

Genistein induces apoptosis and autophagy in human breast MCF-7 cells by modulating the expression of proapoptotic factors and oxidative stress enzymes

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Abstract Breast cancer is one of the common tumors occurring in woman and despite treatment, the prognostic is poor. Genistein, a soy isoflavone, has been reported to have chemopreventive\chemotherapeutic potential in multiple tumor types. Here, we investigated the genistein antiproliferative effect in MCF-7 breast cancer, underlying the molecular mechanisms involved in this effect. MCF-7 cancer and CCD1059sK fibroblast cells were treated with estradiol (10 nM) or genistein (0.01–100 μ M) for 24, 48, and 72 h and the cell proliferation was investigated by MTT; membrane cell permeability was evaluated by LDH and PI incorporation; apoptosis was investigated by

externalization of phosphatidylserine by FACS; and presence of autophagy was detected by LC3A/B immunostaining. The expression of apoptotic proteins and antioxidant enzymes was evaluated by qPCR. The results demonstrate that genistein (100 μ M) for 72 h of treatment selectively reduced MCF-7 cell proliferation independent of estrogen receptor activation, while no cytotoxicity was observed in fibroblast cells. Further experiments showed that genistein induced phosphatidylserine externalization and LC3A/B immunopositivity in MCF-7 cells, indicating apoptosis and autophagy cell death. Genistein increased in three times proapoptotic BAX/Bcl-2 ratio and promoted a parallel downregulation of 20 times of antiapoptotic survivin. In addition, genistein promoted a decrease of 5.5, 9.3, and 3.6 times of MnSOD, CuZnSOD, and TrxR mRNA expression, respectively, while the GPx expression was increased by 6.5 times. These results suggest that the antitumor effect of genistein involved the modulation of antioxidant enzyme and apoptotic signaling expression, which resulted in apoptosis and progression of autophagy.

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Introduction

Breast cancer is one of the common tumors occurring in woman, characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung, and liver [1]. Therapy to breast cancer includes surgery, radiotherapy, and chemotherapy [2]. Despite treatment, the majority of breast cancer is incurable and ultimately claims the life of the patient with complications and development of chemoresistance [3]. Therefore, understanding the

molecular mechanism of breast carcinoma progression and the identification of new therapeutic strategies is important for an effective treatment.

Soybeans products have been used as an alternative hormone replacement to overcome the undesired effects of menopause [4]. The association between the lower risk of cardiovascular/neurodegenerative diseases and cancer and the consumption of soy products had led to the widespread interest in isoflavone supplements and foods to which isoflavones have been added [5]. Genistein (4',5,7-trihydroxyisoflavone), a major isoflavone constituent of soybeans and soy products, has been reported to have beneficial effects to human health. Genistein is similar to the primary structure of 17- β -estradiol, exhibiting both estrogenic agonist and weak antagonist activities, which modulate estrogen-regulated gene expression [6]. Moreover, genistein can exert a variety of biological effects in an estrogen receptor independent way, including the alteration of estrogen metabolism via aromatase inhibition [7]. The metabolism of genistein includes absorption from the gastrointestinal tract and elimination in urine. Urinary levels are higher in people like the Japanese and western vegetarians, who eat soy or other vegetables [8]. Significant amounts of genistein in human blood [8] and accumulation of this molecule in breast tissue and in milk also had been shown [9, 10].

The chemopreventive and chemotherapeutic potential of genistein has been described in multiple tumor types [11]. Genistein exerts its anticancer properties through several mechanisms, which include induction of apoptosis, cell cycle arrest, and inhibition of angiogenesis [12, 13]. This isoflavone also promotes the inhibition of protein tyrosine kinase by competing with ATP for binding to tyrosine kinase domain and thereby, decrease the cancer cell proliferation by interferes with tyrosine kinase cascade activated by mitogens [14, 15]. In ovarian cancer cells, genistein not only initiated apoptosis, but also induced autophagy [16]. Autophagy is mediated by double- or multi-membrane autophagosomes and, in addition to its basic role in the turnover of proteins and organelles, this process has multiple pathophysiological functions including cell differentiation, immune defense, and cell death [17, 18]. Although genistein anticancer properties have been investigated in several cell types, little is known with regard to its ability to induce autophagy in breast cancer cells.

The present study examined the molecular mechanism involved in the antiproliferative effect of genistein in human MCF-7 breast cancer cell line. Our data show that genistein decreased the MCF-7 cell proliferation and modulated expression of genes related to antioxidant enzymes and apoptosis; it induced cell death by apoptosis and progression of autophagy. Taken together, these results

suggest genistein as a potential strategy in cancer prevention and treatment.

Materials and methods

Cell culture and 17 β -estradiol (E2) or genistein treatments

Human breast adenocarcinoma (MCF-7) and human fibroblast (CCD1059sK) cell lines were obtained from American Type Culture Collection (ATCC, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; Gibco BRL, USA) at 37 °C in a humid atmosphere containing 5 % CO₂. E2 and genistein (Sigma Chemical Co., USA) were dissolved in DMSO at concentration of 10 and 100 mM, respectively, and further diluted in DMEM/10 % FBS to obtain 10 nM, final E2 concentration and 0.01, 0.1, 1, 10, or 100 μ M, final genistein concentrations. Appropriate controls containing 0.5 % DMSO were performed.

MTT cell viability and lactate dehydrogenase assays (LDH)

MCF-7 or CCD1059sK cells were subcultured into 96-well plates (5×10^3 cells/well) and exposed to E2 (10 nM) or genistein (0.01–100 μ M) for 24, 48, and 72 h of treatment. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical Co., USA) assay was performed following manufacturer's instructions. LDH activity was measured in the supernatants using in vitro toxicology assay kit (Labtest, BR) in accordance with the manufacturer's instructions.

Propidium iodide assay

MCF-7 cells were subcultured into 24-well plates (20×10^3 cells/well) and were treated with E2 (10 nM) or genistein (10, 100 μ M) for 72 h. Then, cells were incubated with propidium iodide (PI) (7.5 μ M) for 1 h. PI fluorescence was excited at 515–560 nm using an inverted microscope (Olympus IX71, Japan) fitted with a standard rhodamine filter. Images were captured using a digital camera connected to the microscope.

Annexin-V binding assay

MCF-7 cells were subcultured into six-well plates (50×10^3 cells/well) and were treated with genistein (100 μ M) for 72 h. Control and treated cells were trypsinized, and externalized phosphatidylserine was labeled with Guava

Nexin reagent (Guava Technologies, USA) following the manufacturer's instructions. Viable (annexin⁻/7-AAD⁻), apoptotic (annexin⁺/7-AAD⁻), and late apoptotic/necrotic (annexin⁺/7-AAD⁺) cells were characterized as previously described [19].

Gene expression evaluation by real-time PCR

MCF-7 breast cancer cells were treated with genistein (100 μ M) for 72 h and the total RNA extraction, cDNA synthesis, and real-time PCR (qPCR) were carried out as previously described [20] using specific primers for Cu,Zn-superoxide dismutase (CuZnSOD), Mn-superoxide dismutase (MnSOD), Thioredoxin reductase (TrxR), Glutathione peroxidase (GPx), BAX, Bcl-2, and Survivin described at [21].

LC3A/B Immunofluorescent staining (IF)

MCF-7 breast cancer cell cultures were fixed in 10 % phosphate-buffered formalin/acetone and sections were incubated 90 min at RT with the primary antibody rabbit monoclonal anti-LC3A/B (1:200) (Epitomics, Inc., USA). They were then incubated with FITC-conjugated goat antirabbit (1:1,000) (Kirkegaard Perry Laboratories, USA) for 60 min at RT. Sections were counterstained with DAPI blue (1:10,000). Images were captured using a digital camera connected to the microscope (Olympus, Japan).

Statistical analysis

Data were expressed as mean \pm SD and subjected to one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc test. The statistical significance between DMSO-treated and genistein-treated groups and other two-group analyses was carried out by means of unpaired Student's *t* test (Prism GraphPad Software, USA). Differences between mean values were considered significant when $P < 0.05$.

Results

Genistein selectively decreases the MCF-7 breast cancer cell proliferation but not in CCD1059sK fibroblast cells

We determined the genistein effect on cell proliferation of breast cancer cells by exposing MCF-7 cell line to increasing genistein concentrations (0.01–100 μ M) for 24, 48, and 72 h. E2 (10 nM) was used to compare its actions with genistein. At the end of incubations, MTT and LDH assays were performed. In parallel, CCD1059sK fibroblast cells were used as a non-transformed cell model to evaluate the selectivity of genistein.

As shown in Fig. 1, exposition of MCF-7 to genistein at concentrations ranging from 1.0 to 100 μ M for 72 h resulted in a decrease of cell proliferation by \sim 40 % compared to untreated cells (Fig. 1a), whereas fibroblast viability was not significantly altered at same condition (Fig. 1b). Genistein exposition for 24 and 48 h did not alter the cell proliferation (data not shown). In addition, the treatment with E2 (10 nM) did not promote significant alterations on MCF-7 or CCD1059sK cell growth (Fig. 1a, b). These results suggest that genistein target selectively cancer cells and that this effect is estrogenic receptor independent.

In contrast with the decreases in cancer cell proliferation, genistein did not promote a significant leakage of LDH into culture medium (Fig. 2a, b), indicating no alterations in cell membrane integrity. In addition, the membrane cell permeability was also evaluated by PI incorporation. As show in Fig. 3, genistein (10 and 100 μ M) exposure for 72 h promoted a marked decrease in the cell density and

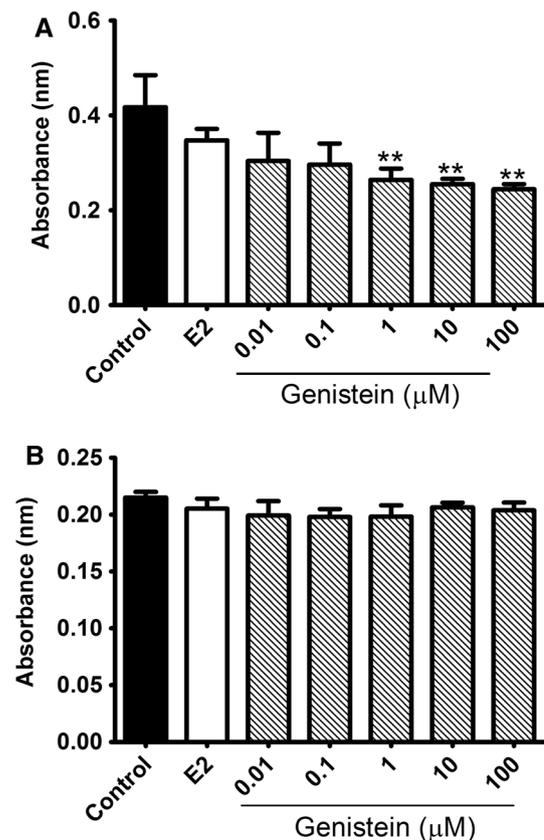


Fig. 1 Effect of genistein treatment on breast cancer and fibroblast cell proliferation. **a** MCF-7 cancer and **b** CCD1059sK fibroblast cell cultures were exposed to E2 (10 nM) or to increasing genistein concentrations (0.01–100 μ M) for 72 h, and the cell proliferation was assessed by the MTT. Appropriate controls containing 0.5 % DMSO were performed. The values represent the mean \pm SD of at least three independent experiments carried out in triplicate. Data were analyzed by ANOVA followed by post hoc comparisons (Tukey–Kramer test). **Significantly different from control and E2 treated cells ($P < 0.01$)

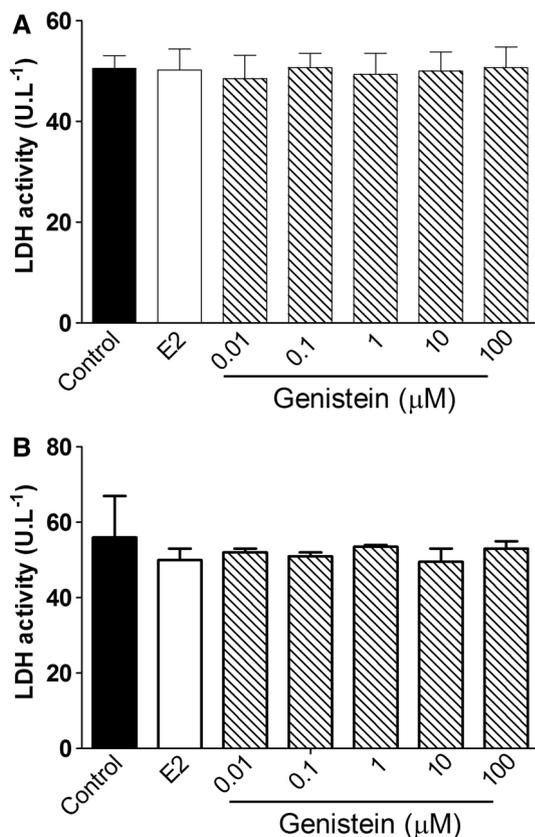


Fig. 2 Effect of genistein treatment on LDH release by breast cancer and fibroblast cell cultures. **a** MCF-7 cancer and **b** CCD1059sK fibroblast cell cultures were exposed to E2 (10 nM) or to increasing genistein concentrations (0.01–100 μ M) for 72 h, and the cell linkage was assessed by LDH release in the culture medium. Appropriate controls containing 0.5 % DMSO were performed. The values represent the mean \pm SD of at least three independent experiments carried out in triplicate. Data were analyzed by ANOVA followed by post hoc comparisons (Tukey–Kramer test)

significant morphological alterations, mainly at higher genistein concentration (100 μ M), including cell detachment and vesicle formation. However, no significant PI incorporation was observed in MCF-7 treated cells. These results suggest that the antiproliferative effect of genistein is not mediated by necrosis and that others cell death pathways such as apoptosis and/or autophagy may be involved.

Genistein promotes MCF-7 cell death via apoptosis and autophagy-dependent pathways

To better evaluate the mechanisms involved in the suppression of cancer cell proliferation, MCF-7 cells were exposed to genistein (100 μ M) for 72 h and then the externalization (flip-flop) of phosphatidylserine and/or 7-AAD staining was evaluated by flow cytometry (Fig. 4). Genistein promoted a reduction by \sim 40 % of MCF-7 viable cells and elicited early apoptosis in 10 % cell population (Annexin⁺/7-AAD⁻), necrosis in 12 % (Annexin⁻/7-AAD⁺), and late apoptosis in

majority of MCF-7 cells, totalizing 27 % of death cells (Annexin⁺/7-AAD⁺). Recent studies point to the cross-talk between apoptosis and autophagy in the cell death regulation and its relevance in either protumor or antitumor effects mediated by phytochemicals, as genistein [11]. Based in the morphology changes promoted by genistein in MCF-7 cells (Fig. 3) and in the genistein potential to induce autophagy in ovary and colon cancer cells [16, 22], the expression of microtubule-associated protein-1 light chain-3 (LC3) was evaluated by immunofluorescent staining. As shown in Fig. 5, the presence of LC3-positive puncta characteristic of autophagosome formation [23] was observed in the MCF-7 cells exposed to genistein (100 μ M) for 72 h, while no LC3 positive staining was observed in control or E2 (10 nM) treated cells. Taken together, these results suggest that genistein induced MCF-7 cell death by modulating apoptosis and autophagy pathways.

Genistein modulates apoptotic- and antioxidant-related gene expression in MCF-7 cancer cells

To further investigate the mechanisms of genistein-induced MCF-7 cell death, the gene expression of anti/proapoptotic and antioxidant enzymes in treated and control cancer cells was assessed by qPCR. When compared to control, genistein (100 μ M) treatment for 72 h did not alter significantly the expression of BAX and Bcl-2, a proapoptotic and antiapoptotic genes, respectively (data not shown). However, genistein promoted a three times increase of BAX/Bcl-2 ratio and an important reduction of 20 times of antiapoptotic survivin (Fig. 6a). The expression of caspase 3, 8, and 9 was not altered by treatment suggesting that the apoptosis induced by genistein in MCF-7 cells is caspase-independent (data not shown). In addition, genistein also altered the antioxidant enzyme expression. As shown in Fig. 6b, this isoflavone reduced the expression of CuZnSOD, MnSOD, and thioredoxin reductase (TrxR) by 9.3, 5.5, and 3.6 times, respectively, while the expression of glutathione peroxidase (GPx) was increased by 6.5 times. Importantly, TrxR upregulation and GPx downregulation have been related to breast cancer progression [24, 25] and the modulation of the enzyme expression by genistein may be involved in MCF-7 cell death. Moreover, these results suggest that the effect of genistein on MCF-7 cell death may be dependent on both modulation of pro/antiapoptotic gene expression and alterations on redox state of cancer cells.

Discussion

The present work demonstrates a novel function of genistein in the control of breast cancer progression. First, we investigated the effect of genistein on MCF-7 and

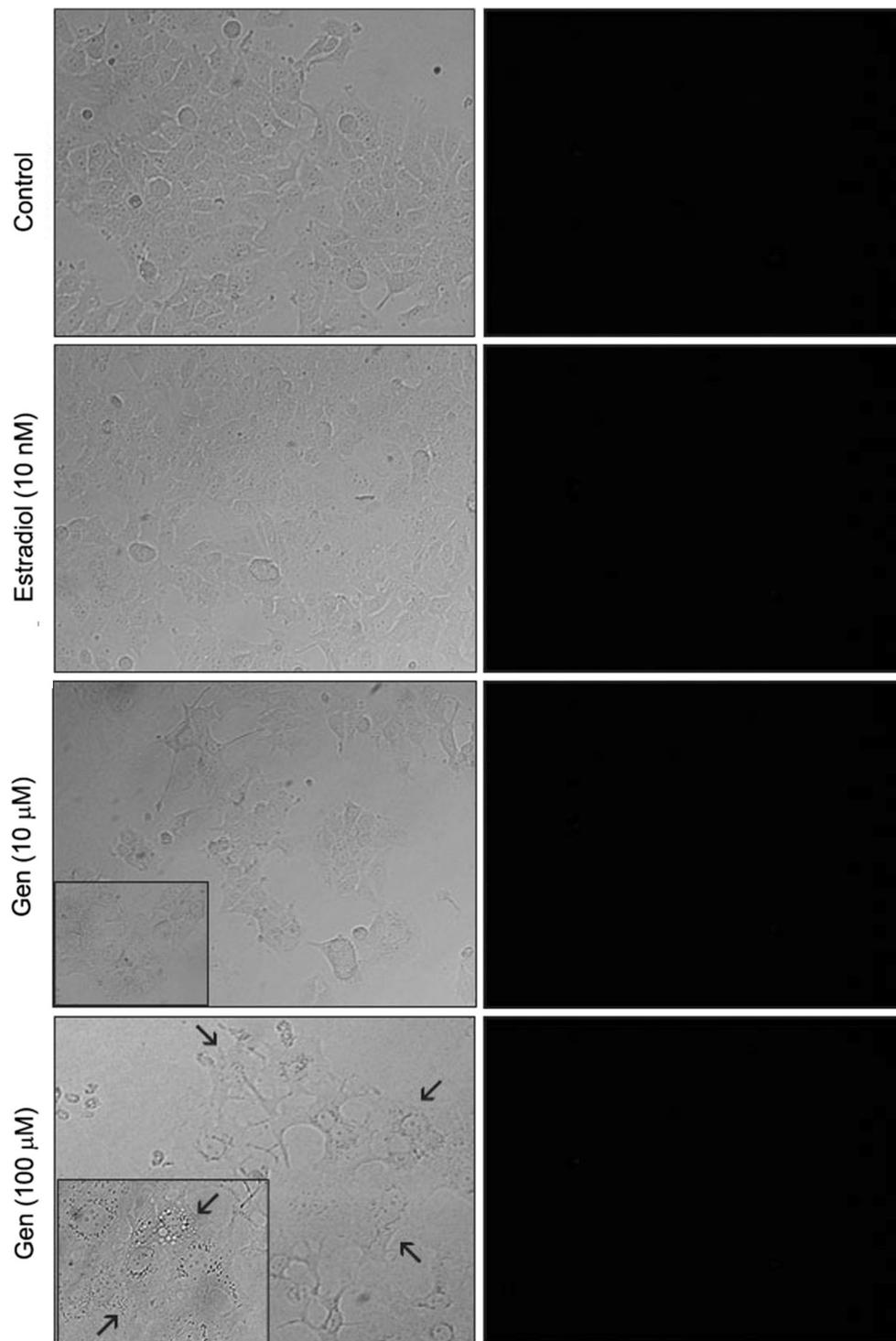


Fig. 3 Analysis of propidium iodide (PI) incorporation in MCF-7 cells following genistein treatment. Cell cultures were exposed to E2 (10 nM) or genistein (10 or 100 μM) and after 72 h of treatment the cells were incubated with PI diluted in culture medium. Fluorescence (*right panel*) and phase contrast (*left panel*) microphotographs were

taken using an Olympus inverted microscope. *Arrows* indicate morphologic changes, including cell detachment and vesicle formation in MCF-7 cancer cells following 100 μM genistein exposure ($\times 20$ magnification; *inset* $\times 40$ magnification)

CCD1059sK fibroblast cell proliferation. While no alterations were observed in fibroblast cells, genistein treatment reduced selectively MCF-7 cell proliferation. We also

characterized the cell death pathway involved in antitumor activity of genistein. Genistein treatment did not promote alterations on MCF-7 cell membrane integrity suggestive

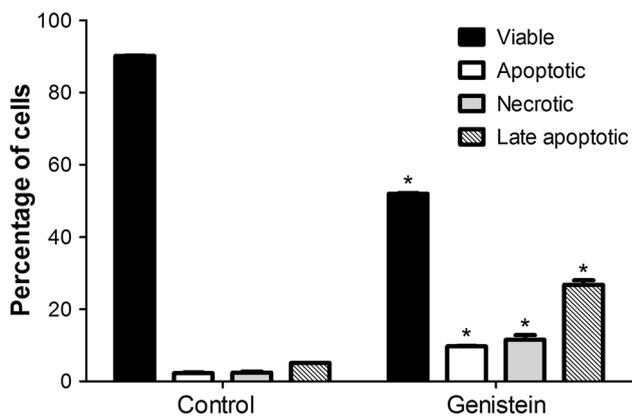


Fig. 4 Analysis of genistein effect on MCF-7 cancer cell death. Cell cultures were exposed to genistein (100 μ M) for 72 h and the MCF-7 cell death was evaluated by Annexin V-FITC bound phosphatidylserine and fluorescence of DNA-bound 7-AAD in individual breast cancer cells by flow cytometry. The values represent the mean \pm SD of at least three independent experiments. Data were analyzed by Student's *t* test. *Significantly different from control cells ($P < 0.05$)

of necrosis, as evidenced by the lack of LDH release and PI incorporation. We further demonstrate that the antitumor effect of genistein was due to apoptosis induction and progression of autophagy via downregulation of antiapoptotic survivin, upregulation of BAX/Bcl-2 ratio, and alterations on redox state of cancer cells.

Mounting evidence links the intake of isoflavones in soy rich foods to lower rates of prostate and breast cancers in Asian countries than in Western population [5, 11]. Genistein is the predominant isoflavone found in soybean enriched foods and, in addition to its anticancer properties, genistein has an excellent bioavailability via oral administration and safety use for clinical transition [26]. In line with this information, we started our work evaluating the antitumor activity of genistein in MCF-7 breast cancer. Genistein reduced selectively the MCF-7 cell proliferation, while no cytotoxicity was observed in CCD1059sK fibroblast cells or in E2-treated cells. Genistein is structurally

similar to E2, binding with higher affinity to estrogen receptor β (ER β) than to estrogen receptor α (ER α) [27]. MCF-7 cell line applied in this study is ER α -positive/ER β -negative breast cancer [28]. Therefore, considering the lack of E2 effect on the analyzed parameters, the antitumor effect of genistein may be independent of nuclear receptor pathways.

The mechanisms involved in genistein antitumor activity were further investigated. Genistein induced cancer cell death preferentially by apoptosis and progression of autophagy. We speculate that genistein antitumor activity was linked to modulation of antiapoptotic and proapoptotic protein expression, including BAX, Bcl-2, and survivin. Accordingly, genistein promoted an increase in BAX/Bcl-2 ratio and a parallel decrease of antiapoptotic survivin. These data are in agreement to evidence suggesting that BAX/Bcl-2 expression ratio is more decisive to apoptosis outcome than the expression level of each Bcl-2 member isolated [29]. Moreover, survivin downregulation by genistein was already reported in other tumor kinds [30] and may be of therapeutic interest. Indeed, survivin plays an essential role in the regulation of cell cycle and apoptosis and its differential expression in cancer cells have been related to increased tumor malignancy and progression [31].

In accordance with the role of polyphenols in modulation of antioxidant and prooxidant defenses [32], we observed that treatment with genistein markedly reduced the expression of antioxidant enzymes, namely CuZnSOD, MnSOD, and TrxR, while the GPx expression was increased. These data suggest that genistein induces disequilibrium in the antioxidant response, which may favor an oxidative stress in MCF-7 with consequent apoptosis and autophagy induction. These results are in agreement with recent works showing that the apoptosis and autophagy induction by natural compounds in MCF-7 cells were associated with reactive oxygen species and it was prevented by the overexpression of CuZn-SOD/Mn-SOD or by treatment with antioxidant molecules [33, 34].



Fig. 5 Detection of autophagosomes in MCF-7 cancer cells following genistein treatment. Cell cultures were exposed to E2 (10 nM) or genistein (100 μ M) for 72 h and they were subjected to LC3A/B immunostaining, as described in “Materials and methods” section.

Note the presence of LC3A/B expression in genistein treated MCF-7 cancer cells (arrows), while no staining was seen in control or E2 treated cells (magnification $\times 40$; inset magnification $\times 100$)

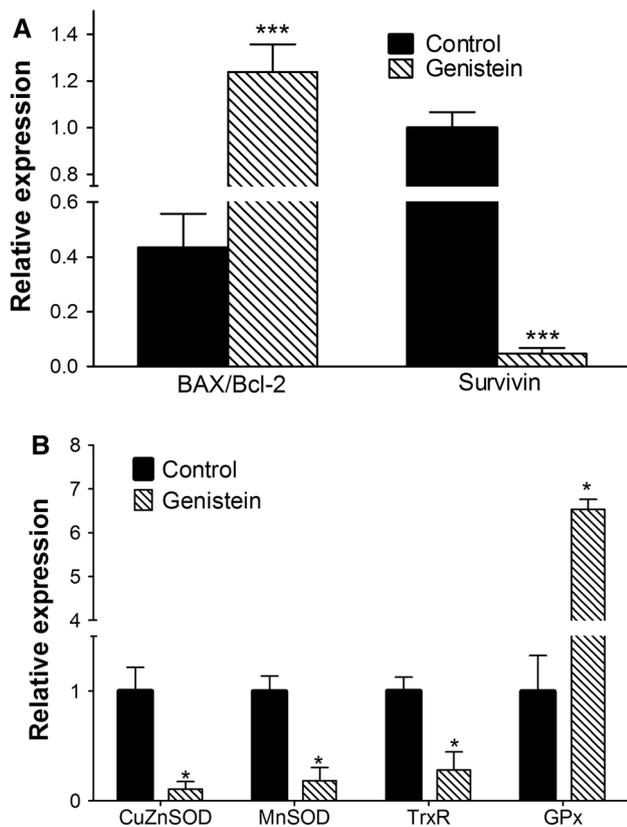


Fig. 6 Analysis of apoptotic- and antioxidant-related gene expression in genistein-treated MCF-7 cancer cells. Cancer cell cultures were treated with genistein (100 μ M) for 72 h and the gene expression was evaluated by qPCR as described in “Materials and methods” section. **a** Antiapoptotic Bcl-2 and survivin; proapoptotic BAX. **b** Antioxidant enzymes: CuZnSOD, MnSOD, thioredoxin reductase (TrxR), glutathione peroxidase (GPx). The values represent the mean \pm SD of at least three independent experiments. Data were analyzed by Student’s *t* test. *, ***Significantly different from control cells ($P < 0.05$; $P < 0.001$)

Importantly, genistein also modulated the TrxR and Gpx expression. The differential expression of these antioxidant enzymes is known to promote tumor progression [35, 36]. TrxR is one of the main components of thiol reducing system and its high expression has been associated with worst prognosis in human breast cancer. By other hand, low levels of GPx expression had been reported in cancer tissues [37, 38]. Therefore, TrxR downregulation and GPx upregulation by genistein could be a therapeutic alternative not only for breast cancer, but also for other tumors that exhibit alterations on antioxidant enzymes. Accordingly, other polyphenols with antitumor activity, including resveratrol, gallic acid, (-)-epigallocatechin-3-gallate, quercetin, and curcumin also modulate the expression levels of antioxidant enzymes, reinforcing the importance of these enzymes as potential targets to cancer treatment [39–42].

In conclusion, our results demonstrate that genistein selectively induced MCF-7 cell death independent of

estrogen receptor activation. The antitumor effect of genistein involved the participation of oxidative stress, with consequent increase of proapoptotic BAX/Bcl-2 ratio and parallel downregulation of antiapoptotic survivin which, in turn, resulted in induction of apoptosis and progression of autophagy. In addition, the ability of genistein to differentially modulate the expression of survivin, TrxR, and GPx may be a therapeutic alternative for adjuvant treatment of oncologic patients. Although further studies are necessary to examine the autophagy and apoptotic process induced by genistein in an in vivo breast cancer model, the data reported here reinforce the potential of dietary phytochemicals as novel strategy in cancer prevention and treatment.

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Conflict of interest The authors declare that there are no conflicts of interest.

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