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Circulating Levels of Genistein in the Neonate, Apart from Dose and Route, Predict Future Adverse Female Reproductive Outcomes

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Abstract

Developmental exposure to estrogenic compounds can disrupt sexual differentiation and adult reproductive function in many animals including humans. Phytoestrogens (plant estrogens) in the diet comprise a significant source of estrogenic exposure to humans, particularly in infants who are fed soy-based infant formula. Animal models have been developed to test the effects of phytoestrogen exposure on the developing fetus and neonate. Here we review studies quantifying the amount of phytoestrogen exposure in human adults and infants and discuss the few available epidemiological studies that have addressed long-term consequences of developmental phytoestrogen exposure. We then describe in detail rodent models of developmental exposure to the most prevalent phytoestrogen in soy products, genistein, and the effects of this exposure on female reproductive function. These models have used various dosing strategies to mimic the phytoestrogen levels in human populations. Serum circulating levels of genistein following each of the models and their correlation to reproductive outcomes are also discussed. Taken together, the studies clearly demonstrate that environmentally relevant doses of genistein have significant negative impacts on ovarian differentiation, estrous cyclicity, and fertility in the rodent model. Additional studies of reproductive function in human populations exposed to high levels of phytoestrogens during development are warranted.

Keywords

Ovary; Oviduct; Uterus; Fertility

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 [1-8]. The most well studied of these estrogenic chemicals is the potent synthetic estrogen, diethylstilbestrol (DES). Rodent models have been developed to replicate and predict many of the adverse effects of DES seen in similarly exposed humans [1, 9-12].

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Naturally occurring phytoestrogens (estrogenic chemicals found in plants) comprise a significant source of environmental exposure to estrogens. The adverse effects of phytoestrogens on reproduction in animals have been known for decades. One of the first reports was that sheep grazing on red clover, a food source high in phytoestrogens, exhibited infertility due to estrogenic effects of the clover [4]. Captive cheetahs exhibited reduced fertility while eating soy-based diets containing very high levels of phytoestrogens; replacement of soy protein with chicken protein restored their fertility [13]. These examples support the idea that phytoestrogens exist in our environment at concentrations high enough to be active during development and can have long-term adverse effects on reproduction in adult animals.

Since these initial observations, numerous studies have been performed in rodents and other animal models to evaluate the effects of phytoestrogens on development and reproduction. Adverse effects observed on the female reproductive system include, but are not limited to, altered estrous cyclicity, altered ovulation, subfertility, infertility and cancer. Rodent models of developmental exposure have also been used to test many other environmental endocrine disruptors that have estrogenic activity for their effects on the developing reproductive system [14-20]. Together, these studies suggest that developmental exposure to phytoestrogens or other environmental estrogens can have permanent adverse effects on female reproduction.

Human Adult and Infant Exposure to Phytoestrogens

Naturally occurring phytoestrogens are readily available in the diet, particularly in soy products [21-23]. The major class of phytoestrogens found in soy is isoflavones and the most abundant isoflavone is genistein (reviewed in [24]). Information on isoflavone content in many foods is also maintained on a USDA website (http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Data/isoflav/Isoflav_R2.pdf). The glycosylated form of genistein, genistin, makes up greater than 65% of the isoflavone content of soy products [25]. Isoflavones are found in high levels in soy food products that are substantial components of most vegetarian diets (reviewed in [24]); therefore, human fetuses can be exposed to these compounds during *in utero* development and during infancy via breast milk [26]. Isoflavone levels in adults vary considerably depending on ethnicity and diet. Serum genistein has been measured in the range of 25 µg/L (92.5 nM) in Asian women, slightly lower than this in vegetarian women, and under 2 µg/L (7 nM) in non-vegetarian women [27].

Infants are exposed to much higher levels of phytoestrogens than adults by direct intake of soy-based infant formulas and soy-based foods that are often specifically marketed for children [25, 26]. For their weight, infants exclusively fed soy-based formula have the highest mean daily consumption of total isoflavones, ranging from 6 to 9 mg/kg/day in 4 month old infants [25]. Infants consuming soy-based formulas have extremely high circulating levels of genistein, indicating that genistein is efficiently absorbed from soy formula [25]. Additional evidence that infants absorb isoflavones from soy-based formula is that glucuronidated genistein metabolites are found in their urine [28].

There are only two published studies of blood genistein levels measured in infants. A study of seven 4-month old infants exclusively consuming soy-based formula demonstrated serum genistein levels that ranged from 1 to 5 µM [25]. A more recent study of 27 infants ranging in age from 0-12 months confirmed these findings and found some levels to be even higher [29]. In the Cao et al. study, the 25th, 50th, and 75th quartiles of blood genistein levels were 405.3, 890.7 and 1455.1 ng/mL (1.5, 3.3 and 5.3 µM) and the 95th quartile was 10.2 µM (2800 ng/mL; personal communication, Kristina Thayer, NIEHS). Of note, neither study

provided exact time intervals from feeding to blood genistein measurement, and made single measurements per infant subject, and therefore could not address what the peak and trough genistein levels in infants might be.

Even at the 1-5 μM range, the serum genistein concentrations in soy formula-fed infants are 10 to 50-fold higher than in Asian women and 100 to 700-fold higher than in non-vegetarian US women. Perhaps more importantly, these levels are at least 13,000 to 68,000-fold higher than normal estradiol levels measured in infants from birth to age 2 [30]. The relative estrogenic potency of genistein is estimated to be 1000 to 10,000-fold lower than 17β -estradiol [31]; therefore, these blood genistein levels are clearly within a range to have significant biological estrogenic effects in infants. In contrast to soy-based infant formulas, only negligible amounts of phytoestrogens are found in cow's milk or human breast milk, even in vegetarian mothers [32-34], and so infants are unlikely to have significant estrogenic exposure via these nutritional sources.

Soy formula has been estimated to constitute approximately 20-25% of the infant formula market [35, 36] and at least one report suggests that for women who would prefer to breast feed, but are unable, soy formula is their substitute of choice [35]. Soybeans also have an extremely variable isoflavone content depending on variety and environmental conditions such as growing season and location [37]; the USDA reports highly variable amounts of genistein in soy products (see website listed above). This variability could result in even higher levels of genistein exposure than expected in particular lots of soy-based products.

Over the last few years, public and scientific interest in phytoestrogens has increased because of its proposed beneficial effects on human health. It must be kept in mind that there are likely to be dramatic differences in the effects of phytoestrogens on adults as compared to their effects on the developing fetus or infant, i.e., developmental exposure. Susceptibility to teratogens depends on critical periods in the the development of specific organs or tissues. One example of this is the susceptibility of the developing brain to teratogens that spans the time period from early fetal life all the way through several years of age because the brain develops and differentiates over that extremely long time period [38]. Sex steroids have dramatically different effects on the course of pregnancy and fetal and neonatal development depending on the exact timing of exposure, from interruption of pregnancy, to disorders of gonadal and external sexual differentiation, to abnormalities in hypothalamic-pituitary function [39].

From studies in animal models, there are conflicting reports on the effects of developmental exposure to genistein, with both beneficial and adverse effects reported depending on the timing of exposure, dose, and endpoints examined. For example, two studies report that prenatal exposure to genistein prevents carcinogen-induced mammary gland cancer in rats [40, 41], whereas another study reports an increase in mammary gland cancer if the developmental window of exposure is shifted to prepubertal life [42]. We have shown that mammary gland expansion and ramification are altered following neonatal genistein treatment [43]. These studies generally concur that mammary gland development is altered by either prenatal or neonatal genistein exposure. Neonatal genistein exposure also leads to the induction of uterine adenocarcinoma in mice later in life, similar to that previously described following neonatal DES exposure [16]. These studies highlight the importance of the exact developmental window of exposure to estrogenic compounds in determining specific outcomes for particular tissues.

There is a sparse but growing body of human epidemiological data regarding developmental exposures to soy products containing phytoestrogens. Beneficial effects were suggested by one study that reported improved cholesterol synthesis rates by human infants consuming

soy-based formulas [44]. On the other hand, another study demonstrated that pregnant women consuming a vegetarian diet during pregnancy give birth to male offspring with an increased incidence of hypospadias, suggesting an adverse effect of soy exposure that was likely related to high maternal levels of soy isoflavones [45]. A 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 as compared to their cow's milk-based formula fed counterparts [46, 47]. These findings are remarkable considering the small sample size (cow's milk-based formula group, 268 women; soy-based formula group, 128 women). A more recent study showed an increased incidence of physician-diagnosed fibroids by age 35 in women who consumed soy-based formulas as infants compared to either breast fed or cow's milk-based formula [48]. These data are from a large cohort of women (~20,000) who are sisters of women with breast cancer. Taken together, these epidemiological studies support the premise that developmental exposure of fetuses or infants to soy products could have long-term consequences for human health.

Comparison of Dose and Route of Exposure of Genistin and Genistein

Because humans cannot be used to directly test the effects of developmental exposures to isoflavones on various health outcomes, animal models have been used instead. The most common has been the rodent model, with various isoflavones administered either prenatally to the pregnant dam or postnatally to the pups either orally or by subcutaneous injection. Interpretation of these studies and their importance to human health outcomes has been highly controversial because of differences in opinion regarding what are the appropriate routes of administration and dosing. We have long believed that the relevant exposure information is the final serum concentration of the active isoflavone, not the exact dose administered, because this measurement corrects for differences in absorption and metabolism. For this reason, the doses of genistein used in our rodent studies have been chosen such that the serum levels measured would span the range of human exposure levels during development, from fetuses and infants of vegetarian mothers during pregnancy and lactation to babies on soy-based infant formulas.

The form of genistein in soy-based infant formulas and other dietary sources is predominantly the glycosylated form, known as genistin. Genistin is quickly hydrolyzed to the unconjugated (aglycone) active form, genistein, in the digestive system. Genistein is readily absorbed in infants because glucuronidated and other genistein metabolites are found in their urine after oral exposure to soy-based infant formula [28]. Although human infant exposure to genistein is primarily through genistin in soy formula, it is not practical to expose neonatal mice to genistein via soy formula because they cannot take in enough soy formula to generate circulating serum levels of genistein similar to those observed in human infants. Administration of genistein to pups via breast milk after maternal exposure is not possible because of the limited lactational transfer of isoflavones [49, 50].

For the reasons stated above, most studies in our laboratory have been carried out using subcutaneous injections of genistein in corn oil at doses of 0.5 to 50 mg/kg/day on days 1-5 of life. However, because of the ongoing controversy over the relevance of a subcutaneous route of genistein exposure, we directly compared the estrogenic activity and subsequent effects on female reproductive outcomes of orally administered genistin to subcutaneous injection of genistein [51]. Female CD-1 mouse pups were treated on postnatal days 1-5 by subcutaneous injection of genistein in corn oil at doses of 0, 12.5, 20 and 25 mg/kg/day or by oral administration (suckling from a pipet tip) of genistin in corn oil at doses of 0, 10, 20, 40 and 60 mg/kg. Because genistin is made up of genistein linked to a large sugar group that

accounts for 37.7% of the molecular weight of genistin, genistin dosing will be expressed as the genistein aglycone equivalent dose (0, 6.25, 12.5, 25 or 37.5 mg/kg/day).

Uterine wet weight, the gold standard bioassay of estrogenic activity, was measured on the 5th day of treatment. A significant increase in uterine wet weight was observed following subcutaneous injection of genistein at the 20 and 25 mg/kg/day doses and also following oral exposure to genistin at the 25 and 37.5 mg/kg/day doses. Oral exposure to genistin 25 mg/kg/day increased uterine wet weight to a similar extent as the subcutaneous injection of genistein 20 mg/kg/day. Because the vast majority of subcutaneously injected genistein enters the circulation, these data suggest that approximately 80% of the oral genistin dose is absorbed into the circulation as indicated by the similar biological response. These findings demonstrate that subcutaneous injection of genistein is a suitable surrogate for oral exposure to genistin.

Pharmacokinetic analysis of subcutaneous exposure to genistein and oral exposure to genistin or genistein has been performed by our laboratory and others (summarized in Table 1). Neonatal female mice treated by subcutaneous injection of genistein (50 mg/kg) have a peak serum circulating genistein level of 6.8 μ M [52]. Neonatal female mice exposed orally to genistin (37.5 mg/kg) have a higher peak circulating genistein level of 19.2 μ M [51]. Although oral exposure led to peak circulating levels of total genistein that were higher than following subcutaneous injection (19.2 μ M vs. 6.2 μ M [52]), the total area under the curve was very similar, 83% of the subcutaneous dose. Serum circulating genistein levels following oral exposure to genistin were about 50% of the subcutaneous dose but the biological activity was around 80%. These data suggest that resulting biological activity is complex and that both the peak and total circulating levels may contribute to the overall biological effect.

In the same study, the pharmacokinetics of an orally administered dose of genistein (37.5 mg/kg) was assessed. There was 90% less genistein found in circulation following oral genistein exposure as compared to the same dose of genistein administered subcutaneously. Oral genistein exposure generated a peak serum genistein level of only 1.1 μ M compared to 19.2 μ M following oral genistin exposure. These data suggest that genistin is much more bioavailable after oral exposure than the aglycone form, genistein.

Another laboratory took the approach of mixing genistein (50 mg/kg) with a combination of soy-based infant formula and corn oil as their method of exposing neonatal mouse pups orally to genistein (Cimafranca, 2010). This rather complex route of exposure resulted in a peak genistein level just over 3 μ M at 1 hour following the dose, and is somewhat higher than our finding of 1.1 μ M genistein following oral exposure to genistein in corn oil [51]. However, these data also confirm the lower bioavailability of genistein following an oral exposure to the aglycone form compared to oral exposure to the glycosylated form, which is the predominant form found in soy products.

Importantly, the genistein levels observed in all of these studies are within the range of those measured in human infants consuming soy-based infant formulas (μ M range). Peak circulating levels in infants are not known because pharmacokinetic studies cannot be conducted in infants; instead the available information is from single point values at unknown time intervals after feeding. The peak circulating genistein levels in rodent studies following oral administration occurs between 30 min and 1 hour after dosing. It is unlikely that blood collection is routinely performed so close to feeding in infants. The available data from infants support this idea because of the large range (~10 fold) of single point values (25th quartile, 1.5 μ M – 95th quartile, 10.2 μ M) {Cao, 2009 #30; and personal communication, Dr. Walter Rogan, NIEHS}. Because young infants are fed every 3-4 hours,

elevated genistein levels likely persist throughout the entire day, unlike in rodent studies where there is a single peak after dosing followed by many hours of very low levels. As a result, overall genistein exposure in human infants via soy-based formula is likely to be somewhat higher than that observed in rodent models where single daily doses are given.

Another point of interest in the pharmacokinetic studies in neonatal mice is the relatively high circulating levels of the aglycone form of genistein (low μM range). This form of genistein exhibits the strongest estrogen receptor (ER) binding activity and is the most estrogenic [53]. This level is approximately 10-fold higher than that found in adult rats following similar exposure [54], suggesting that neonates are exposed to higher levels of the estrogenically active form of genistein compared to an adult. The higher fraction of aglycone form of genistein has also been shown in rats treated perinatally with genistein [55] supporting the idea that glucuronidation of genistein is lower during the neonatal period compared to adulthood. This observation may be explained by the lower UDP glucuronosyltransferase (UGT) activity observed in neonatal mice [56-58]. Many human UGT isoforms exhibit a pattern of expression similar to that in rodent development with lower activity in the neonatal period [59, 60]. Although the serum circulating levels of the aglycone form of genistein in human infants is not known, an elevated fraction of the aglycone form when compared to adult humans is likely.

Another important experimental consideration is the diet consumed by the mother during the time of neonatal treatment. The NIH-31 lab chow our mice are fed contains low levels of phytoestrogens, with total genistein and daidzein content of $98 \mu\text{g/g}$ [61]. This adds up to about 16.7 mg/kg/day for a 30 g mouse. While the mother consumes the diet directly, the neonatal pups do not. Because only a small fraction of circulating isoflavones are found in milk [50], neonatal mice are only exposed to very low levels of genistein in milk and these levels are far below the treatments used in our studies. Indeed, serum genistein is not detectable in neonatal pups suckling from mothers on NIH-31 lab chow [20, 51]. The levels of daidzein and equol were also measured in our oral genistein exposure study and neither of these compounds was detectable in any treatment group. These findings suggest that any estrogenic activity observed in these studies is due solely to genistein or genistein metabolites.

Neonatal Estrogenic Activity

Several studies from our laboratory [16, 51] and a recent study by Cimafranca et al. [62] have examined the estrogenic activity (uterine weight gain) in neonatal mice following subcutaneous administration of genistein or oral administration of genistein and genistin. These data are plotted for comparison in Figure 1. All of the studies were done using outbred CD-1 mice except the Cimafranca study that used inbred C57Bl/6 mice. There is a clear dose-dependent increase in uterine wet weight on neonatal day 5 following both subcutaneous injection of genistein and oral exposure to genistin. Compared to subcutaneous injection of genistein, it takes approximately 20-33% more oral genistin to elicit a similar uterine wet weight increase compared to controls. No significant increase in uterine weight was observed following oral exposure to genistein in corn oil at 25 and 37.5 mg/kg/day , whereas a small but significant increase was observed at 75 mg/kg/day . These data suggest that much less oral genistein is bioavailable compared to oral genistin.

The Cimafranca study was done using an oral dose of genistein mixed in a soy-based infant formula and corn oil suspension instead of corn oil alone [62]. The data from this study show an increased uterine weight that is higher than our study using a similar dose of oral genistein in corn oil [51]. However, the peak serum circulating level of total genistein ($\sim 3 \mu\text{M}$) in the Cimafranca study was higher than what we observed following oral genistein

exposure, suggesting that the biological response depends on total serum genistein levels. One obvious difference between the studies is the vehicle used for oral genistein administration. Perhaps the bioavailability or absorption is affected by one vehicle versus the other but further studies would have to be performed to determine this. Another explanation for the slightly different outcomes is that different mouse strains were used in these studies. Indeed, neonatal exposure to DES has widely disparate effects depending on mouse strain. For example, over 90% of CD-1 mice treated neonatally for 5 days with DES develop uterine adenocarcinoma, whereas C57Bl/6 mice are completely resistant to this treatment [12, 63]. However, despite some subtle differences, all of these genistein studies demonstrate that serum circulating genistein levels similar to those observed in human infants on soy-based infant formulas and have estrogenic activity as indicated by a uterotrophic bioassay in the mouse model.

Ovarian Differentiation and Development

Several laboratories have documented adverse effects of neonatal genistein exposure on the developing mouse ovary [62, 64, 65]. Both CD-1 and C57Bl/6 mice treated neonatally by subcutaneous injection of genistein in corn oil at doses of 0.5, 5 and 50 mg/kg/day showed a dose-dependent increase in the number of mice with multi-oocyte follicles (MOFs) in the ovary prior to puberty [65]. Almost all of the mice in the highest dose group had MOFs. MOFs were also observed in CD-1 mice treated orally with genistein at doses of 12.5, 25 and 37.5 mg/kg [51], and in C57Bl/6 mice treated orally with 50 mg/kg/day genistein in soy formula/corn oil [62], suggesting similar biological activity in the ovary regardless of route of exposure.

The mode of action of genistein in inducing MOFs can be attributed to its estrogenic activity in the developing ovary. This was documented in studies using mice lacking either estrogen receptor alpha (ER α) or beta (ER β) [65]. Mice lacking ER α developed MOFs when treated neonatally with genistein, whereas mice lacking ER β did not, suggesting that genistein interacts with ER β to cause MOFs. The formation of MOFs was further characterized by observing ovarian development during the time of neonatal genistein treatment [64]. At birth, mice have large oocyte clusters or nests in which oocytes are linked by intercellular bridges. These oocyte nests dissociate during the first week of life as individual oocytes become surrounded by granulosa cells to form primordial follicles [66]. Treatment with genistein at a dose of 50 mg/kg on neonatal days 1-5 inhibits this differentiation process, leaving many oocytes together in nests and still attached to each other by intercellular bridges. There was also a higher percentage of unassembled oocytes (those not completely surrounded with granulosa cells) in ovaries of treated mice as compared to controls, further supporting the idea that neonatal genistein treatment limits ovarian differentiation.

MOFs have also been found in rats treated neonatally with genistein, suggesting that this outcome is not limited to mice [15]. The presence of MOFs has also been noted in humans [67] supporting the idea that this effect can be seen in humans although the cause in humans is still not known. Not surprisingly, several other estrogenic compounds cause MOFs if exposure occurs during development; these include 17 β -estradiol, DES and Bisphenol A [18, 68-70]. One study demonstrated that mouse oocytes derived from MOFs have reduced quality because in vitro fertilization of these oocytes is markedly reduced when compared to oocytes derived from follicles supporting a single oocyte [68]. This finding suggests that an increased number of MOFs could be related to decreased fertility later in life.

To further study the effects on the ovary, oocyte quality was examined in adult mice following neonatal exposure to genistein [71]. For this study, mice were treated by subcutaneous injection with genistein (50 mg/kg/day) on days 1-5. Because genistein treated

mice are anovulatory, control and genistein treated females (8 weeks of age) were given pregnant mare serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG) to induce ovulation. Equal numbers of eggs were ovulated from both control and genistein treated mice, and egg morphology including meiotic spindle structure appeared normal. Eggs retrieved from genistein treated mice following superovulation were capable of being fertilized in vitro equally as well as eggs from control mice. In vitro fertilized embryos from both treatment groups also developed into blastocysts equally as well suggesting that poor egg quality is not a major contributor to the infertility seen in these mice. One caveat to this statement is that the mice used in these studies were only 6-8 weeks of age and perhaps eggs obtained from older genistein-treated mice could be of poor quality. In addition, this study did not specifically differentiate between eggs that came from MOFs or single oocyte follicles, so although most eggs were fertilized and developed into healthy embryos, we might not have detected subtle differences in egg quality. Therefore while it is clear that neonatal genistein exposure can lead to the formation of MOFs by impairing primordial follicle formation in the neonatal period, the long-term impact of this finding on fertility is not certain.

Adult Ovarian Function

Numerous studies have demonstrated altered ovarian function following developmental exposure to genistein. A 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 [20]. Doses of genistein lower than 50 mg/kg/day caused enhanced ovulation rates as indicated by increased numbers of oocytes ovulated following exogenous gonadotropin exposure at 26 days of age [65] and increased numbers of CLs at 4 months of age [20]. 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 HPG-related mechanism is most likely for the following reasons. First, exogenous gonadotropins restore ovarian function in mice treated with the high dose of genistein as evidenced by similar numbers of oocytes ovulated compared to controls. Second, neonatal exposure of rats to genistein alters pituitary responsiveness to gonadotropin releasing hormone (GnRH) [72]. Higher neonatal doses of genistein are associated with decreased pituitary responsiveness as indicated by lower luteinizing hormone (LH) levels in response to GnRH stimulation [72]. The LH surge is necessary for ovulation, so lower LH levels could explain the lack of ovulation observed in mice treated with higher genistein doses. Interestingly, rats treated with lower doses of genistein (0.01 mg/kg/day) are hyper-responsive to GnRH stimulation. This response leads to enhanced ovulation rates similar to those observed in younger mice treated neonatally with low doses of genistein, and similar to older mice who have increased CLs at 4 months of age. Together, these data suggest that disruption of HPG axis function following developmental exposure to genistein is responsible for the observed abnormalities in ovarian function.

Estrous Cyclicity

Estrous cyclicity is dependent on proper function of the HPG axis and disruptions described above would alter cyclicity. Indeed, this is true because several studies have shown altered estrous cyclicity in rodents following prenatal or neonatal treatment with genistein [15, 18-20, 73]. As an example, a study 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 and increasing age [20]. 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, indicating that these mice were not cycling. Mice exposed orally to either genistein or genistin also exhibit

altered estrous cyclicity [15, 51, 62] suggesting again that route (oral vs. subcutaneous) and compound (genistein vs. genistin) are less important than the fact that there are biologically relevant circulating levels of genistein present during neonatal development. Other investigators have shown similar estrous cycle alterations in experimental animal models including a study by Nikaido et al. showing that several environmental estrogens given during prenatal development (genistein, resveratrol, zearalenone, and bisphenol A) caused extended estrous cycles when the animals became adults [17]. Prolonged periods of time in estrus have been observed in rats following neonatal exposure to genistein [73]. This finding is similar to estrous cycle abnormalities observed in mice exposed perinatally to DES [10], further supporting the idea that developmental exposure to estrogenic compounds causes disruptions in estrous cyclicity.

Effects of genistein on the HPG axis in relation to estrous cyclicity have also been examined. Rat pups were exposed on neonatal days 1-4 by subcutaneous injection of genistein (10 mg/kg/day) and estrous cyclicity was followed over time [19]. Females lost their ability to maintain regular estrous cycles as they aged. These physiological changes were accompanied by an impaired ability to stimulate GnRH neuronal activity (as measured by the immunoreactivity of both of GnRH and Fos) following ovariectomy and hormone priming [19]. This would be expected since estrogen exposure during the neonatal period typically induces a similar disruption in the organization and signaling from the female HPG axis (for more details on this topic, see [74, 75]).

Reproductive Function

Several studies have demonstrated that developmental exposure to genistein can cause subfertility or complete infertility. Fertility disruption has been observed in rats treated neonatally with 100 mg/kg/day genistein [15]. Female mice treated neonatally with genistein at doses of 0.5 and 5 mg/kg/day had no difference in fertility compared to controls at 2 and 4 months of age [20]. However, by 6 months of age, there was a significant reduction in the percentage of mice delivering live pups in both of these treatment groups as compared to controls. In addition, there was a reduction in the number of live pups in the mice that had litters; these findings suggest early reproductive senescence. Mice treated neonatally with genistein at 25 mg/kg/day had reduced fertility when tested at 2 months of age, whereas after a 50 mg/kg/day neonatal dose the mice were completely infertile as adults despite successful mating as indicated by the presence of vaginal plugs. Examination of the uteri of these mice on days 6, 8, and 10 after mating demonstrated that less than half of the mice had visible implantation sites [20]. The implantation sites that were present were smaller than in controls, and some resorption sites were evident, indicating that the implantation process was disrupted in mice treated with this dose of genistein.

One explanation for the implantation defect is that blastocysts derived from the genistein-treated mice were not developmentally competent. However, when blastocysts from control mice were transferred into pseudopregnant genistein-treated mice, no normal implantation sites were observed on day 8 of pregnancy whereas appropriate numbers of normal-appearing implantation sites were observed in control recipients [71]. A second explanation for the implantation defect is that the ovaries did not generate sufficient steroid hormones to support implantation. However, serum estradiol, progesterone, and testosterone levels during pregnancy were no different in the genistein-treated animals as compared to controls [20]. Mice treated neonatally with high doses of DES are unable to respond appropriately to estrogen stimulation [76] suggesting that the developmental estrogen exposure renders the uterus unresponsive to steroid hormones required to support endometrial receptivity and pregnancy. This altered uterine response is also observed in mice treated neonatally with genistein as indicated by a dampened uterine wet weight response to estrogen challenge

prior to puberty [77]. This is not surprising because uterine cellular differentiation continues during the neonatal period in many animals and can be altered by estrogen exposure during the differentiation process [78, 79]. This reduced estrogen responsiveness could contribute to abnormal implantation and the resulting infertility.

Further studies to determine the cause(s) of infertility in neonatal genistein-treated mice demonstrated that in addition to abnormal implantation, these mice have defects in the ability of the oviduct to support preimplantation embryo development [71]. There is a 50% loss of embryos during transit through the oviduct of genistein treated mice, whereas embryos removed from the oviduct after fertilization and then cultured *in vitro* develop efficiently into blastocysts. These data suggest that there is a permanent change in the function of both the oviduct and uterus that leads to complete infertility in these mice.

The fertility of female mice exposed orally to genistin is also impaired but they are not completely infertile [71]. Mice exposed to oral genistin (25 and 37.5 mg/kg) had reduced number of mice delivering live pups but only at 6 months of age following two previous litters. They also had reduced litter sizes at the 6 month time point. However, if the data from all of the time points was combined, there were significant reductions in the percent of vaginal plug positive females that delivered live pups at the 12.5, 25 and 37.5 mg/kg dose. The Cimafranca study did not find any deficits in fertility after an oral exposure to genistein (50 mg/kg/day) in soy formula/corn oil [62]; however, the mice were only bred for one 7 day period at 6 months of age and only 6/12 vehicle controls and 6/15 genistein treated mice delivered live pups. The fertility of these mice when compared to complete infertility of mice treated subcutaneously with the same dose of genistein is likely explained by the differences in route of administration leading to lower serum genistein levels in the Cimafranca study. If one compares the estrogenic activity during the time of treatment (Figure 1) of oral exposure to genistein with oral exposure to genistin, the Cimafranca study using oral genistein 50 mg/kg/day should be similar in response to our oral genistin 25 mg/kg/day dose. This dose in our study showed fewer vaginal plug positive mice delivering live pups and fewer pups per litter, but only at the 6-month time point and only after two previous litters. Although it is not known what impact previous parity has on the reproductive tract of these mice, perhaps mice exposed orally to genistin develop uterine problems following pregnancy and a difference in fertility can only be seen in subsequent pregnancies. As mentioned previously, the strains of mice used in these two studies were also different, so slight differences in outcomes are not unexpected.

An intriguing finding in our recent study using oral exposure to genistin was that mice treated orally with genistin (12.5, 25 and 37.5 mg/kg) exhibited delayed parturition, in some cases by as long as two days [51]. This was not observed in our previous studies, most likely because the subcutaneous genistein studies did not include a dose that generated intermediate serum genistein levels similar to those in the oral genistin study. Perhaps delayed parturition is a more subtle indication of uterine abnormalities that explain in part why fertility decreases with time and subsequent pregnancies. The uterus may not be functioning at full capacity and while the first pregnancy may be successful, the uterus could be damaged or refractory to hormones in some way. Future studies are needed to determine the mechanism underlying delayed parturition in these mice.

Summary and Conclusions

The data presented herein demonstrate that environmentally relevant doses of genistein disrupt female reproductive development and function. In addition, these data indicate that a subcutaneous injection of genistein is a suitable surrogate for oral exposure to genistin because both routes and compounds cause biological estrogenic activity that depends on the

serum circulating genistein level achieved. Our laboratory and others have showed altered estrous cyclicity, altered ovarian function and subfertility or infertility in female mice exposed neonatally to genistein. Further, we have shown altered ovarian differentiation following neonatal exposure to genistein. Studies using other phytoestrogens including coumestrol [23, 80], daidzein [25, 73] and red clover [81, 82] 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 17 β -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 development exposure to genistein [16] suggest that additional studies of human populations exposed to high levels of phytoestrogens during development is warranted.

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Figure 1.

Summary of uterine weight on neonatal day 5 following treatment with genistein by subcutaneous injection or oral exposure or genistin by oral exposure. Data summarized and re-plotted from the following studies [16, 51, 62]. Original data were uterine weight/body weight ratios.

Table 1

Circulating levels of genistein following various models of exposure.

Model	Route	Dose	Circulating level (uM)	Total (AUC)
Neonatal CD-1 mouse	Subcu. genistein (corn oil vehicle)	50 mg/kg	6.8 (peak, [52])	147.0
Neonatal CD-1 mouse	Oral genistein (corn oil vehicle)	37.5 mg/kg	19.2 (peak, [51])	90.4
Neonatal CD-1 mouse	Oral genistein (corn oil vehicle)	37.5 mg/kg	1.1 (peak, [51])	12.8
Neonatal C57Bl6 mouse	Oral genistein (corn oil + soy formula vehicle)	50 mg/kg	~3.1 (peak, [62])	Not calculated
Human infant	Oral soy isoflavones (genistin and daidzin)	6-9 mg/kg/day	1 - 5 (range, [25]) 1.5 (25 th percentile, [29]) 3.3 (50 th percentile, [29]) 5.3 (75 th percentile, [29]) 10.2 (95 th percentile, Thayer personal communication)	Unknown – only have single spot values at unknown times post feeding.