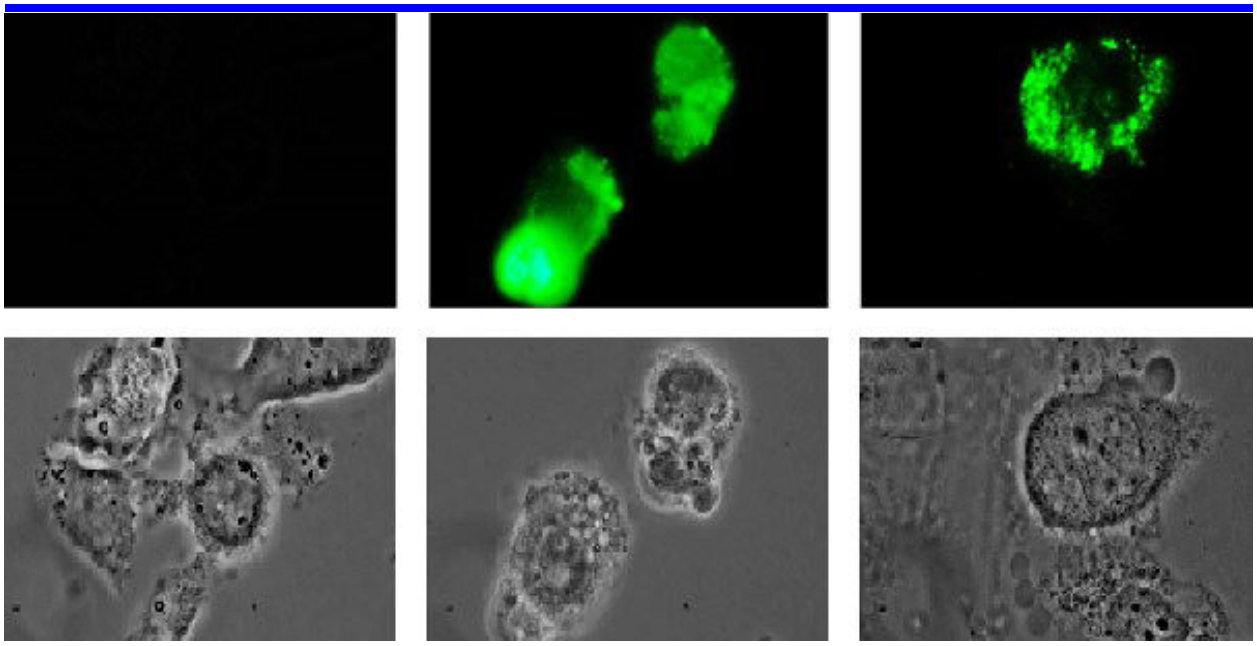

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Equol induces apoptosis in chemoresistant ovarian cancer cells via external pathway

Xue-Mei Gong[1], Cheng-Jiu Hu[2], Quan-Jing Zhao[2] and Dong-Mei Shi[3,4]

[1]Department of Gynecology, Jining No. 1 People's Hospital, Jining, Shandong, China; [2]Department of Pathology, Jining No. 1 People's Hospital, Jining, Shandong, China; [3]Department of Mycology, Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, Jiangsu, China; [4]Department of Dermatology, Jining No. 1 People's Hospital, Jining, Shandong 272 011, China.

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Abstract

Polyphenolic compounds present in fruits, vegetables and grains are bioactive molecules which elicit a wide range of responses both *in vivo* and *in vitro*. The aim of this study is to investigate whether the soybean isoflavone, equol could induce apoptosis in ovarian cancer cells, SKOV-3. In this study, we evaluated molecular events associated with apoptosis induced by equol and paclitaxel (PTX). To assess whether growth inhibition was due to apoptosis, flow cytometry, colorimetry experiments, immunoblot analyses through measuring DNA fragmentation, the level of TRAIL, the cleavage of poly(ADP-ribose) polymerase (PARP) and the activation of caspase-3, -8 and -9 were also performed. Results showed that apoptosis was induced by extrinsic pathway triggered by TNF family members. Overall results suggested that equol induces apoptosis in SKOV-3 cells via a TRAIL and caspase 8-dependent pathway whereas paclitaxel leads to smaller apoptotic events when compared to that of equol.

Introduction

Ovarian cancer is considered as one of the most common cancers and represents the most lethal gynecological cancer. Due to the lack of specific symptoms and absence of reliable screening strategies, this kind of cancer is usually diagnosed at an advanced stage (Bruning and Mylonas, 2011). The biochemical and molecular mechanisms explaining this resistance are not completely known (Coukos and Rubin, 1998).

Despite the introduction of new drugs for the treatment of ovarian cancer, the overall survival of patients suffering from this malignancy is far from satisfactory. Therefore, novel therapeutic approaches are urgently needed for ovarian carcinoma.

Flavonoids are one of the principal plant hormones and are known to possess diverse functional roles including the regulation of plant apoptosis and cell cycle kinetics. Phytoestrogens were originally recognized as plant-derived materials with estrogen-like activity and intensive studies revealed protective effects of phytoestrogens on a variety of diseases, such as prostate and breast cancer (Denis et al., 1999, Lee et al., 1991), osteoporosis (Potter et al., 1998) and cardiovascular diseases (Bakhit et al., 1994).

Equol [7-hydroxy-3-(4'-hydroxyphenyl)-chroman] is a bioactive metabolite of daidzein that is formed by intestinal bacteria. Several actions of equol, including its estrogenic and antioxidant properties and its proliferative and antiproliferative effects, suggest that exposure to the compound may have implications for cancer risk (Magee et al., 2006, Atkinson et al., 2005). In this study, we tested the whether the equol has biological effects on the growth of human ovarian cancer cells.

Materials and Methods

Reagents

Equol was purchased from LC Laboratories® (LC Laboratories®, MA, USA) and dissolved in DMSO (final concentration 0.1% in medium). PTX was supplied by Sequoia Research Products (UK). Medium (RPMI 1640) and fetal bovine serum (FBS) were supplied by Cambrex (Switzerland) and trypsin-EDTA were supplied by Sigma (USA). Caspase-3 Activity Assay was from Calbiochem (USA), Human TRAIL, caspase-9 fluorometric assay kit and Z-LEHD-FMK inhibitor, annexin V-biotin apoptosis detection kit, and Apo-BrdU *in situ* DNA fragmentation assay kit were supplied by BioVision Inc. (USA), caspase-8 assay kit was from Sigma–Aldrich (USA).

Ovarian cancer cell culture and drug treatment

The human SKOV-3 cell line used in the experiments was obtained from American Type Culture Collection (ATCC) (USA). The cells were routinely screened for *Mycoplasma* contamination and maintained as a monolayer in RPMI 1640 supplemented with 10% heat-inactivated FBS. Cell culture was carried out in an environment of 5% CO_2 and 95% normal air at 37°C. Cells were plated out on 96-well microplates at a density of 3×10^5 cells per well on 60 mm petri dishes for double staining Hoechst 33258/propidium iodide and annexin V/propidium iodide assay (3×10^6 cells) and 100 mm petri dishes for immunofluorescence assay of P-gp activity (10^6 cells/dishes). Petri dishes with a diameter of 100 mm were also used for the activity of caspase-3, caspase-9 and caspase-8 assays (10^6 cells/dish), TRAIL assay, and TUNEL assay (2×10^6 cells/dish). After 24 hours, different concentrations of drugs (equol or PTX) were added and the cells were incubated with the drugs in a CO_2 incubator for different periods of time (2–48 hours), depending on the assessment method.

MTT assay

The cytotoxicity of the drugs to SKOV-3 ovarian cancer cells was estimated by the standard microplate MTT colorimetric method. Logarithmically growing cells of the cell line tested (25×10^3 cells/well) were seeded on 96-well microplates, and 24 hours later they were incubated with drugs for 48 hours. At the end of incubation, the medium was removed and washed twice with PBS, 50 µL of MTT (at a final concentration of 0.5 mg/mL) was added to the cells.

Annexin V-FITC labeling

The plasma membrane changes characteristic of apoptosis were analyzed by double staining with annexin V-FITC and propidium iodide using an LSRII flow cytometer (BD Biosciences). Cells were cultured with drugs for 2–48 hours. At certain time points, cells were removed from the culture dishes and suspended in annexin binding buffer. To the cell suspension, annexin V-FITC and propidium iodide were added (in accordance with the manufacturer's instructions) and then the cells were incubated at room temperature for 10 min in total darkness. Three populations of cells were observed: (1) viable cells: Annexin V-FITC negative and propidium iodide negative; (2) apoptotic cells: Annexin V-FITC positive and propidium iodide negative; (3) necrotic cells: Annexin V-FITC positive and propidium iodide positive. Cell observations were made using an Eclipse E-600 epifluorescence microscope (Nikon, Japan). All images were recorded at exactly the same integration time using a DS-Fi1 CCD camera (Nikon, Japan). Cell fluorescence was measured by flow cytometry (LSRII, BD Biosciences, FL2 channel).

Double staining with Hoechst 33258 and propidium iodide

To determine the ratio between the live, apoptotic, and necrotic cell fractions, simultaneous cell staining with Hoechst 33258 and propidium iodide was conducted. These fluorescent dyes vary in their spectral characteristics and ability to penetrate cells. The analysis was done with the Olympus IX70 fluorescence microscope. Cells were cultured with IC_{50} concentrations of the drugs for 2–48 hours. At certain time points, cells were removed from culture dishes by trypsinization, centrifuged and suspended in PBS at a final concentration of 1×10^6 cells/mL. One microliter of Hoechst 33258 (0.13 mM) and 1 µL of propidium iodide (0.23 mM) were added to 100 µlof cell suspensions. The cells were then incubated at room temperature for 10 min in total darkness (Gasiorowski et al., 2001). At least 300 cells were counted on each slide and each experiment was done in triplicate. The percentages of particular cell types were determined by the total number of cells.

Immunofluorescence assay of P-gp activity

The mouse monoclonal antibody against human MDR-1 (UIC2 mAb) reacts with the extracellular moiety of P-gp and inhibits the P-gp-mediated efflux of the majority of the tested chemotherapeutic drugs. It has been shown that the reactivity of UIC2 mAb with P-gp was enhanced in the presence of P-gp substrates due to protein conformational changes (Chaoui et al., 2006). Cells were analyzed by flow cytometry (LSRII, BD Biosciences) using FL2 channel.

Measurement of caspases-3, -8 and -9 activity

Activities of caspases-3, -8, and -9 were determined with assay kits [caspase-3 Z-DEVD-R110 assay kit (Molecular Probes, USA); Ac-IETD-AMC caspase-8 assay kit (Sigma–Aldrich, Germany); caspase-9 LEHD-pNA assay kit (Molecular Probes, USA)] according to the manufacturer's protocols. SKOV-3 cells (3×10^6/

sample) were incubated with drugs for 4, 24, or 48 hours (IC_{50} concentrations) and after this period of time cells were briefly lysed. Additionally in the control, all cell lysates were incubated for 1 hour with the proper reversible caspases inhibitors to confirm that the observed fluorescence in both the control and the drug-treated cells is due to caspase-3,-9,-8 presences in the samples. Caspase-3 inhibitor: Ac-DEVD-CHO, 1 µL of the 1 mM; caspase-9 inhibitor: Z-LEHD-FMK, 1 µL of the 10 mM; caspase-8 inhibitor: Ac-IETD-CHO; 2 µL of the 25 µM. Cystein protease activity was expressed as a ratio of fluorescence (-3, -8) [Fluoroskan Ascent FL plate reader (Lab system, Sweden)] or absorbance (-9) (Power Wave microplate reader (BioTek, USA)) of the drug-treated samples relative to the corresponding untreated controls taken as 100%.

TRAIL assay

Human TRAIL Enzyme-linked Immunosorbent Assay was used to measure the TRAIL level. The SKOV-3 cells were cultured with IC_{50} concentrations of the drugs for 2 and 48 hours. Cell culture supernatant after centrifugation (1500 rpm, 10 min, 4°C) was tested at certain time points. Human TRAIL present in the sample was bound to antibodies (anti-human TRAIL) adsorbed to the microwells. Then, biotin-conjugated anti-human TRAIL antibody was added and bound to human TRAIL captured by the first antibody and incubated. Further, streptavidin–horseradish peroxidase (HRP) was added and bound to the biotin-conjugated anti-human TRAIL antibody. Followed by this, the tetramethyl benzidine solution was added to react with HRP and the reaction was terminated by using acid and then, the absorbance was measured at 450 nm.

Western blotting

In accordance with the manufacturer's protocol (Invitrogen), the cells were lysed in the cell extraction buffer containing the protease inhibitors cocktail and PMSF. Bradford Method was used to determine the protein concentration. The SDS–polyacryamide gel electrophoresis (10%) and the transfer of proteins (100 µg per lane) to nitrocellulose membranes were performed using standard procedures. Membranes were incubated with rabbit monoclonal antibody against PARP-1 at a dilution of 1/5000 (Anti-PARP-1, Millipore) after blocking the nonspecific sites. Rabbit anti GAPDH polyclonal antibody was used as internal control at a dilution of 1/200 (AbD Serotec). Signals were detected using an alkaline phosphatase conjugated secondary antibody (goat anti-rabbit, Millipore) diluted 1/400 and colorimetric detection system (Vector Laboratories, Inc.). The integrated optical density of the bands, in a digitized picture was measured. Gel-Pro® Analyzer software (Media Cybernetics Inc., USA) was used for densitometry analysis of protein bands. Relative expression is given as the ratio of the test band's densitometric volume to that of the respective GAPDH band.

Comet assay

The cells treated with equol or PTX and control cells were collected at 2, 4, 24 and 48 hours after culture initiation. The cells were allowed to recover from the induced damage by washing in PBS and incubating at 37°C with fresh media for 30, 60 and 120 min before being harvested for the repair assay. Randomly selected cells from each slide were measured using the image analysis (Nikon, Japan). The percentage of DNA in comet tail was estimated for a direct comparison of the influence of equol or PTX on the DNA damage. The comet assay was performed under alkaline conditions according to the previous report (Singh et al., 1988).

TUNEL assay

The cells were fixed in 4% paraformaldehyde (Polysciences) prepared freshly in PBS. Then cells were incubated for 1 hour at 37°C in DNA labeling solution containing TdT reaction buffer, terminal deoxynucleotidyl transferase (TdT) and Br-dUTP (bro-molated deoxyuridine triphosphate). The cells were then resuspended in an antibody solution containing anti-BrdU-FITC antibody (in total darkness for 30 min at room temperature) and then incubated with the propidiumiodide/RNase solution. Both the drug-treated and the control cells were processed according to the Apo-BrdU *in situ* DNA fragmentation assay kit protocol supplied by the manufacturer (BioVision). The cell fluorescence was measured with flow-cytometry (Becton Dickinson equipped with an UV-argon laser). The number of TUNEL positive cells was expressed as a percentage of the total number of cells in the sample.

Statistical analysis

Data are expressed as the mean ± SD. Analysis of variance (ANOVA) with the Tukey post hoc test was used for multiple comparisons. All statistical tests were calculated using the Statistica software (StatSoft, Tulsa, OK, USA), and the significance level was set at p-values of <0.05.

Results

MTT assay

The cytotoxicity of the tested substances was estimated by the MTT assay based on the intracellular reduction of yellow dye MTT to purple formazan crystals, a conversion occurring only in viable cells. Figure 1 presents the percentage of viable SKOV-3 human ovarian cancer cells treated with equol or paclitaxel. With increasing drug concentration, a significant decrease in cell growth was recorded. The cytotoxicity of the drugs was determined on the basis of the IC_{50} value. It has been found that the SKOV-3 cell line exhibits a more sensitivity to equol. It has been also observed that these cells were less susceptible to PTX.

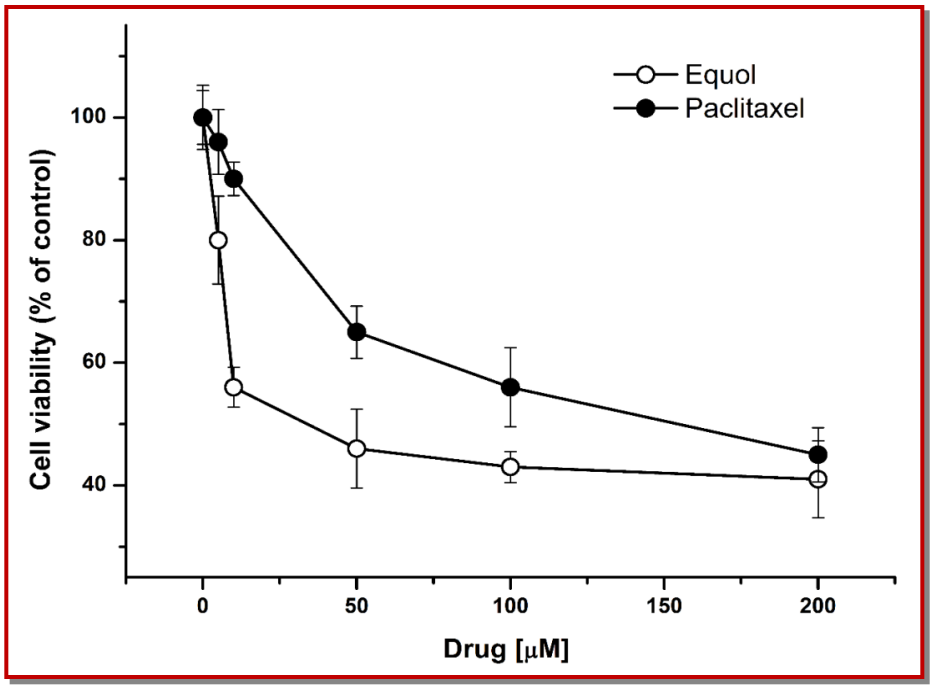

Figure 1: MTT assay results

The IC_{50} parameters determined for equol, and PTX were 20.5 ± 1.4, and 124.2 ± 21.6 μM, respectively. This confirms that equol were more cytotoxic to SKOV-3 cells than PTX. The differences between the IC_{50} values of equol and PTX were statistically significant ($p< 0.0001$).

Double staining with annexin V-FITC/PI and Hoechst 33258/PI: The assessment of apoptosis and necrosis

Two kinds of double staining were used to determine the percentage of apoptotic and necrotic cells that appeared after treatment of the cells with equol or PTX. Early apoptotic changes were presented in Figure 2. Results showed that the number of annexin-positive cells for the tested drugs increased linearly with time. It was observed that after 24 hours incubation with equol, the fraction of annexin-positive cells amounted to ~28% (necrotic ~14%) and increased to 36% (necrotic ~20%) at 48 hours of incubation whereas only minor changes were observed in PTX-treated cells. An increase in the number of annexin-positive cells was found at 24 hours (28%) and 48 hours (~29%). The level of necrotic cells was increased significantly (18%) (Figures 2A and 2B) at the same time of incubation. Significant differences between equol and PTX in terms of annexin-positive cell levels were observed at 48 hours. Externalisation of phosphatidylserine monitored under a fluorescence microscope was shown in Figure 2C. Control cells stained negative for annexin V-FITC. Equol and PTX treatment resulted in cells with strong annexin V-positive fluorescence.

Figure 3 shows the quantitative analysis results of the cell fractions measured by double staining with Hoechst 33258 and propidium iodide. After 48 hours, the significant changes were observed in the treatment with equol (34% of apoptotic cells) whereas a lower level of apoptotic cells was determined for PTX (28%). Tested results indicate that the drugs were shown to induce both apoptosis and necrosis

Activation of P-gp

Immunofluorescence analysis was performed to confirm the presence of P-gp in the SKOV-3 ovarian cells. Figure 4 shows that P-gp activation was found in PTX-treated cells but in contrast equol did not significantly increase the expression of P-gp in SKOV-3 ovarian cell line.

Activation of caspases

Paclitaxel in SKOV-3 cell line leads to the activation of both initiator caspases (-8 and -9). At 24 hours of incubation (216% of control value), the largest increase in activation of caspase-9 was found but upon the prolonged time of incubation with the drug (48 hours, 203%), the gradual decrease in activation was observed as shown in Figure 5. A smaller, but also statistically significant, increase in caspase-8 activity after PTX treatment was observed at early time points like 4 hours (124%). Activation of caspase-8 was also similar to caspase-9. At 24 hours of cell treatment it reached 134%, whereas after 48 hours, it decreased to 118%. We observed fast activation of caspase-8 and -9 at just 4 hours of incubation with equol, at the level of 131% and 113% respectively. Caspase-8 expression decreased in time in order to achieve a level of control at 48 hours. Maximal increase of caspase-3 was at 48 hours of equol treatment (224%).

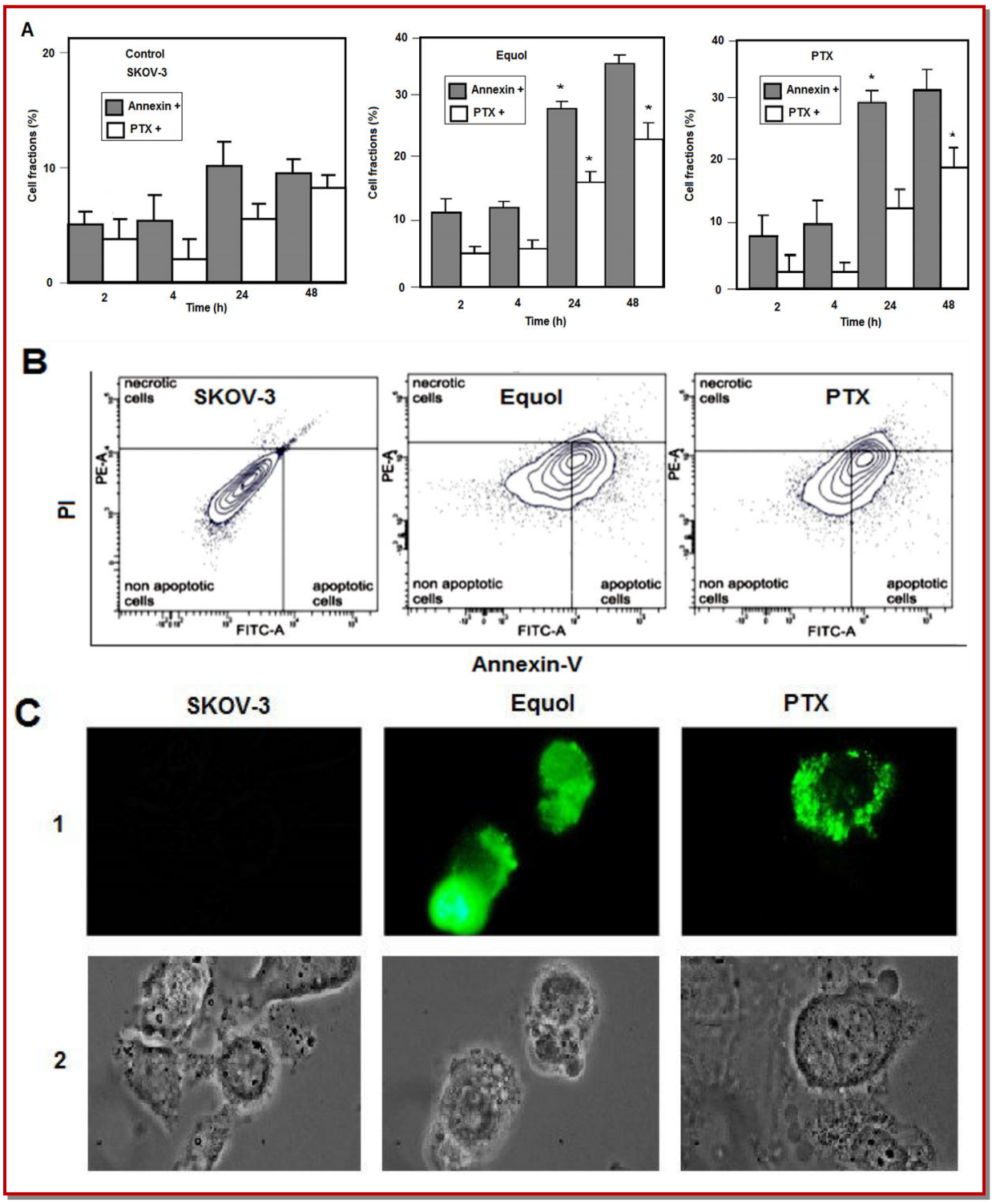

Figure 2: Flow cytometric analysis. Apoptosis in SKOV-3 cells measured using an annexin V-FITC apoptosis detection kit. (A) Quantitative results of the effects of the drugs. (*) Statistically significant differences relative to control cells, taken as 100%, $p< 0.05$. (#) Statistically significant differences between samples incubated with equol and PTX, $p<0.05$. (B) Histograms for control cells and incubated with IC_{50} of equol or PTX for 48 hours. (C) Fluorescence images of SKOV-3 control cells and incubated with equol or PTX (48 hours). Additionally, the images in contrast phase conditions were presented (panel 2)

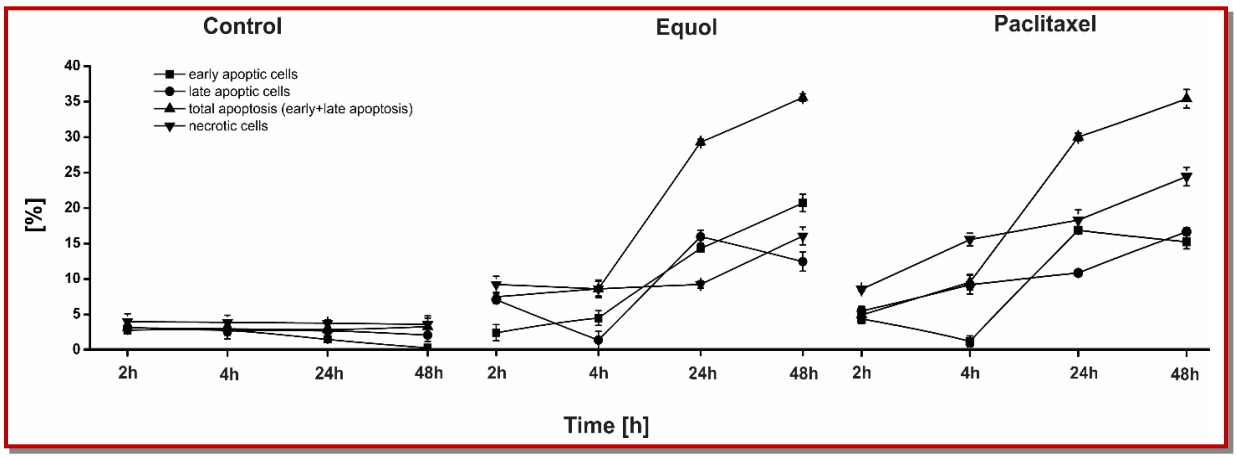

Figure 3: Fraction of the cells in early and late apoptosis and necrosis at different time points following the treatment of SKOV-3 cells with IC_{50} concentration of equol and PTX. Results are presented as means ± S.D. of four experiments

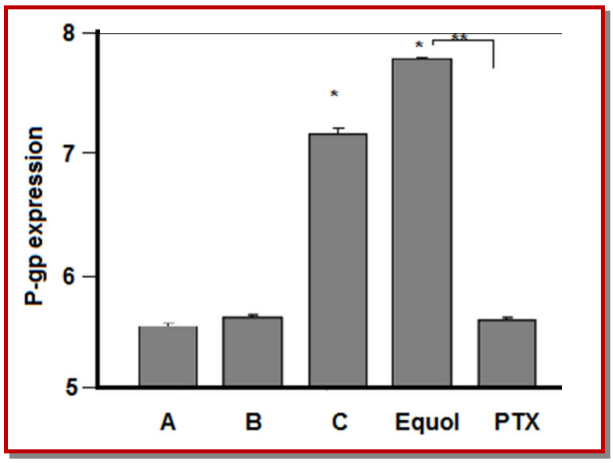

Figure 4: P-gp expression in SKOV-3 cells. (A) control, (B) isotype-matched negative control, (C) vinblastine-positive control

TRAIL level

TRAIL expression in two-time points– after 2 hours, an earlier time than the time of activation of caspase-8 and at 48 hours was measured in this experiment. From the results, we observed that equol lead to a fast increase of TRAIL level when compared to that PTX – after 2 hours of cells treatment (116%). At 48 hours, both drugs mediate a TRAIL level increased of about 126% (Figure 6).

Apoptosis in SKOV-3 cells

Figure 7A shows the specific cleavage of PARP protein induced by equol and PTX. At 24 hours time point, the appearance of an *M*r~ 25,000 polypeptide was clearly observed, that was recognized by the specific antibody anti-PARP-1. Results of the densitometric analysis were shown in Figure 7B. The results showed that the PARP expression was found to be 1.8-fold higher in equol treated cells when compared to that of PTX treated cells. Figure 8 shows the results of TUNEL assay for the determination of DNA fragmentation. It was found that both drugs lead to an increased level of TUNEL-positive cells in a time-dependent manner (4–48 hours). At 24 hours, equol (24%) generates TUNEL-positive cells, quicker when compared to that of PTX (14%). The maximum increase in the percentage of apoptotic cells with DNA strand-breaks was detected 48 hours after treatment with both drugs of about 37%.

DNA damage

Figure 9A showed the quantitative results obtained from comet assay. It clearly demonstrated that equol is able to induce DNA damage in SKOV-3 cells same as that of PTX. The observed increase in DNA damage after 2–48 hours of incubation with both drugs was statistically significant. The highest percentage of the DNA in the tail was observed after 48 hours of equol incubation (40%) whereas PTX generated a similar level of DNA damage of about 28%, even after 48 hours. The level of the DNA damage was found to be higher for equol than that obtained for PTX at all investigated times of incubation. The differences between the drugs after 48 hours incubation were statistically significant and were at the level of 12% (48 hours). Figure 9B showed the measurement of kinetics of DNA damage repair in tumor cells exposed to IC_{50} dose of equol and PTX. The time-dependent significant differences were observed in the mean efficacy of the DNA repair between equol or PTX treated cells and the cells incubated in drug-free medium up to 2 hours.

Higher efficacy of DNA repair was observed after treatment with PTX but when the drug was removed from the medium, the percentage of the DNA in tail drastically decreased to the level of 8% when the cells were preincubated with the PTX 48 hours. When the cells were treated with equol and then the medium was removed, the damages to the DNA remained at a high level, even after the prolonged incubation of 2 hours, and were at the level of 25% (48 hours).

Discussion

The aim of the present study was to approach the

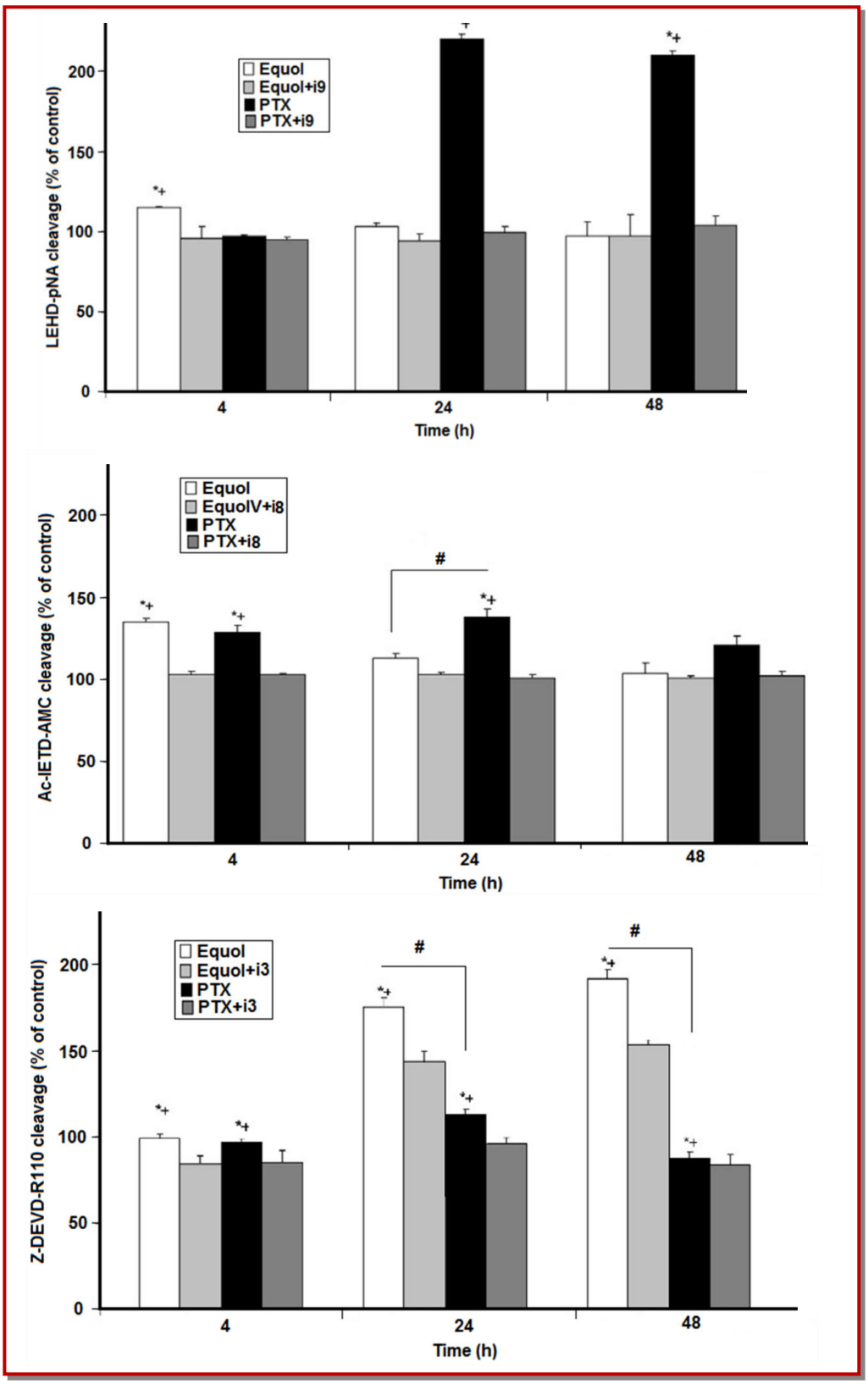

Figure 5: Time-dependent changes in caspase-9, -8 and -3 activity in SKOV-3 cells treated with an IC_{50} dose of equol and PTX for 4 –48 hours in the presence or absence of the caspase-9 inhibitor (Z-LEHD-FMK, i9), caspase-8 inhibitor (Ac-IETD-CHO, i8) or the caspase-3 inhibitor (Ac-DEVD-CHO, i3). The results represent mean ± SD of four independent experiments. (+) statistically significant changes in comparison to the probes incubated with and without caspase inhibitor

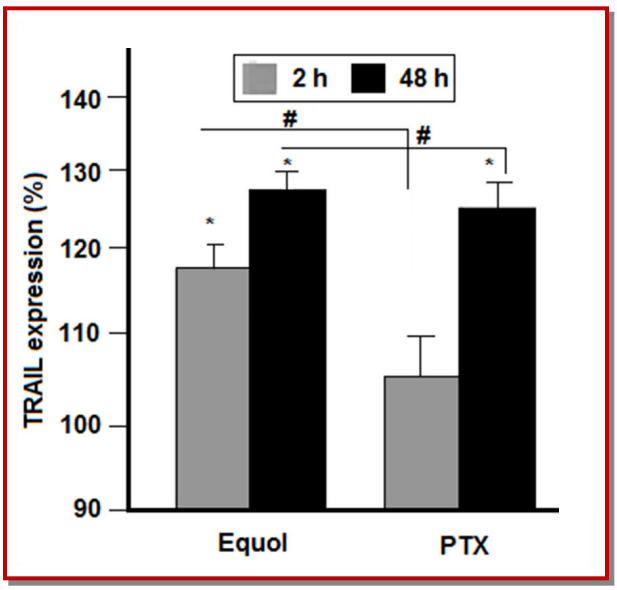

Figure 6: Effect of equol or PTX on TRAIL expression

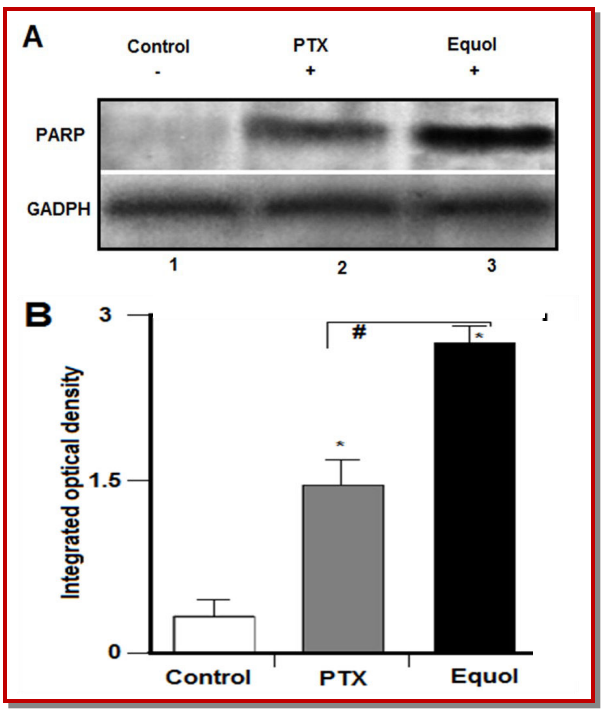

Figure 7: Representative immunoblot and quantitative analysis of PARP protein expression. (A) Western blot analysis showing cleavage of PARP in SKOV-3 cells upon treatment with an IC_{50} dose of equol and PTX. The PARP was detected using specific antibodies. Rabbit Anti-PARP-1 (cleaved p25) monoclonal antibody was attached to the secondary goat anti-rabbit antibody, conjugated with alkaline phosphatase at a dilution of 1/2000. Rabbit anti-GAPDH antibodies were used as internal controls. The blot was developed with BCIP/NBT. (B) The densitometric volume of the PARP bands is expressed relative to the densitometric volume of the loading control gene GAPDH

correlation of the apoptosis induced by PTX and equol, and to determine the signaling pathway leading to cell death in a model of human ovarian carcinoma. We studied the P-gp expression in SKOV-3 cells using the MDR shift assay (Anselmo et al., 2012). We observed that PTX serves as a substrate for the MDR1 P-glycoprotein drug-efflux pump when compared to that of equol. Similar results were obtained in the uterine serous papillary carcinoma cell line (Paik et al., 2010). So far, only a few clinically relevant agents have been shown to trigger bonafide immunogenic cell death. Our results reveal that equol had a greater influence on the TRAIL expression than PTX. The tumor necrosis factor-related apoptosis-inducing ligand is a death ligand, a cytokine that activates apoptosis through cell surface death receptors. The extrinsic pathway can strengthen apoptosis initiated by the cytotoxic drugs. However, there are reports that other anti-cancer drugs stimulate expression of TRAIL (Barbetti et al., 2013, Wennerberg et al., 2013). Actually, equol is one of the most potent anti-oxidant among isoflavones (Arora et al., 1998). In previous studies, it was demonstrated that LPS-induced production of NO and gene expression of iNOS was suppressed by equol.

Stimulation of TRAIL results in death receptors aggregation (DR4, DR5) and Fas-associated death domain-containing protein (FADD). The death domain of FADD binds to an analogous domain of caspase-8 to form the death-inducing signaling complex and thus activates downstream caspases and leads to apoptosis (Xu et al., 2013). Thus, we evaluated the activation of initiator caspase-8 in addition with caspase-9 and -3. We observed that both drugs lead to activation of this caspase, but in different time periods. In the case of PTX, a small early expression of TRAIL, increasing in time, was probably sufficient to activate caspase-8 in the test interval, from 6 to 48 hours. PTX leads to the activation of procaspase-9 at a high level, reaching a maximum at 24 hours of treatment. At 4 hours of PTX incubation, caspase-9 was not observed, which suggests that the first appearing caspase was caspase-8. Moreover, the delay in the expression of TRAIL after treatment with PTX suggests independent of TRAIL activation of caspase-9. Early activation of caspase-9 by equol was sufficient to activate caspase-3. We observed a high activation of caspase-3 after equol, starting 4 hours after treatment. Significant differences between drug-activated capsase-3 levels were observed at 24 hours and the maximal increase was at 48 hours. Active caspase-3 can cleave proteins like PARP or DFF. This leads to morphological changes and DNA damage (Griffin et al., 2003). We observed PARP-1 cleavage in SKOV-3 cells after treatment with both equol and PTX. DNA fragmentation is considered as one of the defining biochemical events of the apoptotic pathway. Both drugs induce TUNEL-positive cells, however, equol leads to a higher level of apoptotic cells than PTX. Equol induces statistically higher changes in the level of DNA damage at prolonged times of incubation in comparison to PTX. Higher DNA damage induced by equol is also in correlation with the level of apoptotic

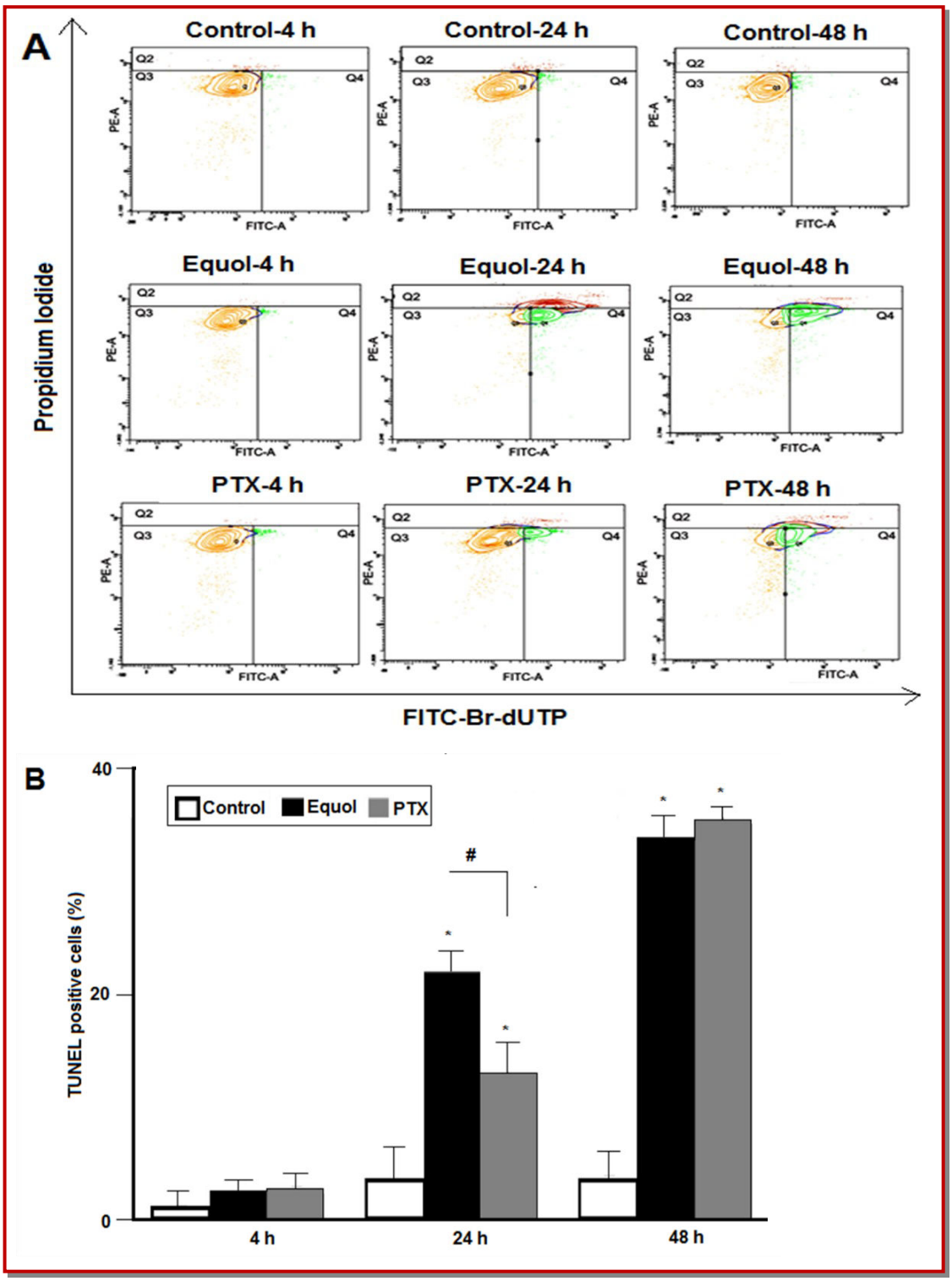

Figure 8: Induction of apoptosis in SKOV-3 cells after treatment with IC_{50} equol or PTX concentration evaluated by TUNEL assay. (A) Typical cytometric histogram obtained after 4 hours, 24 hours and 48 hours of incubation with the drugs. Q2-TUNEL positive cells, PI positive; Q3-TUNEL negative, PI negative cells; Q4-TUNEL positive, PI negative cells. (B) Quantitative results of the effect of the drugs on the level of TUNEL positive cells

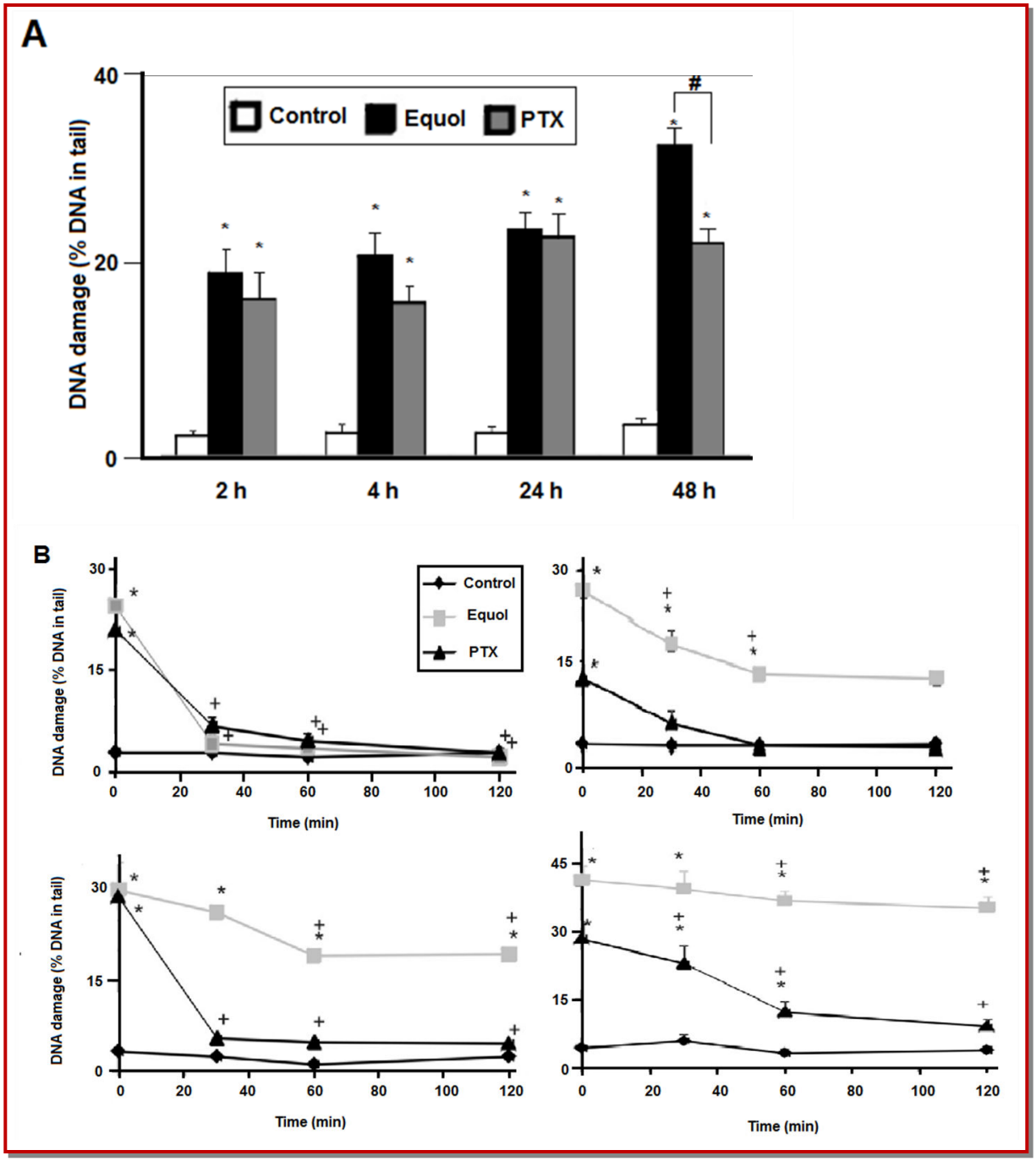

Figure 9: (A) Mean basal endogenous DNA damage measured as the mean comet tail DNA of control cells (without visual disturbances) which were treated with IC_{50} concentration of equol and PTX. The number of analyzed cells in each treatment was 100, and the analysis was repeated four times. (B) Representative kinetics of DNA damage repair in SKOV-3 cells. DNA damage was induced by incubation with IC_{50} concentration of equol and PTX for (1) 2 hours, (2) 4 hours, (3) 24 hours and (4) 48 hours. Repair incubation was in a fresh medium after washing out drugs for 30, 60 and 120 min. Error bars denote SEM

cells indicated based on morphological changes. The levels of apoptotic cells (early and late apoptosis) were higher after equol.

Conclusion

Paclitaxel in ovarian cells induces extrinsic apoptosis pathway as the first line of action. Moreover, for the first time, we demonstrated that equol induces apoptosis via a TRAIL/caspase 8-dependent pathway. The genotoxic effects of equol, as DNA damage, are higher than for PTX and, to a lesser extent, reversible.

Acknowledgement

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Author Info
Dong-Mei Shi (Principal contact)
e-mail: shidongmei13@hotmail.com; Tel: 0086-25-85470580. Fax: 0086-25-85414477

First two authors contributed equally

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