

Full-length article

Effect of genistein on mouse blastocyst development *in vitro*Wen-hsiung CHAN¹, Hsiang-yu LU, Nion-heng SHIAO*Department of Bioscience Technology and Center for Nanotechnology, Chung Yuan Christian University, Chung Li 32023, Taiwan, China***Key words**

genistein; blastocyst; development; implantation

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Abstract

Aim: To examine the cytotoxic effects of genistein, an isoflavone compound, on early postimplantation embryonic development *in vitro*. **Methods:** Mouse blastocysts were incubated in medium with or without genistein (25 or 50 $\mu\text{mol/L}$) or daidzein (50 $\mu\text{mol/L}$) for 24 h. Cell proliferation and growth was investigated by dual differential staining, apoptosis was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, and apoptotic or necrotic cells were visualized by Annexin-V and propidium iodide (PI) staining. Implantation and postimplantation development of embryos were measured by *in vitro* development analysis. **Results:** TUNEL staining and Annexin-V/PI staining showed that genistein dose-dependently increased apoptosis in mouse blastocysts, while daidzein, another soy isoflavone, had no such effect. The pretreatment of the blastocysts with genistein caused fewer cells than the control group and this effect was primary in the inner cell mass. The genistein-pretreated blastocysts showed normal levels of implantation on culture dishes *in vitro*, but significantly fewer genistein-pretreated embryos reached the later stages of embryonic development versus the controls, with many of the former embryos dying at relatively early stages of development. In addition, genistein treatment decreased the development of morulas into blastocysts, and dietary genistein was found to induce cell apoptosis and decrease cell proliferation in an animal assay model of embryogenesis. **Conclusions:** Our results collectively indicate that genistein treatment of mouse blastocysts induces apoptosis, decreases cell numbers, retards early postimplantation blastocyst development, and increases early-stage blastocyst death *in vitro*, while dietary genistein appears to negatively affect mouse embryonic development *in vivo* by inducing cell apoptosis and inhibiting cell proliferation. These novel findings provide important new insights into the effect of genistein on mouse blastocysts.

Introduction

Apoptosis, which is widely observed in different cells of various organisms, is a unique morphological pattern of cell death characterized by chromatin condensation, membrane blebbing, and DNA fragmentation^[1]. Apoptosis plays an important role in the homeostasis of multicellular organisms. Moreover, abnormal apoptotic function has been associated with multiple human diseases, including neurodegenerative disorders and cancers^[2]. During normal embryogenesis, apoptosis functions to remove abnormal or redundant cells

from preimplantation embryos^[3,4]; during normal mouse embryonic development, this occurs only after the blastocyst stage^[5]. Premature apoptosis, such as that triggered by exposure to teratogens, causes improper embryonic development^[6,7]. We recently reported that several natural phytochemical compounds, including curcumin and ginkgolides, were able to prevent or induce apoptosis in embryonic stem cells and/or embryos^[8–10]. The effects of genistein on human embryo development are unclear. In the present study, we investigated whether another natural compound, genistein, had cytotoxic effects on embryonic development.

Our study will provide important new insights about the effect of dietary genistein on embryogenesis. The effect of genistein on embryo development *in vitro* must be studied because it is especially in relation to the dietary use of isoflavones (such as genistein) in pregnant women.

Genistein, a natural isoflavone compound found in soy products, has been shown to inhibit protein tyrosine kinase and topoisomerase-II activity^[11,12], leading to numerous effects on diverse cell functions. For example, genistein has been shown to inhibit tumor cell proliferation and induce tumor cell differentiation^[13-16], and may also trigger cell cycle arrest and apoptosis in some cell types^[17-19], while blocking apoptosis under other circumstances^[20-22]. Recent studies have shown that genistein can cause cell cycle arrest and developmental injury in embryos via its function as a tyrosine kinase inhibitor^[23-25]. It is thought that genistein inhibits epidermal growth factor (EGF) receptor tyrosine kinase activity, inhibiting the positive effect of EGF on glucose uptake. Since glucose is the main exogenous energy source for the early development of the preimplantation embryo, the genistein-induced inhibition of glucose uptake would be expected to dramatically affect early embryonic development^[23]. Genistein has also been suggested to inhibit tyrosine phosphorylation of the cadherin-catenin complex, which mediates compaction, adhesive functions, and embryonic cleavage from the 2 to 4 cell stages in the mouse embryo^[26]. In addition, mitochondria were observed to form numerous tiny clusters uniformly distributed in the cytoplasm in genistein-treated embryos, causing G2-stage arrest in the 2 cell stage of the embryo, suggesting that genistein may inhibit embryonic development by altering mitochondrial structure and function^[24]. However, although multiple studies have addressed the inhibitory effects of genistein on embryonic development due to the inhibition of tyrosine kinases or mitochondria-based mechanisms, no previous work has examined the apoptotic effects of genistein on the early stages of mammalian embryonic development, especially during the blastocyst stage.

In the present study, we exposed mouse blastocysts to genistein and examined apoptosis in the inner cell mass (ICM) and trophectoderm (TE), and also observed early postimplantation embryonic development on culture dishes *in vitro*. Finally, the *in vivo* effects of dietary genistein on cell apoptosis and proliferation were investigated in an animal model of embryogenesis.

Materials and methods

Chemicals and reagents Pregnant mares' serum gona-

dotropin (PMSG), sodium pyruvate, genistein, and daidzein were purchased from Sigma (St Louis, MO, USA). Human chorionic gonadotropin (hCG) was obtained from Serono (NV Organon Oss, the Netherlands). The Annexin-V-FLUOS staining kit and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) *in situ* cell death detection kit were obtained from Roche Molecular Biochemicals (Mannheim, Germany) and the CMRL-1066 medium was from Gibco Life Technologies (Grand Island, NY, USA).

Animals and collection of embryos ICR (a strain of mice) virgin albino mice (6–8 weeks old) were induced to superovulate by injection of 5 IU PMSG followed 48 h later by an injection of 5 IU hCG. The mice were then mated overnight with a single fertile male of the same strain. Female mice with vaginal plugs were separated and used for the experiments. All mice were maintained on breeder chow and kept under a 12 h day/12 h night regimen, with food and water available *ad libitum*. All animals received humane animal care, as outlined in the Guidelines for Care and Use of Experimental Animals (Canadian Council on Animal Care, Ottawa, Canada, 1984). The day a vaginal plug was found was defined as d 0 of pregnancy. Morulas were obtained by flushing the fallopian tubes on the afternoon of d 3, and the blastocysts were obtained by flushing the uterine horn on d 4; in both cases the flushing solution consisted of CMRL-1066 culture medium containing 1 mmol/L glutamine and 1 mmol/L sodium pyruvate.

Genistein treatment The embryos were collected in uncoated plastic 35 mm culture dishes and washed a minimum of 3 times. The expanded blastocysts from different females were pooled and randomly selected for the experiments. The blastocysts were incubated at 37 °C for 24 h in CMRL-1066 medium with and without the indicated concentrations of genistein or daidzein, and were then used for further experiments as described below.

Blastocyst cell counting The blastocysts were incubated with culture medium containing 0–50 µmol/L genistein. Twenty-four hours later, the blastocysts were washed with genistein-free medium, and dual differential staining^[27] was used to facilitate counting of cell numbers in the ICM and TE. The blastocysts were incubated in 0.4% pronase in M₂ medium containing 0.1% bovine serum albumin (M₂-BSA medium) for the removal of the zona pellucida. The denuded blastocysts were exposed to 1 mmol/L trinitrobenzenesulphonic acid in BSA-free M₂ medium containing 0.1% polyvinylpyrrolidone (PVP) at 4 °C for 30 min, and then washed with M₂ medium^[28]. The blastocysts were further treated with 30 µg/mL anti-dinitrophenol-BSA complex antibody in M₂-BSA at 37 °C for 30 min, and then with M₂ medium supple-

mented with 10% whole guinea pig serum as a source of complement, 20 $\mu\text{g}/\text{mL}$ bisbenzimidazole, and 10 $\mu\text{g}/\text{mL}$ propidium iodide (PI) at 37 °C for 30 min. The immunolysed blastocysts were gently transferred to slides and protected from light before observation. Under UV light excitation, the ICM cells (which take up bisbenzimidazole, but exclude PI) appeared blue, whereas the TE cells (which take up both fluorochromes) appeared orange-red. As multinucleated cells are not common in preimplantation embryos^[29], the number of nuclei was considered to represent an accurate measure of the cell number.

Detection of cell apoptosis For TUNEL staining, the embryos were washed in genistein-free medium, fixed, permeabilized, and subjected to TUNEL labeling using an *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's protocol. Photographic images were taken using a fluorescence microscope under bright light (Olympus BX 51, Tokyo, Japan). For Annexin-V staining, the blastocysts were incubated in 0–50 $\mu\text{mol}/\text{L}$ genistein for 24 h, washed with genistein-free culture medium, and then stained with an Annexin-V-FLUOS staining kit (Roche, Germany), according to the manufacturer's instructions. Briefly, the blastocysts were incubated in M_2 -BSA for the removal of the zona pellucida, washed well with phosphate buffer saline (PBS) plus 0.3% BSA, and then incubated for 60 min with a mixture of 100 μL binding buffer, 1 μL fluorescein isothiocyanate (FITC)-conjugated Annexin-V and 1 μL PI. After incubation, the embryos were washed and photographed under a fluorescence microscope. Cells staining Annexin-V⁺/PI⁻ were considered apoptotic, while those staining Annexin-V⁺/PI⁺ were considered necrotic.

Observation of *in vitro* implantation and postimplantation development The blastocysts were cultured according to a modification of the previously reported method^[30]. Briefly, the embryos were cultured in 4 well multidishes at 37 °C. For group culture, 3 embryos were cultured per well. The basic medium consisted of CMRL-1066 supplemented with 1 mmol/L glutamine and 1 mmol/L sodium pyruvate, plus 50 IU/mL penicillin and 50 mg/mL streptomycin (hereinafter called culture medium). For the treatments, the embryos were cultured with the indicated doses of genistein for 24 h without serum supplementation. Thereafter, the embryos were cultured for 3 d in culture medium supplemented with 20% fetal calf serum, and for 4 d in culture medium supplemented with 20% heated-inactivated human placental cord serum, for a total culture time of 8 d from the onset of treatment. The embryos were inspected daily under a phase contrast microscope (Olympus IMT-2, Tokyo, Japan), and the developmental stages were classified according to established methods^[31]. Develop-

mental parameters, such as hatching through the zona pellucida, attachment to the culture dish, trophoblastic outgrowth, and differentiation of the embryo proper were recorded daily. To decrease observer bias, all data were analyzed using the following previously published criteria^[32]: an implanted blastocyst was defined by attachment to the culture dish, followed by outgrowth; an early egg cylinder (EEC) was defined as an embryo that had reached stage 9 or 10 by d 4; a late egg cylinder (LEC) was defined as an embryo that had reached stage 11, 12, or 13 by d 6 of culture; and an early somite (ES) was defined as an embryo that had reached stage 14 or 15 by d 8^[32].

Effect of genistein on mouse embryo development *in vivo*

Female mice were fed a standard diet supplemented with or without genistein (10 $\mu\text{mol}/\text{L}$) in the drinking water for a total of 5 d. Twenty-four hours after the initiation of genistein exposure, the mice were mated overnight with a single fertile male of the same strain. The blastocysts were obtained by flushing the uterine horn on d 4 after mating, and cell apoptosis and proliferation were analyzed as described above.

Statistics The data were analyzed using one-way ANOVA and *t*-tests, and are presented as the mean \pm SD. $P < 0.05$ was considered significant.

Results

Effects of genistein on cell apoptosis in mouse blastocysts To study the apoptotic effects of genistein on embryos, we treated mouse blastocysts (150 blastocysts in each group) with 0–50 $\mu\text{mol}/\text{L}$ genistein at 37 °C for 24 h, and then measured cell apoptosis. TUNEL staining revealed that treatment with 25 and 50 $\mu\text{mol}/\text{L}$ genistein dose-dependently increased apoptosis in the mouse blastocysts (Figure 1A), and quantitative analyses revealed 4 to 6.7 fold more apoptotic cells in the genistein-treated blastocysts versus the untreated controls (Figure 1B). For the comparison, we used similar methods to analyze the cytotoxic effects of daidzein, another type of soybean isoflavone, on mouse blastocysts. Interestingly, TUNEL staining revealed that daidzein did not induce cell apoptosis in mouse blastocysts under our experimental conditions (Figure 1A, 1B). Additional TUNEL staining experiments revealed that the effect of genistein on cell apoptosis was more pronounced in the ICM than in the TE (Figure 1C), and Annexin-V staining revealed a higher rate of Annexin-V-positive cells in the ICM of treated blastocysts versus the controls, but similar rates of positivity in the TE (Figure 1D). These results indicate that genistein is a potent inducer of apoptosis in mouse blastocysts, primarily in the ICM, whereas daidzein does not appear to induce apoptosis

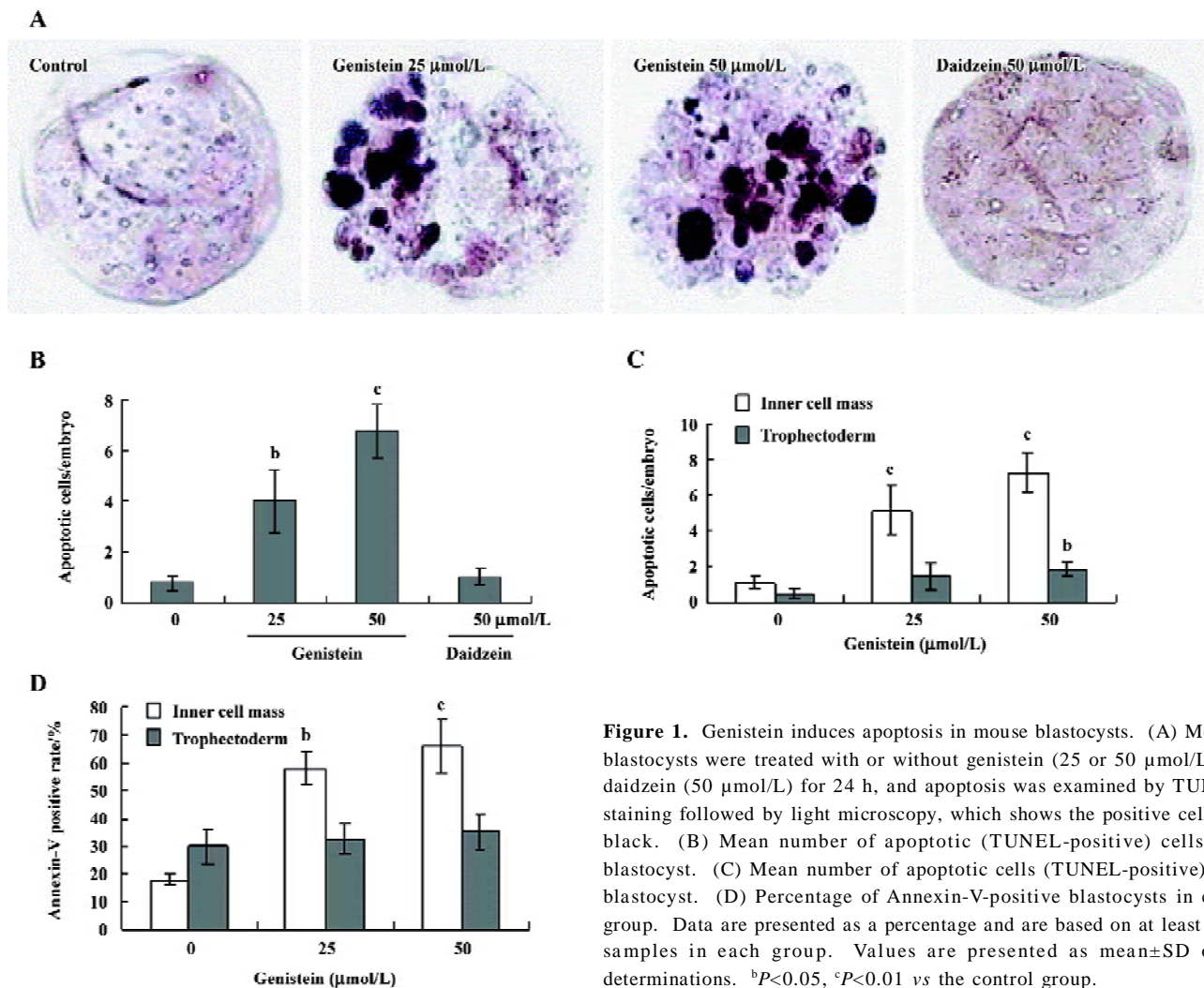


Figure 1. Genistein induces apoptosis in mouse blastocysts. (A) Mouse blastocysts were treated with or without genistein (25 or 50 μmol/L) or daidzein (50 μmol/L) for 24 h, and apoptosis was examined by TUNEL staining followed by light microscopy, which shows the positive cells in black. (B) Mean number of apoptotic (TUNEL-positive) cells per blastocyst. (C) Mean number of apoptotic cells (TUNEL-positive) per blastocyst. (D) Percentage of Annexin-V-positive blastocysts in each group. Data are presented as a percentage and are based on at least 150 samples in each group. Values are presented as mean±SD of 5 determinations. ^bP<0.05, ^cP<0.01 vs the control group.

in mouse blastocysts.

Effects of genistein on the blastocyst cell number To investigate the effect of genistein on cell proliferation in embryos, we treated mouse blastocysts (120 blastocysts in each group) with or without genistein (25–50 μmol/L) for 24 h, and then analyzed proliferation using differential staining followed by cell counting. Our results revealed that the blastocysts treated with 25 or 50 μmol/L of genistein contained fewer cells than the control blastocysts; this effect was more pronounced in the ICM than in the TE (Figure 2).

Effects of genistein on implantation and postimplantation development The blastocysts treated with 25 or 50 μmol/L of genistein (148–173 blastocysts, as indicated) and 148 control blastocysts were assessed for *in vitro* implantation and development. Implantation was similar in both the treatment

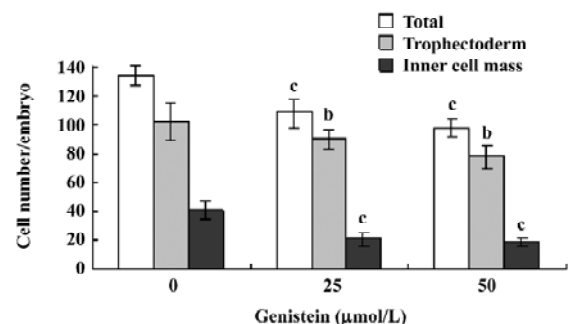


Figure 2. Effects of genistein on blastocyst viability. Mouse blastocysts were treated with or without genistein (25 or 50 μmol/L) for 24 h. The total number of cells per blastocyst and differential cell numbers in the inner cell mass and trophectoderm lineages were counted. Data are presented as a percentage and are based on 120 samples in each group. Values are presented as mean±SD of 5 determinations. ^bP<0.05, ^cP<0.01 vs the control group.

and control groups (Figure 3A). However, the genistein-treated blastocysts showed reduced formation of the 2 layer ICM and ectoplacental cones, and fewer instances of embryos developing to the advanced egg cylinder stages (LEC and ES stages) when compared with the control group (Figure 3A). Morulas exposed to 25 or 50 $\mu\text{mol/L}$ genistein showed 55%–65% development into blastocysts, whereas 86% of control morulas developed into blastocysts under our experimental conditions (Figure 3B).

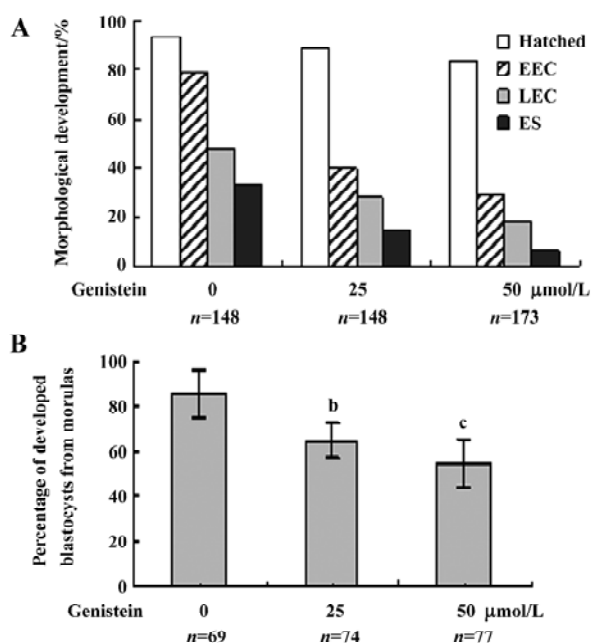


Figure 3. *In vitro* development of mouse embryos exposed to genistein at the blastocyst stage. (A) Mouse blastocysts were treated with or without genistein (25 or 50 $\mu\text{mol/L}$) for 24 h and observed in culture for 7 d post-treatment. Morphological assessment of hatched, EEC, LEC, and ES embryos was made according to established methods^[31,32]. (B) Mouse morulas were treated with or without genistein (25 or 50 $\mu\text{mol/L}$) for 24 h, and then cultured for an additional 24 h at 37 °C. Blastocysts were counted and percentages were calculated. Values are presented as mean \pm SD of 5 determinations. ^b P <0.05 and ^c P <0.01 vs the control group.

Effect of dietary genistein on mouse blastocyst development *in vivo*

The ability of genistein to trigger apoptosis and developmental injury *in vitro* led us to suspect that it might have the same activity *in vivo*. To evaluate this possibility in an animal model, we fed female mice a standard diet supplemented with or without genistein (10 $\mu\text{mol/L}$) in the drinking water, 24 h prior to mating and during the first 4 d of embryonic development. An analysis of flushed blastocysts (280 per group) revealed that dietary genistein significantly induced apoptosis (Figure 4A) and decreased cell prolifera-

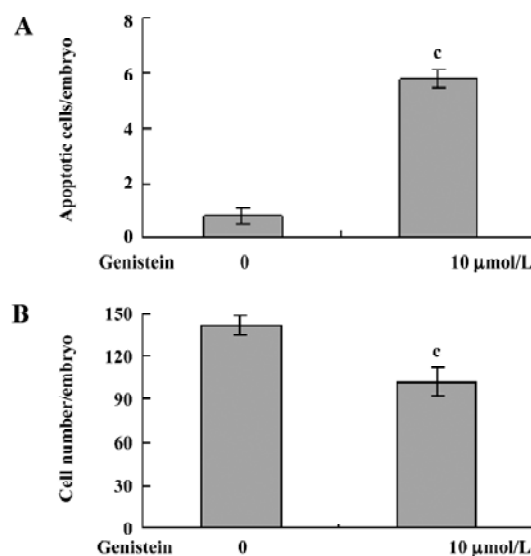


Figure 4. Effect of dietary genistein on cell apoptosis and proliferation in blastocysts assessed in an animal assay model. For the duration of the experiment, female mice were given a standard diet and drinking water supplemented with or without genistein (10 $\mu\text{mol/L}$). Twenty-four hours after induction of genistein exposure, the female mice were mated overnight with a single fertile male of the same strain. Blastocysts were obtained by flushing the uterine horn on d 4 after mating (280 blastocysts were examined per group). (A) Mean number of apoptotic cells (TUNEL-positive) per blastocyst. (B) Total number of cells per blastocyst. ^c P <0.01 vs the genistein-free group.

tion (Figure 4B) in mouse blastocysts. Collectively, these studies suggest that genistein can induce apoptosis and inhibit the proliferation of embryonic cells, and appears to cause embryonic developmental injury *in vitro* and *in vivo*, indicating that genistein may have a hazardous effect on embryonic development.

Discussion

Embryonic development is a complex process during which chemical injury can lead to abortion or embryonic malformation. Thus, it is important to examine the possible teratogenic effects of commonly used plant extracts. We herein show that genistein can decrease the viability of mammalian blastocysts via the increased induction of apoptosis (Figure 1). Our results revealed that treatment of mouse blastocysts with 25 and 50 $\mu\text{mol/L}$ genistein dose-dependently induced 4 to 6.7 fold increases in apoptosis, as assessed by TUNEL staining (Figure 1A, 1B). In contrast, another soy isoflavone, daidzein, had no such effect (Figure 1A, 1B). Annexin-V staining revealed that genistein-induced cell apoptosis occurred more commonly in the ICM than in

the TE (Figure 1C, 1D). Although the treated blastocysts could implant on culture dishes *in vitro*, postimplantation development was retarded, leading to embryonic death.

Previous reports have shown that genistein can either induce or prevent apoptosis, depending on the cell type or experimental conditions^[33,34]. For example, genistein induced apoptosis in immature human thymocytes^[19], leukemia cell lines^[18], human stomach cancer cells^[14], and bladder carcinoma cell lines^[35], but inhibited ionization radiation-induced apoptosis of human B lymphocyte precursors^[36], taxol-induced apoptosis of a human ovarian tumor cell line^[37], anti-CD3-induced apoptosis of mouse thymocytes^[20], didemnin B-induced apoptosis of human HL-60 cells^[22], and UV irradiation-induced apoptosis of a human epidermal carcinoma cell line^[38]. In addition, other studies have demonstrated that genistein can inhibit proliferation and induce differentiation in tumor cells^[39-41]. In the present work, we used TUNEL and Annexin-V staining to show that genistein promotes apoptosis in mouse blastocysts (Figure 1).

During embryonic development, cells are often poised between proliferation and apoptosis. Studies by several other groups and our laboratory have demonstrated that genistein can modulate cell apoptosis, but no previous work has examined the apoptotic effects of genistein on embryonic cell death. We herein report that genistein treatment of mouse blastocysts decreased cell numbers, increased apoptosis, and triggered developmental delays in postimplantation mouse embryos *in vitro*, and further report that dietary genistein appeared to have a teratogenic effect *in vivo*. Taken together, our novel observations in conjunction with the previous reports suggest that the actions of genistein depend on the cell type and treatment duration/dosage. Clearly, additional studies will be required to assess the molecular mechanism of action by which genistein affects cell apoptosis.

Although the effect was more pronounced in the ICM, some genistein-induced cell loss and apoptosis was observed in the TE as well (Figures 1C, 2). The TE arises from the trophoblast at the blastocyst stage, and subsequently develops into a sphere of epithelial cells surrounding the ICM and the blastocoel; these cells contribute to the placenta and are required for the development of the mammalian conceptus^[42]. Although we observed that genistein treatment slightly reduced the cell number and increased apoptosis in the TE of mouse blastocysts in this study (Figures 1C, 1D, 2), embryonic implantation did not appear to be affected *in vitro* (Figure 3A). Future work will be required to assess the effects of genistein on differentiation and giant-cell formation *in vitro* and *in vivo*.

Genistein has been shown to act as an agonist for estrogen receptor (ER) α and ER β ^[43]. The ability of genistein to protect against mammary carcinomas has been attributed to its anti-estrogenic effects^[44], while other studies demonstrated that the estrogenic and/or anti-estrogenic activities of genistein may reduce or enhance estrogen-dependent tumor growth, depending on the dose and timing of exposure^[45,46]. To test whether genistein treatment could affect ER expression, we used real time PCR to investigate the effect of genistein treatment on the expression levels of ER α and ER β in mouse blastocysts. Our results revealed no significant differences in ER α and ER β expression levels between the genistein-treated and the control blastocysts (data not shown), suggesting that genistein-induced cell apoptosis does not act via the alteration of ER α and ER β expression levels in the studied mouse blastocysts.

Researchers recently investigated the effects of phytoestrogens such as genistein on the production of the protein hormone hCG in trophoblasts. Their results revealed that hCG production was decreased by genistein treatment in the trophoblasts of term placentas^[47]. Another study showed that genistein affected the production of hCG and the steroid hormone progesterone in the BeWo and Jeg3 trophoblast cell lines^[48]. Importantly, the report noted that different treatment dosages of genistein could cause different effects on the production of progesterone and hCG. The result showed that a correlation between the effects on the proliferation and the production of progesterone and hCG at high concentrations of genistein (>1 mmol/mL). With low concentrations of genistein treatment, the study found that a stimulation of the production of hCG and a weak inhibition of proliferation in both cell lines BeWo and Jeg3^[48]. These results seem to imply that exposure to genistein during sensitive periods of development may alter reproductive functions and influence fertility. These reports, in conjunction with the present findings, suggest that genistein acts as a teratogen, negatively impacting embryonic development via the inhibition of trophoblast cell proliferation and hCG production, the inactivation of tyrosine kinases, and the induction of cell apoptosis.

In summary, we show for the first time that genistein can decrease the viability of mammalian blastocysts by inducing apoptosis in the ICM. Although additional work will be required to fully elucidate the action mechanisms of genistein, especially in relation to the dietary use of isoflavones (such as genistein) in pregnant women, this study provides the first evidence that genistein could have teratogenic effects through the induction of apoptosis.

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