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Review

Disruption of the developing female reproductive system by phytoestrogens: Genistein as an example

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Studies in our laboratory have shown that exposure to genistein causes deleterious effects on the developing female reproductive system. Mice treated neonatally on days 1–5 by subcutaneous injection of genistein (0.5–50 mg/kg) exhibited altered ovarian differentiation leading to multioocyte follicles (MOFs) at 2 months of age. Ovarian function and estrous cyclicity were also disrupted by neonatal exposure to genistein with increasing severity observed over time. Reduced fertility was observed in mice treated with genistein (0.5, 5, or 25 mg/kg) and infertility was observed at 50 mg/kg. Mammary gland and behavioral endpoints were also affected by neonatal genistein treatment. Further, transgenerational effects were observed; female offspring obtained from breeding genistein treated females (25 mg/kg) to control males had increased MOFs. Thus, neonatal treatment with genistein at environmentally relevant doses caused adverse consequences on female development which is manifested in adulthood. Whether adverse effects occur in human infants exposed to soy-based products such as soy infant formulas is unknown but the neonatal murine model may help address some of the current uncertainties since we have shown that many effects obtained from feeding genistin, the glycosolated form of genistein found in soy formula, are similar to those obtained from injecting genistein.

Keywords: Fertility / Ovary / Ovulation / Reproductive tract / Transgenerational effects

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1 Introduction

Exposure to estrogens during critical periods of development has numerous long-term consequences on the reproductive system of males and females of many species including rodents and humans [1–7]. The potent synthetic estrogen, diethylstilbestrol (DES) is the best studied of these estrogenic chemicals. Rodent models have been developed to replicate and predict many of the adverse effects seen in similarly exposed humans [1, 8–11]. Adverse consequences on the female reproductive system include altered estrous cyclicity, altered ovulation, subfertility/infertility, and cancer. The developmentally exposed rodent model has also been used to test many other environmental endocrine disruptors with estrogenic activity for potential adverse effects on the developing reproductive system [12–16].

Estrogens are known to regulate multiple cell functions in target tissues, including growth and differentiation *via* nuclear receptor-mediated pathways. Furthermore, aberrant temporal or overstimulation of the estrogen signaling pathway during development has long been known to result in multiple long-term abnormalities in the reproductive tract, including neoplasia [1, 8, 10, 17]. Until recently, the majority of estrogen actions were thought to be mediated *via* a single form of nuclear estrogen receptor (ER). However, the discovery of a second ER, termed ERβ (to differentiate from the original, now termed ERα), has prompted a re-evaluation of the physiology and toxicology of the estrogen signaling system.

The biological significance of two ER subtypes remains unclear but may explain the selective and divergent actions of estrogens that occur in various target tissues. Whereas ERα appears to be the predominant ER form in the müllerian-derived structures of the female reproductive tract [18–22], easily detectable levels of both ERs are present in the gonads of both sexes [23–25]. In the reproductive tract of

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Abbreviations: ER, estrogen receptor; **MOF,** multioocyte follicles

late gestational and neonatal female mice, ERβ immunoreactivity is localized to the stromal and epithelial cells of the uterus and the interstitium of the ovary, whereas ERβ immunoreactivity is limited to the ovarian granulosa cells, with little to no detectable expression in the uterus [23, 24, 26]. Other investigators have also reported the presence of ERβ in the human ovary, predominantly in the granulosa cells [27, 28]. No detectable immunoreactivity for ERβ has been found in oocytes, despite previous descriptions of detectable ERα transcripts in mouse and human oocytes [29, 30]. The divergent expression patterns of ERα and ERβ in the developing and adult reproductive tract indicate the complexity of the estrogen signaling system and suggest the two receptors likely play different physiological roles.

One group of compounds that has received significant concern in the last few years is naturally occurring phytoestrogens (estrogenic chemicals found in plants). These compounds are readily available in the diet, particularly in soy products [31–33]. The major class of phytoestrogens found in soy is isoflavones and the phytoestrogen that has received the most attention is genistein. The glycosylated form of genistein (genistin) is found in soy products and makes >65% of the isoflavone content [34]. In addition, phytoestrogens are found in high levels in soy products known to be present in the human diet and are likely substantial components of vegetarian diets; therefore, human fetuses may be exposed to these compounds during *in utero* development as well as infancy through lactation [35]. Furthermore, infants are exposed to high levels phytoestrogens through soy-based infant formulas and soy-based foods marketed for children [35, 36]. Infants on soy-based formulas have high circulating levels of genistein (1–5 μM) indicating that this compound is readily absorbed from the gastrointestinal tract [34]. This has been confirmed in a recent study showing glucuronidated metabolites of genistein as well as other genistein metabolites in the urine of babies on soy-based infant formulas [37]. It is estimated that adults consuming a diet modest in soy isoflavones are exposed to approximately 1 mg/kg/day, whereas, infants consuming a diet of soy-based formulas are exposed to 6–11 mg/kg/day which is much greater than typical adult exposures [34]. Soybeans also have an extremely variable isoflavone content depending on variety and environmental conditions such as growing season and location [38]; the USDA reports highly variable amounts of genistein in soy products (USDA (1999) USDA-Iowa State University Database on the Isoflavone Content of Foods., United States Department of Agriculture) which could lead to even higher levels of genistein exposure than expected in particular lots of soy-based products.

Another layer of complexity of estrogen signaling has been recently realized with differential binding affinities between the two receptors. For example, 17β-estradiol (E_2) exhibits a similar *in vitro* binding affinity for both ERs, while several synthetic and naturally occurring xenoestrogens exhibit a binding preference for one of the two receptors although this binding is not typically as strong as E_2 [39–42]. For example, it takes 20 times as much of the phytoestrogen genistein to elicit similar binding affinity to ERα as E_2 [40]. In addition, some chemicals have a higher binding affinity to either ERα or ERβ; genistein exhibits preferential binding to ERβ over ERα. In fact, genistein binds to ERβ 20–80 times better than to ERα [40–42]. Since phytoestrogens have been shown to bind to both ERα and ERβ and to have estrogenic activity *in vitro* as well as *in vivo* assays, concern has risen over human exposure to these estrogenic chemicals.

Adverse effects of phytoestrogens on fertility have been known for years since sheep grazing on red clover exhibit infertility; this is thought to be due to estrogenic substances found in clover [4]. Another study showed that captive cheetahs exhibited reduced fertility while on soy-based diets containing very high levels of phytoestrogens; replacement of soy protein with chicken protein restored fertility [43]. These studies provide examples that phytoestrogens exist in our environment at concentrations high enough to be active and have adverse effects.

2 Phytoestrogen exposure

Over the last few years, public and scientific interest in phytoestrogens, like genistein, has increased because of their proposed beneficial effects. Soy supplements and health foods containing concentrated amounts of phytoestrogens have been marketed for children. Diets for children have also been supplemented with soy because of its cost effectiveness. However, there is conflicting scientific data on exposure to genistein especially during development suggesting some beneficial effects but also adverse effects depending on the timing of exposure, dose level, and endpoints examined. For example, two studies using rats report that prepubertal exposure to genistein prevents carcinogen-induced mammary gland cancer [44, 45] while another study shows an increase in mammary gland cancer if the developmental window of exposure is shifted to prenatal life [46]. These studies generally agree that the development of the mammary gland is altered by either prenatal or prepubertal exposure to genistein. A recent study from our laboratory using outbred CD-1 mice supports this idea since mammary gland expansion and ramification are altered following neonatal genistein treatment [47]. Studies from our laboratory also showed that neonatal exposure to genistein leads to the induction of uterine adenocarcinoma in mice later in life, similar to that previously described following neonatal DES exposure [14]. Unfortunately, there is sparse human data with dietary exposures during development to soy products containing phytoestrogens. One group reported improved cholesterol synthesis rates of infants consuming soy-based formulas [48] suggesting beneficial

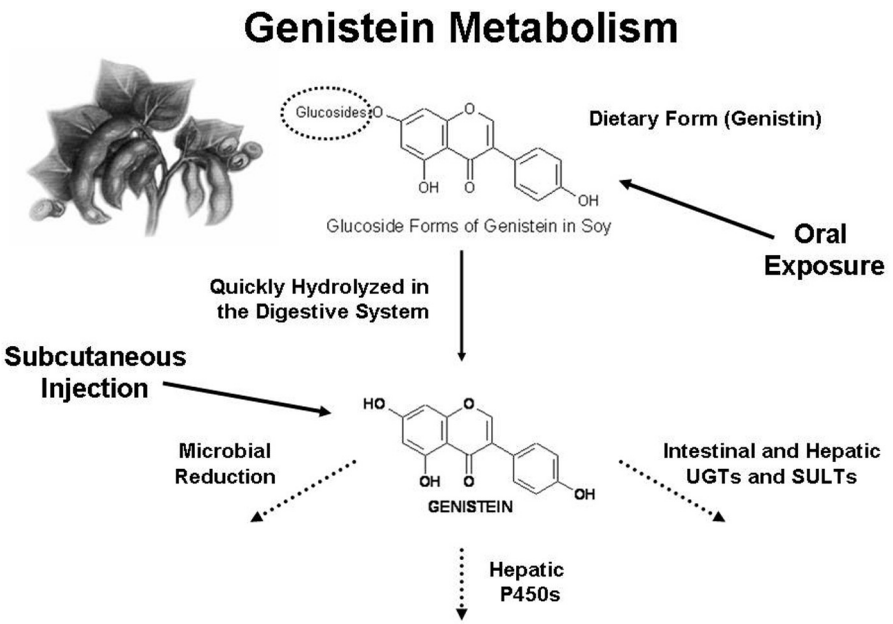

Figure 1. Structures and hydrolysis of genistin to genistein. The form of genistein found in the diet including soy-based infant formulas is the glycosylated form, genistin. This form is quickly hydrolyzed to the aglycone form, genistein. The form and routes used in the studies included in this manuscript are indicated on the diagram.

effects. On the other hand, suggestions of adverse effects of genistein can be seen in an epidemiology study which shows that pregnant women consuming a vegetarian diet during pregnancy give birth to male offspring with an increased incidence of hypospadias; this may be related to high maternal levels of soy isoflavones [49]. Further, an epidemiology study of health outcomes in young adults who were fed soy-based formulas as infants reported an increase in use of allergy medicines in both men and women, and longer menstrual bleeding and more discomfort during the menstrual cycle in women than their cow-based formula fed counterparts [50, 51].

3 Comparison of dose and route of exposure of genistin or genistein

The doses of genistein used in our laboratory studies (0.5–50 mg/kg) designed to investigate potential effects during development, were chosen to span the range of human exposure levels during development, from fetuses and infants during pregnancy and lactation in vegetarian mothers to babies on soy-based infant formulas. Human exposure to genistein is predominantly from soy products in the diet including soy-based infant formulas. While the form of genistein found in soy-based infant formulas and other dietary sources of genistein is predominantly the glycosylated form (genistin), recent studies have shown that genistin is quickly hydrolyzed to the unconjugated active form, genistein in the digestive system (Fig. 1). This form is readily absorbed in infants since glucuronidated metabolites and other genistein metabolites were found in their urine [37]. Although human exposure to genistein is primarily through the diet, it is not practical to expose neonatal mice through the diet since their sole source of nutrition is from the mother's milk; previous studies have shown that exposure of the mother to genistein in the diet has limited lactational transfer to the pups, thus not allowing much of the compound to actually get to the pup [52, 53]. For example, mice exposed to genistein at a dose of 16 mg/kg orally during lactation have a serum circulating level of genistein of 1.8 (g/mL but the level found in the milk was only 0.04 (g/mL which is 45 times less than circulating levels in the mother.

Previous studies in our laboratory were carried out using subcutaneous injections of genistein at doses of 0.5–50 mg/kg in corn oil on days 1–5. The relevance of the subcutaneous route of exposure to genistein has been recently met with controversy since human infants are primarily exposed orally to genistin. Therefore, a recent study was conducted in our laboratory to directly compare the estrogenic activity of orally administered genistin with a subcutaneous injection of genistein (Fig. 1; Table 1). Female CD-1 mouse pups were left untreated or treated on days 2–5 by subcutaneous injection of corn oil or genistein in corn oil at doses of 0, 12.5, 20, and 25 mg/kg or by orally pipeting corn oil or genistin in corn oil at doses of 0, 5, and 25 mg/kg (8 mice *per* group). The dose of genistin is based on the actual amount of genistein in the dose, not the portion of the molecule that is the sugar group (37.7%). Mice were sacrificed on day 5 and body weights and uterine wet weights

Table 1. Uterine wet weight response at 5 days of age following subcutaneous injection of genistein or oral exposure to genistin

Treatment (mg/kg/day)	Uterine wet weight (mg)
Subcutaneous injection	
Corn oil	3.51 ± 0.35
Genistein-12.5	3.54 ± 0.15
Genistein-20	4.56 ± 0.11[a)]
Genistein-25	6.41 ± 0.46[a)]
Oral Exposure	
Corn oil	3.20 ± 0.24
Genistin-5	3.71 ± 0.10
Genistin-25	4.89 ± 0.22[a)]

Female pups were treated on neonatal days 2–5 either by subcutaneous injection with genistein in corn oil (20 μL *per* pup) or by orally administering genistin in corn oil by pipet (pups received 2.5 μL *per* gram). Uterine tissues were collected and weighed 4 h following the last treatment at 5 days of age (data presented in this table was taken from Jefferson *et al. 2007*.

a) Significance at $p < 0.05$ using Dunnett's test.

were taken. The data from this experiment are shown in Table 1 [54]. Data were analyzed by ANOVA followed by Tukey's using JMP (SAS, Cary, NC) and $p < 0.05$ was considered significant. There was no significant difference in body weight between any of the treatment groups suggesting that both oral and subcutaneous treatment methods had no apparent adverse effect on growth of the mice (data not shown). There was a significant increase in uterine wet weight following subcutaneous injection of genistein at 20 and 25 mg/kg as well as an oral exposure to genistin at a dose of 25 mg/kg. While the oral exposure to genistin 25 mg/kg did not increase uterine wet weight to the level achieved by the subcutaneous injection of genistein 25 mg/kg, the increase was comparable to the subcutaneous injection of genistein 20 mg/kg. Since the vast majority of the subcutaneously injected genistein enters the circulation, these data suggest that approximately 80% of the oral dose is absorbed into the circulation causing a similar biological response. This experiment demonstrates that a subcutaneous injection of genistein is comparable to an oral exposure to genistin.

Another study from our laboratory supports this finding since neonatal female mice treated by subcutaneous injection with genistein 50 mg/kg have a serum circulating level of genistein of 6.8 ± 1.4 μM [29]. This is comparable to human infants on soy-based formulas (oral exposure to genistin, ~4–7 mg/kg) with circulating levels ranging from 1 to 5 μM [34, 55]. Neonatal rats treated by subcutaneous injection at a dose of 40 mg/kg/day also showed similar circulating levels of genistein [53]. Another point of interest in our pharmacokinetic studies is the high circulating levels of the aglycone form of genistein (~2 μM). This level was approximately ten-fold higher than that found in an adult rat following similar exposure [56] suggesting the neonate is exposed to higher levels of the estrogenically activity form of genistein compared to an adult; the aglycone form has been shown to exhibit strong ER binding activity [57]. The higher fraction of aglycone form of genistein has also been shown in rats treated perinatally with genistein [58] supporting the idea that glucuronidation of genistein is lower during the neonatal period compared to adulthood. This is most likely due to lower UDP glucuronosyltransferase (UGT) activities in neonatal mice [59, 60]. Many human UGT isoforms exhibit a similar pattern of expression as seen in rodent development with lower activity in the neonatal period [61, 62]. Although the serum circulating levels of the aglycone form of genistein in the neonatal human infant is not known, an elevated fraction of the aglycone form compared to adults is likely. These data suggest that circulating levels of genistein following our treatment method is comparable to what human infants are exposed.

Another important consideration is the diet our mice are fed during the time of treatment. We have previously discussed the role of diet used in our studies since the NIH-31 lab chow our mice are fed contains low levels of phytoestrogens. This diet contains approximately 98 μg/g of genistein and daidzein which is about 16.7 mg/kg/day for a 30 g mouse [63]. It has been shown that mice exposed orally to genistein at a dose of 16 mg/kg through lactation have a serum circulating level of 1.8 μg/mL of genistein, about 45 times less than that found in milk (0.04 μg/mL). Therefore, the amount of genistein that is consumed by the mother from the diet would result in very low exposure to the pups [53] and is far below the treatment levels used in our studies.

4 Altered ovarian differentiation and development

Studies from our laboratory have shown adverse effects on the developing mouse ovary following neonatal exposure to genistein [64, 65]. Mice treated neonatally by subcutaneous injection of genistein at doses of 0.5, 5, and 50 mg/kg dissolved in corn oil showed a dose-dependent increase in the number of mice with multioocyte follicles (MOFs) in the ovary prior to puberty with most of the mice in the highest treatment group having MOFs [65]. Since genistein has properties in addition to its estrogenic activity such as tyrosine kinase inhibitory activity, several experiments were carried out to determine the mechanism of action responsible for the formation of MOFs. The tyrosine kinase inhibitor, lavendustin was used to rule out genistein's tyrosine kinase inhibitory activity as a cause of MOFs and two transgenic mouse models lacking either ERα or ERβ were used to determine which receptor subtype was involved if genistein's estrogenic activity was responsible. Since mice treated with lavendustin did not develop MOFs, the tyrosine

kinase inhibition property of genistein was determined not to be responsible for this effect. Mice lacking ERα still developed MOFs when treated neonatally with genistein while mice lacking ERβ did not, suggesting that genistein interacts with ERβ to cause MOFs.

The formation of MOFs was further characterized by observing ovarian development during the time of neonatal treatment [64]. At birth, mice have large oocyte clusters or nests; these nests dissociate into individual oocytes surrounded by granulosa cells during the first week of life [66]. Treatment with genistein at a dose of 50 mg/kg on days 1–5 inhibits this differentiation process leaving the oocytes together in nests and still attached to each other by intercellular bridges. Our study also showed a higher percentage of unassembled oocytes (those not completely surrounded with granulosa cells) further supporting the limited differentiation of the ovary in genistein treated mice. Therefore, the presence of MOFs later in life is an indication that developmental exposure to genistein permanently altered ovarian differentiation.

MOFs have also been found in rats treated during development with genistein suggesting that this occurrence is not limited to mice [15]. Further, the presence of MOFs has also been noted in humans supporting the idea that this effect can be seen in humans although the cause in humans is still not known. Several other estrogenic compounds have been found to cause MOFs if exposure occurs during development including 17β-estradiol, DES, and Bisphenol A [12, 67–69] supporting the idea that estrogenic substances alter ovarian differentiation. It has also been shown that oocytes derived from MOFs have reduced quality since *in vitro* fertilization of these oocytes is markedly reduced compared to single oocyte follicles [67]. This suggests that an increased number of MOFs may be related to decreased fertility later in life.

5 Altered ovarian function

Numerous studies have shown altered ovarian function following developmental exposure to genistein. A recent study from our laboratory showed the complete lack of corpora lutea (CL) and anovulation at 4 months of age following neonatal exposure to genistein at 50 mg/kg on days 1–5 indicating ovarian function was disrupted. Doses of genistein lower than 50 mg/kg showed enhanced ovulation rates as evidenced by increased numbers of oocytes ovulated following exogenous gonadotropins at 26 days of age [65], as well as, increased numbers of CLs at 4 months of age [70]. Whether these effects are due to a direct effect on the ovary or an indirect effect on the hypothalamic-pituitary-gonadal (HPG) axis is not fully elucidated, but an indirect mechanism is most likely. First, exogenous gonadotropins restored ovarian function in mice treated with the high dose of genistein as evidenced by similar numbers of oocytes ovulated compared to controls although the quality of these oocytes is not known. Second, another study showed that neonatal exposure of rats to genistein altered pituitary responsiveness to gonadotropin releasing hormone (GnRH) [71]. Higher neonatal doses of genistein were associated with decreased pituitary responsiveness by producing less luteinizing hormone (LH) in response to GnRH stimulation [71]. The LH surge is necessary for ovulation so lower levels of LH may explain the lack of ovulation seen in the high dose treated mice. Interestingly, in that same study rats treated with lower doses of genistein (0.01 mg/kg) were hyper-responsive to GnRH stimulation leading to enhanced ovulation rates similar to data from our laboratory using younger mice treated neonatally with low doses of genistein and again in older mice with increased CLs at 4 months of age [65, 70, 71]. Together, these data suggest that the HPG axis is disrupted following developmental exposure to genistein.

6 Altered estrous cyclicity

Several studies have shown altered female reproductive function following developmental exposure to genistein. In fact, several reports showed alterations in estrous cyclicity in rodents following prenatal or neonatal treatment with genistein. As an example, a study done in our laboratory showed that mice treated neonatally with genistein spend significantly longer periods of time in the estrous phase of the cycle; this abnormality increases in severity with increasing dose as well as increasing age. Over half of the mice treated with genistein 50 mg/kg (5/8) and one mouse treated with genistein 5 mg/kg (1/8) exhibited signs of persistent estrus by 6 months of age, suggesting these mice were not cycling. Other investigators have shown similar estrous cycle alterations in experimental animal models including a study by Nikaido *et al.* [13] showing several environmental estrogens given during prenatal development including genistein, resveratrol, zearalenone, and bisphenol A caused extended estrous cycles when the animals became adults. Data from another laboratory showed similar alterations in estrous cycles of rats following neonatal exposure to genistein with prolonged periods in estrus [72]. This is similar to mice exposed perinatally to DES [10, 73] further supporting the idea that developmental exposure to estrogens causes disruptions in estrous cyclicity.

7 Altered reproductive function

Knowing that developmental exposure to genistein caused adverse developmental effects on the reproductive tract and ovaries, the fertility of these mice was examined. Female mice treated with genistein at doses of 0.5 and 5 mg/kg

showed no difference in the numbers of mice delivering live pups compared to controls at 2 and 4 months of age. However, by 6 months of age, a reduction in the percentage of mice delivering live pups in both treatment groups compared to controls (control, 100%; genistein 0.5 mg/kg, 60%; genistein 5 mg/kg, 40%) was seen as well as a reduction in the number of live pups in the mice that delivered; these findings suggest early reproductive senescence [70]. In a group of mice treated neonatally with genistein (25 mg/kg), there were signs of reduced fertility at 2 months of age with only four out of eight (50%) plug positive mice delivering live pups. Female mice treated neonatally with genistein (50 mg/kg) did not deliver any live pups at 2 months of age (0/8 mice) suggesting mice exposed developmentally to this dose are infertile. A study from another laboratory supports our findings since rats treated with genistein (100 mg/kg) also showed disruption of fertility [15].

Since mice treated with genistein 50 mg/kg did not deliver live pups, we conducted additional studies to characterize the source of infertility. Again, mice were treated neonatally with genistein (50 mg/kg) and then bred to control males at 2 months of age, and the uterus collected at 6, 8, and 10 days after mating [70]. Less than half (47%) of the genistein treated mice showed signs of pregnancy following vaginal plug positive (20/43) compared to 93% of the controls (40/43). In addition to low numbers of pregnancies, the females that were pregnant had smaller and fewer implantation sites (9.0 ± 0.8 sites *per* uterus) compared to controls (14.0 ± 0.4 sites *per* uterus). There were also visible reabsorption sites in two of the genistein treated mice.

One possible explanation for these implantation defects is that the environment of the uterus or the hormonal milieu is not suitable for implantation. However, serum hormones measured during pregnancy did not reveal any deficiencies in hormones needed to maintain pregnancy such as progesterone and estradiol suggesting that these are not a likely cause of the implantation problems. Previous studies have shown that mice treated neonatally with high doses of DES lack the ability to respond to estrogen stimulation [74] suggesting that the developmentally estrogen-treated uterus may not be able to respond appropriately to the hormones of pregnancy. A recent study confirmed this altered uterine response in the genistein treated mice [54]. When neonatally genistein treated mice were challenged prior to puberty with estrogen, the uterine response was attenuated. There was a seven to eight-fold increase in uterine wet weight in control mice stimulated with estrogen similar to previously reported data [75], however, genistein treated mice did not respond to the same magnitude with only a five-fold increase in uterine wet weight ($p < 0.05$ by Fisher's exact test, compared to stimulated controls). Although the uterus of the genistein treated mice was capable of responding to estrogen, the response was dampened and may contribute to reduced ability to maintain pregnancy. Further studies to determine decidualization response in these mice as well as embryo transplantation of control blastocyst into genistein treated mice will help determine if genistein treated mice are fully capable of supporting pregnancy.

Another possibility for early pregnancy loss is that the oocyte itself is of poor quality. Additional evidence that neonatal genistein may lead to poor oocyte quality can be found in the study reported by Iguchi *et al.* [67] showing that oocytes collected from MOFs following DES treatment were much less "fertilizable" than oocytes from single oocyte follicles. Since there is a high incidence of MOFs in neonatal genistein treated mice, fewer oocytes may be competent for fertilization, or if fertilized, not capable of normal development. Culture of fertilized oocytes will help to determine if the fertilization rate or the oocyte quality is affected by genistein exposure during development.

8 Mammary gland

Several laboratory studies have shown that exposure to genistein perinatally causes alterations in mammary gland development as well as carcinogenic potential later in life and the timing of exposure, route, and dose of genistein appear to be important factors in the overall effect on the mammary gland [44–46, 76, 77]. For example, one study exposing rats to genistein (~1–10 mg/kg/day) through the diet from gestation day 0 through postnatal day 21 (weaning) showed resistance to dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumors later in life [76]. There was also reduced terminal end bud (TEB) development as well as immature terminal ductal structures that remained until adulthood. TEBs are responsive to local and systemic hormones that drive ductal morphogenesis [78]. The cap cells of the TEBs are normally present during neonatal and prepubertal–pubertal growth periods of the mouse, but they are thought to play a role in mammary gland carcinogenesis. Another study from the same laboratory showed that exposure to high levels of genistein (~500 mg/kg/day) in the diet on prepubertal days 16, 18, and 20 reduced mammary tumors following DMBA treatment in adulthood [45, 77]. Another laboratory showed similar protective effects when rats were exposed on postnatal days 7–20 to genistein ~1 mg/kg [46]. These protective effects of genistein were attributed to advanced mammary gland differentiation and less TEBs thus making the tissue less susceptible to carcinogens. However, premature development and differentiation of the mammary gland is not recommended as prevention for breast cancer. In contrast, a recent report from Hilakivi-Clarke's [44] laboratory showed that rats treated prenatally with lower doses of genistein (~0.1, 0.5, or 1.5 mg/kg/day) on gestation days 15–20 had an increased susceptibility of mammary gland tumors induced by DMBA. Therefore, the effects of genistein on the mammary gland are complicated because of dose-dependency and timing of

Table 2. Effects of genistein on mammary gland morphogenesis (details of measurements of endpoints can be found in Padilla-Banks *et al.* [47])

Treatment (mg/kg/day)	TEB (mm)	Elongation (mm)	Number of branching points	Area (pixels)
5 wk				
Control	13 ± 1.7	1.60 ± 0.24	75.75 ± 3.97	34 751 ± 5900
Gen-0.5	11 ± 0.8	1.40 ± 0.22	52.75 ± 8.01	31 231 ± 3323
Gen-5	11 ± 1.6	1.72 ± 0.44	41.67 ± 7.42[a)]	44 822 ± 6470
Gen-50	8 ± 0.9[a)]	0.64 ± 0.19	36.50 ± 4.86[a)]	28 827 ± 3947
6 wk				
Control	14 ± 1.3	3.15 ± 0.20	75.50 ± 9.92	54 477 ± 8833
Gen-0.5	11 ± 1.0	4.46 ± 0.32[a)]	92.00 ± 4.10	58 572 ± 2923
Gen-5	9 ± 1.4[a)]	3.63 ± 0.31	85.33 ± 11.89	56 035 ± 9727
Gen-50	9 ± 0.7[a)]	2.94 ± 0.25	69.00 ± 4.06	41 228 ± 5617

a) Significance at $p < 0.05$ using Dunnett's test.

exposure. Certainly, if any potential benefits of genistein on the mammary gland are observed, they have to be considered in combination with its adverse effects on reproductive tract tissues and the ovary before determining an appropriate risk/benefit analysis for genistein treatment.

A recent study from our laboratory examined the effects of neonatal exposure to genistein (0.5, 5, and 50 mg/kg) on the development of the mammary gland [79]. Mammary glands (number 4) were removed, fixed in Carnoy's fixative, and stained with carmine for whole mount analysis at 4, 5, and 6 wk of age. Following a procedure described by Ball [78], the number of TEBs in the peripheral leading edge of the differentiating ductal mass was counted and the furthest length of ductal expansion was measured. Mammary glands examined prior to puberty (4 wk of age) showed no differences between control and genistein treated mice. Data taken from Padilla-Banks *et al.* [47] is summarized in Table 2. There was an increase in TEBs from 5 to 6 wk of age in control and genistein treated mice indicating active ductal morphogenesis, however, mice treated with the lowest dose of genistein 0.5 mg/kg had higher overall numbers of TEBs than controls as well as increased ductal expansion. Strikingly, the genistein 5 mg/kg/day dose group showed a different pattern of mammary gland development with more TEBs present at 5 wk of age but less TEBs at 6 wk of age suggesting an advance in differentiation at this dose; ductal expansion was increased at both time points compared to controls. In addition, measurements of hormone receptor levels showed increased levels of progesterone receptor protein and ERβ mRNA in genistein 0.5 mg/kg treated mice compared to controls; ERα expression was decreased following all doses of genistein compared to controls [47]. Overall, mice treated with the two lower doses of genistein showed advanced mammary gland morphogenesis. In contrast, mice treated with genistein 50 mg/kg showed a similar number of TEBs as controls, but mammary gland morphology was abnormal with smaller ducts, less ductal branching, and reduced expansion through the fat pad. This stunted development in the highest dose was evident throughout the life of the animal, remaining apparent even at 18 months of age.

While the development of the mammary gland was altered during the time of puberty in the genistein 0.5 and 5 mg/kg treatment groups, the ultimate function of the mammary gland appeared to be intact since mice from these treatment groups at 2 months of age were able to deliver live pus, lactate, and maintain pup growth [47]. This would be expected since the morphological changes observed in the low dose of genistein treatment would have most likely led to expansion of the mammary gland through the fat pad at a faster rate but not necessarily affecting the function of the mammary gland. The lactational function of the mammary gland in the highest genistein dose could not be assessed since these mice are infertile.

Examination of mammary gland morphology at a later time point (9 months) revealed alterations in both the genistein 5 and 50 treatment groups [47]. There was less branching and less alveolar development compared to controls. In addition, some of the mice in the genistein 5 and 50 groups exhibited the presence of dilated beaded ducts lined with hyperplastic ductal epithelium which were not seen in controls at any age examined. The presence of these structures have been previously reported following prenatal exposure to the xenoestrogen, zearlenone supporting the idea that developmental exposure to an estrogen caused this effect [13]. Whether the lack of tertiary differentiation later in life is due to a direct effect on the mammary gland or due to lack of cyclicity in these mice, in particular lower levels of progesterone, is not known.

9 Maternal behavior

Since developmental exposure to genistein has been well documented to have effects on brain structure and behavior [71, 72, 80–86], we examined the effects specifically on maternal behavior following neonatal exposure to genistein at doses of 0.5 and 5 mg/kg. Mice treated with genistein

Table 3. Time observations of maternal behavior following neonatal treatment with genistein

Treatment (mg/kg)	Time (min/sec)		
	Nesting	Licking	Crouching
Control	2:29	2:44	12:20
Genistein-0.5	1:07	3:03	18:27
Genistein-5	1:48	0:47	12:11

Mice were treated on neonatal days 1–5 by subcutaneous injection of genistein (0.5 and 5 mg/kg). Mice treated with genistein 50 mg/kg was not tested because they are infertile. At 2 months of age, they were bred to control males and allowed to deliver their pups. On the day of birth (day 1), mothers were observed for the length of time spent nesting, licking, and crouching over their pups according to Boufares *et al.* 1993 [86]. The time presented in the table is the average time for each treatment group ($n = 5$ mothers with pups *per* group).

50 mg/kg were not assessed since they were infertile. Female mice were bred to control males and allowed to deliver their pups. On postnatal day 1 (day of birth), maternal behavior was observed as defined by Boufares *et al.* 1993 [86]. Timing for nesting, licking, and crouching were recorded. A summary table of this data is shown in Table 3. The nesting behavior of the genistein 0.5 and 5 mg/kg group was reduced compared to control females. Mice treated with genistein 0.5 mg/kg spent a similar amount of time licking their pups as did the control females while the mice treated with genistein 5 mg/kg spent less time licking. The amount of time crouching was similar for the control and genistein 5 mg/kg females but mice treated with genistein 0.5 mg/kg spent longer amounts of time crouching over their pups. In addition, while the control females licked their pups in the nesting area, the genistein 0.5 mg/kg females did the same but without collecting their pups to a centralized nesting area.

We also observed pup retrieval in the same group of mice on postnatal day 1. The mother was removed from the cage and the pups were dispersed away from the nest. The mother was replaced and time was recorded for the first pup retrieved and placed back into the nest as well as the total time for retrieval of all pups back to the nest. These data can be seen in Fig. 2. All of the control mice retrieved their pups in less than 240 s (4.0 min) while several mice in the two treatment groups took considerably longer to retrieve all of their pups. One mouse in the genistein 0.5 mg/kg group took over 15 min (approximately four times as long) to retrieve all pups and two other mice in that group took ~500 s (8.33 min; approximately two times as long). Two mice in the genistein 5 mg/kg group took ~300 s (5 min). Together these data suggest that maternal behavior is altered following neonatal exposure to low, environmentally relevant doses of genistein. Further studies are needed to determine the long-term effects as well as the mechanism involved in this effect.

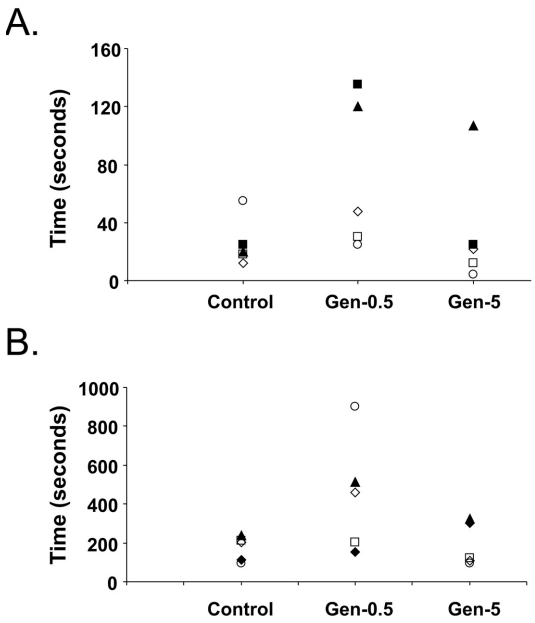

Figure 2. Maternal behavior as determined by pup retrieval following neonatal exposure to genistein. Mice were treated on days 1–5 with genistein 0.5 (Gen-0.5) and genistein 5 mg/kg (Gen-5), weaned at 22 days and bred to control males at 2 months of age. On the day of delivery, mothers were removed from their cage, pups dispersed, and mothers returned to the cage according to Boufares *et al.* 1993 [86] ($n = 5$ mothers *per* group). The time to retrieve the first pup is shown in Panel A and the time to retrieve the entire litter is shown in Panel B (each point on both graphs represents an individual mother). Statistical analysis of the treatment groups did not show any differences from controls using ANOVA followed by Dunnett's test at $p < 0.05$ but that is most likely due to the small sample size.

10 Hormonal carcinogenesis

We have previously shown that the developing reproductive tract is exquisitely sensitive to estrogen exposure and that these exposures lead to lesions of the reproductive tract including uterine adenocarcinoma. Previous work from our laboratory showed that the incidence of these lesions following neonatal DES exposure correlates well with estrogenic activity at 5 days of age as determined by uterine wet weight gain [8, 87]. Increasing doses of DES produced increased uterine wet weight at 5 days of age and a correlating increased incidence of uterine cancer at 18 months of age [87]. In addition, equal estrogenic doses of DES (0.001 mg/kg) and genistein (50 mg/kg) caused a similar incidence of uterine cancer at 18 months of age [DES, 4/13 (31%); genistein, 6/17 (35%)] while no uterine cancer was observed in control mice at the same age [14]. We have also shown that many chemicals with estrogenic activity, if given during neonatal life, cause uterine cancer similar to DES including 17β-estradiol, ethynylestradiol, tamoxifen,

2-OH-estradiol, 4-OH-estradiol, hexestrol, TF-DES, and nonylphenol and the incidence is correlated with the estrogenic activity of these compounds [87]. Oral exposure to genistin (25 mg/kg) caused increased uterine wet weight at 5 days of age suggesting that this chemical and route of exposure would most likely cause uterine cancer similar to a subcutaneous injection of genistein. Future studies in our laboratory will examine this possibility.

11 Transgenerational effects

Recent interest has developed over potential transmission of adverse phenotypes to subsequent generations. Previous work from our laboratory has shown that the deleterious effects of developmental exposure to DES on the male and female reproductive tract are transmitted to subsequent generations [88, 89]. Mice were treated with DES prenatally (2.5, 5, or 10 μg/kg/day) on days 9–16 of gestation, or neonatally (1 μg/kg/day) on days 1–5; these are the highest doses that did not drastically interfere with fertility later in life. When female mice (F1) reached sexual maturity, they were bred to control untreated males. Female and male offsprings (DES-lineage or F2) from these matings were aged to 17–24 months and examined for reproductive tract abnormalities. An increased incidence of uterine adenocarcinoma was seen in DES-lineage females [88]. Further, an increased incidence of proliferative lesions of the rete testis (an estrogen target tissue in the male) and tumors of the reproductive tract was observed in DES-lineage males [89]. The incidence was lower in DES descendants than in their parents; uterine tumor incidence in DES F1 at 18 months was 31% at the neonatal dose of 1 μg/kg whereas it was 11% in their DES descendants. One potential explanation is that alterations occurred in germ cells and were passed to subsequent generations. Interestingly, multigenerational effects of DES have been reported by other laboratories and some of these report transmissions through the paternal lineage [90, 91].

The mechanism(s) involved in transgenerational events are unknown but recent studies have offered clues. Altered methylation patterns in several genes expressed in the uterus have been shown to be permanently dysregulated following developmental DES treatment [92, 93]. The estrogen-responsive proteins lactoferrin (LF) and c-fos were permanently upregulated in the uterus following developmental exposure to DES and the promoter region of these genes was shown to be hypomethylated [92, 93]. Although the consequences of these types of alterations are unclear, studies suggested that methylation patterns can be passed to subsequent generations [94]. A recent report supports this theory since prenatal exposure to other environmental endocrine disruptors, vinclozolin, and methoxychlor, caused adverse effects on testes morphology and male fertility, and these effects were transmitted to subsequent generations [95]. In addition, this report showed that these two chemicals caused epigenetic alterations in the DNA, specifically hyper- and hypomethylation and that these alterations were also observed in subsequent generations [95]. Since response of estrogen regulated genes is set during development, altered hormone response may be transmitted to subsequent generations.

A recent study was conducted in our laboratory to determine if developmental exposure to genistein could lead to adverse effects in subsequent generations. Female mice were treated by subcutaneous injection on days 1–5 with genistein at a dose of 25 mg/kg (F1); this is the highest dose that delivered live pups. Ovaries were collected from one group of mice at 19 days of age for determining the incidence of MOFs in the F1 generation. Another group of F1 mice was allowed to age to 2 months and was bred to control males to generate second generation females (F2). Ovaries were collected at 19 days of age from these F2 female mice and also evaluated for the presence of MOFs. There is a high incidence of MOFs in F1 mice treated neonatally with genistein 25 mg/kg (7/8; 93%) compared to control F1 mice in this study (0/8; 0%). F2 genistein female mice also had MOFs (7/19; 37%) although the incidence was not as high as the F1 genistein treated mice; no control F2 mice had MOFs (0/12; 0%). This finding further supports the idea that developmental exposure to estrogens causes adverse effects and that these effects are transmitted to subsequent generations. Transmission of genistein induced MOFs to subsequent generations provides an opportunity to study the mechanism(s) by which these effects are passed on to the next generation. Since the effect is seen by 19 days of age instead of having to wait many months for cancer to develop and since the incidence is fairly high in both the F1 and F2, the mechanism(s) involved in this transmission can be more easily studied. Future studies in our laboratory are underway to more closely examine the potential mechanisms for transmission of these effects to subsequent generations.

12 Conclusions

The data presented herein demonstrate the ability of genistein to disrupt female development and reproductive function at environmentally relevant doses. In addition, we have shown that a subcutaneous injection of genistein is comparable to oral exposure to genistin since both routes and compounds cause biological estrogenic activity. Our laboratory and others have shown altered estrous cyclicity, altered ovarian function, and subfertility/infertility in female mice exposed perinatally to genistein. Further, we have shown altered ovarian differentiation following neonatal exposure to genistein. Studies using other phytoestrogens including coumestrol [96, 97], daidzein [34, 72], and red clover [98, 99] have also demonstrated disruptions in reproduction

and/or reproductive endpoints supporting the concept that phytoestrogens, although weaker than other more potent estrogens such as DES or 17β-estradiol, can cause adverse effects on the developing reproductive tract. Some of these effects may not be apparent until later in life such as irregular estrous cyclicity, early reproductive senescence, and infertility and therefore would not be detected during the time of exposure. These alterations in reproduction and abnormal ovarian differentiation in experimental animal models combined with prior studies describing an increased incidence of uterine neoplasia following developmental exposure to genistein [14] suggest additional studies with the human population exposed to elevated levels of phytoestrogens during development is warranted.

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13 References

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