

Genistein Induces G2/M Cell Cycle Arrest and Apoptosis in Rat Neuroblastoma B35 Cells; Involvement of p21^{waf1/cip1}, Bax and Bcl-2

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Background : The effect of genistein on different types of cells has been investigated. However, its effect on the nervous system is still unclear. The aim of the present work is to explore the effect of genistein on rat neuroblastoma B35 cells. **Methods :** The effect of genistein on the proliferation of B35 cells, its cytotoxicity, the cell-cycle distribution, the ultra-structural changes and the induction of apoptosis were determined using MTT assay, LDH assay, Flow-cytometric analysis, transmission electron microscopy and Hoechst staining, respectively. Furthermore, Real-time quantitative RT-PCR and Western blotting were used to examine the transcriptional and post-translational alterations of the G2/M cell-cycle arrest marker cyclin-dependent kinase inhibitor p21^{waf1/cip1} and the apoptosis-related genes after genistein treatment. **Results :** Genistein significantly inhibits cell survival, slightly elevates the release of lactate dehydrogenase and induced apoptosis in B35 cells. Genistein increased the number of cells at S-phase and induced cells to accumulate at the G2/M phase. These G2/M arrested cells are associated with a marked up-regulation of p21^{waf1/cip1} at both the mRNA and protein levels. We observed that genistein up-regulates pro-apoptotic Bax with concurrent down-regulation of the anti-apoptotic Bcl-2 protein. **Conclusion :** These observations suggest that the anticancer effect of genistein on B35 neuroblastoma cells is mediated through multiple cellular pathways including G2/M cell-cycle arrest and the induction of apoptosis.

Key Words : B35 Neuroblastoma; Genistein; G2/M cell-cycle arrest; Apoptosis; p21^{waf1/cip1}

The principal soy isoflavone genistein is well known to have both proliferative and anti-proliferative activities depending on the dose.¹ The estrogenic behavior of genistein is clearly shown by the increasing cell proliferation and reduction of apoptosis in an estrogen receptor-dependent manner at low concentrations.^{2,3} In addition, genistein acts as a potent anti-oxidant.⁴ On the other hand, at high doses, genistein displays its anti-proliferative activity via multiple cellular pathways including inhibition of the oncogene products and the tyrosine kinase activity of growth factor receptors such as the epidermal growth factor receptor,⁵ suppression of telomerase activity,⁶ controlling the transcription of several target genes involved in cell proliferation and metastasis by forming a stable complex with DNA topoisomerase II,⁷ and the induction of cell-cycle arrest and apoptosis in different types of normal and malignant cells.^{8,9} Additionally, it has been reported that genistein treatment induces *in vivo* and *in vitro* morphological and ultra-structural changes.¹⁰ Genistein induces growth inhibition through modulating the cell-cycle progression and inducing cell-cycle arrest.¹¹ The mechanism of cell-

cycle arrest induced by genistein has been subjected to extensive investigations. It has been found that genistein induces cell-cycle arrest at the G2/M phase through the up-regulation of the cyclin-dependent kinase inhibitor p21^{waf1/cip1} in different cancer cells,¹²⁻¹⁴ and consequently inactivation of CDK1/cyclin-B complex, which is essential for promoting cell-cycle progression and entering mitosis.¹⁵ Regarding the neuroprotective and the cytotoxic effects of genistein, it has been reported that genistein has anti-apoptotic properties for primary cortical neurons, and this is mediated through estrogen receptors at low genistein concentrations.² On the contrary, genistein is also known to induce apoptosis in many cancer cells at relatively high concentrations.⁸

Cancers of the central nervous system (CNS) are responsible for 2.1% of all cancer deaths, the highest rates are observed in the developed countries (Australia, New Zealand, Europe and North America) and the lowest rates are noted in Africa and the Pacific islands.¹⁶ A geographical cancer incidence that may be linked to soy consumption has been reported for several other types of cancer by Lee *et al.*¹⁷ In addition, the effect of genistein

on the nervous system is poorly understood.¹⁸ Therefore, the current study investigated the effect of genistein on the neuronal system represented by rat neuroblastoma B35 cells as an *in vitro* experimental model. The responses of B35 cells to genistein and the molecular mechanisms by which genistein exerts its anti-cancer activity were investigated in this study.

The cell survival inhibitory action, cell-cycle distribution analysis and induction of apoptosis in B35 rat neuroblastoma cells were evaluated after genistein treatment. We also sought to clarify the underlying molecular mechanism by which genistein exerts its anticancer activity. Ultrastructural changes and the P21^{waf1/cip1} mRNA and protein levels were investigated after genistein treatment. Furthermore, the mechanism underlying apoptosis was investigated through the evaluation of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 mRNA and protein levels after genistein treatment.

MATERIALS AND METHODS

Chemicals and reagents

Genistein, MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide), Hoechst 33342 and propidium iodide were purchased from Sigma (St. Louis, MO, USA), Dulbecco's modified Eagle medium [DMEM] was from HyClone (Logan, UT, USA), Trizole was from Invitrogen Life Technologies (Rockville, MD, USA), 2 X SYBR green PCR master mix was from Applied Biosystems (Foster, CA, USA), and RNase-A was from USB (Cleveland, OH, USA). Rabbit polyclonal IgG anti-P21^{waf1/cip1} (sc-397), anti-Bcl-2 (sc-783) and anti-Bax (sc-930) were obtained from Santa Cruz Biotechnology, INC. Mouse monoclonal IgG Anti- β -tubulin antibody was obtained from Sigma; HRP conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were from Pierce (Rockford, IL, USA), and the ECL plus Western Blotting Detection Reagents were from Amersham Biosciences UK Limited (Chalfont St. Giles, Buckinghamshire, UK). All the other reagents were obtained from standard commercial sources.

Cell culture

The rat neuroblastoma B35 cell line was purchased from the American Type Culture Collection (ATCC) and the cells were originally maintained in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic. The cells were

grown at 37°C in a 5% CO₂ incubator before being used in the different experiments. To prepare the genistein stock solution, genistein was dissolved in DMSO at a final concentration of 100 mM.

Cell proliferation assay

The effect of genistein on the survival of B35 neuroblastoma cells was assessed by MTT assay as described previously.¹⁹ In brief; the cells were plated in 100 μ L media in 96-well plates (1×10^4 /well). On the following day, the experimental media containing different genistein concentrations (0.0, 1, 12.5, 25, 50, 75 and 100 μ M) were added, and then the cells were incubated for varying times. Fifty μ L of MTT solution (2 mg/mL in PBS) was added to each well and the cells were incubated for 4 h. After careful removal of the media, 150 μ L of DMSO was added to each well, and then after shaking, the absorbance was read at 570 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, USA).

LDH cytotoxicity assay

Cell death was quantified by measuring the lactate dehydrogenase (LDH) released into the medium over various experimental periods and this was expressed as the percentage of cellular toxicity induced by a final genistein concentration ranged from 1 μ M to 100 μ M. The experimental procedure was done as previously described.² Briefly, the cells were cultured in 96-well plates (1×10^4 /well). The following day, genistein-containing media was added for 1, 2 and 3 days, respectively. A group of wells was treated with 1% Triton X-100 solution for 45 min for obtaining maximum LDH release. The plates were centrifuged at 500 rpm and 100 μ L of the media from each well was transferred to new 96-well plates; 100 μ L of LDH assay mixture (Takara Bio Inc, Otsu, Japan) was added and the cells were incubated at 37°C for 30 min and the LDH release was estimated at 590 nm according to the manufacturer's instructions. Similar results were obtained in three independent experiments, and all treatments were done in triplicates.

Nuclear Hoechst staining

The cells were cultured in 35-mm plates that contained cover slides. On the following day, the cells were treated with genistein at varying concentrations for one and three days, respectively. The cells were fixed in 4% paraformaldehyde, washed with PBS and then stained in Hoechst 33342 (0.5 μ g/mL in PBS) for 10

min. The cover slides were mounted and the cells were examined under a fluorescence microscope (Olympus BX-51, USA).

Transmission electron microscopy (TEM)

To investigate the ultra-structural changes after genistein treatment, a TEM procedure was done as described by Honma *et al.*²⁰ as follows. The cells were plated in 100-mm plates (2×10^6) in DMEM. One day later, two plates were treated with 0.1% DMSO as a positive control; another two plates were treated with 100 μ M genistein and then they were incubated for three days. The cells were collected using trypsin, washed twice in PBS and then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. The cells were washed three times in 0.1 phosphate buffer and this was followed by centrifugation and careful removal of the supernatant to collect the cell pellets. The cell pellets were post-fixed in 1% osmium tetroxide in 0.1 phosphate buffer for 2 h at 4°C and then they were subsequently washed in three changes of phosphate buffer. The cell pellets were dehydrated in a series of ethyl alcohol solutions, washed in propylene oxide for 30 min, embedded in labeled capsules containing freshly prepared resin epon, and then they left for polymerization at 5°C for 72 h. Semi-thin sections were cut and stained with toluidine blue to determine the selected areas, which were subjected to ultra-thin sectioning. The ultra-thin sections were stained with uranyl acetate and lead hydroxide and they were examined with a transmission electron microscope. Two independent experiments were done to confirm the results.

Cell cycle analysis

The DNA content and cell-cycle distribution after genistein treatment were estimated according to the method of Yu *et al.*²¹ with minor modifications as follows. The cells were seeded at 2×10^6 in 100-mm plates in 10% FBS-containing DMEM. On the following day, the cells were treated with genistein at concentrations of 0, 25, 50, and 100 μ M. After three days of treatment, the cells were collected, and 1×10^6 cells were taken from each treatment and washed in cold PBS solution (PBS+2% FBS) for two times. The cells were then fixed by adding 4 mL of -20°C absolute ethanol to the cell suspension in 1 mL PBS in 15 mL tubes and these tubes were kept at 4°C for overnight. The cells were centrifuged, washed twice and the cell suspension was transferred in 1 mL PBS buffer to an e-tube. 100 μ L of 200 μ g/mL RNase-A in 1.12% sodium citrate buffer was added to the cell suspension and then it was incubated in a 37°C water bath for

30 min. 100 μ L of 1 mg/mL propidium iodide was added, and this was incubated in darkness for 10 min at room temperature. 2×10^4 cells were analyzed for their DNA content and the cell-cycle distribution was determined by flow cytometric analysis with using FACScan (Becton & Dickinson, San Jose, CA, USA).

Quantitative real-time RT-PCR

To investigate the fluctuations of the gene expression of selected genistein-regulated genes in B35 rat neuroblastoma cells after genistein treatment, real time quantitative RT-PCR analysis was performed as described by Schreihöfer³ with minor modifications. In brief, 1×10^6 cells were plated per 100-mm dish in DMEM supplemented with 10% FBS. One day later, the cells were treated with varying genistein concentrations for three days. The total RNA was extracted with Trizol according to the manufacturer's instructions. Five μ g of extracted mRNA from three independent experiments were reverse transcribed into cDNA using the first strand cDNA synthesis kit (MBI Fermentas, Hanover, MD, USA) in 20 μ L reaction mixture according to the manufacturer's instructions. The resulting cDNA was diluted 10-fold and kept at -20°C until use. The real-time RT-PCR primers of the different selected genes were designed by using Primer Express 1.5 software (Applied Biosystems, USA). The expression fluctuations of the cyclin-dependent kinase inhibitor p21^{waf1/cip1} and the two apoptosis-related genes (Bcl-2 and Bax) with using GAPDH as a house keeping gene were evaluated. The primer sequences were as follows: (P21^{waf1/cip1}) sense, 5'-GCAAAGTATGCGTCGTCTGTT-3' and antisense, 5'-GCCAAGTCAAAGT-TCCACCG-T-3', (Bax) Sense, 5'-ATGGAGCTGCAGAGGATGATTG-3' and antisense, 5'-CAGTTGA-AGTTGCCATCAGCAA-3', (Bcl-2) sense, 5'-TTTGTGGAAGTATATGGCCCA-3' and antisense, 5'-AGGTATGCACCCAGAGTGATGC-3' and (GAPDH) sense, 5'-ATCTTCTT-GTGCAGTGCCAGC-3' and antisense, 5'-GAAGGCAGCCCTGGTAACC-3'. Real-time RT-PCR was carried out using an ABI Prism 7000 sequence detection system (Applied Biosystems, USA). Ten μ L of SYBR Green PCR master mix, 4 μ L of diluted cDNA, and 200 nM of primer set were used for the amplification in 20 μ L reaction mixtures. All the samples were amplified in triplicates in a 96-well plate and the cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 sec, followed by 1 min at 60°C. Calculations were performed by calculating the values of the Δ cycle threshold (Δ Ct) by normalizing the average Ct value of each treatment compared to its opposite endogenous control (GAPDH), and then calculating $2^{-\Delta\Delta Ct}$

for each treatment and performing statistical analysis for the data as described previously.²²

Western blot analysis

Changes in the protein expression level after genistein treatment were evaluated as described by Waite *et al.*²³ as follows. The cells were plated in 100-mm dishes at a proper fixed number of cells and they were subsequently treated at a final genistein concentration range of 1 to 100 μM for three days. The cells were washed with PBS and then washed in TPK washing buffer (10 mM Tris-HCl, 70 mM NaCl, 0.5 mM CaCl_2 , 0.5 mM MgCl_2 , 0.5 mM Na-O-Vanadate, 1 mM PMSF, 2 $\mu\text{M}/\text{mL}$ leupeptin and 2 $\mu\text{M}/\text{mL}$ aprotinin), and they were immediately lysed in TPK lysis buffer (TPK washing buffer+10% NP-40). The cell lysates were collected in e-tubes and vortexed. The samples were centrifuged at 15,000 rpm for 20 min at 4°C to remove the cellular debris, and then the supernatants were carefully collected. The protein concentrations were estimated using the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The protein samples were subdivided into a number of aliquots of 40 μg protein concentration and these were kept at -80°C until use in western blotting analysis. 40 μg of the protein samples were mixed with gel-loading buffer (0.0625 M Tris-Cl pH 6.8, 10% [v/v] glycerol, 5% [v/v] β -mercaptoethanol, 2% SDS and 0.001% bromophenol blue) and then this was electrophoresed on a 10% or 12% SDS-page gel. The proteins were then transferred from the SDS-Page gel to a nitrocel-

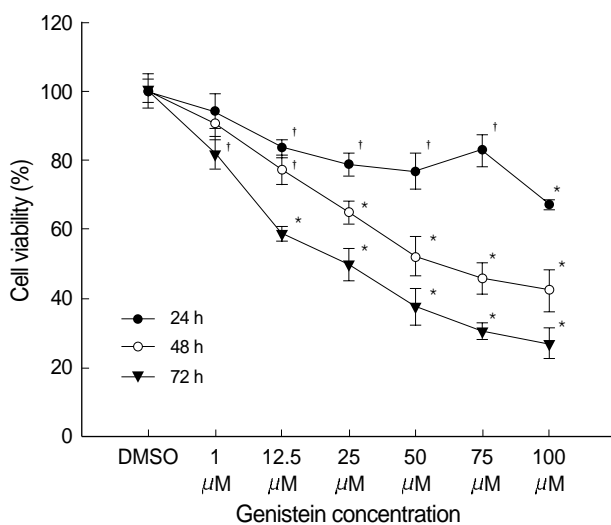


Fig. 1. MTT proliferation assay showing the growth inhibitory effect of genistein on rat neuroblastoma B35 cells in a dose and time dependent manner. Statistical significance was determined using student t-test (* $p < 0.05$, † $p < 0.01$).

lulose membrane according to the method of Towbin *et al.*²⁴ Ponceau S solution (Pierce) was used for confirming equal loading of the protein. The blots were blocked in 5% skim milk in TBS (25 mM Tris base and 150 mM NaCl) overnight at 4°C, and then they were incubated with primary antibody at a 1:500 dilution for 2 h at RT; this was followed by three washes in TBST (TBS+0.01% Tween 20), 10 min for each wash. The appropriate conjugated secondary antibody was used at 1:1,000 dilutions for 1 h at RT and then the membranes were washed three times, 10 min for each time in TBST. The target proteins were detected with ECL plus Amersham detection reagents and developed using X-ray films.

Statistical analysis

The data are expressed as means \pm SEMs; all values were calculated, graphed and then statistically analyzed by the student t-test with using Sigma Plot 8.0 software.

RESULTS

The anti-proliferative effect of genistein

The anti-proliferative activity of genistein on the rat neuroblastoma B35 cell line was assessed using the MTT proliferation assay. It was found that genistein treatment clearly inhibits B35 cell proliferation. In addition, the time- and dose-dependent res-

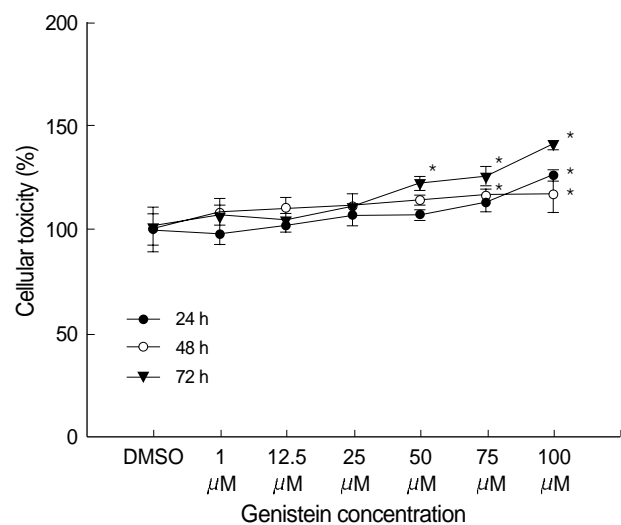


Fig. 2. LDH cytotoxic assay showing the cytotoxic effect of genistein on the rat neuroblastoma B35 cells in a concentration and time dependent manner. * $p < 0.05$ considered statistically significant.

ponses of neuroblastoma B35 cells to genistein indicated that genistein inhibits cell proliferation in a time- and concentration-dependent manner (Fig. 1). However, the inhibitory effect of genistein on the B35 cells did not reach the IC50 (50% inhibitory concentration) even at 100 μM after 24 h of treatment, while after 72 h the IC50 was approximately 25 μM .

Cytotoxic effect of genistein and the induction of apoptosis

To investigate the cytotoxicity and the loss of membrane integrity in B35 cells after genistein treatment, the lactate dehydro-

genase released to the experimental media was evaluated by performing LDH assay. Genistein induced the release of LDH in a dose- and time-dependent manner (Fig. 2). In addition, the induction of apoptosis was observed in the genistein-treated B35 cells by performing Hoechst staining. Apoptosis, as represented by chromatin condensation and nuclear fragmentation, was observed after three days of 25, 50, and 100 μM genistein treatment (Fig. 3). On the other hand, apoptotic cells could not be detected with using Hoechst staining after one day of genistein treatment even at a 100 μM concentration (data not shown).

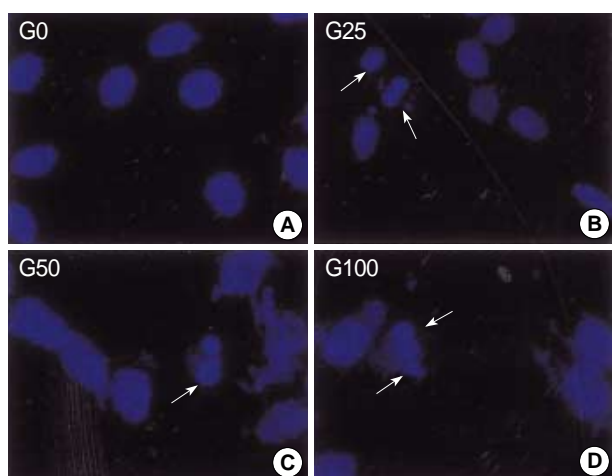


Fig. 3. Chromosome condensation and nuclear fragmentation are observed using Hoechst nuclear staining in genistein-treated neuroblastoma B35 cells (arrows), before genistein treatment (A), and after three days of genistein treatment; 25 μM (B), 50 μM (C), and 100 μM (D). Original magnification: 400 \times .

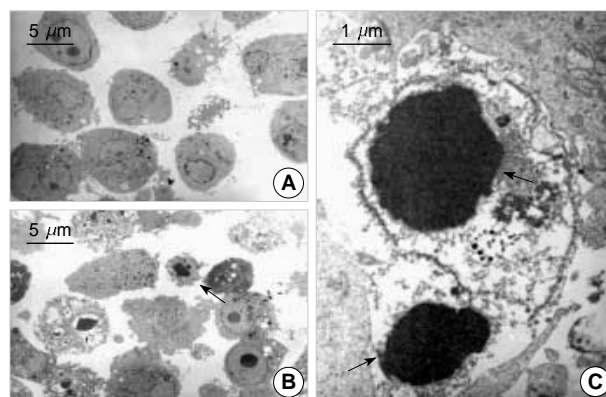


Fig. 4. (A) Transmission electron micrograph of non-treated B35 neuroblastoma cells shows normal cell morphology with open face nuclei. (B and C) Genistein-treated (100 μM , 3 days) rat neuroblastoma B35 cells show chromatin condensation and apoptotic nuclear fragmentation (arrows). (C) High magnification of genistein treated B35 cells show chromatin condensation (arrows).

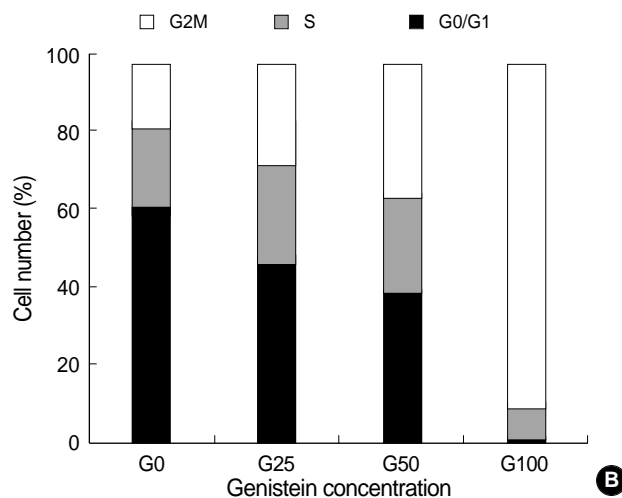
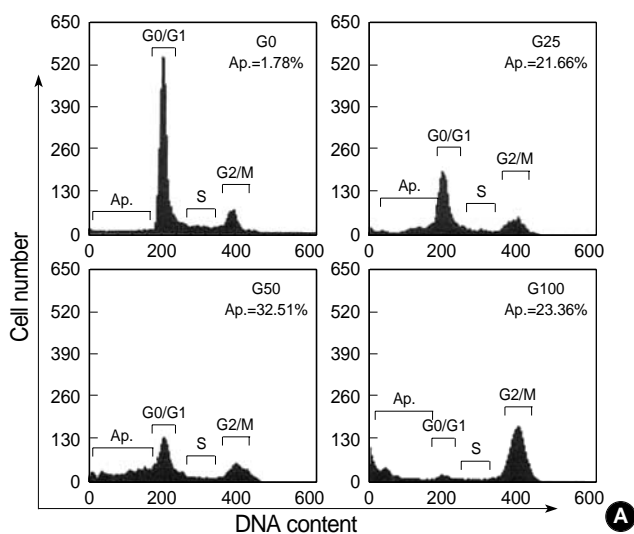


Fig. 5. Flow cytometric analysis of rat neuroblastoma B35 cells before and after three days of genistein treatment (G0, G25, G50 and G100 μM). Genistein induces cell death (apoptosis) represented by the cells accumulated at sub-G0/G1 (A) and the accumulation of cells at G2/M phase in a dose dependent manner (A, B).

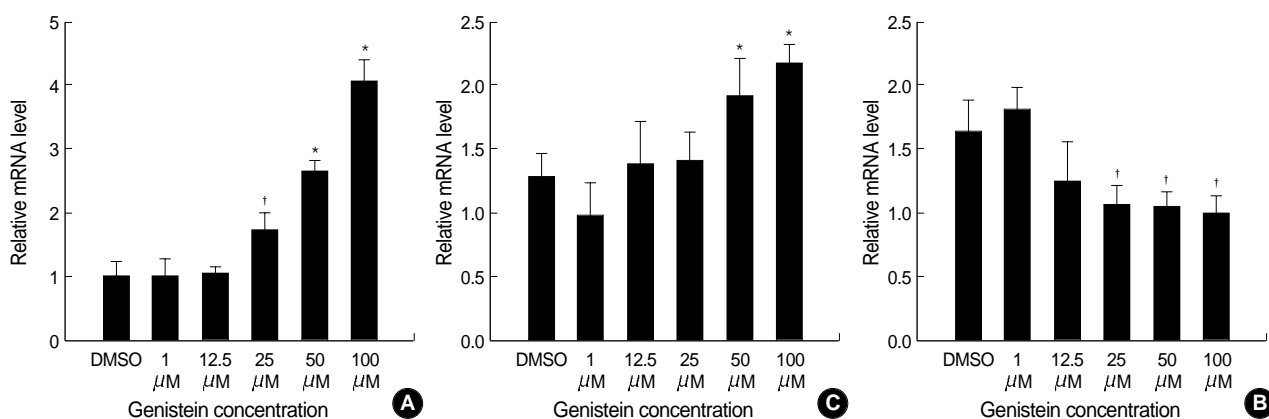


Fig. 6. Quantitative real time RT-PCR analysis of p21^{waf1/cip1}, Bcl-2 and Bax mRNA expression level before and after three days of genistein treatment of rat neuroblastoma B35 cells. Genistein activates p21^{waf1/cip1} (A) and Bax (B) transcription and slightly decreases Bcl-2 mRNA (C) in a dose-dependent manner. Values are means \pm SEM of relative gene expression. * $p < 0.01$, † $p < 0.05$.

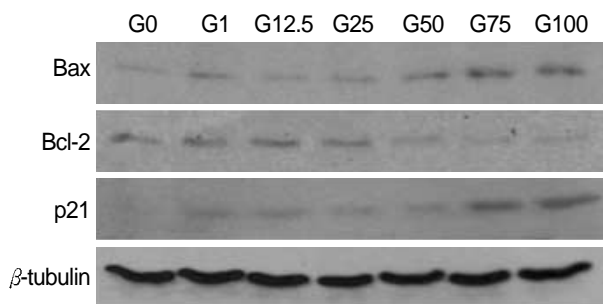


Fig. 7. p21^{waf1/cip1}, Bcl-2 and Bax protein expression in rat neuroblastoma B35 cells before and after three days of genistein treatment (1–100 μ M). Genistein increases p21^{waf1/cip1} and Bax and decreases Bcl-2 protein expression in a dose dependent manner.

The ultrastructural changes after genistein treatment

Similar results were obtained in two independent experiments. The electron micrograph results indicated that the DMSO-treated neuroblastoma B35 cells showed a normal cellular morphology with an open face nuclei (Fig. 4A), while after three days of treatment (100 μ M), the genistein-treated neuroblastoma B35 cells (Fig. 4B, C) showed apoptotic chromatin condensation and nuclear fragmentation (arrows).

The effect of genistein on the cell-cycle distribution

Flow cytometric analyses indicated that genistein induced cell-cycle arrest at the G2/M phase and it prevented cells from going through the mitotic phase with a concurrent decrease of cells at the G0/G1 phase. After three days of treatment, 1.7 fold genistein-induced cells accumulated at the G2/M phase with using the IC50, while there were 6.2 fold genistein-induced cells accumulated at 100 μ M (Fig. 5). These results indicate that genistein

had an effect to induce accumulation of cells at the G2 phase in a concentration-dependent rather than time-dependent manner. Furthermore, at low concentrations where most cells still accumulated at the G0/G1 phase, genistein increased cell-cycle progression at the S-phase. On the other hand, at high genistein concentrations where most cells accumulated at the G2/M phase, there were significant decrease in cell number at the S-phase (Fig. 5B). Also, FACS analysis clarified that genistein accumulated cells at the sub-G0/G1 phase (and this represented dead cells/apoptotic cells) in a concentration dependent manner (Fig. 5A).

Genistein upregulates p21^{waf1/cip1} expression

To analyze the molecular mechanism by which genistein induces cell-cycle arrest at the G2/M phase, we evaluated the expression of cyclin-dependent kinase inhibitor p21^{waf1/cip1}. Therefore, we evaluated the transcriptional and post-translational regulation of p21^{waf1/cip1} after genistein treatment. The genistein-treated G2/M-arrested B35 cells showed the accumulation of the cyclin-dependent kinase inhibitor p21^{waf1/cip1} at both the mRNA and protein levels in a dose-dependent manner, as proven by performing real-time quantitative PCR and western blotting, respectively (Fig. 6A & Fig. 7).

The effect of genistein on the apoptosis-related proteins

Flow cytometric analyses showed the sub-G0/G1 cell accumulation, which expresses cell death after genistein treatment (Fig. 5A). In addition, the transmission electron microscopic results showed that genistein induced neuronal cell death in the form of apoptosis (Fig. 4); this was confirmed by using Hoechst nuclear

staining (Fig. 3). To investigate the regulation of the expression of apoptosis-related proteins after genistein treatment, we analyzed the mRNA and protein levels of both anti-apoptotic Bcl-2 and the pro-apoptotic Bax via real time quantitative PCR and western blotting, respectively. Regarding the pro-apoptotic Bax, genistein treatment significantly increased both its mRNA and protein expressions (Fig. 6B & Fig. 7). Moreover, the protein up-regulation of Bax after genistein treatment was clearly dose-dependent. On the other hand, the anti-apoptotic protein Bcl-2 was markedly decreased in a concentration-dependent manner (Fig. 7). In addition, the real-time PCR results indicated that Bcl-2 transcription was slightly down-regulated in the genistein-treated B35 cells (Fig. 6C). Significantly, a marked increase in Bax and a decrease in Bcl-2 proteins could be detected at relatively high genistein concentrations ($>25 \mu\text{M}$). Moreover, the genistein-treated B35 cells showed decreased Bax and increased Bcl-2 mRNA at low genistein concentrations ($1 \mu\text{M}$).

DISCUSSION

The anticancerous activity of the soy isoflavone genistein has received extensive research attention for its effect on different cancers both *in vivo* and *in vitro*.^{11,12,25} Genistein has been reported to inhibit cell proliferation in both neuronal²⁶ and non-neuronal cancers.¹⁴ In the present study, we investigated the anti-cancer effect of genistein on rat neuroblastoma B35 cells, and we found that the proliferation of the rat neuroblastoma B35 cells that were supplemented with genistein was significantly suppressed in a dose- and time-dependent manner. It is noteworthy that after one day of $25 \mu\text{M}$ genistein treatment, the inhibition of cell proliferation was around 20% while it was 50% (IC50) after three days at the same concentration; this suggests that exposure time plays an important role for getting a significant anti-proliferative effect after genistein treatment, especially at low concentrations. In addition, the active genistein aglycone concentration in the tissues is higher than the physiological concentration ($4 \mu\text{M}$) in the blood stream.²⁷ Therefore, the current results may suggest that chronic supplementation with low genistein concentrations that are near physiological concentrations may significantly suppress malignant cell proliferation.

Several studies have documented that genistein induces cell-cycle arrest at the G2/M phase in different cancer cells.¹¹ On the other hand, G1 cell-cycle arrest in normal fibroblasts and prostate cancer cells has also been reported after genistein treatment.^{28,29} There is no available data on cell-cycle arrest in neuronal cancers

after genistein treatment. Our results indicate that genistein induced cell-cycle arrest at the G2/M phase and cell-cycle progression at the S-phase in rat neuroblastoma B35 cell line; these results are consistent with those obtained for different cancer cells.^{8,29} This induction of cell progression at the S-phase is possibly due to the estrogenic proliferative activity of genistein even at relatively high concentrations (25 and $50 \mu\text{M}$) while at higher concentrations of genistein ($100 \mu\text{M}$), almost all the cells accumulated at the G2/M phase with a concurrent reduction of cells that were accumulated at the G0/G1 and S phases. Cyclin-dependent kinase inhibitor p21^{waf1/cip1} has been widely reported to be up-regulated after genistein treatment at the transcriptional¹³ and post-translational²⁸ levels in several different cancer cells. In contrast, Hewitt and Singletary showed that genistein induced G2/M cell-cycle arrest in F3II mammary adenocarcinoma cells without any change in the p21^{waf1/cip1} expression level.¹² It is interesting that other studies have indicated that the accumulation of p21^{waf1/cip1} is not enough to induce G2/M cell-cycle arrest after genistein treatment in human prostate cancer cells.²⁸ These conflicting results may due to cell-specific or technical detection differences. The current results indicated that genistein causes p21^{waf1/cip1} protein up-regulation and its transcriptional activation. These results are consistent with those obtained in different genistein-treated cancer cells.^{8,11} This accumulation of p21^{waf1/cip1} inhibits the cdc2/cyclin-B1 complex and consequently induces cell-cycle arrest at G2/M.³⁰

Our results demonstrated that genistein slightly stimulated the release of LDH into the media in a time- and dose-dependent manner. In addition, Hoechst staining indicated that genistein induces apoptosis in B35 cells. These results are consistent with several studies that reported the induction of cytotoxicity and apoptosis after genistein treatment in rat cortical neurons.⁹ Interestingly, the ratio of LDH released into the experimental media was correlated with the ratio of apoptosis induction after genistein treatment. Therefore, the induction of apoptosis may be mainly due to the cytotoxic effect of genistein. There are very few previous transmission electron microscopic studies on the ultra-structural changes after genistein treatment. All the available transmission electron microscopic studies have revealed that genistein treatment induces cell death in the form of programmed apoptotic cell death in different cancers.¹⁰ To the best of our knowledge, there are no available studies concerning the ultra-structural effects of genistein on neuronal cells. The present results indicated that genistein induces cell death, and this cell death shows the precise, typical characters of apoptosis. Therefore, the induction of cytotoxicity and apoptosis after genistein treatment

in B35 cells has been confirmed via increased LDH release, and the findings of chromatin condensation and nuclear fragmentation (Hoechst staining and TEM, respectively).

To demonstrate the molecular mechanism by which genistein induced apoptosis, we investigated the well known anti-apoptotic Bcl-2 and pro-apoptotic Bax mRNA and protein expressions after genistein treatment in B35 cells. At low genistein doses (1 μ M), a decreased Bax expression and an increased Bcl-2 mRNA expression indicated that genistein has anti-apoptotic properties at low doses; these results are consistent with the neuroprotective effect of genistein in human cortical neurons.¹⁸ At high genistein doses, we found that genistein decreased the mRNA and protein expression levels of anti-apoptotic protein Bcl-2 and it increased the mRNA and protein expressions of pro-apoptotic protein Bax in a concentration-dependent manner. These results are precisely correlated with the increased induction of apoptosis after genistein treatment in different cancer cells.^{8,13} Therefore, we concluded that genistein induces apoptosis in B35 cells through the down-regulation of anti-apoptotic Bcl-2 protein with the concurrent up-regulation of the pro-apoptotic Bax protein.

We discovered that the mechanism of neuroblastoma B35 cell growth inhibition after genistein treatment involves several different cellular pathways, and it is mainly mediated via modulating cell-cycle progression by arresting cells at G2/M phase and also inducing programmed cell death.

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