ORIGINAL ARTICLE

Exploring the modulation of MLH1 and MSH2 gene expression in hesperetin-treated breast cancer cells (BT-474)

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ABSTRACT

The major mortality factor for women globally is breast cancer, and current treatments have several adverse effects. Hesperetin (HSP) is a flavone that occurs naturally with anti-tumor capabilities and has been investigated as a potential treatment for cancer. This study aimed to investigate the cytotoxic and anti-malignant potential of HSP on breast cancer cells (BT-474) and normal cells (MCF-10a). The results indicated that HSP has dose-dependent cytotoxicity in BT-474 and MCF-10a cells. The elevated concentration of HSP lowered cell viability and proliferation. The half-maximal inhibitory concentration (IC_{50}) of HSP in BT-474 cancer cells after a 48-h exposure was 279.2 μM/ml, while the IC_{50} in normal cells was 855.4 μM/ml. The cytotoxicity of HSP was more significant in cancer cell lines than in normal cell lines and this aspect presents a favorable factor in utilizing the drug for the treatment of breast cancer. The apoptotic effect of HSP in BT-474 cells was investigated, and it was found that the higher the concentration of HSP more the cells underwent apoptosis. Furthermore, the highest concentration of HSP led to overexpression of the MLH1 and MSH2 genes in both breast cancer and normal cell lines. Overall, our study suggests that HSP has an anticancer effect on breast cancer cell lines, and the effect is concentration dependent.

Key words: Apoptosis, breast cancer, cytotoxicity, flow cytometry, half-maximal inhibitory concentration, hesperetin, malignancy, MLH1 gene expression, MSH2 gene expression, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, real-time polymerase chain reaction

INTRODUCTION

Breast cancer is a type of malignant tumor that originates in the cells of the breasts and is characterized by the uncontrolled growth and proliferation of abnormal cells within the breast tissue.[1,2] It is thought that the pathophysiology of breast carcinoma starts in the duct luminal area of the breast, where hyperproliferation occurs and numerous metastatic carcinomas might develop.[3-6] The growth and metastasis of breast cancer are significantly influenced by the stroma, certain cytokines, or macrophages. Breast cancer cells may experience immune escape due to inflammatory cytokines released by macrophages, which can also foster an angiogenic microenvironment. DNA

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methylation has a significant effect on cells and aids in the growth of cancer. Ectopic gene expression have recently been indicated as potential molecular predictors for prognosticating breast cancer patients[1-3] as well as other malignant diseases.[4] Recent research has shown a direct connection between cancer stem cells (CSCs), immune evasion, recurrence, and tumor formation.[7] These CSCs are capable of self-renewal, and stem cell differentiation into progenitors in healthy tissues, and are resistant to standard treatment modalities like radiation and chemotherapy.[8-24]

Hesperidin (HSD) is a type of flavanone that can be found in citrus. Once it is enzymatically hydrolyzed in the gastrointestinal tract, HSD loses its sugar and is converted into hesperetin (HSP).[25] HSP has been the focus of numerous studies in recent years due to its potential effects on cancer pathways. Animal studies have shown that HSP can promote chemically induced colon, bladder, and breast cancers.[9] It has been demonstrated that when exposed to HSP, HER2-positive BT-474 cells undergo greater apoptosis than HER2-negative MD-AMB-231 and MCF-7 cancer cell lines. Western blotting for caspase 3 and caspase 8 activation, which function as crucial apoptosis mediators as executors and initiators of caspases, respectively, revealed that HSP induces apoptosis in the HER2-positive cancer cell line BT-474. Globally, 30% of breast cancer patients have increased HER2 expression, which is linked to cancer aggressiveness and resistance to cell death. Conventional therapy techniques are often ineffective against tumors with the HER2 phenotype. Therefore, it is strongly advised to use targeted chemotherapy and immunotherapy to reduce HER2 expression or stop its kinase activity. However, when the cancer cells gain acquired resistance mechanisms, these treatment methods could subsequently become susceptible. In addition, therapies for HER2-positive tumors may have toxicity problems, including possible side effects for the skin, abdomen, and heart.[10] The aforementioned studies in silico findings show that HSP was capable of establishing long-lasting contact with the ATP-binding region of the tyrosine kinase domain of HER2. Therefore, HSP triggers cellular death and cell cycle interruption in HER2-positive cell lines (e.g. BT-474) via the tyrosine kinase signalling pathway.[11]

Mismatch repair (MMR) is a key biological function that is essential for preserving the integrity and fidelity of the genome. When DNA replication, recombination, or repair occurs, the MMR system detects and corrects DNA mismatches as well as minute insertions or deletions. The proteins that make up the MMR system, including MutSα (MSH2-MSH6), MutSα (MSH2-MSH3), MutL (MLH1-PMS2), and MutL (MLH1-MLH3), work together to detect and correct mismatches.[12] Mismatches and minor insertions or deletions are recognized by the MutSα and MutSα heterodimers, respectively. Small loops and single-base mismatches are recognized by MutSα, whereas larger loops are recognized by MutS. On binding to a mismatch, the MutSα or MutSβ heterodimer recruits the MutLα or MutLβ heterodimer to initiate downstream repair processes. The MutLα complex functions as an endonuclease that nicks the newly synthesized strand near the mismatch, while the MutLβ complex is involved in processing the nicked strand.[13] The stability and function of the MMR system are dependent on the expression and activity of the key proteins MSH2 and MLH1. Mutations or deletions in these genes can lead to genomic instability and cancer predisposition. In particular, loss of MSH2 expression has been associated with increased sensitivity to chemotherapy in some cancer types, while loss of MLH1 expression has been linked to resistance to chemotherapy and drug resistance.[14] It is interesting to note that drug-resistant cell lines and tumors have hypermethylated hMLH1 promoters, which results in MLH1 expression being lost. This highlights the importance of epigenetic regulation in the MMR system and its impact on cancer development and treatment.[15]

In addition, the loss of MMR function increases the frequency of frameshift mutations in DNA, which causes genetic instability at repeat sequences in DNA and results in microsatellite instability (MSI). Multiple cancers, including colorectal, endometrial, and gastric cancers, have been linked to MSI. In general, it is essential to comprehend how MMR proteins, especially MSH2 and MLH1, operate and are regulated in order to design efficient cancer therapies and enhance patient outcomes. The processes governing the regulation of MMR proteins and their effect on the development and therapy of cancer require more study.[16]

The study's goal was to find out how HSP affected various breast cancer cell lines, especially HER2-positive cell lines, which are believed to be the most aggressive form of the disease.

MATERIALS AND METHODS

BT-474 and MCF-10a cell lines were gifted by Pasteur Institute, Baghdad, Iraq. Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin (P/S), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, fetal bovine serum (FBS) were purchased from Gibco, USA. Real-time polymerase chain reaction (RT-PCR) strip was purchased from Gunster Biotech, Taiwan.

Cell culture

The current study used two different breast cancer cell lines, BT-474 and MCF-10a. The cell lines were kept alive in DMEM culture medium with 10% FBS and 1% antibiotic supplement (P/S). A volume of 5 mL of fresh culture medium was added after administration, and the cells' culture medium was removed. The cells were then put

into an incubator with the settings of 37°C and 5% CO_2. The density of the culture flasks was monitored during the week, and cell passage was carried out as necessary.[13]

Cell passage

When the cells in each flask achieved 80% density, cell passage was performed. The cells were examined for infection, morphology, and growth rate before transmission. After the growth media was discarded, the cells were rinsed with 5 mL of phosphate buffer saline (PBS) to get rid of any dead cells. The final step was to remove the remaining live cells from the culture flask using a trypsin-EDTA solution that was administered and settled for 3–5 min. Before being seeded into new flasks at a specific density, the cells were centrifuged, resuspended in fresh media, and the solution was neutralized with a new growth medium.[17] The cell density was calculated using the mathematical procedure shown below:

Cell density (cells/mL) = Total number of cells/volume of medium used for counting (mL)

Cell backup and cryopreservation

After applying 1 mL of trypsin to detach the cells from the culture flask's bottom and removing the supernatant, the cells were prepared for cryopreservation. The supernatant was then eliminated after the cells were rinsed with 5 mL of PBS. The cell plate was reconstituted in 1 mL of the culture medium DMEM. 90% FBS, 10% DMSO, and 2 × 106 cancer cells, 2 cells were added to the cryovial using a neobar slide for cell counting. The temperatures for cryovials were − 20°C for 2 h, −80°C for 24 h, and − 196°C during transfer to nitrogen tanks.[18]

Treatment and cell culture

To study how HSP affects each cancer cell line, the two cancer cell lines were kept in DMEM media with 10% FBS and 1% P/S. Trypan Blue and Lan dye hemocytometers were used to collect and count the cells once they had reached the desired condition. The cells were then given various treatments and incubated for the desired amount of time at 37°C with 5% CO_2. Cells from passage three were utilized in all studies, and 2% FBS was given to the cells to mimic starving.[19]

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability test

Cellular health was evaluated by the MTT test, a colorimetric technique based on the conversion of yellow tetrazolium salt into purple formazan crystals by metabolically active cells. A 96-well plate with 104 cells was seeded with 200 L of DMEM media with 10% FBS and various doses of HSP (100, 200, 400, 600, 800, and 1000 μM/ml) for 48 h at 37°C with 5% CO_2 to perform the MTT test. After 3 h of incubation, the surface culture media was removed from each well and 150 L of MTT solution (0.5 mg/mL) was added. The formazan precipitate was then dissolved using 100 L of DMSO after the MTT solution was withdrawn. A spectrophotometer was used to measure the solution's absorbance at 570 and 630 nm.[20,21] In order to determine cell viability, use the following formula:

Cell viability = (Intensity of control absorption/Intensity of sample absorption) × 100

Real-time polymerase chain reaction

The effects of different HSP concentrations on the expression of the MLH1 and MSH2 genes in BT-474 and MCF-10-A cell lines were assessed by RT-PCR. For this, first, RNA extraction was done in the HSP-treated cell lines, and then reverse transcription was performed to convert the extracted RNA to complementary DNA. The RT-PCR reaction mixture was prepared, containing the cDNA template, gene-specific primers for MLH1 and MSH2 genes, and the PCR reagents. The RT-PCR reaction was carried out through a series of temperature cycles, including denaturation, annealing, and extension, to amplify the MLH1 and MSH2 gene regions of interest. During the PCR amplification, real-time monitoring of the fluorescence signal was performed using a specialized instrument that detects and records the fluorescence emitted by the DNA-binding dye or probe. The obtained RT-PCR data were analyzed using software to determine the gene expression levels relative to reference genes or controls. Statistical analysis was performed to assess the significance of any observed differences.[22,23]

Flow cytometry

For flow cytometry analysis, HSP-treated BT474 cells were harvested using appropriate methods to maintain their integrity. Subsequently, they were washed with a buffer solution to remove any residual media or extracellular components. Annexin V and propidium iodide (PI) staining solutions were prepared according to the manufacturer's instructions. These solutions contained fluorescently labeled annexin V to detect apoptotic cells and PI reagent to assess cell viability. The harvested and washed BT474 cells were then incubated in the staining solutions. After the incubation period, the stained cells were diluted in a buffer solution to ensure a suitable concentration for flow cytometry analysis. A flow cytometer equipped with appropriate lasers and detectors was set up for analysis. The stained cell suspension was loaded into the flow cytometer, and data acquisition was performed by analyzing each individual cell for its fluorescence signals. The instrument captured and recorded data on the annexin V and PI fluorescence intensity for each cell.[24]

RESULTS AND DISCUSSION

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability test

For both the BT-474 and MCF-10-A cell lines, a cell

proliferation test was performed using the MTT uptake technique based on the Mossman methodology. Results demonstrated the viability and proliferation of both cell lines were inversely related to the concentration of HSP. The higher the concentration of HSP, the lower the cell viability and proliferation were observed. The MTT assay showed that HSP exhibited dose-dependent cytotoxicity in both BT-474 and MCF-10a cells. The half-maximal inhibitory concentration (IC_{50}) of HSP was 279.2 µM/ml for the cancer cell line BT-474, while the IC_{50} for the normal cell line MCF-10-A was 855.4 µM/ml after 48 h of exposure to HSP. The viability of dead effector cells decreased with the increase in HSP dose. HSP was able to inhibit the proliferation of the BT-549 cell line within 48 h at different IC_{50} concentrations. The effect of HSP on cancer cell lines was greater than on normal cell lines. The study demonstrated that HSP exhibits dose-dependent cytotoxicity in both cell lines. The IC_{50} of HSP for BT-474 was significantly lower than that for MCF-10-A. Our results indicate that HSP possesses capabilities to be utilized as anti-cancer agent for the management of breast cancer, with minimal effects on normal cells[25] [Figure 1].

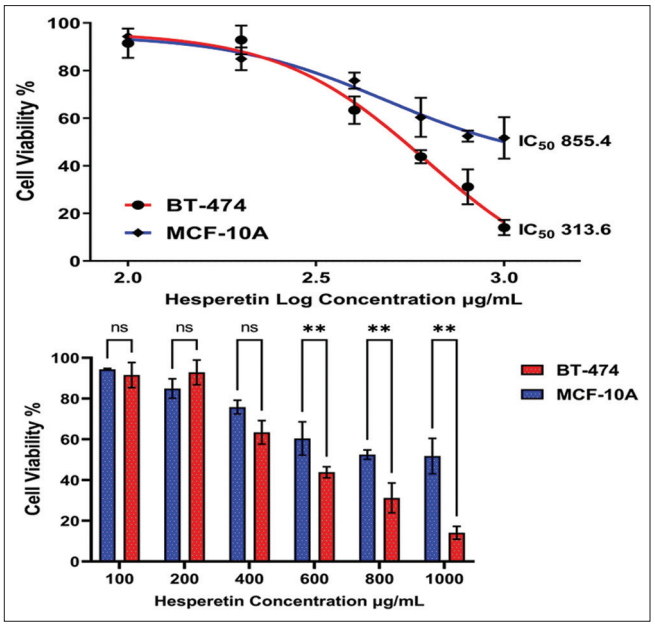

Figure 1: Graphical depiction of cell viability for MSF10-a and BT-549 cell lines treated with hesperetin. IC_{50}: Half-maximal inhibitory concentration. **Represents correlation is significant at the 0.01 *P* value.

Real-time polymerase chain reaction assay

Our findings demonstrated that the MLH1 gene was overexpressed in both cell lines at the highest concentration of HSP. However, only the BT-474 cell line showed a concentration-dependent effect of HSP on MLH1 gene expression, and this effect was comparable to that seen in the MCF-10-A cell line. The statistical analysis's *P* values revealed that there was no discernible change in the expression of the MLH1 gene between the control group and the HSP100 group ($P = 0.667$). The MLH1 gene expression did, however, change significantly between the control group and the HSP200 group ($P = 0.028$*) and significantly ($P = 0.001$**) between the control group and the HSP400 group. Overall, results imply that the expression of the MLH1 gene in the BT-474 cell line was influenced by HSP in a concentration-dependent manner. At higher HSP concentrations (200 and 400 µM), the impact was noticeable, but not at the lowest concentration (100 µM). The use of HSP as a possible therapeutic agent in the treatment of breast cancer may be significantly impacted by these findings[26] [Figure 2].

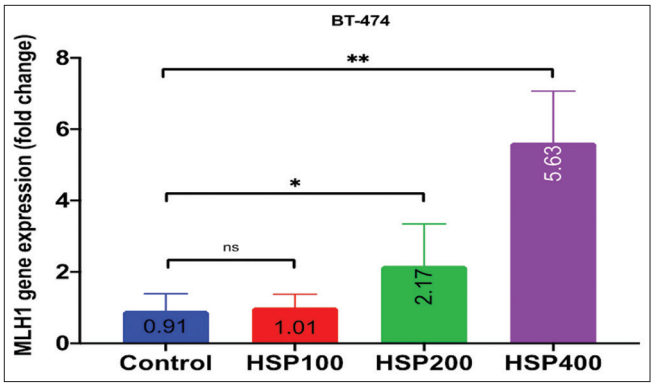

Figure 2: Concentration-dependent effect of hesperetin on MLH1 gene expression in the breast cancer (BT-474) cell lines. HSP: Hesperetin

For the BT-474 cell line, the median expression level of the MSH2 gene was found to increase with increasing concentrations of HSP. Statistical analysis revealed that the differences between the median values of the control group and the HSP-treated groups were significant for HSP200 and HSP400 ($P = 0.012$ and $P = 0.006$, respectively). The HSP100 group and the control group, however, showed no discernible difference ($P = 0.703$). These results imply that the expression of the MSH2 gene in the BT-474 cell line was influenced by HSP in a concentration-associated response. In contrast, there are no appreciable variations in

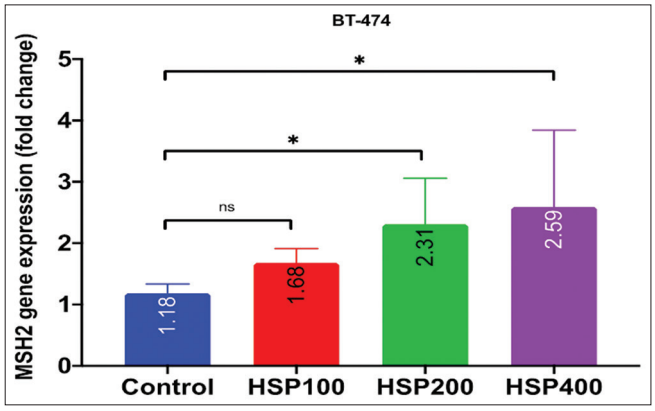

Figure 3: Concentration-dependent effect of hesperetin on MSH2 gene expression in the breast cancer (BT-474) cell line. HSP: Hesperetin. *Represents correlation is significant at the 0.05 *P* value.

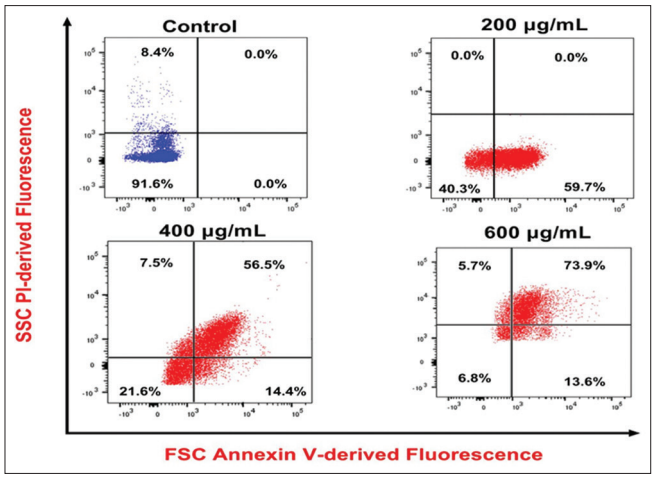

Figure 4: Flow-cytometric analysis of the apoptotic effect of hesperetin on the breast cancer (BT-474) cell line based on concentration-depended manner. PI: Propidium iodide, SSC: Side scatter, FSC: Forward scatter

the median levels of the MSH2 gene expression between the control group and any of the HSP-treated groups for the MCF-10-A cell line (*P* values all > 0.05). All of the groups' median expression levels are close to one and comparable.

The development of innovative therapeutic approaches for breast cancer may be significantly impacted by the increase of MSH2 gene expression in response to HSP therapy. The underlying molecular processes behind this impact of HSP on MSH2 gene expression need more study[27] [Figure 3].

Flow-cytometric analysis

Flow-cytometric analysis, as shown in Figure 4, revealed that treatment with HSP at a concentration of 200 µg/mL significantly induced an early apoptotic effect in BT474 cells by raising the population to 59.7% compared to untreated cells, which showed no signs of apoptosis or necrosis. Increasing the concentration of HSP to 400 µg/mL shifted the cell population to the apoptotic phase by 56.5%. Whereas at 600 µg/mL, most BT474 cells, i.e. 73.9% underwent apoptosis, with an extreme reduction in cell viability down to only 6.8%. These findings suggest that HSP has a concentration-dependent apoptotic effect on BT474 cells.[28]

CONCLUSIONS

The impact of HSP on the breast cancer cell line BT-474 was examined. The findings of the research indicated that the tumor suppressor gene, which may control the progression of the cancer cell cycle, was overexpressed when HSP was present. The findings also suggest that HSP had the capacity to activate both intrinsic and extrinsic pathways associated with apoptosis, the process by which cancerous cells are eliminated. In addition, by inhibiting specific growth factors associated with tumors, HSP may prevent metastasis.

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Conflicts of interest

There are no conflicts of interest.

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