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Effects of Chronic Dietary Exposure to Genistein, a Phytoestrogen, During Various Stages of Development on Reproductive Hormones and Spermatogenesis in Rats

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Developmental, hormonal, and gametogenic parameters were evaluated in male progeny following chronic dietary exposure to the phytoestrogen genistein. Twenty pregnant rats were fed a diet containing genistein (50 μg/d) from d 17 of gestation, and 12 were fed a control diet without genistein. Four litters each of control and genistein-fed rats were euthanized on d 21. The remaining pups were weaned on d 21 and only male rats were used in this study. On d 21, eight litters of genistein-fed rats were placed on control diet (gestational and lactational exposure alone [GL-G]), and the remaining eight continued on genistein diet (lifelong exposure group [LL-G]). These rats were euthanized (four litters/group) on d 70 or 130 of life. Serum testosterone, which was slightly reduced in genistein-exposed rats on d 21, did not differ among treatment and control groups on d 70 and 130. Serum luteinizing hormone of genistein-exposed rats was reduced on d 21 and 130, but not on d 70. Serum follicle-stimulating hormone did not vary among groups at any age. Treatment-related effects of dietary genistein were not observed on the weights of the testes of 21-d-old rats. Except for a slight decrease in testis weight of GL-G rats at 130 d, no significant effect of dietary exposure was observed on the weight of the testes in any other group. However, epididymal weights were significantly reduced in both treated groups at d 130. Testicular sperm count (on d 70 as well as 130) also was not affected in GL-G or LL-G rats. We conclude that gestational plus lactational exposure to genistein and subsequent dietary exposure to genistein have no adverse effects on gametogenic function in male rats.

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Introduction

Studies in laboratory animals and animals in the wild have demonstrated that *in utero* exposure to endocrine-mimicking agents can result in impaired function of the reproductive system in the offspring *(1–5)*. Thus, changes associated with human infertility have been linked to prenatal exposure to a variety of synthetic chemicals including estrogenic and/or antiandrogenic compounds *(6,7)*. For example, it has been reported that sperm counts have fallen by as much as 50% over the last six decades in several human populations *(8)*. Whereas there was some support for this observation *(9)*, others did not find such a decline *(10–13)*. These conflicting reports have raised our awareness to factors within the environment that could be affecting human reproduction. Although considerable scientific uncertainty remains regarding a possible link between environmental agents and an increase in the incidence of certain endocrine-modulated reproductive abnormalities, it is generally accepted that a small perturbance in endocrine function, especially during critical stages of development, pregnancy, and lactation, can lead to profound, long-lasting adverse effects.

Some commonly attributed causative agents that might have an impact on human reproduction include estrogenic substances (xenoestrogens), natural products, as well as environmental contaminants. Among these natural products are estrogens resulting directly from consumption of plants and plant products or indirectly from consumption of meats and meat products of herbivorous animals. These products may have a potent effect on differentiating tissue, particularly if exposure occurs early in life *(14–16)*. These compounds have been shown to mimic estradiol and diethylstilbesterol *(17)*.

In view of the current debate regarding the role of environmental estrogens in causing adverse effects on human

Table 1
The Effects of Dietary Genistein on Body Weight (g)[a]

Group	Day 21	Day 70	Day 130
Control	61 ± 1	342 ± 8	509 ± 12
LL-G	64 ± 3	332 ± 11	434 ± 7*
GL-G	63 ± 1	345 ± 12	451 ± 20*

[a]Values are mean ± SEM. (*) Significant differences ($p < 0.05$) when compared with control rats.

Table 2
The Effects of Dietary Genistein on Weights of Testes and Epididymides and Testicular Sperm Content[a]

	Testis weight (g)	Testicular sperm content (millions)	Epididymis weight (g)
Day 21			
Control	0.17 ± 0.01	ND	ND
Genistein	0.16 ± 0.01	ND	ND
Day 70			
Control	1.5 ± 0.02	212 ± 9	0.35 ± 0.01
LL-G	1.5 ± 0.06	180 ± 23	0.39 ± 0.01
GL-G	1.5 ± 0.06	190 ± 6	0.38 ± 0.02
Day 130			
Control	1.83 ± 0.06	255 ± 13	0.68 ± 0.01
LL-G	1.72 ± 0.04	264 ± 5	0.64 ± 0.01*
GL-G	1.58 ± 0.05*	234 ± 15	0.58 ± 0.02*

[a]Values are mean ± SEM. ND, not done. (*) Significant differences ($p < 0.05$) when compared with control rats.

reproductive function including sperm counts *(6)*, we examined the effects in male rats of early and chronic exposures to genistein, an isoflavonoid found in soybeans *(18)*. Such studies are important because of the controversial nature of issues involved: the dosage, duration, and route of exposure of the test compounds, and the implied detrimental role of phytoestrogens themselves. In an attempt to address some of these issues, a diet containing genistein at concentrations found in natural food items was administered to pregnant rats so that pups were exposed *in utero* (beginning gestation d 17) and during the lactational period (d 1–21 of life) (GL-G rats). On weaning at d 21 of life, a group of pups continued to be exposed to genistein in their diet (LL-G rats). At various stages of reproductive development, we evaluated weights of the testes and epididymides, serum concentrations of testosterone and gonadotropins, steady-state mRNA levels of β-subunits of gonadotropins, and spermatogenic potential (number of sperm/testis).

Results

There were no significant differences in the body weights between control and genistein-exposed rats at d 21. Likewise, there were no significant differences in body weights among control, LL-G, and GL-G rats at d 70. However, at d 130, the control rats were considerably heavier than LL-G and GL-G rats (Table 1).

Dietary exposure to genistein had no effect on the testis weight at d 21 and 70; however, the testis weight of the 130-d GL-G rats was significantly lower than the corresponding control and LL-G rats (Table 2). Exposure to genistein, for any duration, did not affect spermatogenesis (Table 2). At d 70, there were no differences in the weights of the epididymis among the three groups; however, at 130 d, the weight of epididymis was significantly lower in LL-G and GL-G rats (Table 2). Histologic evaluation of testes and epididymides did not reveal any obvious lesions in any group.

At d 21, serum luteinizing hormone (LH) and testosterone were significantly reduced in the genistein group compared with the control group; however, there was no difference in serum follicle-stimulating hormone (FSH). On d 70, serum LH, FSH, and testosterone did not demonstrate any variation among groups. On d 130, whereas serum FSH and testosterone were similar in LL-G and GL-G when compared with control rats, serum LH was significantly reduced in LL-G and GL-G rats (Table 3).

Northern blot analysis and quantification of LHβ and FSHβ mRNA were carried out to determine the pretranslational regulation of LH and FSH during the treatment period. Quantification of the steady-state levels of mRNA of LHβ subunit (Fig. 1A) showed that the level of LHβ mRNA was slightly reduced in genistein-exposed rats when compared with control rats on d 21. However, LHβ mRNA of GL-G and LL-G rats was similar to that of control rats on d 70 and

Table 3
Effects of Dietary Genistein on Serum LH (pg/mL), FSH (ng/mL), and Testosterone (ng/mL)

	LH	FSH	Testosterone
Day 21			
Control	531 ± 72.8	14.2 ± 1.8	1.47 ± 0.23
Genistein	174 ± 15.7*	8.34 ± 5.13	0.88 ± 0.11*
Day 70			
Control	597 ± 110	18.14 ± 5.18	2.11 ± 0.18
LL-G	378 ± 95	12.78 ± 0.78	3.06 ± 0.4
GL-G	304 ± 78	12.71 ± 1.2	2.44 ± 0.35
Day 130			
Control	681 ± 10	18.8 ± 0.9	1.60 ± 0.09
LL-G	640 ± 13 *	17.7 ± 0.6	1.57 ± 0.06
GL-G	587 ± 20 *	20.9 ± 1.3	1.55 ± 0.04

[a]Values are mean ± SEM. (*) Significant differences ($p < 0.05$) when compared with control rats.

130. The decrease in steady-state levels of LHβ mRNA subunit of genistein-exposed rats on d 21 also mirrors the decrease in serum LH concentrations at this age (Table 3). Unlike LHβ mRNA subunit, the steady-state levels of FSHβ subunit mRNA (Fig. 1B) did not differ among groups on d 21, and there were no differences observed thereafter on d 70 and 130.

Discussion

Several studies have reported that estrogenic compounds can adversely affect sexual behavior and gonadal structure and function and decrease reproductive capacity in animals (*19,20*). Recently, attention has been focused on the potential benefit or detrimental effects of phytoestrogens. The effects of exposure to phytoestrogens on spermatogenesis is scanty and, at best, not well explored. The present study was conducted to determine whether *in utero* and lactational exposure to dietary genistein exerts any deleterious effects on male reproductive function. Rats were exposed *in utero* (through their mother) to dietary levels of genistein during the critical period of steroid-mediated development of the reproductive system. The level of genistein fed to pregnant mothers was estimated to mimic human intake. The results of this study demonstrated that *in utero* and lactational exposure of male rats to dietary genistein did not have any negative impact on the pituitary gonadotropin gene expression, serum FSH and testosterone levels, and spermatogenesis at adulthood (reproductive age), although there was a significant reduction in serum LH levels. Whereas the same exposure exerted structural and functional changes in the reproductive system of female rats *(5)*, the reproductive system of the male rats was not compromised.

In the male, changes in spermatogenic potential in adult rats treated with estrogen in earlier studies have been explicable on the basis of impaired LH (and/or FSH) secretion and consequent changes in testosterone *(21–23)*. Steady-state mRNA levels for FSHβ and LHβ as well as serum concentrations of FSH and testosterone were similar among all groups in the present study at d 70 and d 130, but serum testosterone was lower in genistein-exposed animals at d 21. It is not readily apparent why there was a decrease in LHβ mRNA and serum LH leading to a decrease in testosterone levels on d 21 (at weaning). We speculate that early exposure to genistein may have altered the LHβ gene expression during the prepubertal period. In addition, these decreases in genistein-exposed rats may also be a result of the maturational state of the Leydig cells resulting from incomplete maturation of the hypothalamic-pituitary-gonadal axis. Another possibility is that the sensitivity of LHβ mRNA to GnRH pulsatility may have been affected by genistein exposure such that the resulting protein (LH) synthesis is reduced. However, regardless of the slight but significant reduction in serum LH and testosterone on d 21, histologic features of the testes (not shown) did not differ among control, LL-G, and GL-G rats.

At weaning, some of the genistein-exposed rats were placed on a control diet for the rest of their lives (GL-G rats) to determine whether *in utero* and lactational exposure to genistein during the critical period of steroid-mediated reproductive development would permanently alter the reproductive function as adults. Note that as with the GL-G exposure, even the more chronic LL-G exposure did not cause any significant structural or functional changes in the reproductive system. Mating trials of LL-G and GL-G rats with untreated females resulted in normal conception, indicating that the fertility of the genistein-exposed males was not compromised (unpublished observations).

These results suggest that pharmacologic doses of genistein used in earlier studies were probably responsible for structural and functional changes observed in those stud-

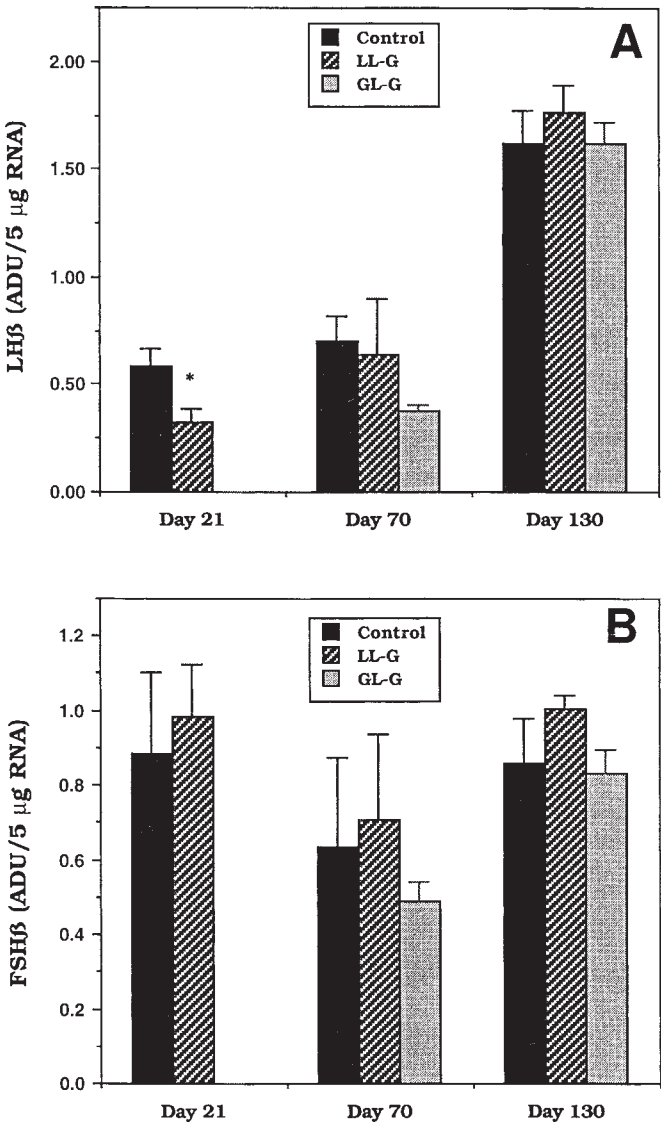

Fig. 1. Effects of genistein exposure on the steady-state mRNA levels of LHβ (**A**) and FSHβ (**B**) at d 21, 70, and 130 of life. Total RNA was isolated from individual pituitary gland, and subunit mRNA levels were determined by Northern blot. The results for four representative rats/group are shown. Each bar represents the mean arbitrary densitometric units (ADU) ± SEM of four autoradiographic bands of Northern blots. The membranes were hybridized with an 18S rRNA probe to control for loading. The specific probe mRNA signal or optical density was (OD) corrected for RNA loading using the 18S signal (OD) and expressed as relative (OD). (*) Significant difference at $p < 0.05$.

ies *(15,24)*, and that genistein administered at levels that might occur naturally appear not to be detrimental to sperm production. In fact, over the past decade, several advantages of plant estrogens such as genistein and daidzein have been reported, and they have become the darlings of the health food enthusiast. Several studies have suggested that these compounds may underlie many of soy's reputed health benefits. For example, it has been shown that genistein and diadzein are found in high concentrations in the urine of Asian women, a population known to be resistant to breast cancer, and found in low concentration in patients with breast cancer *(25–27)*. Similarly, Japanese men who consume a diet high in soy products have a very low mortality from prostate cancer *(28)*. These results would suggest that long-term benefits might ensue from early exposure to phytoestrogens because it could confer protection later in life against hormone-dependent diseases.

Based on the results of this study, we infer that genistein consumed through dietary means does not adversely affect spermatogenesis, even when exposures occur *in utero* and neonatally. Further studies are needed to determine whether there are any subtle effects of this dietary genistein regimen on the development of the reproductive tract and/or function of offspring.

Materials and Methods

Animals

Pregnant Sprague-Dawley rats (Sasco, Omaha, NE) were purchased at d 10 of gestation. Animals were housed individually in a room maintained at 22°C with a constant light-dark cycle (14 h of light and 10 h of darkness), and were provided isoflavanoid-free rat chow and water ad libitum. The studies were performed in accordance with the *Guidelines for the Care and Use of Experimental Animals* and were approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center.

Administration of Genistein

Because commercial rodent chow contains some isoflavonoid *(29)*, all rats were fed the AIN semipurified rat-mouse diet (Bioserve, Frenchtown, NJ), which is devoid of isoflavonoid and contains casein-high nitrogen (20%), DL-methionine (0.3%), cornstarch (15%), sucrose (50%), fiber-celfil (5%), mineral mixture (3.5%), vitamin mixture (1%), and chlorine bitartrate (0.2%), until d 17 of gestation. We chose to administer the genistein diet at a dose of 5 mg/kg of feed based on the data of Barnes et al. *(30)* and Steele et al. *(31)*.

Barnes et al. *(30)* estimated that human intake of genistein is approx 50 μg/d. Steele et al. (*31*) reported that rats given a diet containing 150 mg of genistein/kg of feed had a daily intake of 10.45 mg/kg of body weight. On the basis of these observations, we estimated that the required concentration of genistein in the diet is about 2.5 mg/kg of feed to provide 50 μg/d to a 300-g rat. However, because variations in weight and dietary intake are certain to occur, we elected to supply a dose of 5 mg/kg of feed to ensure that all animals consumed at least 50 μg of genistein/d.

Experimental Design

The critical period of steroid-mediated development of the reproductive system in the rat begins around d 17 of

gestation, and therefore, we began dietary genistein exposure (5 mg/kg of feed) in rats on d 17 of gestation. Eight pregnant rats were fed the semipurified control diet. Animals were weighed weekly throughout the experiment. Daily feed consumption was determined by weighing feed daily at 11 AM. Pups were weaned on d 21 and only male rats were used in the study.

Pups from four litters in the control and genistein-exposed groups were euthanized on d 21; their hormones and reproductive organs were evaluated. On postnatal d 21, eight litters of genistein-exposed rats were given a control diet (GL-G), and the remaining eight litters continued to receive the genistein diet (LL-G) until necropsy (four litters/group) was performed on postnatal d 70 or 130. Eight litters that had been receiving the control diet continued to receive the same diet and served as controls for the 70- and 130-d treated animals.

On d 70 and 130, four litters of rats in each of the control, LL-G, and GL-G groups were euthanized. Trunk blood, pituitary gland, testes, and epididymides were collected. Serum was separated and stored at –20°C for subsequent determination of concentrations of testosterone, LH, and FSH by radioimmunoassay (RIA). Weights of the testes and epididymides were recorded. One testis was fixed in Bouin's solution and processed for histopathologic evaluation. The contralateral testis was used to determine the number of homogenization-resistant elongated spermatids using methods described by Robb et al. (*32*).

Radioimmunoassays

Serum concentrations of gonadotropins (LH and FSH) were measured with the use of a double-antibody RIA as previously described by L'Hernite et al. (*33*). The samples were measured at one time. The intraassay coefficients of variation (CVs) for LH and FSH were 4.7 and 5.3%, respectively.

A previously described RIA procedure was used to determine serum testosterone concentration in duplicate samples *(34)*. The testosterone antiserum GND S250, generously supplied by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO) was used in this assay; the crossreactivity of this antiserum has been reported *(35)*. All serum samples were measured in the same assay. The intrassay CV was 8.6%.

RNA Extraction, Northern Blots, and Probes Used in Hybridization.

Total RNA was isolated from pituitary gland and testis according to the methods of Chomczynski and Sacchi (*36*). Aliquots of total RNA (5 μg) per pituitary gland or equal amount (0.2%) from the pituitary gland were electrophoresed through a denaturing 1.2% agaraose gel and subsequently blotted by capillary action to a nylon filter. Each pituitary blot was sequentially hybridized with LH and FSH probes (generously supplied by Joseph L. Roberts and Richard Maurer, respectively), using conditions previously described *(37)*. After hybridization with LHβ and FSHβ, the membranes were stripped and reprobed with a synthetic oligonucleotide probe (DNA Express, Fort Collins, CO) recognizing the 18S rRNA sequence. Autoradiographs were standardized to account for differences in loading by the 18S rRNA signal. The relative amount of each of the gene transcripts was determined by densitometric scanning of each autoradiograph using an imaging densitometer (model GS-670; Bio-Rad, Hercules, CA). The amount of RNA in each lane (5 μg by OD_{260}) was internally standardized within a blot by determining the amount of 18S rRNA signal and then correcting it for reported LHβ and FSHβ mRNA levels.

Statistical Analyses

One-way analysis of variance was used to detect significant treatment effects among groups. Scheffe's test was used to identify differences among groups (*38*). The level of statistical significance was $P < 0.05$. The data are reported as means ± SEM.

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