the superiority of the third-generation AIs for example letrozole to tamoxifen, which is a selective estrogen receptor. These findings confirm that adjuvant therapy with AIs reduces the recurrence risk in patients with advanced breast cancer (4, 11-13). The AIs response rate is also improved when compared with tamoxifen in postmenopausal women (14). A new class of AIs like LET has been classified as non-steroidal aromatase inhibitors (NSAIs), consists of natural products and their derivatives such as flavonoids (7).

High intake of citrus fruits is associated with a 10% decrease in breast cancer risk in women (15).

The chemopreventive potential of polyphenolic compounds in the recent years has clearly supported their health benefits, including anti-cancer properties. The effects of dietary polyphenols on aromatase may provide an effective strategy for reducing the health burden of cancer in humans (16).

Flavonoids are the most important group of phenolic compounds in plants (17) due to their low toxicity and effectiveness in chemopreventive therapy (8, 18). Flavonoids in citrus have shown a wide range of chemopreventive properties and exhibited anti-proliferative activities toward the human cell lines in numerous in vitro studies (19-21). Nobiletin (NOB) and hesperetin (HES) are the citrus polymethoxyflavone (PMFs) and flavonone which have shown a wide range of biological activities, including anti-inflammatory, anti-carcinogenic, anti-viral, antioxidant, anti-thrombogenic, and anti-atherogenic properties (18, 22).

Combined chemotherapy in the treatment of breast cancer may increase chemotherapeutic agents’ efficacy,
resulting in decreased toxicity for normal tissues (15). Citrus flavonoids increased their cytotoxic effects, modulated cell cycle and induced apoptosis of cancer cells when they were used in a combination with chemotherapeutic agents (15).

To date, various types of flavonoids have been investigated for aromatase inhibition using a microsomal assay (23). However, few studies have assessed the effect of citrus flavonoids on aromatase expression (9). Inhibition of the CYP 19 expression has been reported after exposure to a combination of isoflavones and flavones in human granulose-luteal cells (24).

Since LET is mostly prescribed for patients with breast cancer, assessing other AIs in a combination with LET would be of great value. Nevertheless, information about the possible ability of flavonoids and AIs in a combination is even more limited and until now the effect of citrus flavonoids and AIs in a combination on aromatase has not been examined. Therefore, the present study was carried out to investigate the effect of combinations of HES, NOB and LET on the activity and expression of aromatase in MCF-7 breast cancer cells.

Materials and Methods

Human breast cancer MCF-7 cells (Avicenna Research Institute, Iran) were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco BRL, Grand Island, NY, USA), 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA). The samples were incubated at 37°C in a humidified incubator with 5% CO₂ atmosphere. The cells were routinely subcultured when 80% confluence was reached. Then, the cells were trypsinized using trypsin-EDTA (Gibco BRL, Grand Island, NY, USA). The cells were seeded into 6-well plates at a density of 3 × 10⁵ cells/well for aromatase activity and expression assays and were treated with nobiletin (Sigma-Aldrich, Germany), hesperetin (Sigma-Aldrich, Germany) and letrozole (Ind-Swift-In, India). All compounds were dissolved in dimethyl sulfoxide (DMSO) (Applichem, USA) with final DMSO concentrations less than 0.1% in culture media. The combined groups consisted of letrozole and nobiletin (LET+NOB); letrozole and hesperetin (LET+HES); letrozole, nobiletin, and hesperetin (LET+NOB+HES); nobiletin and hesperetin (NOB+HES). All compounds were added to the culture medium at final concentrations of 1 μM for LET and NOB and 5 μM for HES. Each experiment was performed in duplicate and repeated three times.

Aromatase activity assay

The activity of aromatase enzyme was assessed by measuring the conversion of androgen substrate testosterone to E₂ in cell culture (25). Briefly, MCF-7 cells (3 × 10⁵ cells/well) were seeded in the medium supplemented with 5% FBS. Culture medium was removed after one day and replaced by phenol red-free RPMI medium (Biosera, UK) containing 2% dextran-coated charcoal-stripped (Sigma Chemical Co, St Louis, MO, USA) FBS (DCC-FBS). Testosterone with 100 nM concentration (Sigma Chemical Co, St Louis, MO, USA) was also added as aromatase substrate and incubated overnight. The cells were treated with various combinations for 48 h. Subsequently, the media were collected and stored at −70°C until further analysis. E₂ concentrations were measured using an electrochemiluminescence immunoassay (ECLIA) kit (Roche, Germany) by elecsys 2010 instrument with sensitivity of 18.4 pmol/L, an intra-assay coefficient of variation (CV) of 1.9–5.7% and an inter-assay CV of 2.3–6.2%.

Protein concentrations were determined by the Bradford method (26). Aromatase activity was calculated as picograms of E₂ synthesized per milligram protein per hour (pg E₂/mg protein/hour) and expressed as percentage of control.

RNA extraction and cDNA synthesis

Total RNA was isolated from the cells using a Ribospin kit (GeneAll, South Korea) according to manufacturer’s instructions and stored at -70°C until analysis. RNA quantity and quality were assessed by measuring absorption in 260 nm and 280 nm using Nano Drop spectrophotometer (ND-2000c) (Wilmington, Delaware USA) and by gel electrophoresis, respectively. Complementary DNA (cDNA) was synthesized using a HyperScript™ RT master mix (GeneAll, South Korea) with 1 μg of total RNA in a 20 μl reaction mixture using oligo dT and randomhexamer primers. The reverse transcription (RT) PCR was initiated by pre-heating the mixture 5 min at 65°C to facilitate the reaction by denaturing the secondary structure of RNA, followed by adding master mix solution. The reaction was performed for 5 min at 25°C, followed by 60 min at 55°C. The reaction was terminated at 95°C for 5 min and chilled on ice. cDNA stored at −20°C prior to real-time PCR assay.

Quantitative real-time PCR assay of CYP19 expression

Real-time PCR was performed by a Corbet rotor gene real-time PCR system, utilizing SYBR Green reagents (AmpliQon, Denmark). Primer sequences were as follows: forward and reverse- CYP19 (5’-GAATTCGACCCCTCATCTCCC-3’ and 5’-GCGGAAATCGAAGCTGTAAT-3’); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer (5’-GGGGAAGCTGTAAT-3′); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer (5’-GGGGAAGCTGTAAT-3′); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer (5’-GGGGAAGCTGTAAT-3′); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer (5’-GGGGAAGCTGTAAT-3′); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer (5’-GGGGAAGCTGTAAT-3′); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalizer.

Amplification reactions were run as triplicates. Each reaction was performed in a 20 μl total volume mixture containing 10 μl of SYBR Green reagents, 200 nmol/L of forward and reverse primers and 1 μl of cDNA sample. The reaction was initiated by heating at 95°C for 15 min, followed by 45 amplification cycles of 95°C for 15 s, 62°C for 20 s, and 72°C for 30 s. Melt curves were acquired to ensure the specificity of amplification, after each real time PCR. PCR products were also confirmed by gel electrophoresis.

The threshold cycle (Ct) values were entered into the Relative Expression Software Tool (REST) and were analyzed for significant differences (P <0.05) by pair wise fixed reallocation randomization test using the following formula (1) for calculating the expression ratio:

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\text{Expression ratio} = \frac{(\text{Eff}}{\text{Ct}_{\text{target}})}{(\text{Eff}}{\text{Ct}_{\text{reference}})}
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effects such as the use of combination therapies with ral mechanisms have developed to overcome these side disease in postmenopausal breast cancer patients. Seve
Developing a resistance to AIs results in a relapse of the life side effects are also often seen with the use of AIs. generally related to estrogen deprivation. The quality of pies for the treatment of breast cancer, patients acquire resistance to AIs that consequently cause a challenge in disease management and facing many problems (23, 24). However, the combination of these compounds did not significantly alter the mean differences of E2 concentration before and after treatment between groups.

CYP 19 gene expression
Expression of CYP19 gene was determined using quantitative real-time PCR. No significant changes in the CYP19 gene expression were observed upon exposure to combination of compounds compared with control (Figure 2).

Discussion
Despite the efficacy of AIs or other endocrine therapies for the treatment of breast cancer, patients acquire resistance to AIs that consequently cause a challenge in disease management and facing many problems (23, 27). Synthetic AIs show serious side effects, which is generally related to estrogen deprivation. The quality of life side effects are also often seen with the use of AIs. Developing a resistance to AIs results in a relapse of the disease in postmenopausal breast cancer patients. Seve
ral mechanisms have developed to overcome these side effects such as the use of combination therapies with AIs and other compounds like PCs (23).

Therefore, therapeutic agents that can be used for a treatment of breast cancer and/or enhance sensitivity to AIs would be of great importance (27). Moreover, combined with a chemopreventive agent can increase clinical efficacy and less severe side effects of chemotherapeutic agents and overcome the problem of resistance in cancer cells (18, 23). Thus, co-chemotherapy may lead to better results (15). HES could improve doxorubicin cytotoxic chemotherapy (28). In combination, nobiletin synergistically increases doxorubicin’s cytotoxic activity (15). Flavonoids in combination with AIs could offer reduced drug toxicity to normal tissue and increased effectiveness of AIs (18, 23).

In the present study, MCF-7 cells were observed to express sufficient aromatase activity based on E2 production from testosterone, which is the substrate for the enzyme in breast cancer tissue. This is in agreement with other reports (6, 29). LET as an AI specifically inhibits aromatase enzyme activity and total body aromatization by 99% (30). Previous studies have also reported the ability of some phytochemicals (PCs) such as biochanin A, chrysin, naringenin, apigenin, genistein and quercetin to inhibit aromatase in placental microsomes and breast cancer tissues (10, 31-34). It has been shown HES is able to inhibit aromatase activity in microsomes (23) and in vivo (35). In our previous study, we revealed that NOB at low concentration decreased aromatase activity (36). Similar to the present studies, a combination of some PCs didn't inhibit aromatase in short incubation time (24). However, the combinational effects of HES, NOB, and LET on aromatase activity have not been studied before. In the present study, the combination of NOB, HES and LET had no effect on aromatase. Consistently, Rice et al showed that the combination of isoflavones and flavones did not inhibit aromatase activity after 48 h exposure, although prolonged exposure for120 h inhibited aromatase activity (24). This long term effect suggests an indirect mechanism. In our experiment, the cells were treated for 48 h. Longer incubation time might have been effective on aromatase activity, however, we looked for a direct effect and therefore did not extend the incubation time.

Figure 1. Aromatase activity as the percentage of control (±SE) after incubation of MCF-7 cells for 48 h with 100 nM testosterone without (control) or with letrozole and nobiletin (LET+NOB); letrozole and hesperetin (LET+HES); letrozole, nobiletin, and hesperetin (LET+NOB+HES); nobiletin and hesperetin (NOB+HES). The results are the mean ± SE of duplicate experiments that were repeated at least three times. * P < 0.05 vs. control.

Figure 2. Relative aromatase expression (±SE) compared with control after incubation of MCF-7 cells for 48 h with 100 nM testosterone without (control) or with letrozole and nobiletin (LET+NOB); letrozole and hesperetin (LET+HES); letrozole, nobiletin, and hesperetin (LET+NOB+HES); nobiletin and hesperetin (NOB+HES). The results are the mean ± SE of duplicate experiments that were repeated at least three times.
In our previous in vitro study, we demonstrated that HES and NOB at low concentration are inhibitors of CYP19 gene expression (36, 37). Inhibition of the CYP 19 expression has also been observed after 48 h treatment of human granulose-luteal cells with a combination of isoflavones and flavones (genistein, daidzein, and biochanin A) (24). Nevertheless, different cell lines and flavonoids may have resulted in dissimilar outcomes compared to ours. In addition, cell culture conditions are also known to influence gene expression levels (34). In the present study, the combination of LET, HES, NOB did not significantly alter aromatase expression; however, changes in the activity of aromatase and its gene expression followed the same trend.

Moreover, we showed that the inhibitory function of LET was compromised in the presence of HES and NOB. Interactions of flavonoids with cytochromes play an important role in drug metabolism. Flavonoids might induce cytochromes and/or modulate their activity (38). Since letrozole is metabolized mainly by the cytochromes P450 (39), its lack of function in the presence of flavonoids may be the result of its augmented metabolism. Thus, these drugs–flavonoid interactions are suggested to be carefully examined in future studies.

Despite the little information on animal and human study, we investigated the effect of achievable concentrations in vivo (9, 35, 40). While most of the experimental studies have been performed with flavonoids concentrations exceeding usual dietary intake. On the other hand, we could not perform aromatase western analyses to determine the effect, but it could be recommended in future works.

To our knowledge, this is the first time to show that HES, NOB and LET in a combination that has no effect on aromatase activity and expression in MCF-7 breast cancer cells. The lack of any significant inhibition of aromatase suggests that continuous exposure may have more profound effects. Further studies including animal models need to be investigated to identify the effect of NOB, HES and LET in a combination on aromatase transcription in breast cancer.

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References
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