Research Article

Wogonin from *Scutellaria baicalensis*-Induced Radioresistance in MCF-7 Breast Cancer Cell Line

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Abstract

Objectives: Breast cancer shows the highest incidence and cancer-related deaths among women worldwide. Radiotherapy is used for treating different stages of breast cancer. Several polyphenols have increased the effectiveness of radiotherapy. Wogonin is a flavone compound abundant in the root of Chinese skullcap. This compound induced apoptosis and inhibited proliferation distinctively in cancer cells. We studied effect of wogonin on the response of a typical breast cancer cell line to ionizing radiation.

Methods: MCF-7 cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell counting and double staining apoptosis assays were also used to find suitable physiologically relevant concentrations of wogonin. Cells treated with wogonin were exposed to different doses of X-ray and clonogenic survival assay was utilized to determine the effect of wogonin on survival from radiation.

Results: Wogonin treatment decreased the viability of MCF-7 cells in a dose- and time-dependent manner. It decreased number of cells and increased percent of apoptotic cells dose dependently. Cells pretreated with 5 and 10 µM concentrations of wogonin 3 days before radiation showed increased radioresistance compared with cells that were not treated with wogonin.

Conclusion: Treatment of MCF-7 breast cancer cells with wogonin made them more resistant to ionizing radiation.

Keywords: Breast cancer, Flavonoid, MCF-7, Radioresistance, Radiotherapy, Wogonin

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B reast cancer is the most common cancer and also the first cause of cancer deaths among women worldwide with about 2.1 million incidence and 626,679 deaths in 2018.[1] Radiotherapy is a therapeutic modality used for more than 50% of breast cancer patients and is beneficial in preventing locoregional recurrence after surgery.[2] However, radioresistance is a major obstacle to radiotherapy effectiveness and causes tumor recurrence in some patients.[3] A method for overcoming this obstacle is using synthetic or natural compounds in combination with radiotherapy.[4] Among natural compounds, several plant flavonoids pref-

erentially sensitized cancer cells to radiation by targeting pathways involved in radioresistance.[5]

Wogonin (5,7-dihydroxy-8-methoxyflavone) is an O-methylated flavone from the flavonoid category of polyphenols. This compound is derived from the root of *Scutellaria baicalensis* or Chinese skullcap that was used as a traditional Chinese medicine in curing hepatitis, diarrhea, infections, inflammation, hypertension, cardiovascular disease, neurodegeneration, and tumors.[6] Wogonin showed anti-inflammatory, antioxidant, antiviral, and anxiolytic effects.[7] This compound also had anticancer effects as found by nu-

merous studies. This antitumor effect included inhibition of proliferation, invasion, and angiogenesis, as well as induction of apoptosis, autophagy, cell cycle arrest, and differentiation in various carcinoma, sarcoma, hematologic, and nervous system cancer cell lines as well as decrease of tumor size and inhibition of metastasis in mouse models.[8,9] Interestingly, this compound had little or no toxic effects in normal cells and tissues.[10,11] The differential effect of this compound in normal and cancer cells would be of great value in cancer therapeutic strategies regarding its low side effects.

Due to high usage of radiotherapy in different stages of breast cancer, the raidiosensitizing effect of different polyphenols, anticancer effects of wogonin in breast cancer cell lines,[12-14] and its differential effects in normal and cancer cells, we studied the effect of this flavonoid on response of MCF-7 breast cancer cell line to radiotherapy. Pharmacokinetics studies in rats and Beagle dogs indicated that wogonin plasma concentration was <10 µM, 20 min and 4 h after intravenous injection, respectively,[15,16] and was less than 1 µM after intragastric administration.[15,17] To omit the toxic effect of wogonin treatment from the effect of wogonin on the response of cells to radiation, and to use physiologically relevant wogonin concentrations, the3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay, cell counting, and fluorescence microscopic apoptosis assay were utilized. We performed clonogenic survival assay on cells treated with 5 or 10 µM concentrations of wogonin for 72 h and, then, exposed to 2–8 Gy doses of ionizing radiation. To the best of our knowledge, this is the first study on the effect of wogonin on response of cancer cells to ionizing radiation.

Methods

Materials

RPMI-1640 and fetal bovine serum (FBS) were obtained from Gibco (USA). Penicillin/streptomycin solution was purchased from Bioidea (Iran). Wogonin was gained from AdooQ Bioscience (USA). Ethylenediaminetetraacetic acid (EDTA) and trypan blue were acquired from Bio Basic (Canada) and INOCLON (Iran), respectively. Formaldehyde and crystal violet were purchased from Merck (Germany). Other chemicals were obtained from Sigma-Aldrich (Germany). Cell culture flasks and plates were gained from JET Biofil (China) and Iwaki (Japan).

Cell Culture and Treatment

MCF-7 breast cancer cell line was obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Iran). Cells were cultured in RPMI-1640 medium containing10% FBS supplemented with penicillin (100 unit/mL) and streptomycin (100 µg/mL) antibiotics. Cells were incubated at 37 °C and 5% CO_2 in a 95% humidified incubator (Memmert, Germany). Cells were passaged at 80–90% confluence using trypsin (0.5% w/v) and EDTA (2 mM) in phosphate-buffered saline (PBS) solution.

For treatments, cells were cultured at 10^4 cells/cm^2 density. After 2 days of incubation, at the beginning of logarithmic phase of cell growth, cells were treated with different concentrations of wogonin and incubated for 24, 48, or 72 h. Wogonin was dissolved in 0.1% (v/v) dimethyl sulfoxide (DMSO).

MTT Cell Viability Assay

MCF-7 cells were seeded in 96-well plates at 3200 cells/well density,treated with medium (control), DMSO (0.1% v/v), or wogonin (1, 2, 5, 10, 20, 50, or 100 µM) after 2 days, and incubated for 24, 48, or 72 h. Cell viability assay was performed as described previously[18] with some modifications. Briefly, the medium was aspirated and 10 µL of MTT solution (5 mg/mL) was added to each well. After 2 h of incubation, MTT solution was removed and formazan crystals were dissolved in 100 µL DMSO. The absorbance was determined by Elisa reader (BioTek, USA) and normalized to the absorbance of control group.

Cell Counting

MCF-7 cells were cultured in a 96-well plate at 3200 cells/well density. On the 2nd day, the medium was changed with medium (control), DMSO (0.1% v/v), or medium containing a specific concentration of wogonin (1, 2, 5, 10, 20, 50, or 100 µM). After 72 h of treatment, cells were trypsinized and cells of two similar wells were mixed and counted by a hemocytometer (Marienfeld, Germany) under an inverted microscope (micros, Austria).

Apoptosis Assay

Acridine orange/ethidium bromide (AO/EB) double staining was used to detect apoptotic cells.[19] MCF-7 cells were cultured in a 96-well plate at 3200 cells/well density. After 2 days, cells were treated with medium (control), DMSO (0.1% v/v), or wogonin (1, 2, 5, 10, 20, 50, or 100 µM) and incubated for additional 72 h. After collecting floating and adherent cells by trypsinization and centrifugation, cells were suspended in PBS. 25 µL of the cell suspension was mixed with 1 µL of AO/EB solution (100 µg of each dye/ml) and observed under fluorescent microscope (OPTIKA, Italy) connected to a CCD camera. Percentages of viable, early apoptotic, late apoptotic, and necrotic cells were determined. The apoptotic cells were characterized by a condensed or fragmented chromatin structure. The acridine

orange dye made viable and early apoptotic cells green, and ethidium bromide could penetrate late apoptotic and necrotic cells and made them orange.

Clonogenic Assay

For radiation experiments, MCF-7 cells were seeded in five 24-well plates at 19000 cells/well density. Two days after seeding, cells of each plate were treated with DMSO (0.1% v/v), or wogonin (5, 10, or 50 μM). After 72 h, the medium was replaced with fresh medium. Plates were held on ice to Mehraneh Radiotherapy Center (Zanjan, Iran) where radiation exposure was performed by a Siemens Primus linear accelerator X-ray machine (Siemens AG, Erlangen, Germany) with 6 MV X-ray at 300 monitor unit/min dose rate. Plates radiated with either 0, 2, 4, 6, or 8 Gy dose of X-ray were brought back to lab on ice. To study the effect of wogonin on clonogenicity, MCF-7 cells were seeded in a 96-well plate at 3200 cells/well density. After 2 days, cells were treated with medium (control), DMSO (0.1% v/v), or wogonin (5, 10, 20, 50, or 100 μM) and incubated for additional 72 h.

Treated cells were trypsinized and suitable numbers of cells were seeded on wells of 6-well plates in triplicate. After 7 days of incubation, colonies were formed. Colonies were washed with PBS, fixed with formaldehyde (2% v/v) for 35 min, and then stained with crystal violet (0.5% w/v) for 2 h. After washing with distilled water, colonies that contained more than 50 cells were counted under an inverted microscope (micros, Austria). Plating efficiency (PE) was calculated for each well by the following formula: PE (%)=([Number of colonies]/[Number of seeded cells])×100. Surviving fraction (SF) at each radiation dose was calculated as follows: SF=PE/PE of control. In this equation, control was related to the corresponding unirradiated group.[20]

Statistical Analysis

The Student's t-test was performed by Microsoft Excel for comparing wogonin-treated with DMSO-treated cells in MTT, cell counting, and clonogenic assays. A p-value smaller than 0.05 was considered statistically significant. Results were represented as mean±standard error of mean (SEM) or mean±standard deviation (SD).

Results

To find suitable wogonin concentration for radiation experiments, the MTT, cell counting, and apoptosis assays were performed.

The Effect of Wogonin on Viability of MCF-7

MTT assay was performed on MCF-7 cells treated with increasing concentrations of wogonin for 24, 48, and 72 h (Fig. 1). Wogonin affected MCF-7 cells in a dose-and
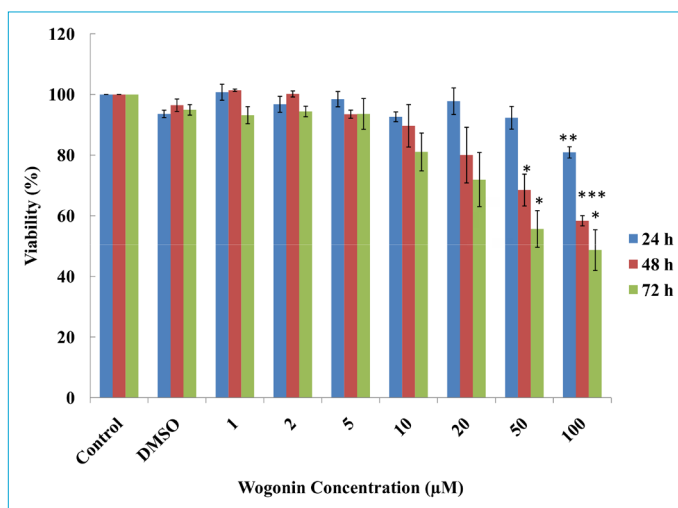

Figure 1. The effect of wogonin on cell viability. Cells in logarithmic phase of cell growth were treated with increasing concentrations of wogonin for 24, 48, and 72 h. MTT assay was performed on treated cells. Data points indicate averages of three independent experiments ± SEM. The significance level, determined by comparison of wogonin-treated with DMSO-treated cells, is represented by *, **, or *** for p<0.05, p<0.01, or p<0.001, respectively.

time-dependent manner. Wogonin treatment for 24 h could significantly reduce cell viability at 100 μM concentration. However 48-and 72-h treatments reduced cell viability from 20 to 10 μM concentrations, respectively. These reductions were significant at 50μM concentration. The half maximal inhibitory concentration was about 140 and 85 μM for 48 and 72 h treatment, respectively. Because the effect of wogonin on MCF-7 cell line was time-dependent, we chose 72 h treatment for further experiments. In this manner, we could use lower and, therefore, more physiologically relevant concentrations of wogonin.

The Effect of Wogonin on MCF-7 Cell Number

To determine whether the observed decrease in absorbance in MTT assay in higher concentrations of wogonin was due to decrease in cell number or decrease in mitochondrial metabolic activity, we counted cells after 3 days of incubation with different concentrations of wogonin (Fig. 2).The treatment with wogonin caused a concentration-dependent decrease in cell number in concentrations higher than 2 μM. This decrease was significant at 20, 50, and 100 μM of wogonin.

The Effect of Wogonin on MCF-7 Apoptosis

We also performed AO/EB apoptosis assay on cells treated with different concentrations of wogonin for 72 h (Fig. 3). Wogonin treatment decreased percent of viable cells in a dose-dependent manner. Early apoptosis was induced even by low doses (1–10 μM) of wogonin. The amount of
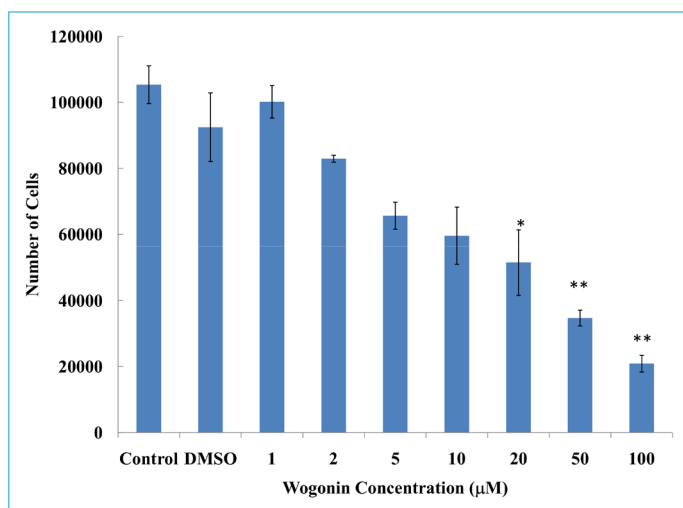
Figure 2. Wogonin decreased number of MCF-7 cells. MCF-7 cells were treated with different concentrations of wogonin. After 72 h, cells were counted. Error bars indicate SEM of four independent experiments. Wogonin-treated cells were compared with DMSO-treated ones; * or ** indicate p<0.05 or p<0.01, respectively.

Figure 3. Wogonin induced apoptosis in MCF-7 cell line. Cells were treated for 72 h with different concentrations of wogonin. AO/EB apoptosis assay was used to determine number of viable, apoptotic (in early and late stages), and necrotic cells. At least 120 cells and on average 264 cells were analyzed for each treatment..

apoptosis at 50 and 100 μM concentration was obviously higher than that at lower doses. In these concentrations, more than 50% of cells were apoptotic. Increase in necrosis was observed in cells treated with 20 μM, 50 μM, and especially with 100 μM concentration of wogonin.

According to MTT, cell counting, and apoptosis assays for 72 h wogonin treatments, cell viability was decreased by wogonin concentrations higher than 10 μM (Fig. 1), cell number was decreased from 5 μM concentrations (Fig. 2), late apoptosis increased from 10 μM concentration and necrosis was low in concentrations less than 20 μM (Fig. 3). Therefore, we chose 5 and 10 μM wogonin as concentrations that started decreasing MCF-7 cell viability.

The Effect of Wogonin on Clonogenic Survival of MCF-7 after Radiation

To determine the effect of wogonin on sensitivity of MCF-7 cells to ionizing radiation, cells were treated with 5 and 10 μM concentrations of wogonin for 72 h and, then, exposed to increasing doses of X radiation. These concentrations were the wogonin concentrations, in which viability and cell number started to decrease (Figs. 1 and 2), and late apoptosis started to increase (Fig. 3). Clonogenic assay was performed for wogonin-treated and control (DMSO-treated) groups and surviving fractions were calculated (Fig. 4). The treatment with 5 μM concentration of wogonin increased surviving fraction in 2, 4, 6, and 8 Gy doses of ionizing radiation and 10 μM concentration of wogonin increased survival of cells at 6 and 8 Gy IR doses. In other words, low doses of wogonin caused radioresistance, especially at higher doses of X-ray.
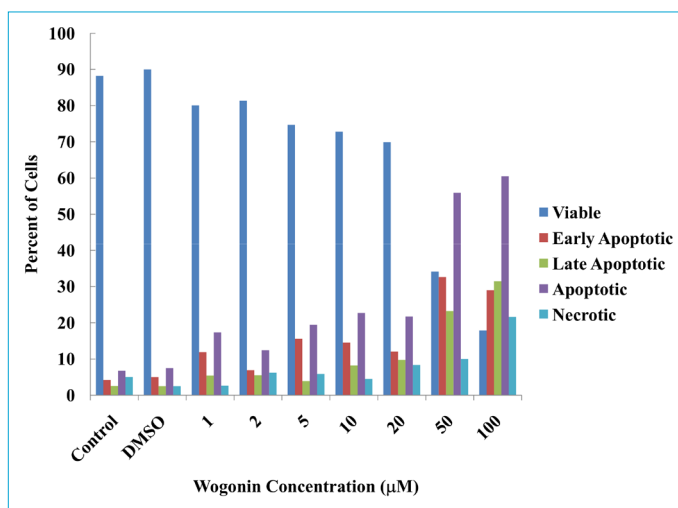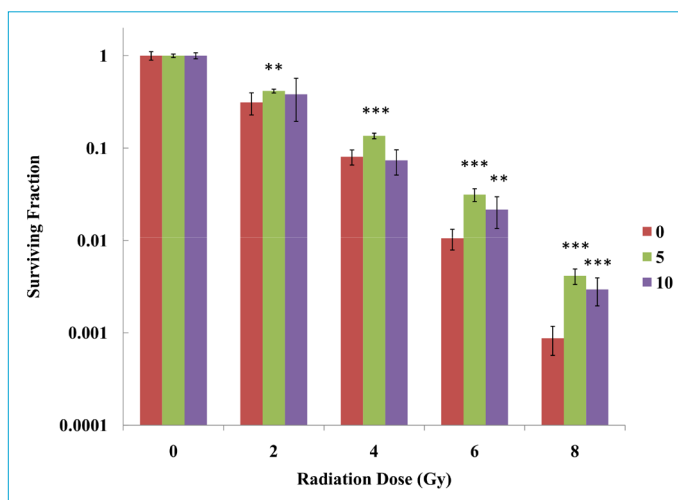

Figure 4. Low concentrations of wogonin increased the radioresistance of MCF-7 cell line. MCF-7 cells were treated with different concentrations of wogonin (0, 5, and 10 μM) for 72 h. Treated cells were exposed to increasing doses of ionizing radiation. Clonogenic assay was performed afterward and surviving fractions were calculated. Error bars indicate SD of data in two independent experiments. In each radiation dose, wogonin-treated cells were compared with the corresponding DMSO-treated control group. ** and *** indicate p<0.01 and p<0.001, respectively.

Discussion

The observed effect of wogonin on the viability of MCF-7 cells (Fig. 1) was consistent with the previous observations. [14,21,22] Cell counting indicated that wogonin treatment for 72 h decreased number of cells in a dose-dependent manner (Fig. 2). This observation implied the cytotoxic or cyto-

static effect of wogonin on MCF-7 breast cancer cell line. Apoptosis assay showed that wogonin induced apoptosis within 72 h, especially at 50 and 100µM concentrations (Fig. 3). Apoptosis induction by treatment of MCF-7 cells with 60 µM or 50 µM wogonin for 24 h or 48 h, respectively, was observed in the previous studies.[14,23]

Wogonin might also have had a cytostatic effect. The treatment of MCF-7 cells with wogonin increased percentage of cells in G0/G1 phase and decreased percent of cells in S phase of cell cycle.[14,22] On the other hand, Tao et al. showed that treatment of MCF-7 cells with wogonin decreased percent of cells in G0/G1 and increased that in G2/M phase of the cell cycle.[21]

We studied whether pretreatment of MCF-7 breast cancer cells with wogonin could affect clonogenicity after exposure to ionizing radiation (Fig. 4). The treatment of MCF-7 cells with 10 and especially 5 µM concentrations of wogonin before radiation increased resistance of the typical ER-positive MCF-7 breast cancer cells. Therefore, we recommend caution in using this compound together with radiotherapy.

It seemed that cells that survived wogonin treatment showed less amount of mitotic or apoptotic death after exposure to ionizing radiation. The treatment of MCF-7 cells with low concentrations of wogonin (5 and 10 µM) might have activated pathways involved in surviving toxic effects of X-ray. Evidence exists for a little hydroxyl radical scavenging property of wogonin.[24] 10 µM concentration of wogonin could increase viability of SH-SY5Y neuroblastoma cells in response to H_2O_2 treatment.[24,25] However, the treatment with high concentrations of wogonin (150 µM for 24 h[21] and 150 and 200 µM for 48 h[23]) increased the level of ROS in MCF-7 cells. The effect of wogonin on the level of free radicals might be concentration-dependent.

On the other hand, it was shown that the colony formation of cultured murine mammary epithelial cells did not decrease during the early stages of p53-induced apoptosis. There was a nearly apoptosis reversibility that occurred through increase in DNA repair activity in the early apoptotic cells. This DNA repair activity decreased during the late stages of p53-induced apoptosis.[26] Wogonin increased the level of p53 protein in MCF-7 breast cancer cell line.[14] Therefore, it is possible that the early apoptosis induced by low concentrations of wogonin (Fig. 3) has potentiated the DNA repair machinery which increased the radioresistance of MCF-7 cells treated with 5 and 10 µM concentrations of wogonin (Fig. 4).

Several flavonoids showed a radiosensitizing activity. The treatment of tumor cell lines with some flavonoids before exposure to radiation and seeding decreased their clono-

genicity compared with control group only exposed to IR. Quercetin sensitized ovarian cancer cells by suppressing the proteins involved in repair of double-strand breaks (DSBs).[27] Pre-treatment of breast cancer cell lines with genistein sensitized them to radiation through decreasing repair, increasing the amount of DSBs, inducing G2/M cell cycle arrest, and increasing apoptosis.[28] Genistein increased the level of radiation-induced ROS and apoptosis in non-small cell lung cancer.[29] There was an increasing DNA damage and apoptosis in equol-treated breast cancer cell lines with respect to control group after exposure to ionizing radiation.[30] Therefore, in the present study, pretreatment of MCF-7 cells with wogonin probably could not increase radiation-induced apoptosis and DNA damage. Comparison of the effect of different flavonoids on radiosensitivity of cancer cells would provide us with clues for designing novel efficient synthetic radiosensitizers.

To the best of our knowledge, this is the first study in which the effect of wogonin was investigated on clonogenic survival of irradiated cancer cells. The treatment of the typical ER-positive MCF-7 breast cancer cells with low concentrations of wogonin increased their radioresistance. We recommend caution in using wogonin and radiotherapy simultaneously. Besides, the present study may provide structural clues in designing synthetic radiosensitizers.

Disclosures

Ethics Committee Approval: No animal/human sample was used in this study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors have no conflicts of interest to declare.

Authorship Contributions: Concept – M.R.; Design – M.R.; Supervision – M.R.; Materials – M.R.; Data collection &/or processing – A.H.D.; Analysis and/or interpretation – M.R., A.H.D.; Literature search – M.R., A.H.D.; Writing – M.R.; Critical review – M.R.

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