

Effects of physiological levels of daidzein on cell proliferation of tumoral and non-tumoral breast cells lines

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Abstract

Introduction

Soy isoflavones are associated with the lower breast cancer risk found in Asiatic populations. They can interfere in various stages of the carcinogenesis process, preventing the development of this pathology and, for this reason, its consumption became popular. Instead of the beneficial effects found in literature, most chemopreventive in vitro studies use non-physiological levels of daidzein (> 30 μM). In our study, the effects of maximal physiological levels of daidzein was investigated in two human breast cell lines: HB4a (non-tumoral) and MCF-7 (tumoral). Experiments to verify cytotoxicity, induction of cell death by apoptosis and, additionally, cell cycle arrest were performed.

Materials and methods

Cytotoxic effect of daidzein (0.1 - 100 μM) was assessed using resazurin-based assay. Flow cytometry tests were performed to investigate apoptosis induction (annexin V-FITC/PI) and cell cycle arrest (propidium iodide staining of DNA content) by daidzein at 10 and 25 μM . Changes in expression of apoptosis related genes after treatment of cells with daidzein (10 and 25 μM) were investigated using quantitative real time PCR.

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Results

Daidzein ranging from 0.1 to 100 μM (for 24 and 48h) were not cytotoxic to both cell lines. Concentrations of 10 and 25 μM did not induce apoptosis in these cells, instead of statistical significant changes in gene expression of some of the target genes (CASP-3, CASP-7, BAX, BCL-xL, COX-2). Both concentrations of daidzein arrested cell cycle in G0/G1 phase only in MCF-7.

Conclusion

Daidzein at maximal achievable physiologic serum levels selectively arrested cell cycle only in the tumoral MCF-7, and did not induce cell death by apoptosis, one of the major effects described in literature. We hope that our study helps in understanding the chemopreventive effects of low levels of soy isoflavones in breast carcinogenesis.

Introduction

It has been more than 30 years since Doll and Peto called attention for the association between lifestyle and cancer¹ and now it is becoming clearer as research continues to show nutrition plays a major role in cancer.

Epidemiological studies also point out this association as many of these show that the incidence and mortality of some kinds of cancer are higher in Western countries with meat-based diets than in Asian countries with plant-based diets^{2,3}. One of the best examples of this situation is the association between the high intake of soy-based food in Asian populations and the reduced risk of breast, ovarian, prostate and other types of cancer and chronic diseases⁴.

Because of these beneficial health effects, soy-based products have gained worldwide popularity and are especially consumed by women with

menopause to alleviate its symptoms^{5,6}.

However there have been concerns that isoflavones (the main compounds found in soybean and soy products), may increase the risk of recurrence or stimulate the growth of existing tumours⁷. Despite significant research in the area, the role of isoflavones in breast and hormone-related cancers remains controversial^{8,9}.

The main compounds found in soy that are responsible for the human health benefits are the isoflavones daidzein (7,4'-dihydroxyisoflavone) and genistein (4', 5, 7-trihydroxyisoflavone). Before ingestion, daidzein and genistein are present as β -D-glycosides namely daidzin and genistin, which according to Setchell¹⁰, are biologically inactive. Only after being hydrolysed by intestinal bacteria, isoflavone glycosides become bioactive aglycones daidzein and genistein and are absorbed by the intestinal tract¹¹.

Daidzein and genistein, also known as phytoestrogens, are able to bind to oestrogen receptors, inducing cell transcription and eliciting biological effects similar to those of other natural oestrogens¹² and, for this reason, interest has focused primarily on their effects on hormone-dependent conditions, such as breast, ovarian and prostate cancers.

Cancer chemoprevention properties of daidzein include induction of apoptosis and cell cycle arrest^{13,14,15}.

However, in most studies, high concentrations of daidzein (> 30 μM) were tested, and they are difficultly found in human plasma after ingestion of soy-based foods or supplements. According to Moiseeva and Manson¹⁶, such studies hinder predictions of efficacy.

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Another limitation to investigate the preventive effects of isoflavones is the majority use of tumoral cell lines, although very well established in most areas¹⁴.

Considering these observations, we have evaluated the cytotoxicity, induction of apoptosis, cell cycle arrest and expression of pro- and anti-apoptotic genes focusing on concentrations of daidzein considered the maximal physiologically (i.e., that have been already found in human plasma) achievable through diet or through pharmacological administration, using two human mammary cell lines: a non-tumoral lineage (HB4a) and a tumoral one (MCF-7).

Materials and methods

The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

Reagents

Daidzein (DAID.) (CAS 446-72-0), camptothecin (CAS 7689-03-4) and resazurin (CAS 62758-13-8) were acquired from Acros Organics (New Jersey, USA). Doxorubicin (CAS 29042-30-6; Adriblastina®) is manufactured by Actavis Italy (Milan, Italy) and distributed by Pfizer. Reagents used to maintain cell cultures and used to perform qRT-PCR were from Invitrogen Corporation (NY, USA): DMEM (Dulbecco's Modified Eagle Medium; cat n. 12800-058), foetal bovine serum, antibiotics (cat n. 15240-062), insulin and trypsin; RNase A, Trizol LS, DNase I, dNTP mix, RNase Out™, M-MLV reverse transcriptase, DEPC water and Platinum® SYBR® Green qPCR SuperMix-UDG kit. Hydrocortisone was from Pharma Nostra (Rio de Janeiro, Brazil). Oligo sequences were purchased from Prodimol Biotecnologia (Belo Horizonte, MG, Brazil). Propidium iodide was from Sigma-Aldrich (St. Louis, MO, USA) and annexin V-FITC from BD Pharmigen™ (San Diego, CA, USA).

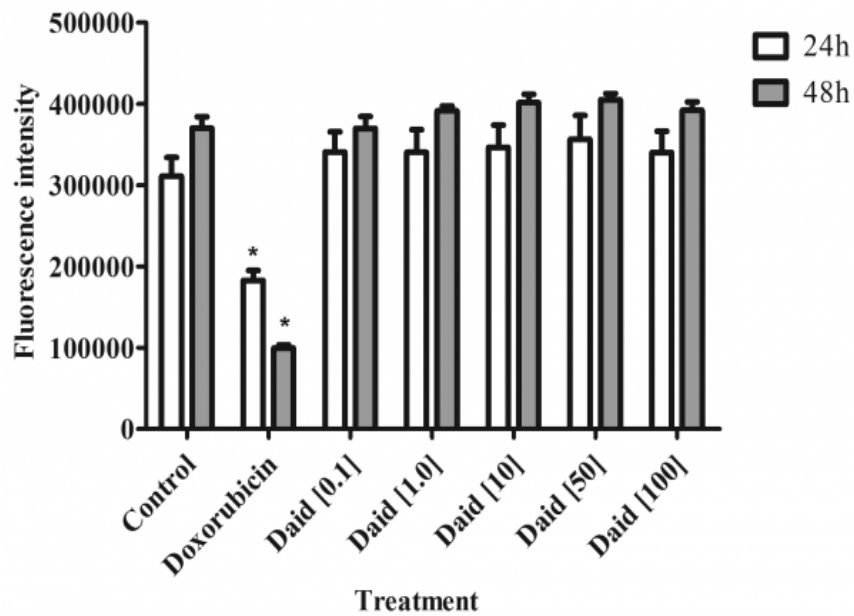


Figure 1: Fluorescence intensity (mean ± SD) measured after treatment of HB4a with different concentrations of daidzein (Daid) in resazurin-based assay (24, 48 h). Daid [0.1] = cells treated with daidzein 0.1 µM; Daid [1] = cells treated with daidzein 1 µM; Daid [10] = cells treated with daidzein 10 µM; Daid [50] = cells treated with daidzein 50 µM; Daid [100] = cells treated with daidzein 100 µM; Doxorubicin = cells treated with doxorubicin 10 µg/mL. * indicates cytotoxic effect ($p < 0.05$; ANOVA followed by Dunnett's test compared to control group; $n=9$).

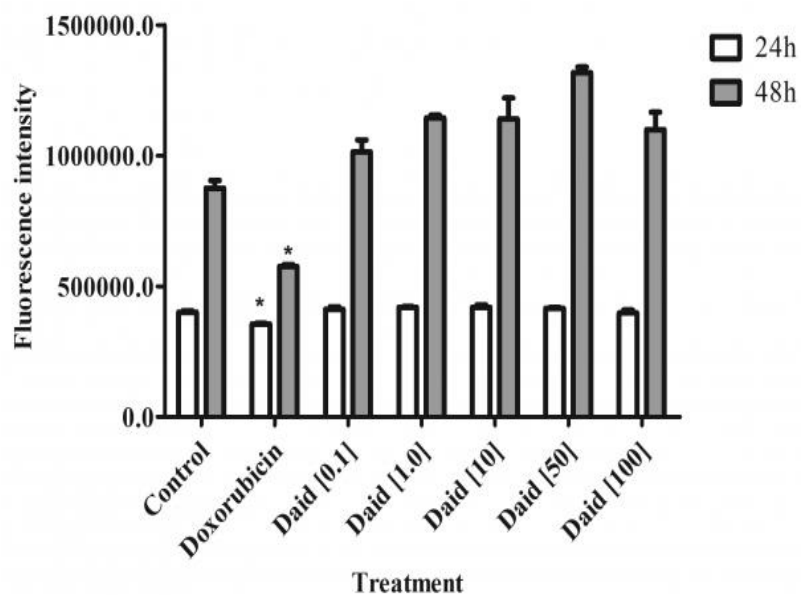


Figure 2: Fluorescence intensity (mean ± SD) measured after treatment of MCF-7 with different concentrations of daidzein (Daid) in resazurin-based assay (24, 48h). Daid [0.1] = cells treated with daidzein 0.1 µM; Daid [1] = cells treated with daidzein 1 µM; Daid [10] = cells treated with daidzein 10 µM; Daid [50] = cells treated with daidzein 50 µM; Daid [100] = cells treated with daidzein 100 µM; Doxorubicin = cells treated with doxorubicin 10 µg/mL. * indicates cytotoxic effect ($p < 0.05$; ANOVA followed by Dunnett's test compared to control group; $n=9$).

Daidzein concentrations

Daidzein was dissolved in dimethylsulfoxide (DMSO) and used in culture with DMSO maximum concentration of 1%. In cytotoxicity assay, we evaluated five concentrations

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of daidzein. In the other tests, we have focused on concentrations of 10 and 25 μM of daidzein, representing, respectively, maximal dietary and pharmacological concentrations found in human plasma according to literature^{8,17,18,19}.

Cell lines and culture conditions

MCF-7 cells (tumoral) were kindly provided by Professor João Ernesto de Carvalho (UNICAMP, São Paulo, Brazil) and HB4a cell line (non-tumoral) was kindly provided by Professor Silvia R. Rogatto from Hospital do Câncer A. C. Camargo (São Paulo, Brazil). MCF-7 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and antibiotics. HB4a cells were cultivated in the same medium supplemented with 5 $\mu\text{g}/\text{mL}$ of insulin and hydrocortisone. Cells cultures were maintained at 37 $^{\circ}\text{C}$, 5% CO_2 and 95% relative humidity.

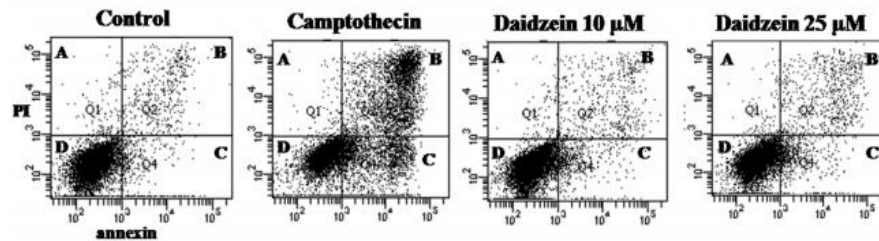
Cell density used in each experiment was determined by pilot-tests and it aimed to avoid detaching of cells resultant from total confluence in the last day of each assay.

Cytotoxicity evaluation - resazurin-based assay

The cytotoxicity assay was standardized based on the protocols described by Nakayama et al., O'Brien et al. and McMillian et al.^{20,21,22}.

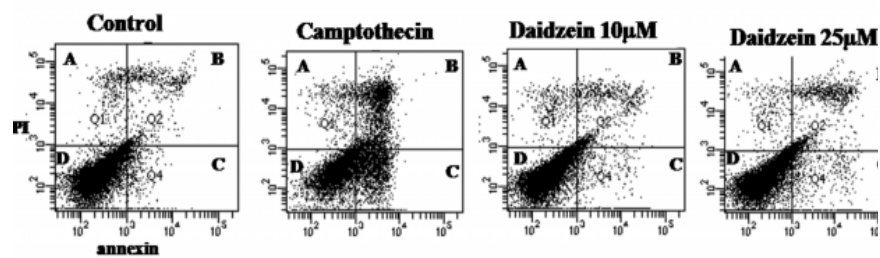
Approximately 4.5×10^4 cells were seeded in each well of a 24 wells-microplate and treated with daidzein for 24 or 48h (0.1; 1; 10; 50 and 100 μM). Control treatment was medium with 1% DMSO and control of cytotoxicity induction was doxorubicin at final concentration of 10 $\mu\text{g}/\text{mL}$. At the end of this period, cells were incubated with resazurin 60 μM for 3h.

Fluorescence was measured with VICTOR 3 (Perkin Elmer) at 530-560 nm of excitation and 580-600 nm of emission ranges. Experiments were performed in biological triplicate with three wells/ each treatment (n=9).



Treatment	Viable cells (D)	Apoptotic cells (C)	Late apoptotic cells (B)	Total of apoptotic cell (B+C)	Necrotic cells (A)
Control	86.66 ± 3.21	6.32 ± 1.93	5.72 ± 1.54	12.04 ± 3.25	1.2 ± 0.4
Camptothecin	36.78 ± 10.79*	32.26 ± 7.81*	30.38 ± 3.90*	62.64 ± 11.3*	0.6 ± 0.51
Daidzein 10 μM	81.66 ± 5.4	9.53 ± 3.92	7.86 ± 2.02	17.4 ± 5.58	0.84 ± 0.27
Daidzein 25 μM	80.7 ± 6.71	8.86 ± 3.34	9.48 ± 4.37	18.35 ± 6.01	0.82 ± 0.19

Figure 3: Annexin V-FITC/PI analysis of apoptosis (flow cytometry) induction in HB4a cells treated with daidzein for 24 hours. *statistical significant ($p < 0.05$; ANOVA followed by Dunnett's test compared to control group; n=6).



Treatment	Viable Cells (D)	Apoptotic Cells (C)	Late apoptotic cells (B)	Total of apoptotic cells (B+C)	Necrotic cells (A)
Control	78.75 ± 6.22	10.51 ± 8.1	6.35 ± 1.34	16.86 ± 7.25	5.58 ± 2.13
Camptothecin	50.3 ± 13.72*	26.35 ± 8.95*	15.4 ± 9.36*	41.75 ± 18.31*	6.17 ± 4.11
Daidzein 10 μM	68.51 ± 8.60	16.01 ± 12.58	9.54 ± 2.23	27.42 ± 9.37	4.9 ± 1.98
Daidzein 25 μM	70.45 ± 8.55	15.45 ± 11.07	9.36 ± 4.09	24.81 ± 9.24	4.43 ± 1.57

Figure 4: Annexin V-FITC/PI analysis (flow cytometry) of apoptosis induction in MCF-7 cells treated with daidzein for 24 hours. *statistical significant ($p < 0.05$; ANOVA followed by Dunnett's test compared to control group; n=6).

Analysis of apoptosis induction by flow cytometry (annexin V-FITC/PI)

Approximately 10^5 cells HB4a or MCF-7 were seeded in each well of a 6 wells-microplate. Cells were treated with camptothecin (4 $\mu\text{g}/\text{mL}$) and daidzein 10 or 25 μM for 24 hours. At the end of the treatment, cells were trypsinized and centrifuged (1000 rpm, 5 min, 4 $^{\circ}\text{C}$). Supernatant was

discarded and cell pellet was resuspended in cold PBS followed by another centrifugation (1000 rpm, 10 min, 4 $^{\circ}\text{C}$). Cells were then labelled with annexin V-FITC and propidium iodide (5 $\mu\text{g}/\text{mL}$), and protected from the light. Ten thousand events were analysed in a BD FACS CANTO™ flow cytometer. Experiments were

Table 1: Relative gene expression (R value) of CASP-3, CASP-7, BAX, BCL-xL and COX-2 after treatment of 10 or 25 μM of daidzein in HB4a cells. R value \pm SD and p value (qRT-PCR).

	Daidzein 10 μM	Daidzein 25 μM
CASP-3(Caspase-3)	1.003 \pm 0.232 p = 0.987	1.208 \pm 0.185 p = 0.031
CASP-7(Caspase-7)	1.114 \pm 0.193 p = 0.522	1.191 \pm 0.149 p = 0.204
BAX(BCL2-associated X protein)	1.124 \pm 0.156 p = 0.461	1.524 \pm 0.176 p = 0.014
BCL-xL(BCL2-like 1)	-1.562 \pm 0.176 p = 0.058	1.335 \pm 0.168 p = 0.010
COX-2(cyclooxygenase-2)	1.477 \pm 0.477 p = 0.11	1.96 \pm 0.478 p = 0.001

Data were submitted to software REST© (PFAFFL *et al.*, 2002). Negative R value means downregulation of the gene.

performed in biological duplicate with three wells/ each treatment (n = 6).

Cell cycle analysis by flow cytometry (propidium iodide staining of DNA content)

The same way as in the previous experiment (annexin V-FITC/PI tests), cells (10^5 /well) were seeded in 6 wells-microplate and treated with camptothecin (4 $\mu\text{g}/\text{mL}$) and daidzein 10 or 25 μM for 24 hours. After trypsinization of the cells and centrifugation (1000 rpm, 5 min, 4°C), it was added to PBS and ice cold ethanol 70% to the pellet. Then, it was resuspended and left at -20°C for 24 hours. At the end of this period, cells were centrifuged (1000 rpm, 5 min, 4°C), supernatant was discarded and it was added to cold PBS and RNase A (10 mg/mL) to each tube. Samples were incubated for 30 minutes at 37°C, followed by staining with hypotonic fluorochrome solution (propidium iodide, 5 $\mu\text{g}/\text{mL}$; sodium citrate 0.1% and Triton X-100 0.1%) one hour before flow cytometry analysis (10.000 events; BD FACS CANTO™).

Experiments were performed in biological duplicate with three wells/ each treatment (n = 6).

Quantitative Real-time PCR (qRT-PCR)

For analysis of gene expression, 10^6 cells were seeded in 25 cm² culture flasks and treated with 10 μM or 25 μM of daidzein for 12 h. Experiments were performed in biological

duplicate and technical triplicate (n = 6).

Total RNA was prepared using Trizol LS reagent followed by DNase I treatment, both according to the manufacturer's instructions.

Complementary DNA (cDNA) was synthesized subsequent to verification of RNA quality in agarose gel (28S and 18S rRNA pattern of bands) and by A260/A280 ratio (Biophotometer -

SuperMix-UDG kit in a total of 20 μL of reaction mix (2 μL cDNA - diluted 1:5; 10 μL SYBR® Green; 0.5 μL of each specific gene primer (10 μM), and 7 μL of H₂O). Target cDNAs were amplified in separate tubes: 3 min at 95°C, then 35 cycles of denaturation (95°C for 20 s), annealing (at 60°C for 30 s) and extension (72°C for 20 s) per cycle.

Melting curves (50 to 95°C ready every 0.5°C) for each PCR reaction were generated to ensure the purity of the

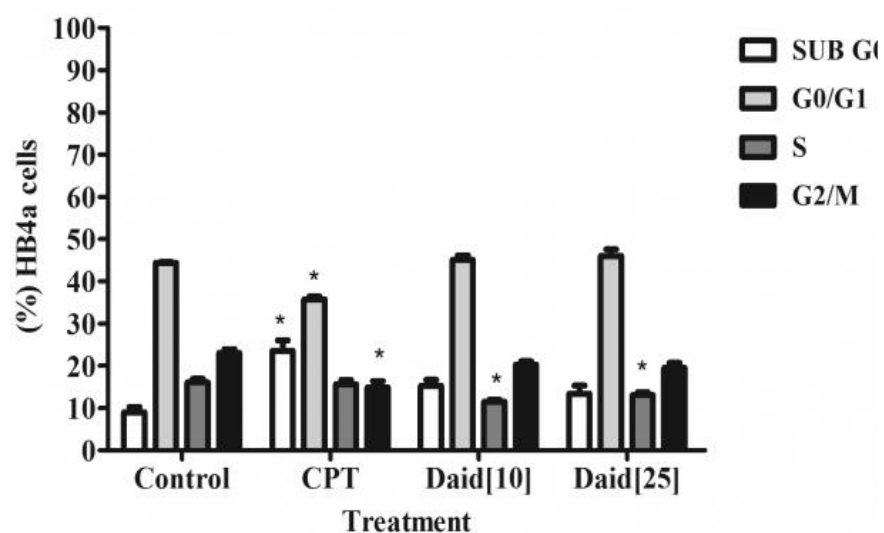


Figure 5: Effects of daidzein (24 h) on cell cycle distribution of HB4a (propidium iodide staining of DNA content – flow cytometry). CPT = cells treated with camptothecin 4 $\mu\text{g}/\text{mL}$; Daid [10] = cells treated with daidzein 10 μM ; Daid [25] = cells treated with daidzein 25 μM . *statistical significant (p < 0.05; ANOVA followed by Dunnett's test compared to control group; n=6).

Eppendorf). Real time PCR experiments were performed in the Cromo 4™ Engine Opticon detection system (Bio-Rad) using the Platinum® SYBR® Green qPCR

amplification product. Primers of CASP-3, CASP-7, BAX and BCL-xL were used, as well as GAPDH as reference gene²³ and COX-224. Primer sequences were designed with the IDT tool⁴⁸ CASP-3

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(NM_032991.2) 5'-GTGCTACAATGCCCTGGAT-3' and 5'-GCCATTTCATTTATTGCTTCC-3' (199 bp); CASP-7 (NM_001227.3) 5'-TCACCATGCGATCCATCAAGACCA-3' and 5'-TTTGTCTGTTCCGTTTCGAACGCC-3' (149bp); BAX (NM_004324.3) 5'-TTTCTGACGGCAACTTCAACTGGG-3' and 5'-TGTCCAGCCCATGATGGTTCTGAT-3' (122bp); BCL-xL (NM_138578.1) 5'-TGGGCTCACTCTCAGTCGGAAT-3' and 5'-ATGTAGTGGTTCTCCTGGTGGCAA-3' (121bp).

Data analysis

Data obtained in cytotoxicity and in flow cytometry tests were submitted to ANOVA followed by Dunnett's test compared to the control group ($\alpha = 0.05$; GraphPad Prism5 software). Relative quantification method of analysis was employed in qRT-PCR using GAPDH as reference gene according to Pfaffl with REST© software (Relative Expression Software Tool)^{25,26,49}.

Results

Cytotoxicity evaluation – resazurin-based assay

Data obtained with the resazurin-based assay showed that none of the tested concentrations of daidzein were cytotoxic, neither for non-tumoral cells (HB4a) nor for the tumoral lineage MCF-7 (Figure 1 and Figure 2).

Analysis of apoptosis induction by flow cytometry

Figure 3 and figure 4 show results obtained in analysis of apoptosis induction by flow cytometry. Both concentrations of daidzein (10 or 25 μM) were not able to induce apoptosis in HB4a or in MCF-7 cells; it was observed only when cells were treated with camptothecin.

Cell cycle analysis by flow cytometry

In this test, we observed that the concentrations of daidzein used (10 or

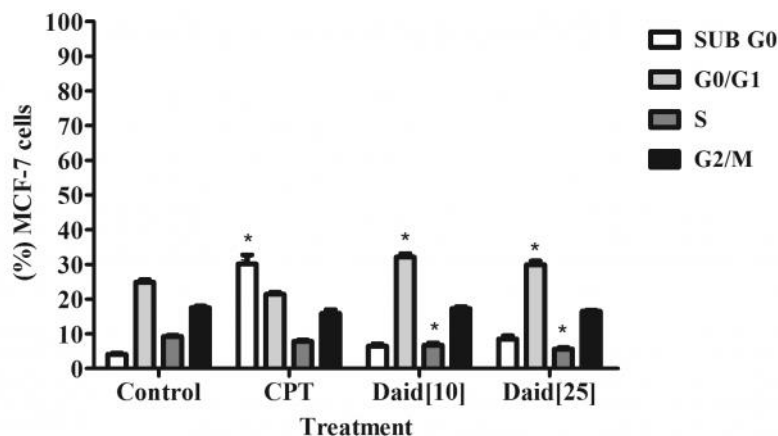


Figure 6: Effects of daidzein (24 h) on cell cycle distribution of MCF-7 (propidium iodide staining of DNA content – flow cytometry). CPT = cells treated with camptothecin 4 $\mu\text{g}/\text{mL}$; Daid [10] = cells treated with daidzein 10 μM ; Daid [25] = cells treated with daidzein 25 μM . *statistical significant ($p < 0.05$; ANOVA followed by Dunnett's test compared to control group; $n=6$).

25 μM) were not able to arrest cell cycle of non-tumoral cells HB4a (Figure 5). For MCF-7 (Figure 6) we can observe a significant increase in number of cells at the G0/G1 phase after treatment of both concentrations of daidzein (10 or 25 μM) for 24 hours. As a consequence of this arrest, the percentage of cells at S phase had decreased.

Quantitative Real-time PCR (qRT-PCR)

Table 1 and table 2 show the values of R (ratio) obtained in the REST© software for gene expression of the selected primers.

For HB4a cells (Table 1), we can observe that dietary concentration of daidzein (10 μM) did not modify mRNA expression of the analysed genes significantly; however, the gene expression ratios for CASP-3, BAX, BCL-xL and COX-2 became statistically significant when pharmacological concentration of daidzein (25 μM) was used to treat these lineage. In MCF-7 cells (Table 2), daidzein 10 μM modified expression of BCL-xL. When cells were treated with 25 μM of daidzein, CASP-3 and CASP-7 expression was altered. It is important to report that COX-2 expression in

mammary tumoral MCF-7 cells was null.

Discussion

Soy based-food and supplements are among the most worldwide consumed because of their various beneficial health effects. They are very popular among women at climacteric, which have been used as soy isoflavones to manage menopausal symptoms⁹.

Although there are a significant number of studies on soy isoflavones, their preventive effects on breast cancer are still under consideration because of the lack of consistent human data from long-term dietary intervention trials and because of the tumour stimulating effects found in in vitro and in vivo studies.

Classical examples are the proliferating effect of tumoral MCF-7 cells in low daidzein concentrations, increase in breast tumour growth in mice fed with daidzein (xenograft mouse model) or even the absence of protective effect of the animals fed with isoflavones^{27,28,29,30,31}.

Besides, there are much more studies and data on molecular mechanisms of the effects of genistein than of daidzein.

Table 2: Relative gene expression (R value) of CASP-3, CASP-7, BAX and BCL-xL after treatment of 10 or 25 μ M of daidzein in MCF-7 cells. R value \pm SD and p value (qRT-PCR).

	Daidzein 10 μ M	Daidzein 25 μ M
CASP-3 (Caspase-3)	1.253 \pm 0.254 p = 0.251	2.773 \pm 0.489 p = 0.045
CASP-7 (Caspase-7)	1.38 \pm 0.297 p = 0.171	2.059 \pm 0.371 p = 0.004
BAX(BCL2-associated X protein)	1.34 \pm 0.238 p = 0.151	-1.107 \pm 0.213 p = 0.418
BCL-xL (BCL2-like 1)	1.467 \pm 0.259 p = 0.037	1.209 \pm 0.218 p = 0.368

Data were submitted to software REST© (PFAFFL *et al.*, 2002). Negative R value means downregulation of the gene. COX-2 expression in mammary tumoral MCF-7 cells was null.

In our study, daidzein at low (0.1-10 μ M) and at high (> 10 μ M) concentrations were not cytotoxic to tumoral MCF-7 cells or to non-tumoral HB4a cells, in both experimental points analysed (24 and 48 h). In the study performed by Röhrdanz *et al.*³² with H4IIE rat hepatoma cells, cytotoxicity was apparent only with concentrations of daidzein above 200 μ M (MTT assay). Daidzein at concentrations of 0.05, 0.5, 5, 50 μ M also did not show cytotoxicity to porcine granulosa cells harvested from large, preovulatory follicles, in the Alamar Blue™ assay³³.

Blay *et al.*³⁴ found a range of non-cytotoxic concentration of 0–25 μ M for daidzein in RAW 264.7 cells. Lee *et al.*³⁵ also did not find cytotoxicity in their study with HCT-116 human colon cancer cells with high concentrations of daidzein (25, 50 and 100 μ M). Moreover, Wang and Kurzer³⁶ found that daidzein 0.01-100 μ M was not cytotoxic and stimulated DNA synthesis (proliferation) in MCF-7. Our results are also in accordance with the data obtained by Park *et al.*³⁷: high concentration of daidzein (200 μ M) also did not induce cytotoxicity to SK-HEP-1 (hepatocellular carcinoma cells) or Chang cells (normal hepatocytes).

Contrary, some authors have found cytotoxic activity of daidzein in different cells types, including breast cancer cells lines. Choi and Kim³⁸ demonstrated that daidzein significantly decreased the proliferation of both MCF-7 and MDA-

MB-453 cells in a dose- and time-dependent manner, with significant decreases at concentrations of 10 μ M in MCF-7 and 1 μ M in MDA-MB-453 after 24 hours of treatment. Jin *et al.*³⁹ also observed cytotoxic effect of daidzein in MCF-7 cells, but they used only high concentrations of 25, 50 and 100 μ M. These contradictory results are in accordance with the well documented biphasic effect of soy isoflavones, i.e., they can promote or inhibit cell proliferation^{17,36}.

Daidzein, like other isoflavones, can exert a wide range of effects through activation/inhibition of several cellular pathways^{14,15}. Soy isoflavones activities on apoptotic and cell cycle cascades are one of the most investigated by researches in searching to obtain a cancer chemopreventive effect. Another desirable characteristic of a chemopreventive agent is to affect only tumoral cells. In our study, at maximal achievable physiologic serum levels (10 or 25 μ M), daidzein was not able to induce apoptosis in the breast cell lines used (HB4a and MCF-7).

Together with qRT-PCR results, we can conclude that daidzein are able to act in an apoptotic cascade, but it was not sufficient to produce a chemopreventive response in the studied cells through this classical pathway of cell death.

Anti-apoptotic effect of daidzein has been found by many authors, and despite this it might not be desirable in cancer chemoprevention, it might

be useful in the prevention/treatment of neurodegenerative diseases.

Adams *et al.*⁴⁰, for example, showed that addition of daidzein attenuated the upregulation of active caspase-3 expression in neurons cultures. Wu *et al.*⁴¹ demonstrated that daidzein (1 or 5 μ M) decreased the rate of apoptosis in MCF-7 cells through downregulation of bax protein. Moreover, daidzein improved antiapoptotic effect in an E2 (17 β -Estradiol) dose-dependent manner, suggesting that daidzein may enhance the deleterious effect of endogenous E2 in hormone-dependent breast cancer⁴¹.

Pro-apoptotic activities of daidzein have also been demonstrating in different cell lines, however with supraphysiological concentrations being investigated in most of the studies, like in the one performed by Park *et al.*³⁷ These authors concluded that daidzein-induced apoptosis (200, 400 and 600 μ M) in SK-HEP-1 cells was a response of down-regulation of Bcl-2 and Bcl-xL up-regulates Bak, triggering the release of cyt c, which may act as an important factor in the regulation and activation of the caspase cascade³⁷.

In another work, Jin *et al.*³⁹ found that the generation of reactive oxygen species (ROS) by daidzein was responsible for the apoptosis observed in their study with MCF-7 cells (25, 50 and 100 μ M). There was a disruption of mitochondrial transmembrane potential, downregulation of bcl-2 and upregulation of bax, which led to the release of cytochrome c, resulting in caspases 9 and 7 activation, and ultimately in cell death³⁹.

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In the study of Choi and Kim³⁸, caspase 9 activity (early biomarker of apoptosis) was increased by daidzein 100 μ M treatment for 72 hours in both MCF-7 and MDA-MB-453, but it only caused the accumulation of cells in sub-G0 phase (apoptotic peak) in MDA-MB-453.

As it can be observed with our results, daidzein arrested cell cycle in a selective manner: both tested concentrations (10 and 25 μ M) increased percentage of cells at G0/G1 phase only for MCF-7. This effect on cell cycle was observed by Casagrande and Darbon⁴² in OCM-1 melanoma cells, however with high daidzein concentration of 150 μ M for 72 hours. Consistently with this result, they also verified CDK2 inhibition (cyclin-dependent kinase protein that controls G1/S transition) and increased expression of p21^{CIP1} (CDK inhibitor)⁴².

Daidzein (50 and 100 μ M) can also arrest cells at the G2/M phase, as demonstrated by Choi and Kim³⁸ in the MCF-7 and in MDA-MB-453, with more pronounced results in the latter. Upregulation of p21^{CIP1} (and G2/M arrest) was also observed in human tumoral prostate cells (PC-3) with soy isoflavone concentrate, which according to the authors reflects the natural composition found in soybeans⁴³.

Lack of COX-2 mRNA expression in our results with MCF-7 cells is consistent with data found in literature^{44,45,46}.

In our experiments with HB4a cells, daidzein caused dose-dependent increase in COX-2 transcripts. This observation, together with the fact of selective cell cycle arrest, lead us to infer that absence of COX-2 expression sensitizes MCF-7 cells to daidzein treatment, thus resulting in cell cycle arrest.

It is well known that various drugs are COX-2 inhibitors, and because of this property, they can arrest cell cycle and induce apoptosis in different

tumoral cell lines. On the other hand, overexpression of COX-2 has been associated with many aspects of tumorigenesis⁴⁷, including mechanisms that confer chemoresistance to cancerous cells.

Conclusion

Taken together, our results and data from literature confirm that daidzein at maximal achievable physiological levels can still be an effective cancer chemopreventive agent, arresting cell cycle only in the tumoral breast cell line (MCF-7) and maintaining the normal balance between survival and cell death signs in non-malignant breast cells (HB4a).

Further studies to comprehend the molecular mechanisms of low levels of daidzein on cancer chemoprevention are necessary, mainly because in recent years, new molecular targets of isoflavones are being discovered, like microRNAs.

We hope that our study alert scientific community and general population to pay attention to the physiological concentrations of soy isoflavones and their real effects on the carcinogenesis of breast tumours.

It is also important to understand if their ingestion is appropriated to all kinds of public (healthy people, cancer patients, women at menopause, pregnant, children, etc), so that people can have better counselling on the consumption of soybean and its derivatives.

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