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Impact of genistein on maturation of mouse oocytes, fertilization, and fetal development

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ABSTRACT

Genistein (GNT), a natural isoflavone compound found in soy products, affects diverse cell functions, including proliferation, differentiation and cell death. An earlier study by our group showed that GNT has cytotoxic effects on mouse blastocysts and is associated with defects in their subsequent development *in vitro*. Here, we further investigate the effects of GNT on oocyte maturation, and subsequent pre- and postimplantation development, both *in vitro* and *in vivo*. GNT induced a significant reduction in the rate of oocyte maturation, fertilization, and *in vitro* embryo development. Treatment of oocytes with GNT during *in vitro* maturation (IVM) led to increased resorption of postimplantation embryos, and decreased placental and fetal weights. With the aid of an *in vivo* mouse model, we showed that consumption of drinking water containing GNT led to decreased oocyte maturation and *in vitro* fertilization, as well as early embryonic developmental injury. Moreover, our findings support a degree of selective inhibition of retinoic acid receptors in blastocysts treated with GNT during oocyte maturation. To our knowledge, this is the first study investigating the impact of GNT on maturation of mouse oocytes, fertilization, and sequential embryonic development.

1. Introduction

Genistein (GNT), a natural isoflavone compound in soy products, inhibits protein tyrosine kinase (PTK) and topoisomerase-II (Topo-II) activities [1,2], consequently affecting diverse cell functions. For example, GNT inhibits proliferation and induces differentiation of tumor cells [3–6], and may also trigger cell cycle arrest and apoptosis in specific cell types [7–9], while blocking apoptosis under other circumstances [10–12]. GNT causes cell cycle arrest and developmental injury in embryos via its activity as a tyrosine kinase inhibitor [13–15]. It is thought that GNT suppresses epidermal growth factor (EGF) receptor tyrosine kinase activity, in turn, inhibiting the positive effect of EGF on glucose uptake. Since glucose is the main source of exogenous energy for early development of the preimplantation embryo, GNT-induced inhibition of glucose uptake would dramatically affect early embryonic development [13]. GNT also inhibits tyrosine phosphorylation of the cadherin–catenin complex, which mediates compaction, adhesive functions and embryonic cleavage from the 2- to 4-cell stage in the mouse embryo [16]. In addition, mitochondria form numerous tiny clusters uniformly distributed in the cytoplasm in GNT-treated embryos, causing G2 stage arrest at the two-cell stage, suggesting that this compound inhibits embryonic development by altering mitochondrial structure and function [14]. We recently reported that several natural phytochemical compounds, including curcumin and ginkgolides, either prevent or induce apoptosis in embryonic stem cells and/or embryos [17–19]. Our group further showed that GNT induces apoptosis, decreases cell numbers, retards early postimplantation development and increases early-stage death in mouse blastocysts *in vitro*, while dietary GNT negatively affects mouse embryonic development *in vivo* by inducing apoptosis and inhibiting proliferation [20]. However, the effects of GNT on human embryo development are currently unclear. In the present study, we investigated whether GNT has cytotoxic effects on mouse oocyte maturation, fertilization, and sequential embryonic development. Knowledge on the effects of GNT on oocyte maturation and fertilization *in vitro* is essential, particularly in relation to the dietary use of this isoflavone by pregnant women.

Oocyte viability is affected by the microenvironment during growth and maturation. Specifically, heat stress, oxygen concentration, and glucose content are determinants of oocyte viability [21–23]. A few researchers have investigated the influence of environmental biological toxins on oocyte maturation *in vivo* and *in vitro*. During normal embryogenesis, apoptosis (a unique morphological pattern of cell death) functions to clear abnormal or

Abbreviations: GNT, genistein; RARs, retinoic acid receptors; COCs, cumulus–oocyte complexes; IVM, *in vitro* maturation; IVF, *in vitro* fertilization; ICM, inner cell mass; TE, trophectoderm.

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redundant cells in preimplantation embryos [24,25]. However, apoptotic processes do not occur prior to the blastocyst stage during normal mouse embryonic development [26], and induction of cell death during oocyte maturation and the early stages of embryogenesis (i.e., via exposure to a teratogen) leads to embryonic developmental injury [17,22,27]. GNT clearly promotes cell apoptosis and developmental injury in blastocysts [20]. Previous studies of isoflavone administration to human subjects have demonstrated that the highest serum GNT levels achievable through ingestion of a soy-rich diet appear to be in the range of 1–5 μM [28–30]. In Wistar rats exposed to dietary doses of GNT in the 0.2–200 mg/kg body weight/day range, serum GNT levels approximately 10 nM to 8 μM [31,32]. Furthermore, in adult rats consuming 500 ppm dietary GNT, total circulating GNT reached levels of approximately 6–8 μM [33]. Thus, studies have shown that rats fed a GNT-containing diet experience circulating GNT levels comparable to those found in humans consuming soy-rich diets. Here, investigate whether such levels of GNT (1–10 μM) could have hazardous effects on oocyte maturation and embryonic development at treatment doses reflecting physiological concentrations achievable through dietary intake.

Retinoic acid receptors (RARs) belong to the class II receptor family. These proteins heterodimerize with the retinoid X receptor, RXR, and further interact with specific DNA-binding motifs. Six known genes encode retinoic acid receptors. During RAR binding to its ligand, heterodimerization generates a RAR/RXR/ligand complex, which further interacts with response target genes and elicits transcriptional responses [34–36]. Expression of mRNA encoding all three retinoic acid receptors (RAR-α, -β and -γ) is observed in blastocysts of early postimplantation mouse embryos [37]. These findings suggest that factors affecting interactions or expression of RAR are involved in the developmental processes of early mammalian embryos through the regulation of specific control genes. Thus, differential expression of RAR appears to be an important step in embryonic cell development. In our experiments, GNT inhibited oocyte maturation, *in vitro* fertilization, and embryonic development. Our results further support a degree of selective inhibition of RARs in blastocysts generated from GNT-treated oocytes.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), genistein, and pregnant mare serum gonadotropin (PMSG) were obtained from Sigma (St. Louis, MO). Human chorionic gonadotropin (hCG) was purchased from Serono (NV Organon Oss, The Netherlands). TUNEL *in situ* cell death detection kits were acquired from Roche (Mannheim, Germany), and CMRL-1066 medium from Gibco Life Technologies (Grand Island, NY).

2.2. COC collection and in vitro maturation (IVM)

ICR mice were acquired from the National Laboratory Animal Center (Taiwan, ROC). This research was approved by the Animal Research Ethics Board of Chung Yuan Christian University (Taiwan, ROC). All animals received humane care, as outlined in the Guidelines for Care and Use of Experimental Animals (Canadian Council on Animal Care, Ottawa, 1984). Mice were maintained on breeder chow (Harlan Teklad chow) with food and water available *ad libitum*. Housing was provided in standard 28 cm × 16 cm × 11 cm (height) polypropylene cages with wire-grid tops, and maintained under a 12 h day/12 h night regimen. Cumulus–oocyte complexes (COCs) were obtained according to a previous protocol [22]. Briefly, COCs were isolated from female hybrid ICR mice (21 days old) injected with 5 IU human chorionic gonadotrophin (hCG) 44 h prior to oocyte collection. COCs were collected in HEPES-buffered α minimum essential medium (MEM) (containing 50 μg/mL Streptomycin sulfate, 75 μg/mL Penicillin G, and 5% fetal bovine serum) by gently puncturing visible antral follicles present on the ovary surface. Germinal vesicle stage oocytes containing an intact vestment of cumulus cells were collected and pooled in at least eight animals. For oocyte maturation, one drop (~100 μL) of buffer (αMEM supplemented with 50 μg/mL Streptomycin, 75 μg/mL Penicillin G, 5% FBS and 50 mIU/mL recombinant human FSH) containing 10 COCs was added under oil in 35 mm culture dishes. COC maturation was analyzed following treatment with or without various concentrations of GNT (1, 5 or 10 μM) for 24 h.

2.3. Maturation status assessment

After *in vitro* maturation (IVM), COCs of each group were treated with 50 U/mL ovine hyaluronidase and gently pipetted for the removal of all cumulus cells. Denuded oocytes were collected, and washed with fresh medium, followed by phosphate-buffered saline (PBS). Oocytes were fixed in ethanol: glacial acetic acid (1:3) for 48 h, and stained with 1% aceto-orcein solution. Nuclear structures were visualized using phase-contrast microscopy.

2.4. In vivo maturation

For obtaining *in vivo* matured oocytes, 21-day-old mice were injected with 5 IU eCG and 5 IU hCG, 61 and 13 h prior to fertilization, respectively. Mature ova were collected from the oviduct into HEPES-buffered αMEM medium.

2.5. Effects of GNT intake on oocyte maturation in an animal model

The effects of GNT on oocytes were analyzed in 6–8-week-old ICR virgin albino mice. Female mice were randomly divided into four groups of 20 animals each, and administered a standard diet with or without 1, 5 or 10 μM GNT in drinking water for 4 days. COCs were collected, and analyzed for oocyte maturation, *in vitro* fertilization, and embryonic development.

2.6. In vitro fertilization

For *in vitro* fertilization, ova were washed twice in bicarbonate-buffered α-MEM medium (containing 50 mg/mL Streptomycin, 75 mg/mL Penicillin G and 3 mg/mL fatty acid free bovine serum albumin), and fertilized in the same medium with fresh sperm (obtained from a CBAB6F1 male donor). After incubation with sperm for 4.5 h, eggs were washed three times in potassium simplex optimized medium (KSOM) without amino acids in the presence of L-alanyl-L-glutamine (1.0 mM). Next, eggs were placed in 20 mL drops of KSOM under oil, and cultured overnight. During cleavage to the 2-cell stage, embryos were transferred to a fresh drop of KSOM under oil, and cultured for another 72 h.

2.7. Fertilization assessment

For the examination of fertilization, ova were incubated with sperm for 4.5 h, followed by 3 h of culture in fresh medium. Zygotes were assessed for the presence of the male pronucleus with orcein staining, as described previously [22].

2.8. Cell proliferation

Cell proliferation was analyzed by dual differential staining, which facilitated the counting of cell numbers in inner cell mass (ICM) and TE [38]. Blastocysts were incubated with 0.4% pronase in M_2-BSA medium (M_2 medium containing 0.1% bovine serum albumin) for the removal of zona pellucida. Denuded blastocysts were exposed to 1 mM trinitrobenzenesulfonic acid (TNBS) in BSA-free M_2 medium containing 0.1% polyvinylpyrrolidone (PVP) at 4 °C for 30 min, and washed with M_2 [39]. Blastocysts were further treated with 30 μg/mL anti-dinitrophenol–BSA complex antibody in M_2-BSA at 37 °C for 30 min, followed by M_2 supplemented with 10% whole guinea pig serum as a source of complement, along with 20 μg/mL bisbenzimide and 10 μg/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, ICM cells (which take up bisbenzimidine but exclude PI) appeared blue, whereas TE cells (which take up both fluorochromes) appeared orange-red. Since multinucleated cells are not common in preimplantation embryos [40], the number of nuclei represent an accurate measurement of cell number.

2.9. TUNEL assay of blastocysts

For TUNEL staining, embryos were washed in GNT-free medium, fixed, permeabilized, and subjected to labeling using an *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's protocol. Photographic images were obtained with a fluorescence microscope under bright-field illumination.

2.10. Blastocyst development following embryo transfer

To determine the ability of expanded blastocysts to implant and develop *in vivo*, embryos generated were transferred to recipient mice. ICR females (white skin) were mated with vasectomized males (C57BL/6J; black skin; National Laboratory Animal Center, Taiwan, ROC) to produce pseudopregnant dams as recipients for embryo transfer. To ensure that all fetuses in pseudopregnant mice were derived from embryo transfer (white color) and not fertilization by C57BL/6J (black color), we examined skin color at day 18 post-coitus. For assessment of the impact of GNT on postimplantation growth *in vivo*, COCs were exposed to 0, 1, 5 or 10 μM GNT for 24 h, followed by fertilization and *in vitro* maturation to the blastocyst stages. Subsequently, 8 embryos were transferred in parallel to paired uterine horns of day 4 pseudopregnant mice. Surrogate mice were killed on day 18 post-coitus, and the