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Original Article

Inhibitory effects of O-methylated isoflavone glycitein on human breast cancer SKBR-3 cells

Bo Zhang[1], Jun-Ping Su[2], Yang Bai[1], Jie Li[1], Yong-Hong Liu[1]

[1]Department of Surgical Oncology, Cangzhou Central Hospital, Cangzhou, China; [2]Department of Endocrinology, Cangzhou People's Hospital, Cangzhou, China

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Abstract: Glycitein is an O-methylated isoflavone which accounts for 5-10% of the total isoflavones in soy food products. Cell proliferation studies on the dietary phytoestrogen, glycitein against human breast carcinoma SKBR-3 cells showed that glycitein exhibits biphasic regulation on SKBR-3 cells. At concentrations of less than 10 mg/mL, cells respond to glycitein by increasing cell growth and de novo DNA synthesis whereas the addition of glycitein at concentrations greater than 30 mg/mL significantly inhibited cell growth and DNA synthesis in a dose-dependent manner. Cells treated with 60 mg/mL of glycitein did not regain normal growth after treatment was stopped. Glycitein was found to be cytostatic at low concentrations and cytotoxic at higher concentrations. Treatment with 100 mg/mL of glycitein severely altered the cell morphology. Collective results showed that glycitein damaged the cell membranes by increasing membrane permeability and suggested possible mechanisms of the action of dietary phytoestrogens on human breast carcinoma SKBR-3 cells.

Keywords: Glycitein, SKBR-3 cells, breast carcinoma

Introduction

Breast cancer remains an overwhelming health burden, with an estimated 232,670 new breast cancer cases and 40,000 deaths among women living in the U.S. in 2014 [1]. Breast cancer in young women is believed to represent a more biologically aggressive disease with a higher frequency of adverse histopathologic characteristics, worse outcomes, and a predisposition to secondary breast or ovarian tumors [2, 3]. Age at diagnosis remains a predictive factor in effective screening, diagnosis, and management of breast cancer, especially in developing countries where late clinical presentation and poor outcome are hallmarks of the disease. Although the factors that affect the risk of breast cancer are diverse and not limited to reproductive history [4], the aging of the population and low fertility rates suggest that women play an indispensable role in the work force, which inevitably favors the delay of the first birth, low parity, and a shorter duration of breastfeeding. Breast cancer has been classified into 5 clinically relevant subtypes based on the immunohistochemical analysis of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression: luminal A, luminal B, HER2 overexpressing, basal-like, and normal breast-like (unclassified) [5].

Many people supplement their diets with soy isoflavones because epidemiologic and animal studies suggest that consumption of soybeans and soy-containing foods may lower one's risk of breast and prostate cancer [6-9]. Soybeans contain a large amount of isoflavones, including genistein, daidzein, glycitein and their glycosides [10]. The chemopreventive effects of soybeans and soy-containing foods may be related to their isoflavone content [11, 12]. Isoflavonoids have a diphenolic structure similar to that of estradiol and have been shown to have a weak estrogenic or antiestrogenic activity in many *in vitro* and *in vivo* systems [13, 14].

Glycitein is a representative isoflavone compound (**Figure 1**). The roles of glycitein in a variety of cell types have been described. Glycitein

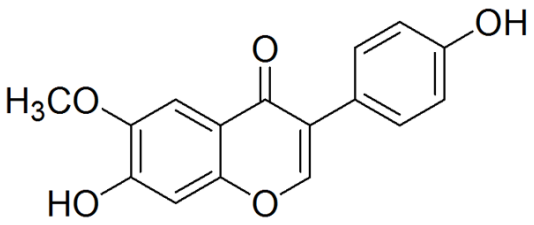

Figure 1. The structure of glycitein.

possesses estrogenic, antioxidant, hypocholesterolemic activities, and has a neuroprotective effect against -amyloid-induced toxicity [15-17]. Several papers have demonstrated that glycitein inhibits cancer cell proliferation or invasion. Glycitein exerts a potent inhibitory effect on invasiveness of MDA-MB-231 breast cancer cells, and inhibits Jurkat T cell invasion through down-regulation of MMP-13 activity and MMP-8 expression. Although the role of glycitein in tumor cell proliferation and invasion has been reported [18, 19], the effect of glycitein on breast cells has not been reported until now. Therefore, in the present study we investigated the effect of glycitein against the breast cancer cells.

Materials and methods

Chemicals

Soybean isoflavone glycitein was obtained from Nichimo (Tokyo, Japan). The SKBR-3 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD USA). DMEM (phenol red free) was obtained from Life Technologies (Rockville, MD). Fetal bovine serum (FBS) was purchased from Filtron (Brooklyn, Australia). Hank's balanced salt solution (HBSS) and an antibiotic/antimycotic mixture were obtained from GIBCO BRL (Gaithersburg, MD USA). A cell proliferation kit (WST-1), a 5-bromo-2'-deoxyuridine (BrdU) DNA synthesis labeling kit, and cell membrane permeability assay kit were purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). The solution of Glycitein was prepared in dimethylsulfoxide (DMSO) and stored at 220°C in the dark. All tissue-culture dishes and flasks were purchased from Becton Dickinson (Franklin Lakes, NJ).

Cell culture conditions

The human breast cancer cell lines, SKBR-3 were grown in phenol red-free DMEM containing a 1X antibiotic/antimycotic mix, 5 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, and 0.37% sodium bicarbonate [20]. Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide and fed every 2 days. The medium was supplemented with either 10% FBS or 3X dextran/charcoal-stripped FBS [21].

Cell proliferation studies

Cells were cultured in phenol red-free DMEM supplemented with 10% FBS until 80 to 90% confluence was reached and the medium was changed to DMEM without serum for an additional 24 hours to synchronize cells to the G_0/G_1 phase of the cell cycle. The cells were removed from the culture flasks with 0.05% trypsin and 3 mM EDTA in HBSS, collected by centrifugation at 500 × *g* for 3 minutes, and the number of cells per milliliter was determined by the trypan blue dye exclusion method.

Cells were seeded in 96-well culture plates in phenol red-free DMEM containing 10% 3X dextran/charcoal-stripped FBS at 1×10^4 cells per well for the experiments. After a 24-hour, the medium was removed and fresh phenol red-free medium supplemented with 10% dextran/charcoal-stripped FBS alone or with the preestablished concentrations of glycitein was added. DMSO, at the same dilution, was added in parallel cultures as a control. Final concentrations of DMSO in the culture medium were kept below 1% (vol/vol), which caused no measurable effects on cell growth or cell morphology. At the end of incubation period, WST-1 reagent was added to determine the cell viability. The formation of formazan dye that exhibits absorbance at the wavelength of 450 nm was quantified using a scanning multi well spectrophotometer [enzyme-linked immunosorbent assay (ELISA) reader (E_{max} precision microplate reader, Molecular Devices, Sunnyvale, CA USA]. Each condition was represented in five separate wells per experiment and was repeated in at least four independent cultures.

DNA synthesis studies

The SKBR-3 cells were grown, synchronized as described, and seeded to 96-well culture plates at the density of 5×10^4 cells per well in phenol red-free DMEM containing 10% dextran/char-

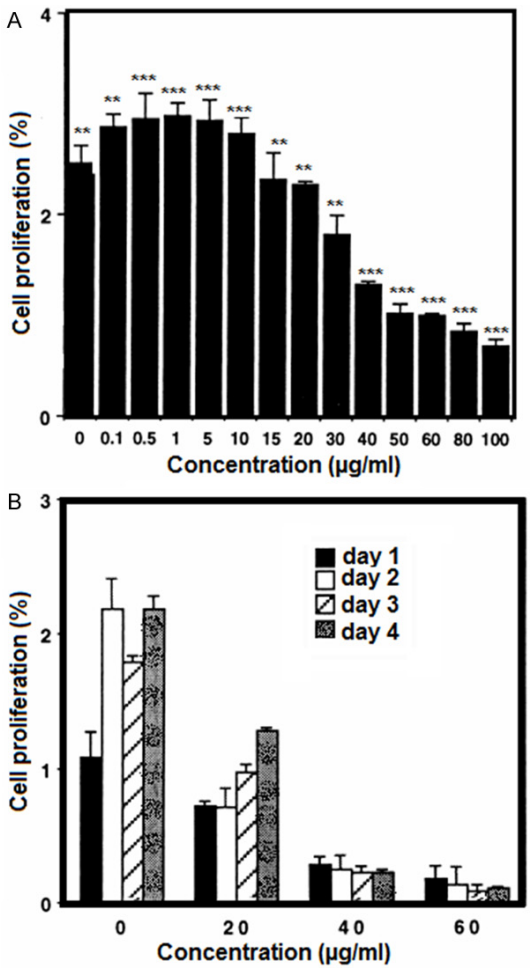

Figure 2. Effects of glycitein on the proliferation of SKBR-3 cells. (A) SKBR-3 cells were treated with glycitein for 2 days at the concentrations indicated or (B) for 0 to 4 days at preestablished concentrations. The values with ** and ***indicate significant difference ($P < 0.005$) and very significant difference ($P < 0.0001$), respectively, compared with the dimethylsulfoxide-treated cells (0 mg/mL of glycitein) as control.

coal stripped FBS. After 24 hours, the medium was removed and fresh medium containing glycitein/DMSO was added to each well. Cells were incubated for various periods of time and the amounts of de novo DNA synthesis in each well was measured by using the BrdU DNA synthesis labeling kit according to the manufacturer's instructions (Boehringer Mannheim Biochemicals). This assay is based on the detection of BrdU incorporated into the genomic DNA of proliferating cells. Cells were labeled by the addition of BrdU, which was incorporated in place of thymidine into the DNA of cycling cells. After removing the labeling medium, cells were fixed and the DNA was denatured. Anti-BrdU-POD antibody, a monoclonal antibody from mouse-mouse hybrid cells (clone BMG 6H6, Fab fragments) against BrdU conjugated with peroxidase (POD), was added and bound to the BrdU that was already incorporated into the newly-synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction by the addition of tetramethylbenzidine and the reaction product was quantified by measuring the absorbance at A_{450} using an ELISA reader. Experiments were repeated at least twice and each condition was represented in four separate wells per experiment.

Cell morphology

Cells were cultured in DMEM containing 10% FBS and synchronized as described above. An aliquot of 5×10^5 cells was seeded into each well of six-well culture plates. After 24 hours of growth, glycitein was added to each well to 100 mg/mL for an additional 4 days. Cells that remained attached to the plate surface were then washed with phosphate buffered saline (PBS) twice and photographed with a Leitz Orthoplan microscope (Wetzlar, Germany). Dead cells in the medium were recovered by centrifugation, washed with PBS, and applied to cover slides using cytospin (Kubota 5800) to allow reattachment to the slide surface before they were viewed and photographed.

Cell membrane permeability assay

Synchronized SKBR-3 cells were grown and treated with glycitein as described. The culture supernatant was collected cell-free and incubated with the substrate mixture. The lactate dehydrogenase (LDH) activity in the supernatant was determined with a coupled enzymatic reaction whereby the tetrazolium salt was reduced to formazan dye that was measured by an ELISA reader.

Statistical analysis

Data were analyzed by the method of least-squares analysis of variance using the GLM procedures for a completely randomized design with glycitein concentration as the treatment factor [22]. Treatment means were compared by the orthogonal contrast with the compari-

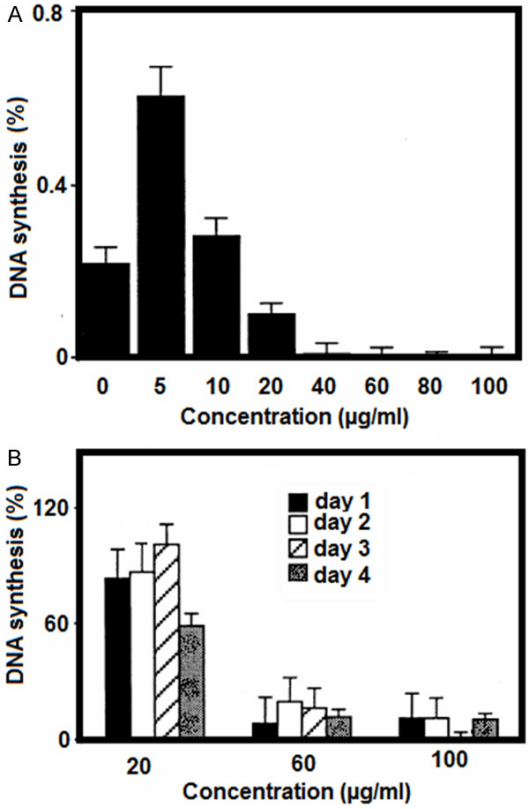

Figure 3. Regulation of DNA synthesis by glycitein in SKBR-3 cells. Cultured cells were treated with glycitein at the concentrations indicated for (A) 4 days or (B) the period of time indicated, and the DNA synthesis was determined. Values are means ± SD.

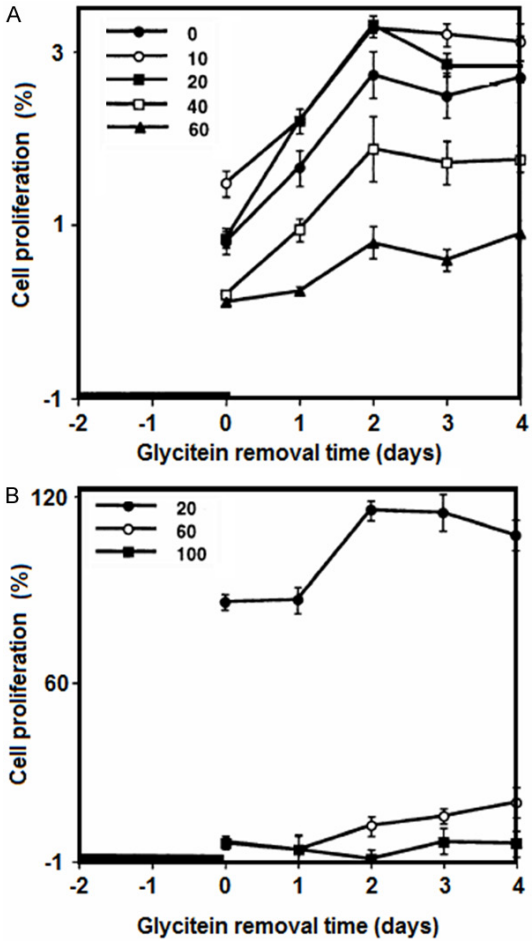

Figure 4. Reversibility in the inhibitory effect of glycitein on cell proliferation and DNA synthesis in SKBR-3 cells. Exponentially growing SKBR-3 cells were treated with glycitein at various concentrations for 48 hours. At the end of the incubation, glycitein -containing medium was removed and cells were washed with fresh Dulbecco's modified Eagle's medium (DMEM) three times to remove any residual glycitein. (A) Fresh phenol red-free DMEM medium supplemented with 10% charcoal-stripped fetal bovine serum was added and (A) cell growth and (B) DNA synthesis were measured after cells were allowed to grow for 0 to 4 days. Values are means ± SD.

sons of each individual glycitein concentration versus control blank.

Results

Effect of glycitein on SKBR-3 cell proliferation

Cell cultures were exposed to 0.1 to 100 mg/mL of glycitein in medium containing 10% charcoal stripped FBS for 1 to 4 days in order to study the kinetics of inhibition in cell proliferation, (**Figure 2**). The results showed that glycitein produced a reproducible biphasic response against SKBR-3 cells. Cell proliferation was stimulated by glycitein, at a concentration less than 10 mg/mL (**Figure 2A**) whereas the cell proliferation was significantly enhanced ($P < 0.0001$) upon treatment with 1 mg/mL of glycitein to approximately 125% that of controls. At the lowest concentration tested (0.1 mg/mL), glycitein induced a 19% growth increase ($P < 0.005$) when compared to that of DMSO-treated control cells. When concentrations greater than 30 mg/mL were used, glycitein exhibited inhibitory activity in the cell proliferation of SKBR-3 cells in a dose-dependent fashion (**Figure 2A**). The SKBR-3 cell proliferation was inhibited approximately 50% at 40 mg/mL and 75% at 100 mg/mL, which was the highest concentration of glycitein tested. The inhibitory effects of glycitein on SKBR-3 cell proliferation were clearly observed after the first day of administration (**Figure 2B**). No further notice-

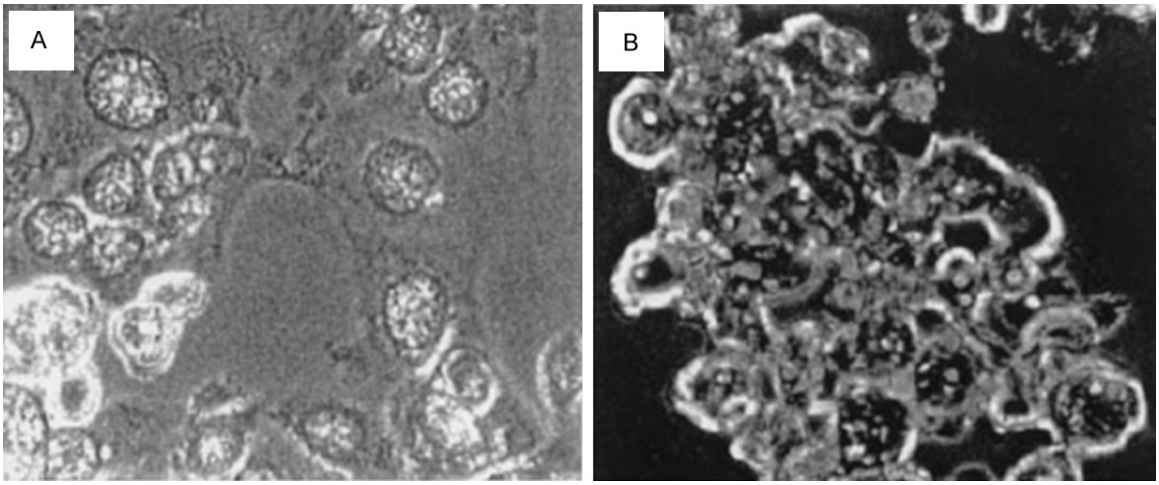

Figure 5. Cell morphology of glycitein-treated cells. A. Control SKBR-3 cells that are attached to the surface of culture plates after being treated with dimethylsulfoxide in parallel and photographed for purposes of comparison. B. After incubation with 100 mg/mL of glycitein for 4 days, SKBR-3 cells that were detached from the culture flask surface and became floated in the medium due to treatment were centrifugated, washed with phosphate buffered saline, reattached to the glass coverslip, and photographed. The bar below the photographs indicates a length for 50 μm.

able inhibition by glycitein on cell proliferation was detected after day 4.

Glycitein altered DNA synthesis in SKBR-3 cells

The effects of glycitein on cell proliferation may have been caused by regulating the cell cycle progression at the G1-S phase. Therefore we examined the de novo DNA synthesis that occurred only during the S phase in SKBR-3 cells treated with glycitein (**Figure 3**). Glycitein significantly influenced DNA synthesis at all concentrations tested (**Figure 3A**). Glycitein exhibited a similar biphasic response in DNA synthesis as observed in the case of cell proliferation. DNA synthesis increased after treatment of glycitein at low concentrations. An increase of 278% in DNA synthesis was observed at a concentration of 5 mg/mL whereas at 20 mg/mL, DNA synthesis in SKBR-3 cells declined to 48% after being treated with glycitein for 4 days. At 40 mg/mL, only residual DNA synthesis (4%) remained after exposure for 4 days. At concentrations greater than 40 mg/mL, no measurable DNA synthesis was present when cells were treated with glycitein. An analysis of the changes in DNA synthesis from day 1 of glycitein treatment in conjunction with the kinetics of glycitein inhibitory effects on DNA synthesis revealed a correlation to cell proliferation (**Figure 3B**).

DNA synthesis in SKBR-3 cells was severely blocked to approximately 10% that in control cells by glycitein at concentrations of 60 and 100 mg/mL even after only 1 day of treatment. We did not detect further inhibition when the cells were exposed to glycitein for an additional 2 to 4 days. The concentration of 20 mg/mL inhibited DNA synthesis to a lesser extent. To further analyze the inhibitory effects of glycitein at the early time points, SKBR-3 cells were treated with glycitein for 7 to 55 hours and the amount of de novo DNA synthesis was determined (data not shown). At 60 mg/mL, significant inhibition in DNA synthesis was evident after cells had been treated with the glycitein for 7 hours.

Reversibility of the effects of glycitein on SKBR-3 cell proliferation and DNA synthesis

In order to identify the reversibility of the effect of glycitein, cells were incubated with various concentrations of glycitein for 48 hours and removed thereafter (**Figure 4**). At concentrations below 40 mg/mL, cells were able to showed regrowth after removal of the drug (**Figure 4A**) whereas at 60 mg/mL, the cells were failed to resume normal growth after the compound was removed for 2 days, although slight regrowth was occasionally observed after 3 days of drug removal. Analysis of the de novo DNA synthesis was done on cells treated with

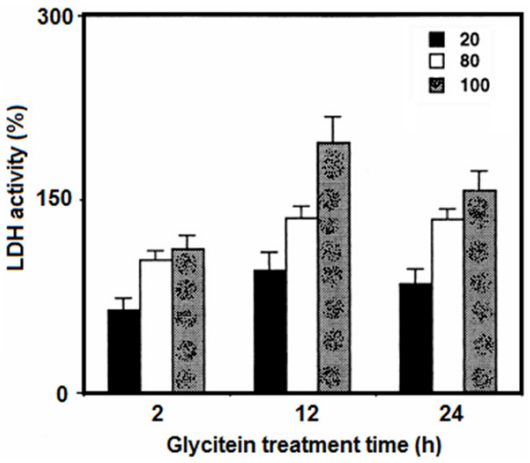

Figure 6. Cell membrane permeability of SKBR-3 cells treated with glycitein. SKBR-3cells were exposed to glycitein at concentrations of 20, 80, or 100 mg/mL and incubated for 2, 12, or 24 hours. Lactate dehydrogenase (LDH) activity in the cell supernatant, which correlates with cell permeability, was determined as described in Materials and methods. The permeability of SKBR-3 cells treated with dimethylsulfoxide for the same period of time was designated 100%. Values are means ± SD.

60 and 100 mg/mL glycitein concentrations (**Figure 4B**). The results showed that DNA synthesis did not return to normal when compared with that of control cells. At 100 mg/mL, DNA synthesis was barely measurable, even when the compound had been removed for 4 days. Therefore, the inhibition of DNA synthesis at high doses of glycitein was found to be irreversible whereas at 20 mg/mL concentration, DNA synthesis was found to be reversible after glycitein was removed.

Glycitein treatment affects cell morphology

The morphology of SKBR-3 cells treated with glycitein was examined microscopically (**Figure 5**). Upon treatment with glycitein for 4 days at 100 mg/mL, a significant portion of the cells became detached from the culture flasks. After the cells floating in the medium caused by the treatment were allowed to reattach to the cover slide surface, the cell morphology of the floating cells was compared with that of DMSO-treated control cells that remained attached to the culture plates (**Figure 5A** and **5B**). Cells that remained attached to the flask surface after glycitein treatment also exhibited altered cell morphology, although to a lesser extent compared with that of floating cells. They appeared to lose some cellular volume, but condensation of the cellular components was not as severe as that in cells that were found floating (data not shown). We did not observe similar morphologic changes in the DMSO-treated control cells (**Figure 5A**).

Glycitein affects cell membrane permeability

Cell membrane permeability was examined to analyze the possible effects of glycitein on cell morphology (**Figure 6**) by the measuring the amount of LDH released from the cells. An increase in the number of cells with damaged membrane results in increased LDH enzyme activity in the culture supernatant. After 12 hours of glycitein treatment, the cell membrane began to show damage as indicated. At 100 mg/mL, glycitein was able to increase the cell membrane permeability to 152% that of control cells upon exposure to the chemical for 24 hours and this damage appears to be dose dependent. Changes in the cell membrane permeability reached a plateau at a 12 hours' incubation with glycitein. The cell membrane permeability were more effective at high concentrations of glycitein (80 and 100 mg/mL) than those compared to that of low concentrations (20 mg/mL).

Discussion

In this study, we have reported the cellular and molecular effects of the phytoestrogen glycitein against SKBR-3 cells. Glycitein is a representative isoflavone compound, which is distributed in the plant of family Leguminosae such as flowers and roots of Pueraria thunbergiana, the bark of Maackia amurensis and soybean [23, 24]. Reports shown that the human intestinal bacteria transform glycitin into glycitein, which is a bioactive compound that can be absorbed into the gastrointestinal tract [25, 26]. The roles of glycitein in a variety of cell types have been described. Recently, our group reported that isoflavone metabolites, such as irisolidone, tectorigenin, and glycitein, inhibit LPS-induced inflammatory reactions in brain microglia [27]. The anti-inflammatory effects of glycitein have also been reported in peripheral systems [28]. From the experimental results it is revealed that glycitein influenced cell proliferation and DNA synthesis in SKBR-3 cells. At < 10 mg/mL concentrations, cell proliferation

and DNA synthesis were stimulated by the treatment of glycitein, whereas these events were inhibited by higher concentrations. The extent to which DNA synthesis was affected by glycitein appears to be more profound than that of cell proliferation.

A flavone derivative, flavopiridol was shown to induce a block at the G1 phase in the cell cycle [29]. Similarly, Glycitein also affected the cell cycle at the same point and influence only cells starting to enter the G1-S phase in the cell cycle at different rates. Because DNA synthesis assays detect only newly-synthesized DNA that occurs in cells entering the S phase and cell proliferation assays measure viable cells in all phases of the cell cycle, the effects of glycitein would likely be more evident for DNA synthesis. Our observation also suggests that cell membrane damages in SKBR-3 cells were produced by glycitein. Cell membrane permeability was altered such that cell morphology had clearly changed.

The biphasic effects reported in this study are similar to those observed by Wang and Kurzer [30]. They showed the effects of phytoestrogens on DNA synthesis in SKBR-3 cells to be variable and concentration dependent. Glycitein enhanced the DNA synthesis at 0.1 to 10 mM and inhibited the synthesis with an IC_{50} value of 36.4 mM. Zava and Duwe showed stimulation of DNA content to 150 to 400% at 0.001 to 10 mM of genistein and inhibition at 10 mM. Other reports have shown stimulation of protein content by daidzein at the concentration of 0.3 mg/mL. In MCF-7 cells, the IC_{50} for biochanin A for DNA synthesis was reported to be approximately 13 mg/mL. The IC_{50} for genistein for cell growth was 10 mg/mL in MDA-468 cells. The potency of glycitein in the proliferation inhibition was achieved after exposure to the chemical for only 1 day. No noticeable further inhibition was evident. The inhibitory kinetics of glycitein in cell proliferation were also present in DNA synthesis and cell membrane permeability. The irreversibility of glycitein treatment at high concentrations may be correlated with infertility in animals that have been grazing estrogenic plants for long periods [31]. Our results showed that cells did appear to recover from the influence of glycitein at low concentrations.

The observation of the biphasic effects of glycitein in estrogen target cells is intriguing. The observed inhibition in cell proliferation of estrogen target cells that are exposed to high concentrations of glycitein may have a correlation to such phenomena. In addition, the biphasic response in cell proliferation suggests that the biological actions of plant estrogens may be mediated through multiple pathways depending on the concentration of glycitein. Recently, phytoestrogens also have been shown to induce apoptosis in culture cells and the reproductive tract of female rats [32]. Further investigations are necessary to clarify the mechanisms responsible for these effects.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yang Bai, Department of Endocrinology, Cangzhou People's Hospital, 16 West of Xinhua Road, Cangzhou 061001, Hebei, China. Tel: 0086-317-2072692; Fax: 0086-317-2072692; E-mail: baiyang05211@gmail.com

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