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Original article

Genistein causes germ cell reduction in the genital ridges of Japanese quail *Coturnix japonica* embryo

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Abstract

Genistein (GEN), an isoflavonoid phytoestrogen, is one of the potent estrogenic compounds derived from plants that can cause disrupting effects on sex organ development in non-mammalian and mammalian species. The present study revealed effect of genistein on germ cell number in the genital ridges during gonadogenesis. Genistein (16 and 24 µg/g egg) was injected into the egg yolk prior to incubation. Effect of genistein on quail-primordial germ cells (PGCs) number was examined by counting the number of *Wisteria floribunda* (WFA)-positive cells localized in both left and right genital ridges compared with the control group. Both concentrations of genistein resulted in significant decrease of PGC number compared with the control group. Percentages of the sterility rate of the embryo treated with 16 and 24 µg of genistein/g egg were 19% and 23%, respectively. These results provide evidence that genistein may be a germ cell toxicant causing sterility later in life of adult birds. This is the first report on the effect of genistein on PGC number in the genital ridges of the avian embryo.

Key words: genistein, primordial germ cell, genital ridges, quail embryo, *Coturnix japonica*

Introduction

Environmental contaminants are able to disrupt the endocrine system of living organisms via endocrine disruption (Safe et al. 2000). Several terms were used to refer the specific action of these chemicals on the endocrine milieu such as endocrine disrupting contaminants (Guillette and Gunderson 2001), environmental signals (Cheek et al. 1998), environmental hormones (Danzo 1998), and endocrine disruptors (Kavlock et al. 1996). Endocrine disruptors or EDs can be either synthetic or natural substances that are able to disrupt the functions of endocrine system as well as endogenous hormones (McLachlan et al. 2001).

Phytoestrogens are classified as natural EDs as they were reported to be able to disturb the function of endogenous estrogen, and produce estrogen-like effects (Kanno et al. 2002). Phytoestrogens are divided into three groups: isoflavones, coumestans and lignans (Turner and Sharpe 1997, Setchell 1998). Isoflavones (i.e. genistein and daidzein) are the most

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potent phytoestrogens that can be found predominantly in soybean, and soy products (Adlercreutz 1995). There have been reports that both genistein and daidzein were able to bind to the estrogen receptor (ER) (Hanafy et al. 2004, Hanafy et al. 2005), and act as estrogen agonists or antagonists (Setchell 1998, Lephart et al. 2002).

Genistein was reported to be an endocrine disruptor being able to produce detrimental effects on development of reproductive organs, particularly germ cell development (Casanova et al. 1999, Nagao et al. 2001, Jung et al. 2004). In mammalian species, genistein caused cytotoxic effects by inducing germ cell apoptosis *in vitro* (Kumi-Diaka et al. 1998, Kumi-Diaka et al. 1999), and decreasing oocyte maturation *in vivo* (Chan 2009). Conversely, isoflavonoid daidzein, but not genistein, was reported to act as an antioxidant in chicken germ cells (Liu et al. 2006, Tang et al. 2006, Mi et al. 2007). However, there is no study on developmental effect of genistein on avian primordial germ cells at the embryonic stage. Only one study on quail at the adult stage reported that male quails treated with high doses of genistein exhibited germ cell apoptosis (Ekinci and Erkan 2012). Thus, the effect of genistein on germ cell development in the avian embryo is still unknown.

Use of the avian embryo as a model in germline stem cell biology was documented (Intarapat and Stern 2013a). Strikingly, the avian embryo exhibits several unique characteristics during embryonic development: asymmetrical gonads develop in female, whereas bilateral gonads develop in male (Romanoff 1960, Smith and Sinclair 2001), male embryos have a greater number of germ cells in the left than the right gonad (Intarapat and Stern 2013b, 2014). There are several advantages of using quail embryo as a model: embryonic development until hatching of quail embryo is shorter than that of chicken embryo (Padgett and Ivey 1960), detailed stages of embryonic development and gonadogenesis were well established (Ainsworth et al. 2010, Intarapat and Satayalai 2014). Moreover, quail embryos and their reproductive organs are recommended for studying reproductive and development toxicology (OECD 1984, 2000). Previous study reported that genistein caused anatomical and histological alterations in the reproductive organs of quail embryo in both sexes after *in ovo* exposure (Intarapat et al. 2014). The present study is aimed to examine the effects of genistein on PGC number in the genital ridges of quail embryo during gonadogenesis.

Materials and Methods

Animals and Chemical treatment

Japanese quail (*Coturnix japonica*) eggs were obtained from the Department of Animal Science, Kasetsart University, Thailand. Genistein (4',5,7-Trihydroxyisoflavone; purity ≥ 98%) was purchased from Sigma chemical (St. Louis, Mo, USA). DMSO (dimethyl sulfoxide; purity ≥ 99%) was obtained from Merck (Darmstadt, Germany) and Mazola corn oil was the product of CPC (AJI, Thailand). The eggs were randomly divided into 3 groups, the control, genistein-16 and genistein-24 µg/g-treated eggs (minimum 20 eggs per group). *In ovo* exposure, genistein preparation and dosing were conducted in accordance with the methods described in a previous study (Intarapat et al. 2014).

Histological examination of the genital ridges of 3-day-old quail embryo

The genital ridges with the mesonephroi of 3-day-old quail embryos were dissected under SZ-PT stereomicroscope (Olympus, Japan) and cleaned with chick Ringer solution. The cleaned embryos were fixed with Rossman's fluid for 24 hr. The fixed embryos were rinsed in 95% ethanol for 2 wk. (until color of picric acid disappeared), then dehydrated in absolute ethanol, cleared in xylene, and embedded in Paraplast (Sherwood Medical Company, St. Louis, MO, USA.). The embedded gonads were cut at 6 µm with a microtome (The Gemmary, Fallbrook, USA). Sections were stained with hematoxylin and eosin (H&E).

Histochemical detection of quail-PGCs in the genital ridges

Lectin from *Wisteria floribunda* (WFA Biotin conjugate, Sigma-Aldrich, MO, USA) was used as a maker for quail-PGCs (Yoshinaga et al. 1992, Intarapat and Satayalai 2014). Histochemical staining was processed according to the methods described in a previous study (Intarapat and Satayalai 2014). Briefly, paraffin sections cut from the region of the hindgut of 3-day-old quail embryos were deparaffinized by immersing sections in graded series of ethanol. Sections were pretreated with 0.3% H_2O_2 (APS, Auburn, Australia) for 30 min to block endogenous peroxidase. The pretreated sections were rinsed in 1X rinse buffer (Tris buffer + distilled water and Tween 80, Fluka, Bunch, Switzerland) 5 times. The rinsed

Table 1. Frequencies of mortality fertility and viability of quail embryo following *in ovo* injection.

Treatment (µg/g egg)	Mortality (%)[a]	Fertility (%)[b]	Viability (%)[c]
Control[d]	9 (2/23)	91 (21/23)	91 (19/21)
Genistein[e] (16)	0 (0/26)	81 (21/26)	100 (21/21)
Genistein[e] (24)	4 (1/26)	96 (25/26)	96 (24/25)

[a] The ratio (in parentheses) represents the number of dead embryos divided by the number of treated eggs. Frequencies of the mortality, fertility and viability in the treatment groups were compared with the frequency in the control group using Fisher's exact test.
[b] The number of fertile eggs divided by the number of total eggs
[c] The number of viable embryos divided by the number of fertile eggs
[d] Eggs-treated with DMSO/corn oil based emulsion.
[e] Genistein was dissolved with DMSO and emulsified with corn oil

Table 2. The PGC number at the genital ridges of 3-day-old quail embryo following *in ovo* exposure to genistein prior to incubation.

Treatment (µg/g egg)	Number of examined embryos	Number of WFA-positive cells in the left and right genital ridges
Control	6	50.49 ± 1.86
Genistein (16)	7	41.09 ± 1.38 ***
Genistein (24)	6	38.82 ± 0.84 ***

The results are expressed as means ± SEM (standard error of the mean)
*** Statistical difference in relation to the control group ($p<0.001$)

sections were then added with blocking reagent (normal goat serum in phosphate buffered saline provided in the kit, Chemicon International, USA) and incubated for 5 min. Sections were rinsed with 1X rinse buffer and then incubated in 20 µl biotinylated WFA lectin for 1 hour at room temperature. Next, the sections were added with 2 drops of streptavidin horseradish peroxidase (Chemicon International, USA) and then incubated for 30 min at room temperature. Finally, sections were incubated in 0.05% diaminobenzidine with 0.3% H_2O_2 (DAB-H_2O_2) (Chemicon International, USA) in TBS for 10 min, rinsed in distilled H_2O, counterstained with hematoxylin, dehydrated and mounted with Permount (Fisher, NJ, USA). For negative control, sections were incubated with the incubation medium omitting the biotinylated lectin. The sections were studied and photographed by PM-10 M3 camera (Olympus, Japan).

Statistical analysis

Frequencies of embryo mortality, fertility, and viability in control and treated groups were compared using Fisher's exact test. To quantify the number of quail-PGCs (WFA positive cells) in the genital ridges, WFA positive cells were counted starting from the first section containing left and right genital ridges to the tenth section (Intarapat and Satayalai 2014). To avoid counting the same cells, one in three sections was counted until the last section of the genital ridges was reached (Intarapat and Satayalai 2014). Index of sterility (IS) was also calculated as IS = (N -X) / N, where N is the number of PGCs in the control and X is the equivalent number of PGCs in treated embryos (Aige-Gil and Simkiss 1991). The quantitative data was analyzed by one-way ANOVA using SPSS software (IBM, USA).

Results

Effects of genistein on embryo mortality fertility and viability

Both concentrations of genistein (16 and 24 µg/g egg) used in this study did not cause embryo mortality (Table 1). Frequencies of fertility of the control, 16 µg genistein and 24 µg genistein-treated eggs were 91, 81, and 96%, respectively (Table 1). Frequencies of embryo viability of the control, 16 µg genistein and 24 µg genistein-treated eggs were 91, 100, and 96%, respectively (Table 1).

Effect of genistein on PGC number

In histological examination, PGCs can be easily distinguished from surrounding somatic cells by hav-

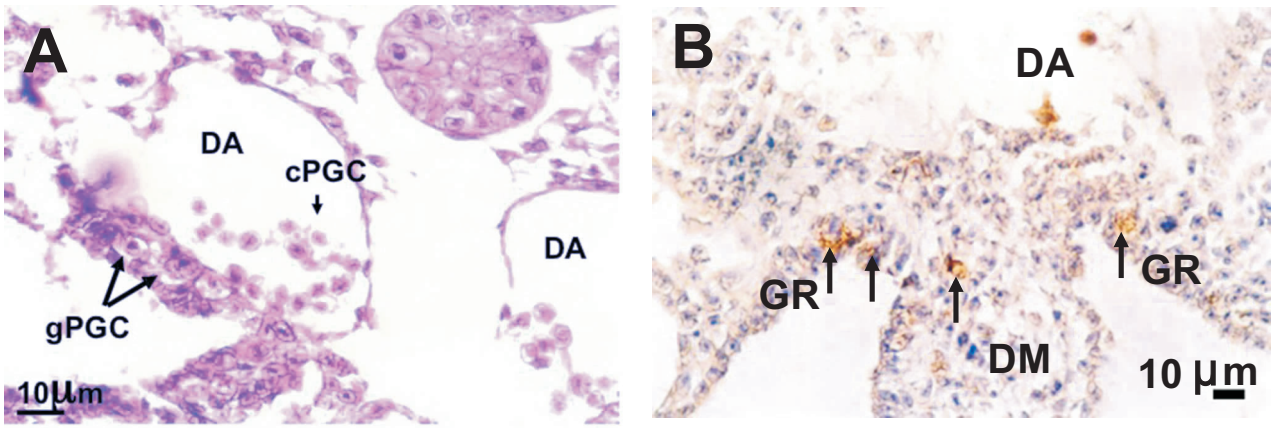

Fig. 1. A: stage of germ cell migration showing PGCs (arrows) in the dorsal aorta and the genital ridge of quail embryo; Bar = 10 µm, H&E staining (x400). B: Cross section of the genital ridges of 3-day-old quail embryo showing WFA-positive cells (arrows) in left and right genital ridges as well as the dorsal mesentery; Bar = 10 µm, Lectin-HC staining (x400). Abbreviations: cPGC : circulating-primordial germ cell, gPGC : gonadal-primordial germ cell, DA : dorsal aorta, DM : dorsal mesentery, GR : genital ridges.

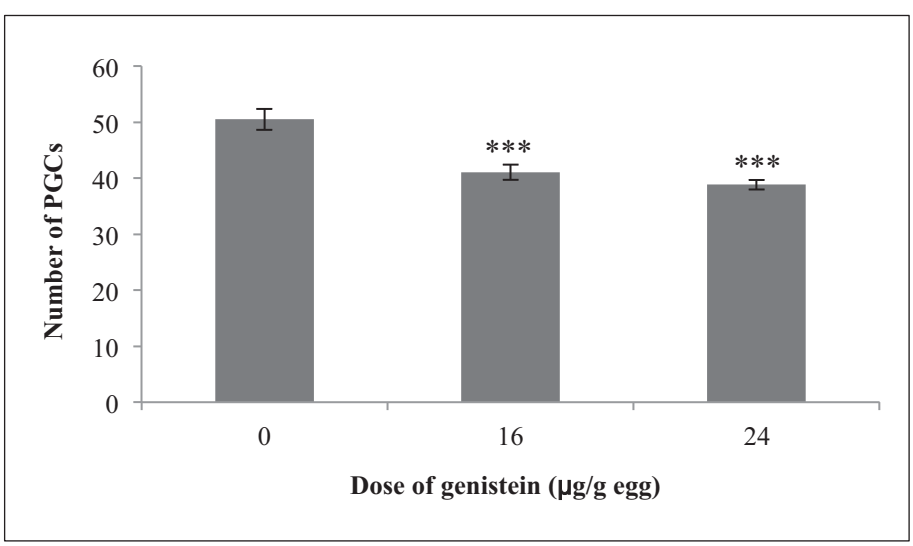

Fig. 2. The PGC number (mean ± SEM) at the geniteal ridges of 3-day-old quail embryo in the control (n=6), 16 µg genistein-treated (n=7) and 24 µg genistein-treated (n=6); (***p<0.001).

ing larger cell body with larger nucleus, and clear cytoplasm (Fig. 1A). WFA-lectin histochemistry revealed quail-PGCs locating in both left and right genital ridges as well as the dorsal mesentery (Fig. 1B). Localization of WFA-lectin can be found in both cell membrane and cytoplasm of quail-PGCs (Fig. 1B). The number of PGCs in the control, 16 and 24 µg genistein-treated groups were 50.49 ± 1.86, 41.09 ± 1.38, and 38.82 ± 0.84, respectively (Table 2). The number of PGCs in the genital ridges of genistein-treated embryos decreased significantly compared with the control group (16 and 24 µg/g egg, $p<0.001$, in left and right genital ridges, Fig. 2). Both concentrations of genistein decreased PGC number in the left and right genital ridges significantly (16 and 24 µg/g egg, $p<0.001$, in the left genital ridge; $p<0.001$, in the right genital ridge) compared with the control group as shown in Fig. 3.

Evaluation of sterility rate of the embryos-treated with genistein

Effect of genistein on sterility rate was observed. A significant increase in the index of sterility of 16 µg genistein-treated and 24 µg genistein-treated embryos were 0.19, and 0.23 ($p<0.001$), respectively, compared with the control group (Table 3). Percentages

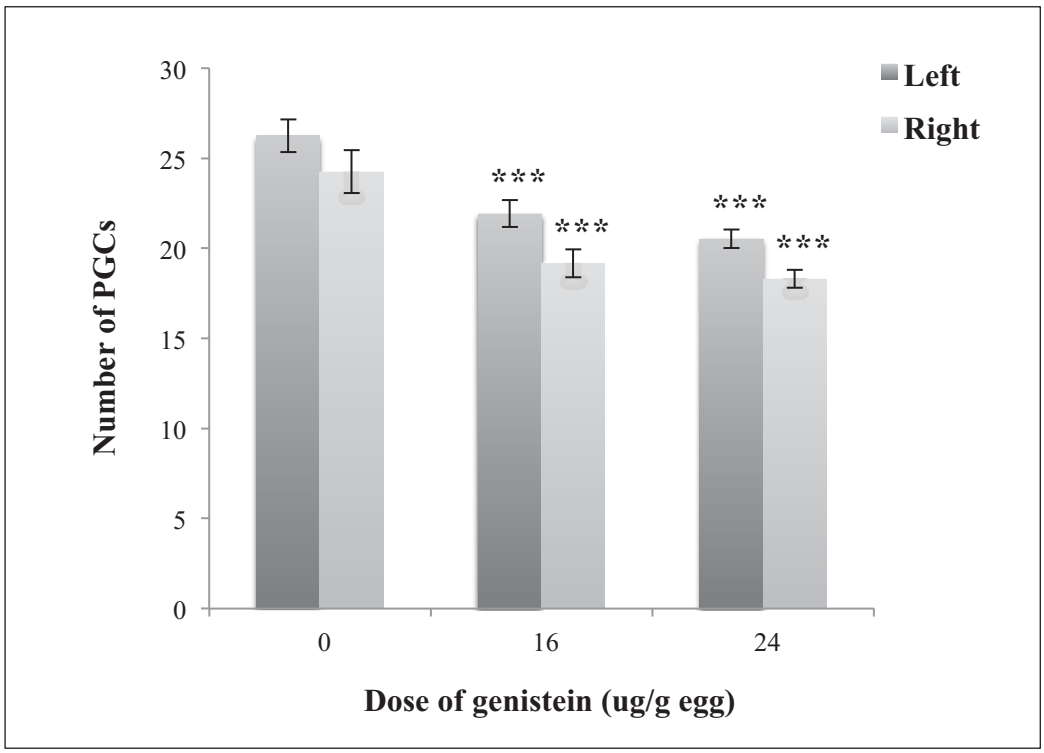

Fig. 3. Comparison of PGC number (mean ± SEM) between left and right genital ridges of 3-day-old quail embryo in the control (n=6), 16 µg genistein-treated (n=7) and 24 µg genistein-treated (n=6); (***p<0.001).

Table 3. Index of sterility (IS) of 3-day-old quail embryo following *in ovo* exposure to genistein prior to incubation.

Treatment (µg/g egg)	Index of sterility (IS)[1]	Percentage of sterility (%)[2]
Control	0	0
Genistein (16)	0.19 ***	19.0
Genistein (24)	0.23 ***	23.0

[1] Index of sterility (IS) = $\frac{\text{PGC number in the control group – PGC number in genistein-treated group}}{\text{PGC number in the control group}}$

[2] Percentage of sterility = Index of sterility (IS) x 100

*** Statistical difference in relation to the control group (p<0.001)

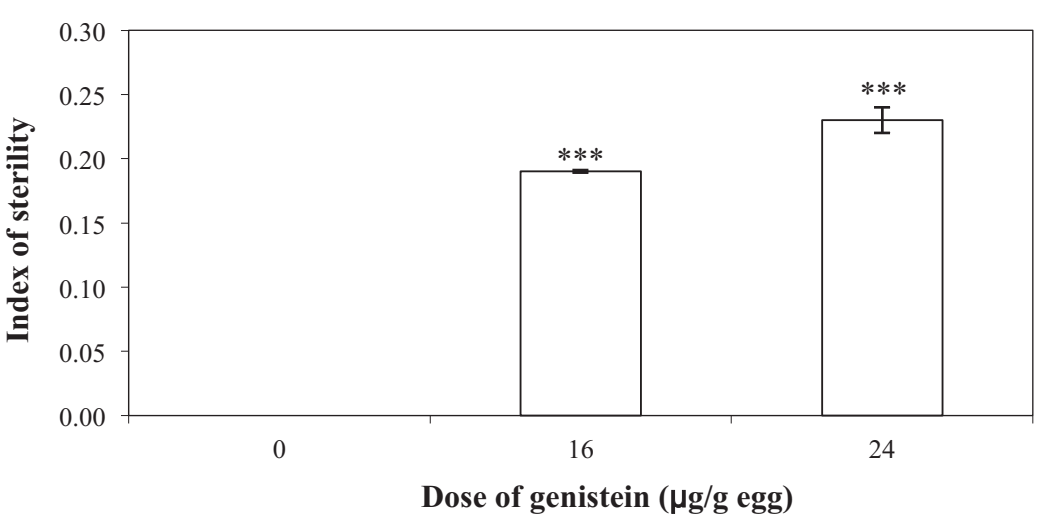

Fig. 4. Index of sterility (IS) (mean ± SEM) of the control and genistein 16 and 24 µg-treated groups; (***p<0.001).

of sterility in the control, 16 and 24 µg genistein-treated groups were 0, 19, and 23%, respectively (Table 3). The IS value of genistein-treated groups increased with the increasing doses of genistein (Fig. 4).

Discussion

Our results revealed the effective doses of genistein that cause germ cell reduction in the genital ridges of 3-day-old quail embryo. We found that the number of quail PGCs was reduced significantly by two doses of genistein. It has been reported that mitotic figures can be found in dividing PGCs during germ cell migration (Swartz and Domm 1972). Thus, genistein may inhibit germ cell division and proliferation. Presumably, genistein caused germ cell reduction by acting via TGF-β signaling pathway. Pervious studies reported that TGF-β1 and activin limited PGC proliferation in 8.5-day-old mouse embryos *in vitro* (Godin and Wylie 1991, Richards et al. 1999). Similar study on chicken reported that TGF-β2 was able to inhibit PGC proliferation in 6-day-old chicken embryo (Fujioka et al. 2004). Genistein was reported to be able to inhibit cell growth by modulating TGF-β signaling pathway (Kim et al. 1998). However, genistein can either activate or inhibit TGF-β signaling pathway depending on cell type (Sathyamoorthy et al. 1998, Richards et al. 1999). Besides TGF-β, stromal cell-derived factor 1 (SDF-1) was reported to be a chemotactic factor as it binds to CXCR4 receptor to play a role in PGC migration (Stebler et al. 2004). A possible mechanism of genistein on germ cell reduction might be acting with TGF-β rather than SDF-1, since SDF-1 plays a role in PGC survival as they colonize the genital ridges (Molyneaux et al. 2003, Stebler et al. 2004). These proposed mechanisms underlying genistein-induced germ cell reduction are considered to be direct effect. Whether genistein induces germ cell reduction by TGF-β signaling pathway in the quail embryo requires molecular studies.

The reduced germ cell number in genistein-treated embryos suggests that genistein may delay migration of PGCs along migratory routes towards the genital ridges. Accumulation of extracellular materials (ECM), fibronectin, and collagen fiber in migratory routes, including the hindgut as well as the dorsal mesentery can impede PGC migration. Possible mechanism underlying delayed germ cells migration might be TGF-β-smad signaling pathway. Genistein was reported to be able to activate such pathway via TGF-β receptor type 1 or ALK5 at the hindgut and the dorsal mesentery leading to increased ECM synthesis, fibronectin, and collagen type I (Larsson et al. 2001, Itoh et al. 2003). It has been reported that TGF-β stimulated fibronectin and collagen syntheses from chicken fibroblasts (Ignotz and Massague 1986). Chuva de Sousa Lopes et al. (2005) have found that PGC number in the genital ridges of *Alk5* -/- mice was greater than that of the wild type due to the reduced deposition of collagen type I surrounding the gut of mutant embryos. The study in non-mammalian species reported that fibronectin and collagen type IV can be found in the dorsal mesentery and the genital ridges as they helped to maintain cell adhesion between PGC and ECM during germ cell migration (Urven et al. 1989). Moreover, altered ECM in migratory routes was a key factor to play a role in PGC migration and colonization of the genital ridges (D'costa et al. 2001).

There are two possible factors, chemotactic factor and ECM, that can possibly reduce PGC number in the genital ridges of quail embryo after genistein exposure. Because we studied germ cell *in vivo*, the putative mechanism of genistein on germ cell reduction would support the study of Chuva de Sousa Lopes et al. (2005). Differences between surrounding environments of PGC, including different types of feeder cells used in *in vitro* studies (Godin and Wylie 1991, Richards et al. 1999, Fujioka et al. 2004) and ECM found in migratory routes of *in vivo* study (Chuva de Sousa Lopes et al. 2005) are likely to be discrepancy. Comparison between *in vitro* and *in vivo* studies of the effects of genistein on ECM synthesis in migratory routes of PGC during germ cell migration requires further study.

There is no study on the effects of phytoestrogen genistein on avian germ cell migration. Only one study reported that phytoestrogen daidzein promoted proliferation of cultured ovarian germ cells by estrogenic action and attenuated ROS-induced toxicity by antioxidant action in embryonic chickens (Liu et al. 2006). However, study of Liu et al. (2006) was conducted in 18 day-old-chicken embryos, whereas 3-day-old quail embryos were used in the present study. In addition, it has been reported that mRNA encoded by estrogen receptor (ER) gene and its protein started to be expressed on day 3.5 and 5.5 of incubation, respectively (Andrews et al. 1997, Smith et al. 1997). Hence, estrogen receptor protein has yet to be found in 3-day-old quail embryos, suggesting that genistein is likely to act via TGF-β signaling rather than ER-dependent pathway. The results of this study suggested that the effect of genistein on germ cell reduction in the genital ridges might be caused by its toxic effect rather than its estrogen-like effect.

In this study, we also found that both concentrations of genistein increased sterility rates in quail-treated embryos by reducing PGC number im-

planted in the genital ridges. Impairments of PGC migration and colonization towards the genital ridges resulted in sterility (Zhang et al. 2002). Busulfan (BU) (1,4-butanediol dimethanesulfonate) was reported to be a germ cell toxicant since quail germ cells were depleted by BU treatment leading to absence of oogonia and spermatogonia in 3-day-old hatching quail (Hallett and Wentworth 1991). Similar study in chicken embryo found that *in ovo* injection of BU caused germ cell cytotoxicity and 100% sterility in 6-day-old chicken embryos (Aige-Gil and Simkiss 1991). These evidences display PGC depletion-induced sterility in avian species.

Both concentrations of genistein, 16 and 24 µg/g egg used in this study were tested for predicting sterility in quail species using index of sterility (IS). We found that percentages of sterility rate of quail embryos treated with 16 and 24 µg/g egg were 19% and 23%, respectively. Similar study reported that sterility rates of chicken embryos treated with 200 and 400 µg of tamoxifen were 16.08% and 16.36%, respectively (Mohsen and Ahmed 2002). Mohsen and Ahmed (2002) have concluded that tamoxifen is a chemosterilizer in chicken embryo. Obviously, percentages of sterility rate of genistein-treated embryos in this study are higher than that of sterility rate of tamoxifen-treated embryos reported by Mohsen and Ahmed (2002). This indicates a higher potency of genistein as a chemosterilizer inducing sterility in avian species. Use of genistein to deplete endogenous germ cells for injecting germ cells isolated from other species for studying interspecific germline transmission requires more studies.

In conclusion, both concentrations of genistein (16 and 24 µg/g egg) decreased PGC number in the genital ridges significantly and increased index of sterility in 3-day-old quail embryo after *in ovo* exposure. Increasing of percentage of sterility rate after genistein exposure at embryonic stage represents a possible cause of sterility and infertility in adult birds.

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