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The Phytoestrogen Genistein Affects Breast Cancer Cells Treatment Depending on the ERα/ERβ Ratio

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ABSTRACT

Genistein (GEN) is a phytoestrogen found in soybeans. GEN exerts its functions through its interaction with the estrogen receptors (ER), ERα and ERβ, and we previously reported that the ERα/ERβ ratio is an important factor to consider in GEN-treated breast cancer cells. The aim of this study was to investigate the effects of GEN in breast cancer cells with different ERα/ERβ ratio: MCF-7 (high ratio), T47D (low ratio), and MCF-7 overexpressing ERβ (MCF7 + ERβ) treated with cisplatin (CDDP), paclitaxel (PTX) or tamoxifen (TAM). Cell viability, ROS production, autophagy, apoptosis, antioxidant enzymes protein levels, and cell cycle were analyzed. GEN treatment provoked an increase in cell viability in MCF-7 cells and in the antioxidant enzymes protein levels in combination with the cytotoxic agents, decreasing ROS production (CDDP + GEN and TAM+GEN) and autophagy (TAM + GEN) or apoptosis (CDDP + GEN and TAM + GEN). Moreover GEN treatment enhanced the cell cycle S phase entry in CDDP+GEN- and TAM + GEN-treated MCF-7 cells and, in the case of CDDP + GEN, increased the proportion of cells in the G2/M phase and decreased it in the subG_0/G_1 phase. Otherwise, in the T47D and MCF7 + ERβ cells the combination of GEN with cytotoxic treatments did not cause significant changes in these parameters, even TAM + GEN-treated T47D cells showed less cell viability due to an increment in the autophagy. In conclusion, GEN consumption may be counterproductive in those patients receiving anticancer treatment with a high ERα/ERβ ratio diagnosed breast cancer and it could be harmless or even beneficial in those patients with a lower ERα/ERβ ratio breast cancer cells. J. Cell. Biochem. 117: 218–229, 2016.

KEY WORDS: GENISTEIN; ERα/ERβ ratio; BREAST CANCER; ROS PRODUCTION; APOPTOSIS; AUTOPHAGY; ANTICANCER TREATMENT

Phytoestrogens are a large group of natural compounds which have been found in more than 300 plants [Farnsworth et al., 1975]. These compounds are characterized by having a close similarity in chemical structure to estrogens and harboring estrogenic activity [Kurzer and Xu, 1997]. It is believed that phytoestrogens have a lot of health-beneficial properties against several diseases such as osteoporosis, immunity and inflammation, reproduction and fertility, cardiovascular disease and cancer [Messina and Loprinzi, 2001; Dixon, 2004; Mense et al., 2008; Orlando et al., 2011].

Genistein (GEN) is the major isoflavone, which is a type of phytoestrogen, found in soybeans. The lower incidence of breast cancer in Asia has been associated with higher consumption of GEN, as soybean intake is higher in Asia and blood levels of GEN in people from Eastern countries are 10 times higher than people from Western countries [Zhou et al., 1998; Adlercreutz, 2002; Morton et al., 2002].

GEN exerts its functions through its interaction with estrogen receptors (ER), ERα and ERβ, and GEN has stronger affinity for ERβ than for ERα [Kuiper et al., 1997, 1998]. The ERα is associated with cell growth and proliferation, while ERβ activation is more related to cytostatic and differentiation processes through inhibition of mammary cancer cell growth as well as counteracting the proliferative effects of ERα activation [Strom et al., 2004; Chang et al., 2006; Sotoca et al., 2008; Rajah et al., 2009].

Conflict of Interest: None.

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However, the GEN consumption presents some controversy, especially once breast cancer is diagnosed. Previous studies in our research group have demonstrated that the ERα/ERβ ratio is an important factor in order to determine the benefits of the GEN consumption. Thus, GEN treatment promoted cell proliferation as well as worsened mitochondrial functionality leading to an increase in ROS production in MCF-7 breast cancer cells, with a high ERα/ERβ ratio [Nadal-Serrano et al., 2013; Pons et al., 2014]. On the other hand, GEN treatment produced a better mitochondrial functionality, less ROS production and cell cycle arrest in T47D breast cancer cells, with a low ERα/ERβ ratio [Nadal-Serrano et al., 2013; Pons et al., 2014]. Finally, GEN treatment hardly affected MDA-MB-231 cells, which is considered negative for ERs, although it has a very low presence of ERβ [Pons et al., 2014]. It is important to note that in these experiments GEN treatment was applied in the absence of 17β-estradiol (E2), the main estrogen in women. Unlike GEN, E2 has higher affinity for ERα than for ERβ [Kuiper et al., 1997, 1998], which may be important in the response to GEN treatment.

Some anticancer treatments have among its mechanisms of action the generation of radical oxygen species (ROS), producing oxidative stress in cancer cells leading to cell death. Cisplatin (CDDP), paclitaxel (PTX) and tamoxifen (TAM), whose main cytotoxic effects on cancer cells are the generation of DNA adducts [Jordan and Carmo-Fonseca, 2000], the microtubule stabilization blocking their disassembly [Luo et al., 2014] and the estrogen receptor antagonism [Jensen and Jordan, 2003], respectively, can affect mitochondria, diminishing the mitochondrial functionality, which results in a rise in ROS production and, in most cases, cell death [Andre et al., 2002; Marullo et al., 2013; Razandi et al., 2013], contributing to the action of these cytotoxic compounds on cancer cells.

The main objective of the current study was to investigate the effects of GEN treatment in breast cancer cells with different ERα/ERβ ratio, MCF-7 (high ERα/ERβ ratio) and T47D (low ERα/ERβ ratio) treated with cisplatin, paclitaxel and tamoxifen. For this purpose we analyzed parameters such as cell viability, ROS production, apoptosis, autophagy and cell cycle in order to find out whether the consumption of soy-related products may be beneficial or detrimental in patients being treated for breast cancer. Moreover, some of the experiments (cell viability, ROS production, apoptosis, and autophagy) were performed in MCF-7 cells over-expressing ERβ (MCF7 + ERβ) to confirm the results obtained in the two breast cancer cells with different ERα/ERβ ratio.

MATERIALS AND METHODS

REAGENTS

Dulbecco's Modified Eagle's medium (DMEM) high glucose was purchased from GIBCO (Paisley, UK). Genistein (*4′,5,7-Trihydroxyisoflavone or GEN*), cisplatin (*cis-Diammineplatinum(II) dichloride or CDDP), paclitaxel (from Taxus brevifolia or PTX) and tamoxifen (trans-2-[4-(1,2-Diphenyl-1-butenyl) phenoxy]-N,N-dimethylethylamine or TAM*) were purchased from Sigma-Aldrich (St. Louis, MO). Primers were purchased from TIB MOLBIOL (Berlin, Germany) and from Metabion (Martinsried, Germany). Routine chemicals used were obtained from Sigma-Aldrich, Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA).

QUANTIFICATION OF 17β-ESTRADIOL OF FETAL BOVINE SERUM

The concentration of 17β-estradiol of the serum was quantified with an Estradiol ELISA kit purchased from DRG Diagnostic (Marburg, Germany), following the manufacturer's instructions. The Estradiol ELISA kit revealed that the fetal bovine serum contains 1 nM of 17β-estradiol (data not shown), which means that in the culture medium there is 0.1 nM of E2 (because of the dilution of FBS in the DMEM at 10%). It is important to note the estrogenic capacity of phenol red, and the concentration of phenol red in the DMEM (0.04 mM) is equivalent to 0.4 nM of E2 [Berthois et al., 1986]. Therefore, the concentrations of estrogenic compounds are equivalent to 0.5 nM of E2.

CELL CULTURE AND TREATMENTS

The MCF-7 and T47D human breast cancer cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA) and routinely cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. For cytotoxic treatments, cells were seeded in 6-well or 96-well plates and treated the next day in confluence with vehicle (0.1% DMSO), 1 μM GEN (the physiological concentration of GEN in a regular consumer of soy enriched foods [Safford et al., 2003]), the cytotoxic treatments 10 μM CDDP, 10 nM PTX or 10 μM TAM (we tested these cytotoxic concentrations in order to find out the IC50 in the MCF-7 cells), and the combination of GEN with each cytotoxic agent for 48 h.

ESTROGEN RECEPTOR β STABLE TRANSFECTION

ERβ cDNA clone was purchased from Origene (Rockville, MD). First of all, ERβ cDNA clone was amplified in *Escherichia coli* DH5αF′ Competent Cells (Life Technologies, Paisley, UK) and isolated with MaxiPrep isolation kit (Life Technologies). Afterwards cDNA quantification using a BioSpec-nano spectrophotometer set at 260 nm (Shimadzu Biotech, Kyoto, Japan), aliquots of 1 μg/ml of cDNA were kept in −20°C for cell transfections. Briefly, MCF-7 cells were seeded in 6-well plates and the next day were transfected with an ERβ cDNA clone following the manufacturer's instructions. Lipofectamine 2000 was used to perform cell transfection according to the manufacturer's protocol. After transfection the complexes were removed and cells were provided with normal growth medium. Two days after cDNA transfection, growth medium was replaced for DMEM (+10% FBS and +1% antibiotics) with 600 μg/ml of G418 (or neomycin) in order to select those cells that have incorporated the ERβ cDNA clone in their genome. After two weeks with 600 μg/ml of G418 in the growth medium, colonies were harvested with cloning discs (Sigma-Aldrich) and plated in 24-well plates (one well per colony). Ten days later, cells were ready to trypsinize and they were plated in 6-well plates. Then, cells were subcultured in order to get cells to perform the experiments. It is important to note that the MCF-7 + ERβ cells obtained with this protocol must be grown routinely at a concentration of 200 μg/ml of G418 in the growth medium, except when these cells were cultured for the experiments of this study.

CELL VIABILITY ASSAY

MCF-7, T47D or MCF-7+ERβ cells were seeded in 96-well plates. Genistein and cytotoxic treatments were applied the day after for

48 h. Crystal Violet was the method used to determine cell viability at the time of 0 h (at the moment of applying the treatments), 24 and 48 h. Briefly, cells were stained with 0.5% (p/v) crystal violet in 30% (v/v) acetic acid for 10 min. After washing with distilled water, the dye was solubilized in 100 μl of methanol and absorbance was measured photometrically (A595nm) using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) to determine cell viability.

CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

MCF-7, T47D or MCF-7 + ERβ cells were seeded in 6-well plates. Genistein and cytotoxic treatments were applied the day after for 48 h. After harvesting the cells with trypsin-EDTA, cells were fixed incubating cells for 1 h at −20°C with methanol 100% and DNA staining was carried out with an RNAase and propidium iodide mix. After 30 min of room temperature incubation in the dark, samples were analyzed using an Epics XL flow cytometer (Beckman Coulter, Miami, FL). The red fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. Ten thousand events were acquired and analyzed with Expo32 ADC analysis software (Beckman Coulter).

FLUORIMETRIC DETERMINATION OF H_2O_2 PRODUCTION (ROS PRODUCTION)

ROS production was measured fluorimetrically by using an Amplex® Red Hydrogen Peroxide/Peroxidase assay kit (Molecular Probes). MCF-7, T47D or MCF-7 + ERβ cells were seeded in 96-well plates. The day after, genistein and cytotoxic treatments were applied for 48 h. The measurement day, cells were exposed to 50 μM of Amplex Red reagent and 0.1 U/ml of horseradish peroxidase, in Krebs-Ringer buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4). Fluorescence was measured with an FLx800 microplate fluorescence reader (Bio-Tek Winooski, VT), set at excitation and emission wavelengths of 571 and 585 nm, to detect the maximum slope of increment in the fluorescence within 1 h of exposure to kit reagents. Thus, the measurement obtained is the H_2O_2 produced (related to ROS production) by the cells for 1 h. Values were normalized per number of viable cells determined by crystal violet assay.

AUTOPHAGIC VACUOLES DETERMINATION

Autophagic vacuoles were measured fluorimetrically by using Monodansylcadaverine (MDC), respectively. MCF-7, T47D, or MCF-7 + ERβ cells were seeded in 96-well plates and, the next day, cells were exposed for 48 h to genistein and cytotoxic treatments. Fluorescence measurement was performed in a FLx800 microplate fluorescence reader (Bio-Tek Winooski) set at excitation and emission wavelengths of 340 and 535 nm after an incubation of 15 min with 50 μM MDC at 37°C. Values were normalized per number of viable cells determined by crystal violet assay.

APOPTOSIS ASSAY

Apoptosis was measured fluorimetrically by using Annexin V method, as described recently by Fiorini et al. [2015]. Briefly, MCF-7, T47D, or MCF-7+ERβ cells were seeded in 96-well plates and treated with genistein and cytotoxic treatments for 48 h. At the end of the treatment, cells were fixed with 2% paraformaldehyde (BD Biosciences, NJ) in PBS at room temperature for 30 min and washed twice with PBS. Cells were then stained with AnnexinV/Alexa-Fluor350 (Life Technologies, Thermo Fisher Scientific, Waltham, MA) in annexin binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) for 10 min at room temperature in the dark. To finish, cells were washed once with annexin binding buffer. Florescence was measured in a FLx800 microplate fluorescence reader (Bio-Tek Winooski) set at excitation and emission wavelengths of 346 and 442 nm, with cells kept in 100 μl of annexin binding buffer. Values were normalized per number of viable cells determined by crystal violet.

REAL-TIME QUANTITATIVE PCR

MCF-7 and MCF-7+ERβ were seeded in 6-well plates and the day after total RNA was isolated from cultured cells by using TriPure® isolation reagent (Roche, Barcelona, Spain) following the manufacturer's protocol and then quantified using a BioSpec-nano spectrophotometer set at 260 nm (Shimadzu Biotech, Kyoto, Japan). 1 μg of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10 μl volume of retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNase inhibitor, and 500 μM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−80°C) until the PCR reactions were carried out.

PCR was performed in triplicate samples by SYBR Green technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). The primers used were forward 5′-ggACACggACAggATTgACA-3′ and reverse 5′-ACCCACggAATCgAgAAAgA-3′ for the 18S ribosomal RNA gene, and forward 5′-TAgTggTCCATCgCCAgTTAT-3′ and reverse 5′-gggAgCCACACTTCACCAT-3′ for the ERβ gene. Total reaction volume was 10 μl, containing 7.5 μl Lightcycler® 480 SYBR Green I Master (containing 0.5 μM of the sense and antisense specific primers) and 2.5 μl of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95°C), followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, 61°C for 18S and 64°C for ERβ), and an extension step (12 s, 72°C min). A negative control lacking cDNA template was run in each assay.

The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

WESTERN BLOT ANALYSIS

MCF-7 and T47D cells were seeded in 6-well plates. The next day, cells were exposed to genistein and cytotoxic treatments for 48h. With the aim of checking the ERβ overexpression in MCF7 + ERβ cells, these cells were seeded as well in 6-well plates and harvested 24 h after seeding. Cell protein extracts were obtained scraping cells with 200 μl of RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 1 mM EDTA, 1mM NaF,

1 mM Na_3VO_4, 10 μM leupeptin and 10 μM pepstatin; finally, 1 mM PMSF was added just before harvesting the cells with the scraper). The lysate was sonicated three times at 40% amplitude for 7 s. Then samples were centrifuged at 14,000*g* for 10 min at 4°C and protein content (supernatant) was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). A 20 μg protein aliquot from the cell lysate was separated on a 12% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. Membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20) for 1 h. Antisera against PARP and LC3A/B (Cell Signaling Technology Inc., Danvers, MA), catalase (Calbiochem, Merck Millipore, Darmstadt, Germany), and Manganese-Superoxide Dismutase (MnSOD) and GAPDH (Santa Cruz Biotechnology, CA) as the house-keeping were used as primary antibodies. Anti-rabbit secondary antibody was from Sigma-Aldrich (St. Louis, MO). Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

STATISTICAL ANALYSIS

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc., Chicago, IL) was used for all statistical analyses. Data ($n = 6$) are expressed as mean values ± standard error of the mean (SEM). Statistical significances between control and the overexpression of ERβ in ERβ mRNA levels were assessed by Student's *t*-test. In the survival curves, Student's *t*-test was performed in order to find out the significance between vehicle- and genistein-treated cells, as well as the significance between cytotoxic- and cytotoxic + genistein-treated MCF-7 and T47D cells. The effects of changes produced by cytotoxic agents and their combination or not with genistein in MCF-7, T47D, and MCF7 + ERβ cell lines were analyzed using two-way analysis of variance (ANOVA), and when results reach significant combinatory effects, Student's *t*-test was performed in order to find out the significance between the experimental groups. Statistical significance was set at $P < 0.05$.

RESULTS

ERβ STABLE OVEREXPRESSION IN MCF-7 CELLS

In order to ensure the levels of ERβ expression, RT-PCR for this gene was carried out in the MCF-7 clone overexpressing ERβ (MCF7 + ERβ) selected to perform the experiments. Results indicated that ERβ mRNA expression levels were increased by 734% compared to MCF-7 *wild-type* cells (data not shown).

GENISTEIN DECREASED BREAST CANCER CELLS SENSITIVITY TO CYTOTOXIC TREATMENTS, ESPECIALLY IN THOSE WITH A HIGH ERα/ERβ RATIO

Figure 1 shows that GEN treatment increased cancer cells viability alone or in combination with any cytotoxic treatment in MCF-7 cells in a time-dependent manner, but in T47D cells GEN treatment even reduced cell viability in TAM + GEN-treated cells at 24 and 48 h. In Figure 2, it can be observed that GEN treatment (1 μM) produced an increase in cell viability (+9.6% in MCF-7 and +4.9% in T47D) in control cells. Moreover, GEN treatment significantly raised cell viability in CDDP- (+5.1%), PTX- (+5.3%) and TAM-treated (+5.6%) MCF-7 cells, but not in T47D. Interestingly, the TAM+-GEN-treated cells in the T47D cell line showed a significant decrease in cell viability in comparison with TAM-treated cells (-3.4%).

GENISTEIN TREATMENT DECREASED ROS PRODUCTION IN COMBINATION WITH CYTOTOXIC AGENTS

As shown in Figure 3 GEN treatment caused a reduction in ROS production in a similar way in the two cell lines (−11% in MCF-7 and −9% in T47D). In MCF-7 cells, the combination of GEN with CDDP and TAM treatment caused a significant reduction in ROS production (−28% in CDDP + GEN-treated cells and −29% in TAM + GEN-treated cells) compared to cytotoxic treatments alone. In T47D, GEN treatment also produced a decrease in ROS production in combination with CDDP- and TAM-treated cells, but it was milder than in MCF-7 cells (−8% and −9% in CDDP + GEN- and TAM + GEN-treated cells, respectively). The combination of GEN and PTX treatment reached no significant difference in both cell lines.

THE COMBINATION OF GENISTEIN TREATMENT WITH CYTOTOXICS AFFECTED AUTOPHAGY ESPECIALLY IN TAM-TREATED BREAST CANCER CELLS

Figure 4 shows that GEN treatment produced a reduction in autophagy in MCF-7 control cells (−4%), which is not observed in T47D cells. Furthermore, autophagic vacuoles formation was reduced especially after TAM+GEN treatment in MCF-7 cells (−28%), which was accompanied by a decrease in the LC3-II/LC3-I ratio (−96%). Interestingly, in the T47D cell line the combination of GEN and TAM triggered an increase in autophagic vacuoles formation in comparison with TAM-treated T47D cells (+21%).

APOPTOSIS IS DECREASED IN CDDP + GEN- AND TAM + GEN-TREATED MCF-7 CELLS AND PARP CLEAVAGE IS INCREASED IN TAM + GEN T47D CELLS WITH RESPECT TO CYTOTOXIC TREATMENT ALONE

For the analysis of apoptosis (Fig. 5), GEN combination with CDDP and TAM provoked a drop in Annexin V Fluorescence (−25% and −20%, respectively) regarding to cytotoxic treatment alone in MCF-7 cells, in addition to a reduction in the cleavage of the PARP protein in CDDP + GEN-treated cells (−112%). However, the combination of GEN and the three cytotoxic treatments studied did not reach any significant difference in Annexin V Fluorescence compared to cytotoxic treatments alone in T47D cells, although in TAM+GEN-treated cells there was an increase in the PARP cleavage (+84%).

GENISTEIN INCREASED THE MnSOD AND CATALASE PROTEIN LEVELS IN MCF-7 CELLS WHEN TREATED IN COMBINATION WITH CISPLATIN AND TAMOXIFEN RESPECTIVELY

The Table I shows that, in MCF-7 cells, MnSOD protein levels were increased in CDDP + GEN-treated cells and catalase protein levels were increased in TAM+GEN-treated cells, compared to cytotoxic treatments alone. In T47D cells the GEN treatment did not produce any statistical significant change in combination with any cytotoxic agent.

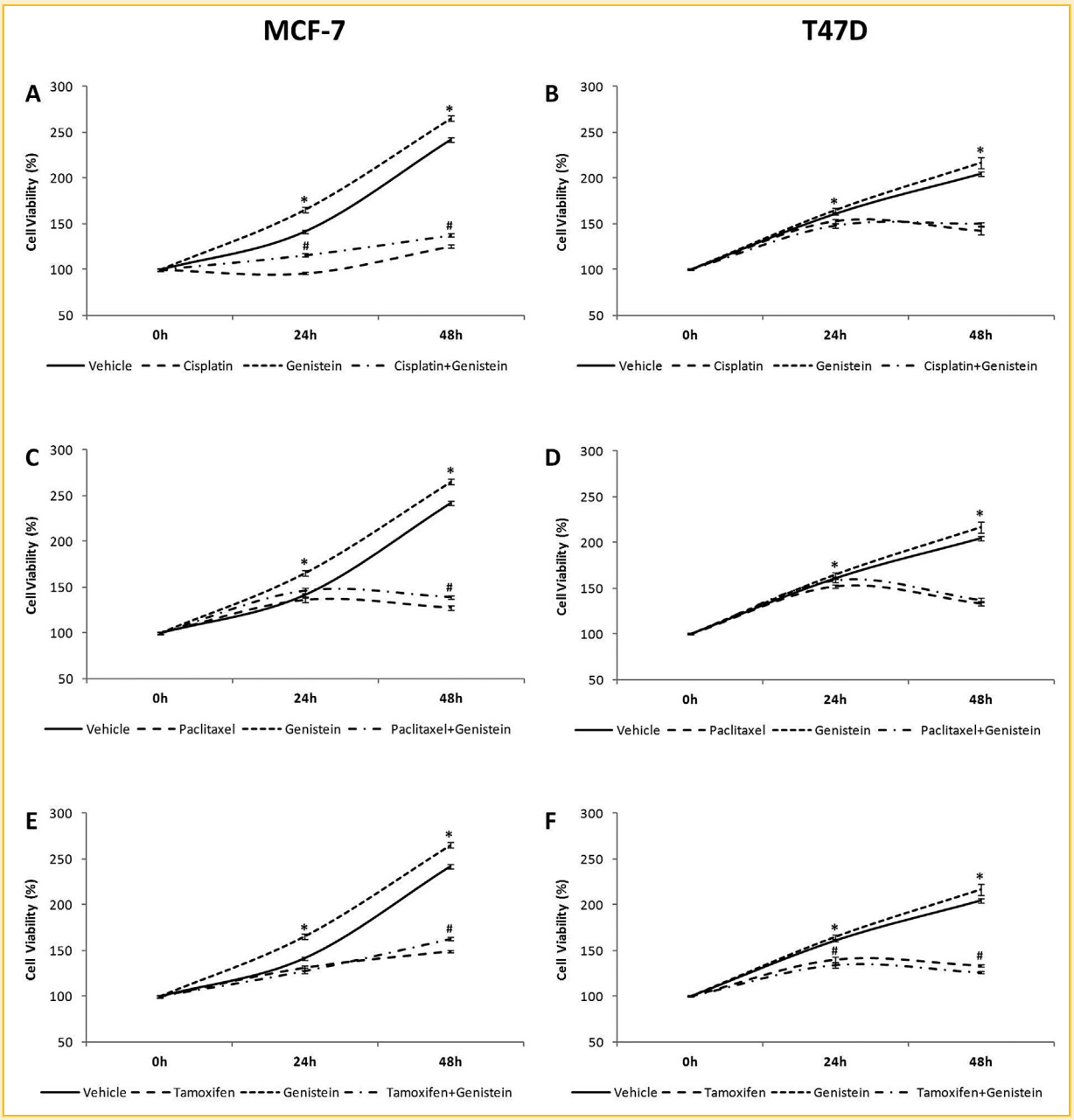

Fig. 1. Survival curves in MCF-7 and T47D cells after genistein and cytotoxic treatments for 0, 24, and 48 h. MCF-7 and T47D cells were treated with vehicle (0.1% DMSO), 10 μM cisplatin, 10 nM paclitaxel or 10 μM tamoxifen in combination or not with 1 μM genistein for 0, 24, and 48 h. Cell viability was analyzed with Cristal Violet method. A: Cisplatin-treated MCF-7 cells. B: Cisplatin-treated T47D cells. C: Paclitaxel-treated MCF-7 cells. D: Paclitaxel-treated T47D cells. E: Tamoxifen-treated MCF-7 cells. F: Tamoxifen-treated T47D cells. Values are expressed as means ± SEM (n = 6), and normalized as percentage of the 0 h value for each cell line and treatment. *Significant difference between Vehicle-treated cells and Genistein-treated cells. #significant difference between cytotoxic-treated cells and cytotoxic + genistein-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

GENISTEIN STIMULATED THE ENTRY INTO THE CELL CYCLE S-PHASE IN MCF-7 CELLS WHEN COMBINED WITH CISPLATIN AND TAMOXIFEN TREATMENTS

Cell cycle analysis (Table II) revealed an increase in cells in S-phase after GEN treatment in vehicle- (+0.9%), CDDP- (+0.9%) and TAM-treated cells (+0.9%). Moreover, despite CDDP provoked an S-phase arrest, the combination of CDDP with GEN increased the proportion of cells in the G2/M phase (+3.2%) and decreased the percentage of cells in subG0 phase (−0.38%).

THE OVEREXPRESSION OF ERβ MODERATED CHANGES IN RESPONSE TO COMBINATION OF GENISTEIN WITH CISPLATIN AND TAMOXIFEN IN MCF-7 CELLS

In MCF7 + ERβ cells, as shown in Figure 6 combination of GEN with CDDP did not reach any significant difference in cell viability

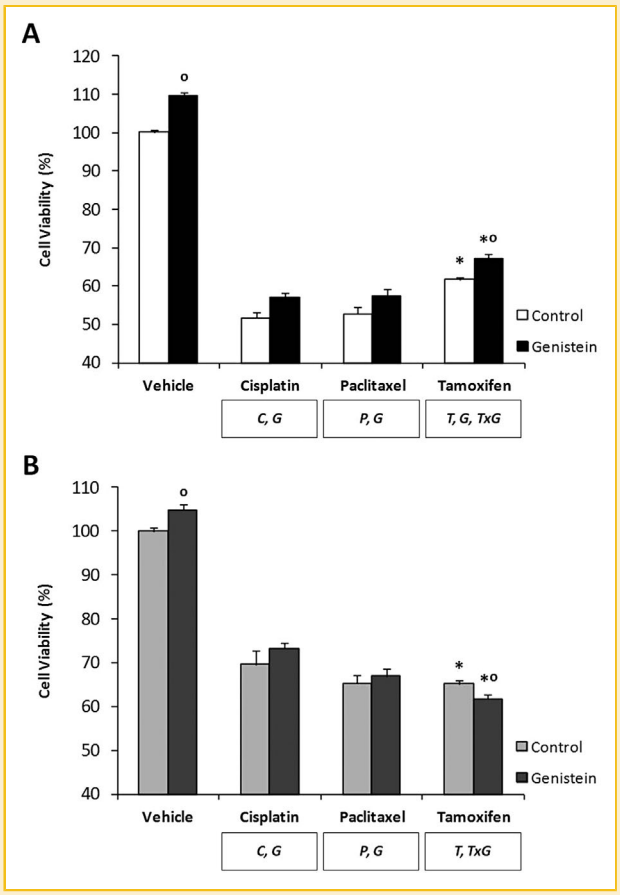

Fig. 2. Effects of genistein and cytotoxic treatments on cell viability. MCF-7 and T47D cells were treated with vehicle (0.1% DMSO), 10 μM cisplatin, 10 nM paclitaxel or 10 μM tamoxifen in combination or not with 1 μM genistein for 48 h. Cell viability was analyzed with Cristal Violet method. A: MCF-7 breast cancer cells. B: T47D breast cancer cells. Values are expressed as means ± SEM (n = 6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and TxG means combinatory effect of tamoxifen treatment and genistein. As a result of combinatory effect, Student's *t*-test ($P < 0.05$, n = 6) was carried out: ° significant difference between control vehicle- and genistein-treated cells; * significant difference between vehicle- and cytotoxic-treated cells; *° significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein.

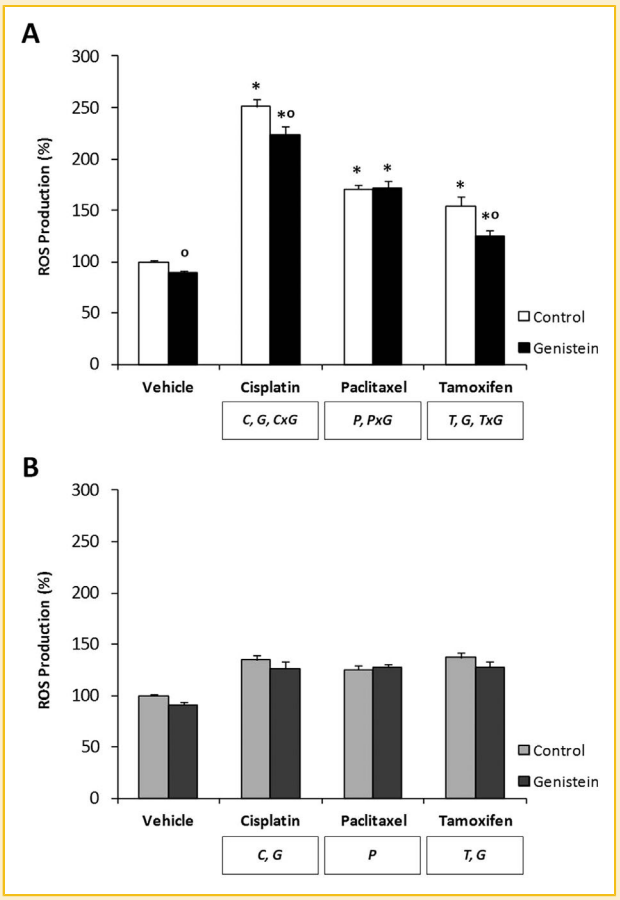

Fig. 3. Effects of genistein and cytotoxic treatments on ROS production. MCF-7 and T47D cells were treated with vehicle (0.1% DMSO), 10 μM cisplatin, 10 nM paclitaxel or 10 μM tamoxifen in combination or not with 1 μM genistein for 48 h. ROS production was analyzed with Amplex® Red Hydrogen Peroxide/Peroxidase assay kit as shown in the Materials and Methods section. A: MCF-7 breast cancer cells. B: T47D breast cancer cells. Values are expressed as means ± SEM (n = 6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and CxG, PxG, or TxG means combinatory effect of cytotoxic treatments and genistein. As a result of combinatory effect, Student's *t*-test ($P < 0.05$, n = 6) was carried out: 0 significant difference between control vehicle- and genistein-treated cells; * significant difference between vehicle- and cytotoxic-treated cells; *0 significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein.

regarding to CDDP-treated cells, but in TAM-treated cells GEN treatment caused a slight increase in cell viability compared to TAM-treated cells (+4%). Moreover, the decrease in ROS production after combination of GEN with cytotoxic agents was milder (−15% in CDDP + GEN-treated cells and −26% in TAM+GEN-treated cells) than in MCF-7 cells. Finally, the study of autophagy and apoptosis revealed that there were no significant changes in the combined treatment with GEN regarding to cytotoxic agent alone.

DISCUSSION

We have studied the effects of the phytoestrogen genistein (GEN) in the efficacy of the anticancer treatments cisplatin (CDDP), paclitaxel (PTX) and tamoxifen (TAM) in breast cancer cell lines with different estrogen receptors (ERα and ERβ) ratio. Thus, the analysis of cell viability revealed that the combination of GEN with any cytotoxic treatment increased the cell survival in MCF-7 cells (high ERα/ERβ ratio) regarding to cytotoxic-treated cells. This increment in cell viability was accompanied by a decrease in ROS production and in the PARP cleavage (an indicator of apoptosis) in CDDP+GEN-treated cells and in autophagy in TAM+GEN-treated cells. Moreover, cell cycle analysis of MCF-7 cells showed a greater entrance in the S phase with GEN treatment and, in the case of CDDP, a lesser proportion of cells in sub-G0/G1 (apoptotic cells) and, furthermore, a

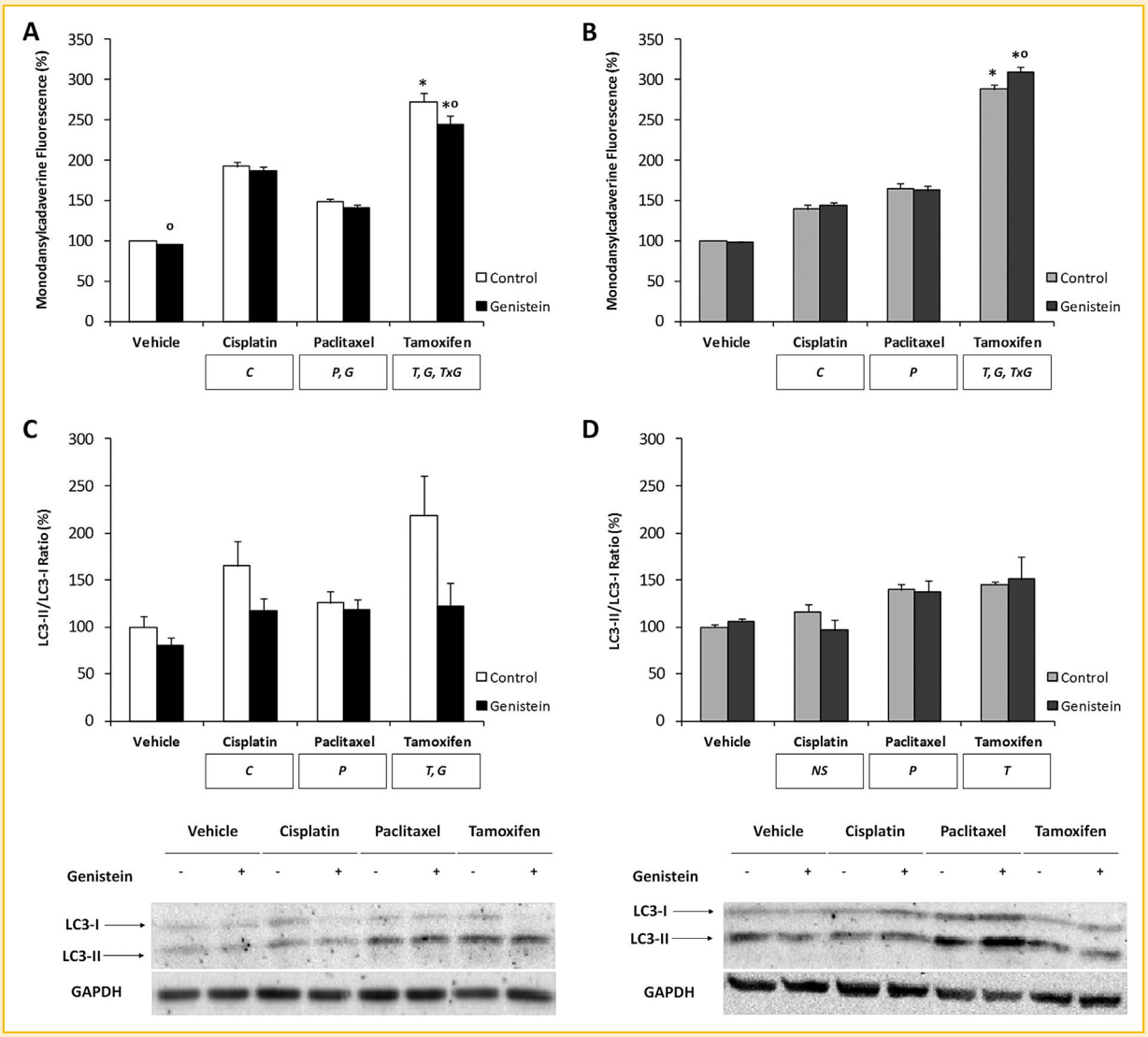

Fig. 4. Effects of genistein and cytotoxic treatments on autophagy. MCF-7 and T47D cells were treated with vehicle (0.1% DMSO), 10 μM cisplatin, 10 nM paclitaxel, or 10 μM tamoxifen in combination or not with 1 μM genistein for 48h. Autophagic vacuoles formation was analyzed with the fluorescence of monodansylcadaverine and the lipidation of LC3-I to LC3-II as mentioned in the Materials and Methods section. A: Autophagic vacuoles formation in MCF-7 cells. B: Autophagic vacuoles formation in T47D cells. C: LC3-II/LC3-I Ratio measured by western blot in MCF-7 cells. D: LC3-II/LC3-I Ratio measured by Western blot in T47D cells. Values are expressed as means ± SEM (n = 6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and TxG means combinatory effect of tamoxifen treatment and genistein. As a result of combinatory effect, Student's *t*-test ($P < 0.05$, n = 6) was carried out: 0 significant difference between control vehicle- and genistein-treated cells; * significant difference between vehicle- and cytotoxic-treated cells; *0 significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein; NS no significant difference in the ANOVA analysis.

higher number of cells in the G2/M phase, explaining the greater cell viability in the CDDP + GEN-treated cells. Nevertheless, in T47D cells (low ERα/ERβ ratio) and in MCF-7 cells overexpressing ERβ (MCF7 + ERβ), the increase in cell viability after GEN treatment was milder than in MCF-7 cells and, in some cases such as TAM+GEN-treated T47D cells, there was a decrease in cell viability after GEN treatment through a raise in the autophagic cell death.

We previously reported that GEN treatment, in a phenol red-free cell culture medium supplemented with 10% charcoal-stripped FBS, affects cancer cells in a different way depending on the ERα/ERβ ratio [Nadal-Serrano et al., 2013; Pons et al., 2014]. In the current study, cells were cultured in medium containing 10% FBS and phenol red, with an equivalent E2 concentration of 0.5 nM, which corresponds to a physiological concentration of E2 [Rajah et al., 2012]. Thus, GEN treatment produced a greater cell survival in MCF-7 cells, probably due to its estrogenic activity through its binding to ERα [Hsieh et al., 1998]. However, the interaction of the GEN with the ERβ maybe the most important in the study of the combination of GEN with the cytotoxic treatments, because it provokes a lower ROS production [Nadal-Serrano et al., 2013] decreasing the apoptosis and, in the case of CDDP+GEN-treated cells, decreasing the cleaved PARP/PARP ratio, a final indicator of apoptosis [Hoffman et al., 2013], in

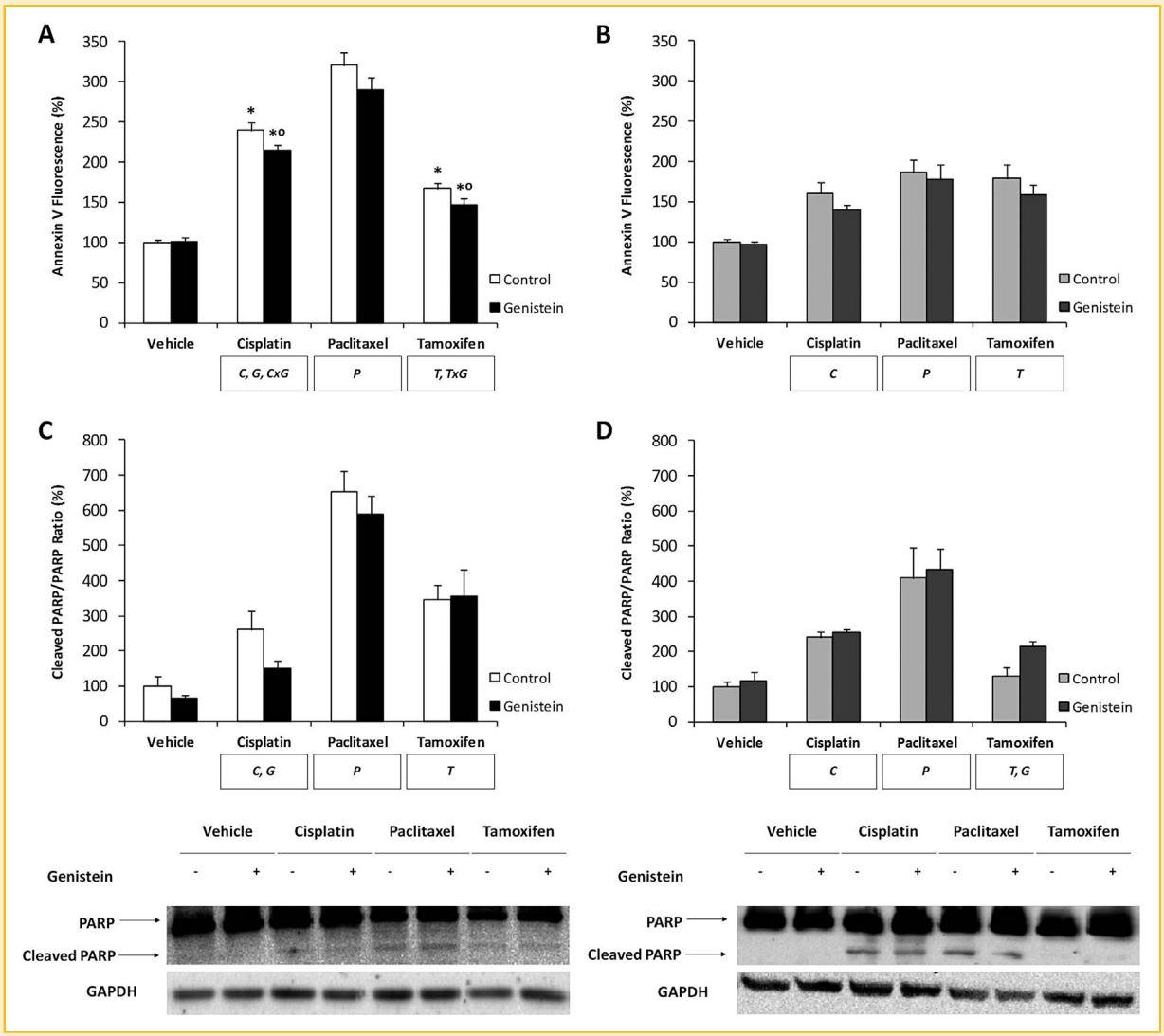

Fig. 5. Effects of genistein and cytotoxic treatments on apoptosis. MCF-7 and T47D cells were treated with vehicle (0.1% DMSO), 10 μM cisplatin, 10 nM paclitaxel, or 10 μM tamoxifen in combination or not with 1 μM genistein for 48h. Apoptosis was analyzed with the fluorescence of Annexin V and the cleavage of PARP as mentioned in the Materials and Methods section. A: Apoptosis in MCF-7 cells. B: Apoptosis in T47D cells. C: Cleaved PARP/PARP Ratio measured by western blot in MCF-7 cells. D) Cleaved PARP/PARP Ratio measured by western blot in T47D cells. Values are expressed as means ± SEM (n = 6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and CxG, PxG or TxG means combinatory effect of cytotoxic treatments and genistein. As a result of combinatory effect, Student's *t*-test ($P < 0.05$, n = 6) was carried out: * significant difference between vehicle- and cytotoxic-treated cells; *0 significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein.

comparison with cytotoxic alone-treated MCF-7 cells. These results, in addition to the increase of MnSOD protein levels, suggest that GEN could reduce ROS production and therefore apoptosis in high ERα/ERβ ratio breast cancer cells cultured with physiological concentrations of E2. Park et al. [2010] demonstrated in prostate cancer cells the relationship between GEN treatment and the decrease in ROS levels, increasing the expression of antioxidant enzymes such as MnSOD and catalase [Park et al., 2010], and more specifically other authors confirmed that the overexpression of MnSOD attenuates the apoptosis in CDDP-treated kidney cells [Davis et al., 2001]. Moreover, GEN treatment in control cells as well as in combination with cytotoxic agents raised the entrance in S phase of cell cycle, as described by Chen and Wong [2004], who demonstrated that GEN treatment at 1 μM stimulated the growth of MCF-7 cells and significantly increased cells in the S phase [Chen and Wong, 2004]. Cell cycle analysis also revealed that, despite CDDP causes cell cycle arrest in the S phase [Wagner and Karnitz, 2009], the CDDP + GEN-treated cells showed an increase in G2/M phase and a drop in subG$_0$/G$_1$ phase (corresponding to apoptotic cells [Chuang et al., 2005]).

On the other hand, the combination of GEN with PTX and especially with TAM produced a decrease in the autophagy, leading to more viable breast cancer cells than cytotoxic alone-treated cells. Precisely, TAM+GEN-treated MCF-7 cells showed a decrease in the LC3 II/LC3 I ratio, an indicator of autophagy

TABLE I. Manganese SuperOxide Dismutase (MnSOD) and Catalase Protein Levels in MCF-7 and T47D Human Breast Cell Lines After Genistein and/or Cytotoxic Treatments (Cisplatin 10 μM, Paclitaxel 10 nM, or tamoxifen 10 μM) for 48 h.

Cell line	Treatment	MnSOD (%)			Catalase (%)		
		Control	Genistein		Control	Genistein	
MCF-7	Vehicle	100±6	120±11	ANOVA	100±5	109±5	ANOVA
	Cisplatin	120±7	140±8	C, G	119± 12	129±13	NS
	Paclitaxel	139±10	138±14	P	109±10	127±7	NS
	Tamoxifen	122±4	129±14	NS	122±7	157±15	T, G
T47D	Vehicle	100±8	95.4±11.0	ANOVA	100±12	82.3±9.3	ANOVA
	Cisplatin	98.1±10.6	92.2±7.4	NS	88.0±13.1	106±24	NS
	Paclitaxel	97.8±9.2	98.1±6.4	NS	104±16	107±11	NS
	Tamoxifen	131±12	111±11	T	80.2±12.2	87.6±15.3	NS

Data represent the means ± SEM (n = 6). Values of vehicle (0.1% DMSO) in control cells (without genistein) in MCF-7 and T47D cell lines were set at 100%. ANOVA analysis was carried out for the analysis of every cytotoxic treatment in combination with genistein, where C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect, G means genistein effect and NS means no significant difference ($P < 0.05$, n = 6)

[Kabeya et al., 2004], confirming the reduction of autophagy after GEN treatment in TAM-treated cells, as other authors have reported previously in pancreas cancer cells showing that GEN treatment produced a decrease in the LC3 II/LC3 I ratio [Suzuki et al., 2014]. The study of apoptosis revealed that there was a decrease in apoptosis after GEN treatment in CDDP and TAM combined treatments and, in the case of CDDP+GEN-treated cells it was confirmed by a decrease in the PARP cleavage. The reduction in the autophagy and apoptosis, and therefore the raise in cell viability after GEN treatment in combination with TAM could be due at least in part to the decrease in ROS production and the increase in the catalase protein levels, as other authors have reported that the antioxidants such catalase may lead to breast cancer cells growth inhibition when treated with tamoxifen

TABLE II. Cell Cycle Analysis Measured by Flow Cytometry in MCF-7 Cells After Genistein (1 μM) and/or Cytotoxic Treatments (Cisplatin 10 μM or Tamoxifen 10 μM) for 48 h

	Control			Genistein			ANOVA	
Cell cycle phase	Vehicle	Cisplatin	Tamoxifen	Vehicle	Cisplatin	Tamoxifen	ANOVA cisplatin	ANOVA tamoxifen
Sub G_0/G_1 (%)	0.90 ± 0.07	1.74 ± 0.17	0.98 ± 0.08	0.78 ± 0.14	1.36 ± 0.10	0.95 ± 0.06	*C*	*NS*
G_0/G_1 (%)	49.4 ± 0.5	43.1 ± 0.7*	54.1 ± 1.2	53.1 ± 0.90	39.4 ± 0.8*°	56.0 ± 1.6	*C, CrG*	*T, G*
S (%)	10.1 ± 0.2	20.0 ± 0.2	4.29 ± 0.21	11.0 ± 0.4	20.9 ± 0.2	5.20 ± 0.33	*C, G*	*T, G*
G_2/M (%)	39.6 ± 0.3	35.2 ± 0.7*	40.6 ± 1.2	35.1 ± 0.70	38.4 ± 0.6*°	37.9 ± 1.3	*CrG*	*G*

Data represent the means ± SEM (n = 6) of the percentage of cells in each cell cycle phase after vehicle (0.1% DMSO) or cytotoxic treatments in combination (Genistein) or not (Control) with genistein treatment (1 μM) for 48 h in MCF-7 breast cancer cells. ANOVA analysis was carried out where C means cisplatin effect, T means tamoxifen effect, G means genistein effect and CxG means combinatory effect of cisplatin and genistein treatments. As a result of combinatory effect, Student's *t*-test ($P < 0.05$, n = 6) was carried out: 0 significant difference between control vehicle- and genistein-treated cells; * significant difference between Vehicle and Cisplatin-treated cells; *° significant difference between Cisplatin-treated cells and Cisplatin-treated cells in combination with genistein; NS no significant difference in the ANOVA analysis.

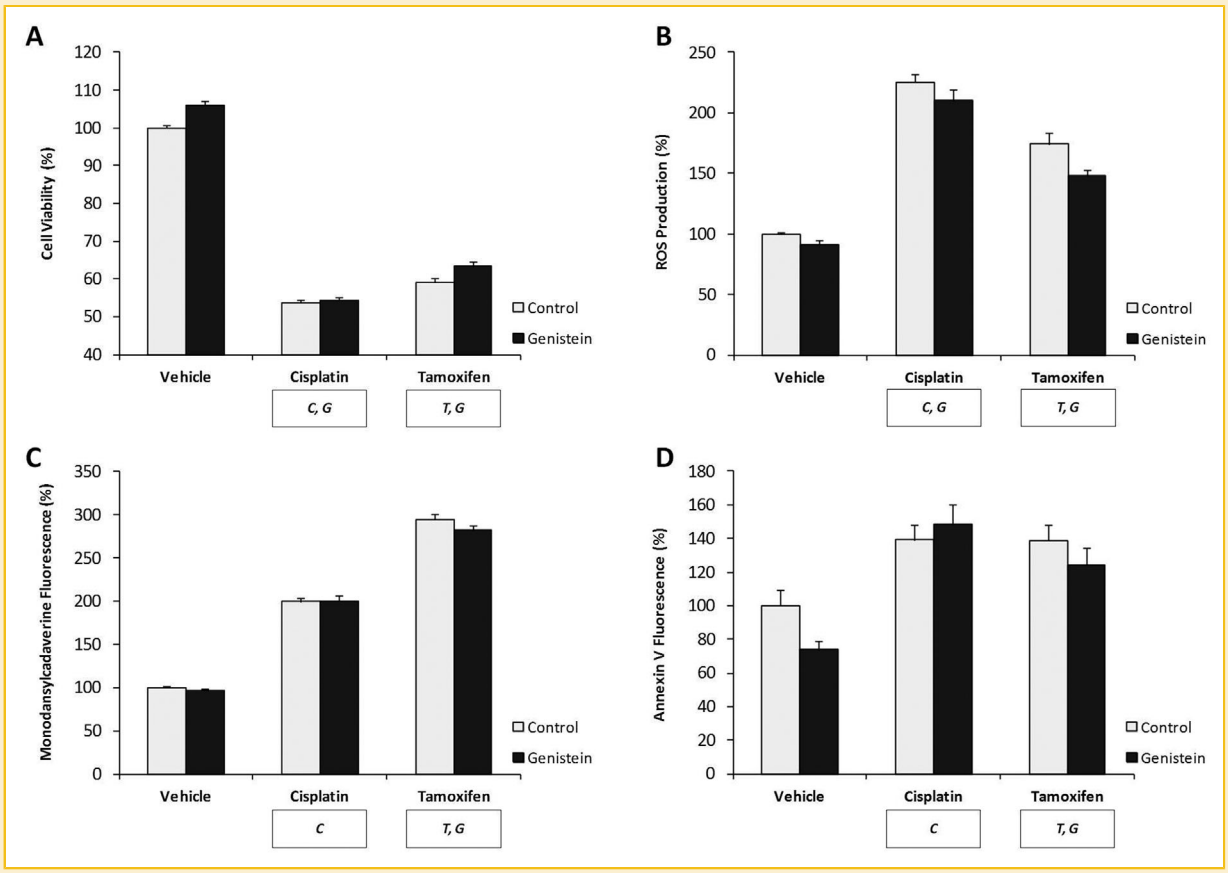

Fig. 6. Effects of genistein and cytotoxic treatments on cell viability, ROS production, autophagy and apoptosis in MCF-7 overexpressing ERβ (MCF7+ERβ). MCF7+ERβ cells were treated with vehicle (0.1% DMSO), 10 μM cisplatin or 10 μM tamoxifen in combination or not with 1 μM genistein for 48 h. A: Cell proliferation was analyzed by Crystal Violet assay. B: ROS production was measured fluorimetrically using Amplex red reagent. C: Autophagic vacuoles formation was measured fluorimetrically using monodansylcadaverine reagent. D: Apoptosis fraction was measured fluorimetrically using Annexin V method. Values are expressed as means ± SEM (n = 6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect and T means tamoxifen effect.

[Gundimeda et al., 1996]. These results do confirm the effects of the combination of physiological concentrations of GEN with cytotoxic agents such as CDDP and TAM, reducing their efficacy. Other authors have demonstrated that GEN reduces CDDP-induced apoptosis in the kidney through the regulation of p53 induction [Sung et al., 2008], and there is literature confirming that dietary GEN negate/overwhelm the inhibitory effect of TAM on MCF-7 tumor growth in mice [Ju et al., 2002].

In those cells with a low ERα/ERβ ratio such as T47D cells and the MCF-7 cells overexpressing ERβ (MCF7 + ERβ), GEN treatment did not produce any significant increment in cell viability when incubated in combination with cytotoxic agents (with the exception of a small rise in the TAM+GEN-treated MCF7+ERβ cells). This is probably due to the E2 present in the medium stimulating the ERβ in these cells; therefore the protective effects of the ERβ against ROS production [Nadal-Serrano et al., 2012] are already occurring, so that the addition of GEN would have no significant effect. Curiously, TAM+GEN-treated MCF7+ERβ cells were the only ones (of those cells with a low ERα/ERβ ratio) that combination of GEN with the cytotoxic agents caused an increase in cell viability, maybe due to the decrease in ROS production, as a drop in this parameter could lead to a greater cell survival [Trachootham et al., 2008].

Interestingly, TAM + GEN-treated T47D cells had lower cell viability than TAM-treated T47D cells through an increase in the autophagic vacuoles formation. Autophagy is a conserved evolutionary process that may enable cells to maintain homeostasis in unfavorable environmental conditions [Cook et al., 2011]. This process allows the cell to recover energy from damaged or unnecessary subcellular components (macromolecules or organelles) [Cook et al., 2011]; therefore it has been considered as a process associated with cell survival. However, if the damage is too severe and a high level of autophagy persists, autophagic cell death or programmed cell death-2 will occur, which is a different phenomenon than apoptosis or programmed cell death-1 [Akar et al., 2008]. So T47D cells treated with GEN+TAM showed an increase in the autophagic cell death, leading to a poorer cell survival than TAM-treated T47D cells. Moreover, PARP cleavage is increased in TAM+GEN-treated T47D cells suggesting an increase in apoptosis as well. The raise in autophagic vacuoles formation and the cleavage of the PARP are contributing to the lesser cell survival than TAM-

treated T47D cells. These results confirm the importance of the presence of ERβ when cancer cells are treated with ERβ agonists, validating the results recently obtained by Ruddy et al. [2014] who demonstrated that apoptosis and autophagy are increased in those breast cancer cells treated with a combination of TAM and an ERβ agonist [Ruddy et al., 2014].

In conclusion, these results indicate that in women diagnosed with breast cancer whose cancer cells have a high ERα/ERβ ratio, the genistein consumption (soy products or dietary supplements) may have a counterproductive effect in the anticancer treatment mainly due to the decrease in the ROS production (in the case of cisplatin and tamoxifen treatments, since ROS production is one of the mechanism of action of these cytotoxic agents). The consequence of that is a reduction in the apoptosis (in cisplatin-treated cells) or the autophagic cell death (in tamoxifen-treated cells), therefore increasing cancer cells viability. However, in those cells with a low ERα/ERβ ratio genistein consumption seems to have a lesser impact in the efficacy of anticancer therapies, and even increases that effectiveness in the tamoxifen-treated cells in combination with the genistein, raising the autophagic cell death. Further studies are necessary to better understand the role of the genistein in the efficacy of the anticancer treatments, as well as the role of the ERα/ERβ ratio in those patients consuming high amounts of genistein in that efficacy.

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