

# Daidzein induces MCF-7 breast cancer cell apoptosis via the mitochondrial pathway

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**Background:** In order to study the anticancer effects and cellular apoptosis pathways induced by daidzein.

**Materials and methods:** We used the human MCF-7 breast cancer cell line as a model and examined the apoptosis by Hoechst–propidium iodide staining fluorescence imaging and flow cytometry.

**Results:** Our data indicated that daidzein induces antiproliferative effects in a concentration- and time-dependent manner. We demonstrated that daidzein-induced apoptosis in MCF-7 cells was initiated by the generation of reactive oxygen species (ROS). Furthermore, we showed that this daidzein-induced ROS generation was accompanied by disruption of mitochondrial transmembrane potential, down-regulation of bcl-2, and up-regulation of bax, which led to the release of cytochrome C from the mitochondria into the cytosol, which, in turn, resulted in the activation of caspase-9 and caspase-7, and ultimately in cell death. The induction of the mitochondrial caspase-dependent pathway was confirmed by pretreatment with pan-caspase inhibitor z-VAD-fmk and antioxidant *N*-acetyl-L-cysteine.

**Conclusion:** Accordingly, daidzein could induce breast cancer cell apoptosis through the mitochondrial caspase-dependent cell death pathway.

**Key words:** apoptosis, breast cancer, daidzein, mitochondria

## Introduction

Although disease-free survival and overall survival of patients with breast cancer have been improved through intensive treatment, breast cancer is still an important public health problem and the second most lethal cancer in women worldwide [1, 2]. A large body of epidemiologic data supports the fact that diet and nutrition play a vital role in carcinogenesis. A recent study confirmed that increasing vegetable and fruit consumption might reduce the risk of breast cancer [3]. Furthermore, diet is considered a primary contributing factor to the huge differential in the prevalence of breast carcinoma. Epidemiological observations found that the incidence of breast cancer is much lower in Asian women than in Western women because the former consume significantly higher amounts of phytoestrogens [4]. A lower incidence of breast cancer is associated with high consumption of phytoestrogens, which are biologically active plant-derived phenolic compounds that structurally mimic the mammalian estrogen, estradiol-17 $\beta$  [5]. Thus, the relationship between phytoestrogens and breast cancer has become a major focus of recent research.

Daidzein belongs to the isoflavone family, which is the most commonly ingested and most intensely studied type of

phytoestrogen, often found in nuts, fruits, soybeans, and soy-based products [6, 7]. Previously, daidzein has been garnered interest as a nontoxic compound capable of inducing tumor cell death in a variety of cancer types [8–10]. Eun et al. [11] showed that daidzein causes cell cycle arrest at the G1 and G2/M phases in human breast cancer cells. They also showed that while caspase-9 activity was significantly increased by daidzein, cyclin D expression decreased. *In vivo*, 9,10-dimethyl-1,2-dibenzanthracene-induced mammary tumors in rats were notably inhibited by daidzein and tumor latency was significantly increased in mouse mammary tumor virus-neu mice [12, 13]. Moreover, Ju et al. [14] revealed that daidzein-induced MCF-7 cell proliferation was blocked by treatment with antiestrogen antibody Faslodex (ICI 182780), demonstrating that daidzein-induced stimulatory effect was estrogen receptor (ER) mediated.

Together, the evidence from *in vitro* [8–11] and *in vivo* [12, 13] studies indicate that the anticancer activity of daidzein in breast cancer is mediated through cell cycle arrest and apoptosis. However, the specific apoptosis mechanisms at work are not yet well understood. Because caspase-9 (an apoptosis biomarker) activity was significantly increased by daidzein, it prompted us to hypothesize that daidzein induces apoptosis through the intrinsic pathway, which regulates apoptotic cascades through signaling convergence in the mitochondrion. Therefore, the aim of the present study was to thoroughly

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research daidzein-induced apoptosis and explore the potential mechanisms at work.

## materials and methods

### reagents

Daidzein (purity 99%) was provided by Tauto Biotech (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) (final concentration 100 mmol/l in medium and stored at  $-20^{\circ}\text{C}$ ). DMSO (Sigma-Aldrich, St. Louis, MO) was used as negative control. The final concentration of DMSO was 0.1% in cell culture experiments. Materials used included Annexin V–Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Becton Dickinson, Franklin Lakes, NJ); [3,3'-dihexyloxycarbocyanine iodide] (DiOC6(3)) (Merck, Darmstadt, Germany); Hoechst–propidium iodide (PI) staining assay kit (Molecular Probes, Beyotime Institute of Biotechnology, Shanghai, China); 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology, Shanghai, China); ApoAlert® Cell Fractionation Kit (Clontech, Japan); bcl-2, bax, cytochrome C, anticlaved caspase-9, anticlaved caspase-7, and  $\beta$ -actin (anti-human) (Santa Cruz Biotechnology, Santa Cruz, CA); the pan-caspase inhibitor, z-VAD-fmk (Promega Biotec, Madison, WI); and antioxidant *N*-acetyl-L-cysteine (NAC; Amresco, Solon, OH).

### experimental procedures

**cell culture.** The MCF-7 cell line was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (Hyclone) in a fully humidified incubator (US Autoflow; Nuair, Plymouth, MN) at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . All cell lines were treated in phenol red-free RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% charcoal-stripped FBS (Hyclone), 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM L-glutamine for 48 h at the beginning of each experiment. The cells were kept in an exponential growth phase during experiments.

**cell growth inhibition study using the MTT assay.** Cell growth inhibition by daidzein was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MCF-7 cells were seeded in 96-well plates at a density of  $6 \times 10^3$  cells per well. After treatment with 25–100  $\mu\text{M}$  daidzein for 24, 48, or 72 h, 20  $\mu\text{l}$  MTT (5 mg/ml) was added. Four hours later, 100  $\mu\text{l}$  DMSO was added to each well to dissolve the resulting formazan crystals. Absorbance was read at 490 nm using an enzyme-linked immunosorbent assay reader (SpectraMax; Molecular Devices, Sunnyvale, CA). Data were collected from three separate experiments and the percentage of daidzein-induced cell growth inhibition was determined by comparison to DMSO-treated control cells.

**examination of daidzein-induced apoptosis by Hoechst–PI staining fluorescence imaging.** To certificate apoptosis phenomenon, the Hoechst 33342 and PI staining assay was carried out,  $2 \times 10^4$  MCF-7 cells per well were cultured overnight in 24-well plates and treated with 0, 25, 50, and 100  $\mu\text{M}$  daidzein for 24 h. At the 24-h point, cell morphology was assessed using an Apoptosis and Necrosis Assay Kit (Beyotime Institute of Biotechnology). Briefly, cells in 24-well plates were trypsinized, washed twice with PBS (phosphate-buffered saline, pH 7.4), and 100  $\mu\text{l}$  of the cell suspension was transferred to black 96-well plates. The cells were then stained with Hoechst 33342 (10 ng/ml) and PI (10 ng/ml) for 20 min at  $4^{\circ}\text{C}$  in the dark. The condensed or fragmented nuclei of apoptotic cells were observed using fluorescence microscopy [excitation (Ex), 365 nm and emission (Em), 480 nm].

**examination of daidzein-induced apoptosis by flow cytometer.** Cells were incubated with 0, 25, 50, and 100  $\mu\text{M}$  daidzein for 24 h, washed twice with

PBS, adjusted to 100  $\mu\text{l}$  of the solution and transferred to a 1-ml centrifuge tube ( $1 \times 10^5$  cells). Then, 5  $\mu\text{l}$  of Annexin V–FITC and 5  $\mu\text{l}$  of PI were added and cells were gently vortexed. Cells were then incubated for 15 min at room temperature (RT) ( $25^{\circ}\text{C}$ ) in the dark and 400  $\mu\text{l}$  of 1 $\times$  binding buffer was added to each tube. Finally, cells were analyzed by flow cytometer (Becton Dickinson) (Ex, 488 nm and Em, 530 nm).

Cells were grown in 6-well plates for 24 h before treatment with pan-caspase inhibitor z-VAD-fmk (100  $\mu\text{M}$ ) or antioxidant NAC (5 mM) for 1 h before exposing them to 100  $\mu\text{M}$  daidzein for 24 h. Then, 5  $\mu\text{l}$  of Annexin V–FITC and 5  $\mu\text{l}$  of PI were added and cells were incubated for 15 min at  $25^{\circ}\text{C}$  in the dark before being analyzed as described above.

**measurement of mitochondrial transmembrane potential with DiOC6(3).** Cells cultured as described above were resuspended with culture medium to a concentration of  $1 \times 10^5$  cells/ml and incubated with DiOC6(3) (20 nM) at  $37^{\circ}\text{C}$  for 30 min. Then DiOC6(3) fluorescence was immediately analyzed with a flow cytometer (Becton Dickinson).

**determination of intracellular reactive oxygen species.** Reactive oxygen species (ROS) were measured on the basis of the intracellular peroxide-dependent oxidation of DCFH-DA to form the fluorescent compound 2',7'-dichlorofluorescein (DCF). Cells were seeded on to 24-well plates at a density of  $2 \times 10^4$  cells per well and cultured for 24 h. After washing twice with PBS, fresh medium containing 0–100  $\mu\text{M}$  daidzein was added and cells were incubated for 24 h. Then, 20  $\mu\text{M}$  DCFH-DA was added and cells were incubated for 30 min at  $37^{\circ}\text{C}$ . The cells were washed twice with PBS, 400  $\mu\text{l}$  of PBS were added to each well, and fluorescence intensity was determined with a flow cytometer.

**subcellular fractionation and western blot analysis.** Cells were separately washed, collected and homogenized in a lysis buffer (10 mM Tris–HCl, pH 8, 0.32 mM sucrose, 5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethyl sulfonylfluoride, and 1% Triton X-100), and centrifuged (16 000 g, 10 min,  $4^{\circ}\text{C}$ ). The protein-containing supernatant was used to detect cleaved caspase-9, cleaved caspase-7, bcl-2, and bax. To examine the subcellular location of cytochrome C, preparations of cytosolic extracts were carried out according to the manual provided in the ApoAlert® Cell Fractionation Kit. To ensure that an equal amount of protein was loaded in each case, western blots were also carried out, using the Bradford protein assay. Equal amounts of proteins (50  $\mu\text{g}$ ) were subjected to electrophoresis in a sodium dodecyl sulfate–polyacrylamide gel (10%). The gel-separated proteins were transferred to Nitropure nitrocellulose membranes (Santa Cruz Biotechnology) and the membranes were blocked with 10% bovine serum albumin in TBST [10 mM Tris–HCl (pH 8.0), 137 mM NaCl, and 0.05% Tween-20 by vol] overnight at  $4^{\circ}\text{C}$  and probed with primary antibodies at RT ( $25^{\circ}\text{C}$ ) for 2 h. Each of the targeted proteins was immunostained using specific antibodies. The antibodies used were anti-bcl-2 (1 : 500 dilution), anti-bax (1 : 500 dilution), anti-cytochrome C (1 : 200 dilution), anticlaved caspase-9 (1 : 500 dilution), anticlaved caspase-7 (1 : 500 dilution), and anti- $\beta$ -actin (1 : 500 dilution) from the Santa company. The membranes were washed three times with TBST and then incubated for 1 h at RT with alkaline phosphatase-conjugated secondary antibodies (Santa) before being visualized by using a chemiluminescence detection kit (Promega).

### statistical analysis

Experiments were carried out three times and statistical evaluation was carried out using one-way analysis of variance tests with the SPSS software system. Data are presented as mean values  $\pm$  standard deviation. *P* values of  $<0.05$  were considered statistically significant.

## results

### MCF-7 cell growth inhibition

Results from MTT assays indicated that the viability of MCF-7 cells was significantly inhibited by daidzein at concentrations  $\geq 25 \mu\text{M}$  (Figure 1). The treatment of MCF-7 cells with 0 (control), 25, 50, or 100  $\mu\text{M}$  of daidzein resulted in a dose- and time-dependent inhibition of cell growth, with inhibition rates of  $12.4 \pm 1.1\%$ ,  $15.87 \pm 0.92\%$ , or  $19.27 \pm 0.95\%$ , respectively, at 24 h, compared with control (all  $P$  values  $< 0.01$ ),  $21.9 \pm 2\%$ ,  $28.37 \pm 1.11\%$ , or  $32.54 \pm 2.16\%$ , respectively, at 48 h ( $P < 0.01$ ), and  $34.88 \pm 1.76\%$ ,  $42.63 \pm 2.30\%$ , or  $49.22 \pm 1.56\%$ , respectively, at 72 h ( $P < 0.01$ ).

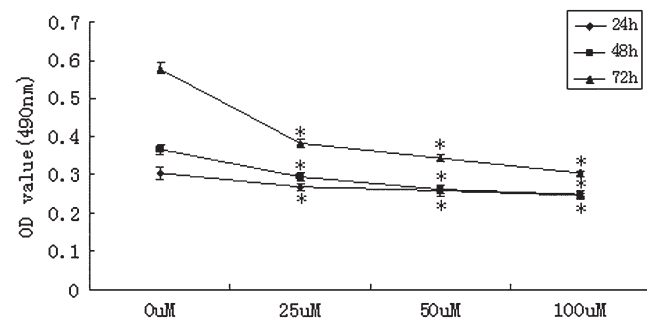
### daidzein-induced apoptosis measured by Hoechst-PI staining fluorescence imaging

Figure 2B shows the results of the Hoechst 33342 and PI staining assay, which was used to confirm daidzein-induced cell apoptosis. In this case, the apoptotic cells, characterized by condensed nuclei, were observed after the exposure to 25, 50, and 100  $\mu\text{M}$  of daidzein for 24 h. Numbers of apoptotic cells increased along with the concentration of daidzein.

### daidzein-induced apoptosis examined by flow cytometry

Flow cytometry assays showed marked changes in cell profiles after treatment with 0, 25, 50, and 100  $\mu\text{M}$  daidzein, which strongly indicated that daidzein could induce apoptosis (Figure 2A). All daidzein treatment groups showed significant increases in apoptosis compared with control groups ( $P < 0.01$ ).

Apoptotic rates ranged from  $0.32 \pm 0.1\%$  to  $29.8 \pm 2.15\%$  (Figure 2A). The 100- $\mu\text{M}$  daidzein group was the highest in all experiments (compared with control group,  $P < 0.01$ ). When we added the antioxidant NAC (5 mM) for 1 h before exposing cells to 100  $\mu\text{M}$  daidzein for 24 h, apoptotic rates decreased from  $29.8 \pm 2.15\%$  to  $15 \pm 2.49\%$  ( $P < 0.01$ ). Additionally, when we added a pan-caspase inhibitor, z-VAD-fmk (100  $\mu\text{M}$ ) for 1 h before exposure to 100  $\mu\text{M}$  daidzein for 24 h, apoptotic rates decreased to  $9.93 \pm 0.55\%$  ( $P < 0.01$ ).



**Figure 1.** Daidzein inhibits MCF-7 cell proliferation. MCF-7 cells were treated with or without different concentrations of daidzein for 24, 48, or 72 h. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out as described. Data are presented as means  $\pm$  standard deviation from three independent experiments. Each experiment was conducted in triplicate.  $*P < 0.01$ .

### measurement of mitochondrial transmembrane potential with DiOC6(3)

As shown in Figure 3A, mitochondrial transmembrane potential (MMP) decreased in response to daidzein in a dose-dependent manner (Figure 3A). Cells treated with 25  $\mu\text{M}$  daidzein showed a  $15.97 \pm 1.78\%$  decrease in membrane potential ( $P < 0.01$ ). Cells treated with 50  $\mu\text{M}$  daidzein showed a  $26.77 \pm 3.52\%$  decrease in membrane potential ( $P < 0.01$ ). Finally, cells treated with 100  $\mu\text{M}$  daidzein showed a  $38.03 \pm 2.79\%$  decrease in membrane potential ( $P < 0.01$ ) (Figure 3B).

### ROS generation in daidzein-treated MCF-7 cells

Daidzein-induced intracellular ROS generation was evaluated using intracellular peroxide-dependent oxidation of DCFH-DA to form fluorescent DCF. DCF fluorescence was detected after cells were treated with 0–100  $\mu\text{M}$  daidzein for 24 h (Figure 4A). The group without DCHF-DA addition was a negative control (N-CON). Rosup-induced intracellular peroxide production was used as a positive control. ROS production was significantly increased upon treatment with 25–100  $\mu\text{M}$  daidzein compared with control ( $P < 0.01$ ) (Figure 4B).

### subcellular fractionation and western blot analysis

To examine whether the daidzein-induced release of cytochrome C resulted in activation of cleaved caspase-9 ( $\sim 37$  kDa) and caspase-7 ( $\sim 17$  kDa), western blots of cells lysates were carried out. The release of cytochrome C ( $\sim 15$  kDa) (Figure 5A) into the cytoplasm and generation of activated fragments of the caspase-9 and caspase-7 at 24 h was clearly shown (Figure 5B) in daidzein-treated cells compared with controls.

In order to determine whether daidzein-induced apoptosis occurred through alterations in expression of the pro- or antiapoptotic proteins bax ( $\sim 26$  kDa) or bcl-2 ( $\sim 28$  kDa), respectively, we carried out western blot analyses for bax and bcl-2. bcl-2 expression was reduced, while bax expression was increased upon treatment with 25–100  $\mu\text{M}$  daidzein for 24 h (Figure 5B). Pretreatment with NAC for 1 h before incubation with 50 or 100  $\mu\text{M}$  of daidzein for 24 h reduced daidzein-induced cytosolic cytochrome C and cleaved caspase-9 and cleaved caspase-7 (Figure 5C).

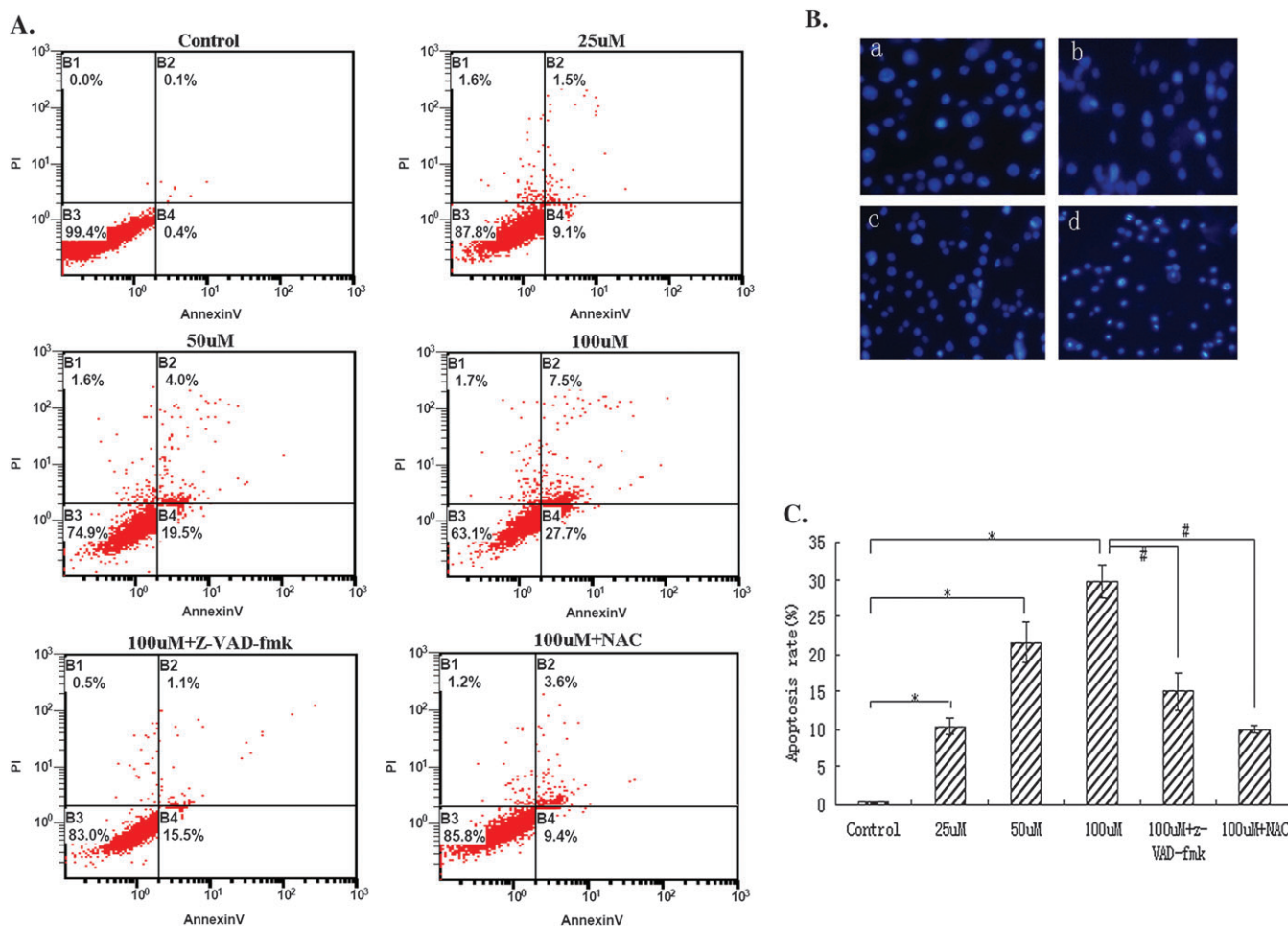
## discussion

We have shown that the phytoestrogen daidzein is effective in decreasing cell numbers in the human MCF-7 breast cancer cell line through growth inhibition and/or apoptosis. Moreover, we determined the exact mechanism of daidzein-induced apoptosis.

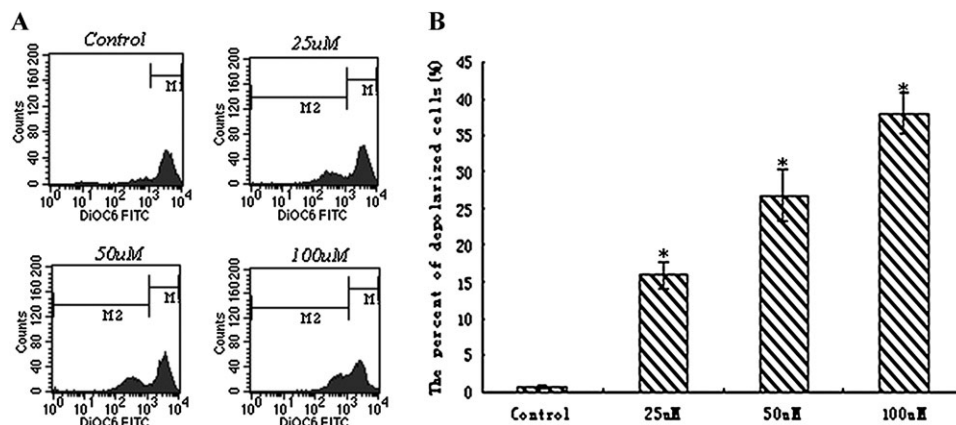
In this study, we showed that MCF-7 cell growth was inhibited by daidzein at different concentrations, ranging from 25 to 100  $\mu\text{M}$ , for 24, 48, and 72 h. Daidzein significantly decreased the proliferation of MCF-7 cells in a concentration- and time-dependent manner (Figure 1). These results are in agreement with previous studies treating MCF-7 and MDA-MB-453 cells with various concentrations of daidzein [11].

To understand the association between daidzein and apoptosis, we examined levels of various apoptotic markers in

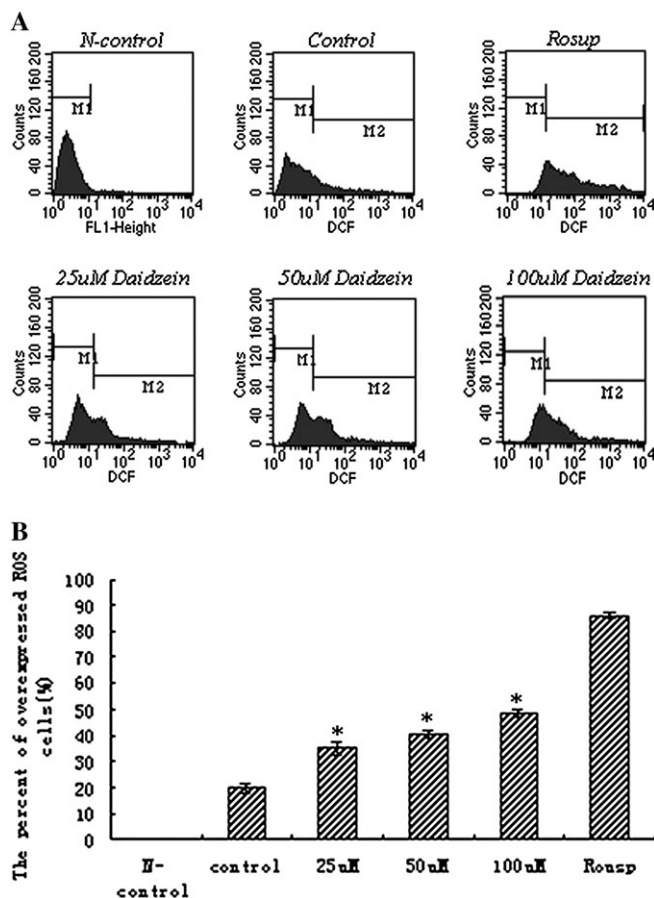




**Figure 2.** Daidzein induces apoptosis in MCF-7 cells. (A) Representative flow cytometric analysis of MCF-7 apoptotic cells stained for Annexin V + propidium iodide (PI) after treatment with 0–100 μM daidzein, 100 μM daidzein + pan-caspase inhibitor z-VAD-fmk (100 μM), or antioxidant N-acetyl-L-cysteine (5 mM) for 24 h. In each panel, the lower right quadrant contains apoptotic cells (positive for Annexin V and negative for PI). (B) Representative photomicrographs (Olympus, Tokoy, Japan; magnification ×200) of MCF-7 cells stained with Hoechst 33342 and PI fluorescent dye after exposure of the cells to 0 μM (a), 25 μM (b), 50 μM (c), and 100 μM (d) daidzein for 24 h. Apoptotic cells were characterized as having condensed or fragmented nuclei. Numbers of apoptotic cells increased with daidzein concentration. (C) Data are representative of at least three independent experiments with similar results, \*, # significant differences from control at  $P < 0.01$ .



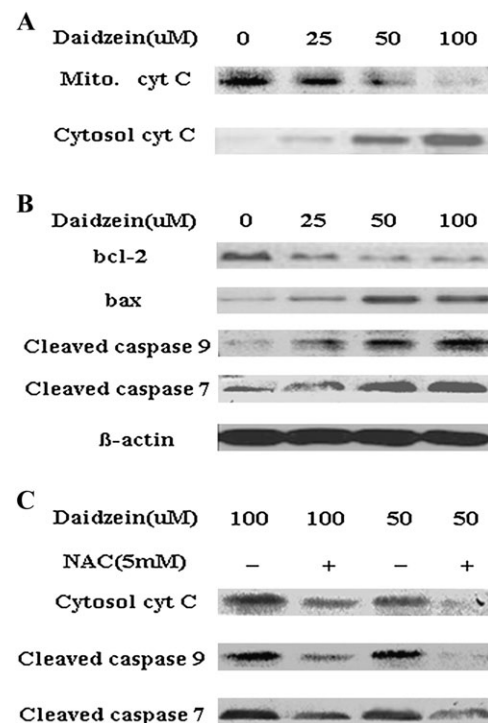
**Figure 3.** Daidzein-induced mitochondrial membrane hyperpolarization in MCF-7 cells. (A) Cells were treated with various concentrations of daidzein for 24 h and then stained with DiOC6 and incubated at 37°C for 30 min. The mean DiOC6 fluorescence intensity was detected using a flow cytometer. M2 represented the percent of low-mitochondrial transmembrane potential cells. (B) Data are expressed in percentage of cells displaying mitochondrial depolarization in daidzein-treated groups compared with controls. Data are presented as means ± standard deviation of three independent experiments, and each experiment was carried out in triplicate (\* $P < 0.01$ ).



**Figure 4.** Daidzein-induced reactive oxygen species (ROS) generation in MCF-7 cells. (A) MCF-7 cells were incubated with 0–100  $\mu\text{M}$  daidzein 24 h followed by incubation with 20  $\mu\text{M}$  2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min. The level of intracellular peroxide was detected by DCFH-DA assay using flow cytometric analysis. The percentage of ROS-overexpressing cells under different treatments was detected. A decrease in intracellular fluorescence intensity was detected in MCF-7 cells without DCFH-DA addition as a negative control (N-CON). Rosup-induced intracellular peroxide production was used as a positive control. (B) Data are expressed as the percentage of ROS-overexpressing cells in each group compared with controls. Data are presented as means  $\pm$  standard deviation of three independent experiments, and each experiment was carried out in triplicate (\* $P < 0.01$ ).

MCF-7 cells exposed to 25, 50, and 100  $\mu\text{M}$  daidzein. We found that apoptosis was induced in a concentration-dependent manner through flow cytometry (Figure 2A) and Hoechst-PI staining fluorescence imaging (Figure 2B).

Generally speaking, apoptosis is initiated by either an extrinsic (activated caspase-8) or an intrinsic pathway (activated caspase-9) [15]. The extrinsic pathway can directly activate caspase-8 through death receptors on the cell surface. The intrinsic pathway regulates apoptotic cascades by the signaling convergence in the mitochondrion, which results in the alteration of the MMP, the release of cytochrome C into the cytosol, and the activation of caspase-9. Since caspase-9 was activated by daidzein [11], we presumed the mitochondrial-mediated cell death pathway was being activated. Disruption of mitochondrial membrane potential and the subsequent release of apoptosis-promoting factors such as cytochrome C are considered key cellular events



**Figure 5.** Effects of daidzein on the expression of apoptosis-related proteins. (A) Western blot analysis of the effect of daidzein on cytochrome C release in MCF-7 cells treated with corresponding doses of daidzein for 24 h. Cytosolic protein and mitochondrial protein were separately extracted. (B) Representative western blots for the expression of bax, bcl-2, cleaved caspase-9, and cleaved caspase-7 in MCF-7 cells following exposure to different concentrations of daidzein for 24 h. Equal amounts of whole-cell extracts were separated in sodium dodecyl sulfate–polyacrylamide gels.  $\beta$ -actin ( $\sim 42$  kDa) was used as a loading control. Results are representative of two independent experiments. (C) Cells pretreated with 5 mM *N*-acetyl-L-cysteine (NAC) were incubated with 50 or 100  $\mu\text{M}$  daidzein for 24 h. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and cytochrome C, cleaved caspase-9, and cleaved caspase-7 were detected using western blotting.

that trigger the intrinsic apoptotic pathway. The observed disruption of mitochondrial membrane permeability (Figure 3) and the increased cytochrome C release (Figure 5A) confirmed our above hypothesis.

ROS are generated in and around mitochondria and are regarded as the byproducts of normal cellular oxidative processes. It has been indicated that they can regulate initiation of apoptotic signaling [16, 17]. High levels of ROS produced by the MCF-7 cells after daidzein treatment were observed (Figure 4) while pretreatment with NAC for 1 h blocked this increase (Figure 2A). Moreover, release of cytochrome C and cleavage of caspase-7 and -9 were significantly suppressed when cells were pretreated with NAC for 1 h before treatment with daidzein (Figure 5C). These results confirmed that ROS were crucial in the induction of apoptosis and acted as upstream signaling molecules to initiate cell death.

Similar to the role of mitochondria in the control of cell death, bcl-2 and bax, survival or apoptotic factors can also prevent or facilitate the release of apoptogenic factors such as cytochrome C [16–18]. The loss of the bcl-2 protein promotes

the opening of the mitochondrial permeability transition pore, leading to cytochrome C release into the cytosol. Disruption of mitochondrial membrane potential by bcl-2 has been shown in several forms of breast cancer [19–21]. We observed a decrease in expression of bcl-2, as well as an increase in expression of bax, in MCF-7 cells after treatment with different concentrations of daidzein. This change in bcl-2 and bax expression may be enough to facilitate pore opening.

The loss of membrane potential is an early event in mitochondrial-mediated apoptosis [22]. After the reduction of membrane potential and the release of mitochondrial cytochrome C, a critical step is the formation of apoptosomes, which ultimately cleave procaspase-3 to form active caspase-3. Caspases play critical roles in the execution of apoptosis [23]. It has been shown that MCF-7 cells lack caspase-3 activity due to a point mutation in the gene coding for this protein [24]. Caspase-7, which is a member of the caspase-3 subfamily, is involved in apoptosis in caspase-3-deficient MCF-7 cells [25]. Through the western blot analysis of caspase-9 and -7, we showed that daidzein-induced cell death in MCF-7 cells was still caspase dependent. Further, the involvement of the caspase-dependent pathway was confirmed by pretreatment with pan-caspase inhibitor z-VAD-fmk, which significantly inhibited daidzein-induced apoptosis. Consequently, our findings indicate that the mitochondrial-mediated caspase-dependent pathway is one possible mechanism involved in daidzein-induced apoptosis. Additionally, we have found the MDA-MB-231 cell line, an ER-negative human breast cancer cell line, can induce apoptosis by the mitochondrial-mediated caspase-dependent pathway (data not shown). From this, we conclude that ER had no connection to apoptosis induction by daidzein.

In conclusion, we have demonstrated that daidzein induces apoptosis through the generation of ROS that perturb mitochondrial function, leading to mitochondrial permeability, cytochrome C release, and caspase activation. The loss of the antiapoptotic mitochondrial bcl-2 protein was also partly responsible for the opening of the mitochondrial permeability transition pore. We have demonstrated that daidzein-introduced apoptosis in MCF-7 cells is mediated by the caspase-dependent cell death pathway. Accordingly, further research should be carried out to elucidate the relationship between daidzein and breast cancer cell apoptosis.

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## disclosure

The authors declare no conflict of interest.

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