

## Dietary Genistein Exerts Estrogenic Effects upon the Uterus, Mammary Gland and the Hypothalamic/Pituitary Axis in Rats<sup>1</sup>

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**ABSTRACT** These studies were undertaken to assess the estrogenic and antiestrogenic effects of dietary genistein. To determine estrogenic effects, genistein was mixed into a modified AIN-76 or AIN-93G semipurified diet at 0 (negative control), 150, 375 or 750  $\mu\text{g/g}$  and 17,  $\beta$ -estradiol at 1.0  $\mu\text{g/g}$  and fed to ovariectomized 70-d-old Sprague-Dawley rats. Estrogenic potency was determined by analyzing uterine weight, mammary gland development, plasma prolactin and expression of uterine c-fos. Dietary genistein (375 and 750  $\mu\text{g/g}$ ) increased uterine wet and dry weights ( $P < 0.05$ ). Mammary gland regression following ovariectomy was significantly inhibited by dietary genistein at 750  $\mu\text{g/g}$  ( $P < 0.05$ ). Plasma prolactin was significantly greater in ovariectomized rats fed genistein (750  $\mu\text{g/g}$ ) compared with comparable rats not receiving genistein. The relative binding affinity of genistein to the estrogen receptor (ER) was  $\sim 0.01$  that of estradiol. Genistein (750  $\mu\text{g/g}$ ) induced the uterine expression of c-fos. To evaluate potential antiestrogenic effects, genistein and estradiol were mixed into the modified AIN diets at the doses noted above and fed to ovariectomized rats. Dietary genistein (375 or 750  $\mu\text{g/g}$ ) did not inhibit the effects of estradiol on uterine weight, mammary gland development or plasma prolactin. Serum concentration of total genistein (conjugated plus free) in rats fed 750  $\mu\text{g/g}$  was 2.2  $\mu\text{mol/L}$  and free genistein was 0.4  $\mu\text{mol/L}$ . Administration of dietary genistein at 750  $\mu\text{g/g}$  can exert estrogenic effects in the uterus, mammary gland and hypothalamic/pituitary axis. Dietary genistein (750  $\mu\text{g/g}$ ) did not antagonize the action of estradiol in estradiol-supplemented ovariectomized rats or in intact rats. J. Nutr. 127: 263–269, 1997.

**KEY WORDS:** • rats • genistein • uterus • mammary gland • prolactin

Phytoestrogens include the isoflavones, lignans and other nonsteroidal chemicals found in plants and plant products. These compounds can bind to the estrogen receptor and are thought to exert their estrogenic effects through mechanisms similar to those of estradiol.

The consumption of certain plants and plant products can result in impaired reproductive function in livestock. Over five decades ago, clover disease, a syndrome with effects ranging from temporary to permanent infertility, was described in sheep foraging upon subterranean clover in western Australia (Bennets et al. 1946). Additional studies have documented impaired reproductive function in a number of species (reviewed in Price and Fenwick 1985) including desert quail feeding upon desert brush (Leopold et al. 1976). Furthermore, a decrease in reproductive performance was observed in female rats fed either a soy-based diet or a diet supplemented with genistin at 2 g/kg diet (Carter et al. 1955), and in male mice fed genistin at 36 mg/(mouse  $\cdot$  d) (Matrone et al. 1955). All of these effects were attributed to the phytoestrogen content of the diets.

It was later discovered that genistein was responsible for the impaired reproductive performance seen in sheep ingesting

subterranean clover (Bradbury and White 1951). Genistein is an isoflavone (4',5,7-trihydroxyisoflavone), which has estrogenic activity (Folman and Pope 1966), present in various plants including soybeans (Naim et al. 1974). Since the initial discovery of its estrogenic activity, there have been a number of studies in which the effects of soy and genistein on the uterus of mice and rats were evaluated (Carter et al. 1953): all of these studies demonstrated estrogenic effects except the work of Farmakalidis and Murphy (1984). In their study, the potent estrogen agonist diethylstilbestrol also did not promote uterotrophic effects in this strain of mouse (CD-1).

The estrogenic effects of genistein in the uterus are well documented. However, few experiments have been conducted to assess estrogenic effects in other tissues, and to our knowledge, none have examined the effects of dietary genistein on the mammary gland or the hypothalamic/pituitary axis. These studies were undertaken to provide additional insight into the actions of dietary genistein by analyzing its effects on the uterus, pituitary gland and mammary gland. In addition, the plasma concentration of genistein responsible for these estrogenic effects was also determined.

### MATERIALS AND METHODS

**Chemicals.** Genistein was synthesized by the demethylation of biochanin A or from organic precursors as described by Chang et al. (1994). In both processes, chemical identity was assessed by nuclear

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magnetic resonance (NMR)<sup>3</sup> and purity assessed at >98%. <sup>3</sup>H-17,  $\beta$ -Estradiol (3.59 TBq/mmol) and <sup>32</sup>P-deoxycytidine 5'-triphosphate, tetra(triethylammonium) salt (111 TBq/mmol) were purchased from Dupont New England Nuclear (Boston, MA). All other chemicals, unless otherwise specified, were purchased from Sigma, St. Louis, MO.

**Animals.** All rats were maintained according to guidelines in the *Guide for the Care and Use of Laboratory Animals* (NRC 1985). Intact and ovariectomized 60-d-old Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing from 200 to 225 g were used in Experiments 1 and 2. In Experiment 3, intact 30-d-old rats weighing from 80 to 90 g were used. Upon receipt, rats were weighed and sorted to equalize animal weights within each treatment group. Unless otherwise noted, rats were acclimated to the animal care facility and diet for 7 d prior to initiating the studies. Rats were maintained in the animal care facility with temperature  $22 \pm 2^\circ\text{C}$ , relative humidity (40–70%), and a 12-h light:dark cycle. Rats were housed in polycarbonate cages (3 rats/cage) with aspen woodchip bedding and were allowed unrestricted access to food and water.

**Diets.** Animals were fed the American Institute of Nutrition-93G (AIN-93G) or modified AIN-76 diets prepared in our facilities. For the AIN-76 diet cornstarch was substituted for sucrose to lower the sucrose concentration from 50 g/100 g to 10 g/100 g of the diet. Semipurified diets are required because the potential presence of genistein and other phytoestrogens in the soy portion of commercial nonpurified diets could confound the experimental results. Genistein and 17,  $\beta$ -estradiol were mixed into the AIN-76 or AIN-93G diets at the concentrations specified in Experiments 1, 2 and 3.

**Experiment 1: estrogenic effects of dietary genistein and estradiol.** Forty-two rats were ovariectomized at 56 d of age and dietary treatments were begun at 70 d of age. The ovariectomized rats (6/treatment) were given free access to food and water for a period of 5 d. Dietary treatments consisted of genistein at 150, 375 and 750  $\mu\text{g/g}$  or estradiol at 0.5, 1.0 and 1.5  $\mu\text{g/g}$ . Eight intact and six ovariectomized 70-d-old rats were fed a modified AIN-76 diet and served as positive and negative controls, respectively. Estrogenic activity was assessed by uterine wet and dry weights.

**Experiment 2: anti-estrogenic activity of dietary genistein.** Forty-six rats were ovariectomized at 56 d of age and fed the modified AIN-76 diet until 70 d of age at which time dietary treatments were begun. Rats, six per group, were given free access to food and water for a period of 21 d. Dietary treatments included estradiol at 1.0  $\mu\text{g/g}$ , genistein at 750  $\mu\text{g/g}$ , genistein at 150, 375 and 750  $\mu\text{g/g}$  plus estradiol at 1.0  $\mu\text{g/g}$  and an untreated control group. Ten ovariectomized rats were killed at the start of the experiment to obtain baseline values for uterine weight and mammary gland development. Estrogenic activity was assessed by comparing uterine weight, mammary gland development, plasma prolactin and uterine expression of an estrogen responsive gene, *c-fos*, to those of ovariectomized, untreated controls. Antiestrogenic activity of genistein was assessed by comparing the uterine weight, mammary development and plasma prolactin of rats consuming diets with varying combinations of genistein and estradiol to the rats fed the diet containing 1.0  $\mu\text{g/g}$  estradiol.

**Experiment 3: effects of dietary genistein on the development of the mammary gland and uterus of intact rats.** Thirty-four 30-d-old rats were fed the AIN-93G diet for 1 d and treatments began on the second day. Rats, eight per group, were given free access to food and water for a period of 14 d. Dietary treatments consisted of genistein at 375 and 750  $\mu\text{g/g}$ . Eight rats fed the AIN-93G diet without genistein served as positive controls. Ten rats were killed at the start of the experiment to establish base-line values for uterine weight and mammary gland development.

**Analysis of uteri.** Rats were weighed, anesthetized by CO<sub>2</sub> exposure and then killed by cervical dislocation. Uteri were excised, trimmed of fat and connective tissue, weighed and immediately placed in liquid nitrogen for later RNA analysis. Wet uterine weight was determined in all experiments. In Experiment 1, dry weight was also

determined for each uterus by incubating approximately one-half of the uterus at 100°C for 16 h.

**Analysis of mammary glands.** Rats were weighed, anesthetized by CO<sub>2</sub> exposure and then killed by cervical dislocation. Mammary glands were prepared according to the procedure of Banerjee et al. (1976). The inguinal mammary gland was excised, fixed in a 3:1 (v/v) solution of ethanol:acetic acid for 2 h, transferred to 70% ethanol for 3 d with ethanol changed each day, rinsed in water and stained in alum-carmin for 16 h. The glands were then rinsed in water, placed in 70, 95 and 100% ethanol for 30 min each, transferred to toluene for 24 h for clarification and then stored in absolute methyl salicylate. Lobulo-alveolar and ductal structure (extent of side branching) were assessed using a dissecting microscope and assigned values from 0 to 4, with the higher number representing maximal development. Analyses were performed twice on each gland with the identity of the glands unknown to the analyst.

**Northern blot analysis of *c-fos*.** Total RNA was isolated by the procedure of Chirgwin et al. (1979) as modified by Helfferich et al. (1990). Uteri from four rats in each group (randomly selected from the following groups: ovariectomized control, 1  $\mu\text{g/g}$  estradiol and 750  $\mu\text{g/g}$  genistein) were removed from liquid nitrogen, placed in 4.0 mL of 4.0 mol/L guanidinium thiocyanate (GITC) and homogenized on ice with a polytron (Brinkman Instruments, Westbury, NY). *N*-Lauryl sarcosyl was added (100  $\mu\text{L}$  10% solution per milliliter GITC), the mixture vortexed and cellular debris removed by centrifugation at  $12,000 \times g$  (g max) for 10 min. The supernatant was removed and layered over 1.0 mL of 5.7 mol/L CsCl followed by centrifugation at  $110,000 \times g$  (g max) for 16 h. The pellet was resuspended in 7 mol/L guanidine-HCl, 20 mmol/L sodium acetate, 1 mmol/L dithiothreitol, 10 mmol/L iodoacetic acid and 1 mmol/L Na<sub>2</sub>EDTA and transferred to a 1.5-mL microfuge tube. RNA was precipitated with 2 volumes absolute ethanol and 0.1 volume 300 mmol/L sodium acetate at  $-20^\circ\text{C}$ . RNA precipitates were washed once with 3 mol/L sodium acetate and 10 mmol/L iodoacetate, pH 5.0 at  $4^\circ\text{C}$ , then with 66% ethanol and 33 mmol/L sodium acetate, pH 5.0, and then with absolute ethanol at  $-20^\circ\text{C}$ . Ethanol was removed and the RNA pellet dissolved in Tris-EDTA (pH 8.0) and spectrophotometrically quantified at 260/280 nm. Ten micrograms of RNA was loaded onto a 1.2% agarose formaldehyde denaturing gel and electrophoresed for 8 h at 35 V. The RNA was transferred to membrane (Hybond-N, Amersham Life Sciences, Cleveland, OH), and the membrane exposed to UV light at 120 kJ/cm<sup>2</sup> for 2 min. Rat *c-fos* cDNA, 2100 bp, was kindly provided by Tom Curran (Roche Institute of Molecular Biology, Nutley, NJ) and probes made by random priming (Boehringer Mannheim, Indianapolis, IN) with incorporation of <sup>32</sup>P-deoxycytidine 5'-triphosphate. The membrane was blocked by prehybridizing with herring sperm DNA at  $42^\circ\text{C}$  for 4 h followed by hybridization for 12 h and subsequent washing at  $65^\circ\text{C}$  with 2X saline sodium citrate buffer (SSC) + 0.1% sodium dodecyl sulfate (SDS) for 30 min each, then 0.3X SSC + 0.1% SDS for 25 min. The hybridized membrane was exposed to Kodak X-OMAT film at  $-70^\circ\text{C}$  for 3 d and then developed.

**Plasma prolactin analysis.** Plasma prolactin concentration was determined in the laboratory of Keith Lookingland at Michigan State University utilizing a double antibody RIA employing reagents and procedures of the National Institute for Diabetes and Digestive and Kidney Disease (NIDDK) assay kit (generously supplied by A. F. Parlow and S. Ratti, National Hormone and Pituitary Program, Rockville, MD). NIDDK rat PRL RP-3 was used as the standard. Using a 100- $\mu\text{L}$  aliquot of plasma, the lower limit of sensitivity was 10.0  $\mu\text{g/L}$ . Samples from each rat were assayed in duplicate at two different dilutions in a single radioimmunoassay.

**Competitive binding analysis.** Competitive binding analysis with <sup>3</sup>H-estradiol was performed using uterine cytosol prepared from untreated rats consuming a nonpurified diet (22/5 Rodent Diet (w) 8640, Harlan-Teklad, Madison, WI). Rat uteri were placed in ice-cold TEDG (10.0 mmol/L tris-HCl pH 7.4, 1.5 mmol/L EDTA pH 7.4, 1.0 mmol/L dithiothreitol added fresh, and 10% glycerol) and homogenized with a polytron (Brinkman Instruments). The homogenate was centrifuged at  $800 \times g$  for 10 min, and the resulting supernatant removed and centrifuged at  $110,000 \times g$  (g max) for 1.5 h at  $4^\circ\text{C}$ . Protein concentration was 5.0 g/L as determined by the Bradford assay (Bradford 1976). Aliquots were quickly frozen in a isopropanol/

<sup>3</sup> Abbreviations used: DCC, dextran-coated charcoal; ER, estrogen receptor; GITC, guanidinium thiocyanate; NIDDK, National Institute for Diabetes and Digestive and Kidney Disease; NMR, nuclear magnetic resonance; TEDG, tris-HCl, EDTA, dithiothreitol, glycerol.

dry-ice bath and stored at  $-70^{\circ}\text{C}$ . Binding assays were composed of 200  $\mu\text{L}$  uterine cytosol (1.0 mg total protein), TEDG, and genistein or estradiol in ethanol vehicle bringing the total volume to 500  $\mu\text{L}$ . Assays containing 5.0 nmol/L  $^3\text{H}$ -estradiol and 0–20 nmol/L 17, $\beta$ -estradiol or 0–50  $\mu\text{mol/L}$  genistein were incubated at  $4^{\circ}\text{C}$  for 3 h. After incubation, the contents were removed and placed in a microfuge tube containing the pellet from 250  $\mu\text{L}$  of dextran-coated charcoal (DCC) solution (5% Norit A, 0.5% dextran T-70 in TEDG) to remove the unbound  $^3\text{H}$ -estradiol. The microfuge tube was vortexed to disperse the DCC pellet and incubated for 3 min at  $23^{\circ}\text{C}$  followed by centrifugation at  $13,000 \times g$  to pellet the DCC. Two 200- $\mu\text{L}$  aliquots of the supernatant were collected and counted on a scintillation counter (Beckman Instruments Model LS100, Fullerton, CA). The counts were averaged, divided by the total counts and expressed as a percentage of the total radioactivity.

**Serum genistein analysis.** Rats were anesthetized with diethyl ether, and blood ( $\sim 6$  mL/rat) was collected from the tail artery. Blood was placed at  $4^{\circ}\text{C}$  for 16 h to allow clotting. The blood was then centrifuged at  $350 \times g$  for 10 min and the serum removed and stored at  $-70^{\circ}\text{C}$ . For genistein analysis, 50  $\mu\text{L}$  of serum, from each of four rats fed 750  $\mu\text{g/g}$  genistein in Experiment 2, was sampled in duplicate with one set receiving 5  $\mu\text{L}$  (515 units) of B-glucuronidase Type H-1 (Sigma). All aliquots were incubated in 0.5-mL microfuge tubes at  $37^{\circ}\text{C}$  for 48 h. Following the incubation, 50  $\mu\text{L}$  of absolute methanol was added to each tube, the tubes vortexed and then centrifuged at  $15,000 \times g$  for 10 min. Approximately 75  $\mu\text{L}$  was removed and placed at  $-20^{\circ}\text{C}$  until analysis. For analysis, the samples were centrifuged at  $15,000 \times g$  for 15 min and 20  $\mu\text{L}$  injected onto a  $\text{C}_{18}$  column (Microsorb-MV, 5  $\mu\text{m}$  100A, Rainin Instrument, Woburn, MA) with a flow rate of 1.0 mL/min of 50:50 methanol:water, with 17 mmol/L acetic acid. Recovery was determined by spiking serum from control rats with genistein and then assessing recovery of genistein. Mean recoveries were determined to be 74% (SEM 1.68%). The data presented are corrected for recovery. No genistein was detected in control rats fed the AIN-76A diet.

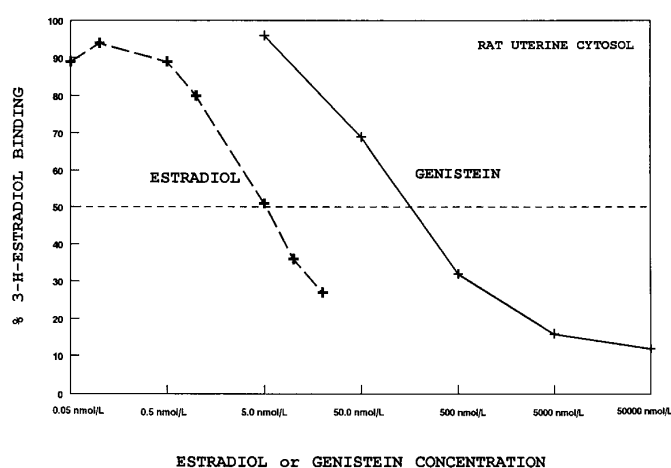
**Statistical analyses.** All statistical tests were performed using a PC-based version of the Statistical Program for the Social Sciences (SPSS) Version SPSS/PC 2.0, Chicago, IL 60611. Uterine weight and plasma prolactin data were analyzed by one-way ANOVA. Variances in uterine weights [Tables 1 and 2] were nonhomogeneous with respect to treatment; thus these data were log transformed prior to ANOVA. Data are means and standard errors before transformation. When a significant ( $P < 0.05$ ) treatment effect was detected, treatment means were compared using the least significant difference method (Steel and Torrie 1980). Mammary gland data were analyzed by the Kruskal-Wallis nonparametric test (Shavelson 1988). When a significant ( $P < 0.05$ ) treatment effect was detected, treatment rank means were compared using the least significant difference method (Steel and Torrie 1980). Values in the text are means  $\pm$  SEM.

## RESULTS

**Competitive binding analysis.** Competitive binding studies utilizing rat uterine cytosol,  $^3\text{H}$ -estradiol, unlabeled estradiol and genistein were performed to determine the relative binding affinity of genistein to the estrogen receptor. The concentration of unlabeled 17, $\beta$ -estradiol required to displace 50% of the bound  $^3\text{H}$ -17, $\beta$ -estradiol in rat uterine cytosol in this study was  $\sim 5$  nmol/L (Fig. 1). Competitive binding analysis indicated that the relative binding affinity of genistein was  $\sim 0.01$  that of 17, $\beta$ -estradiol.

**Uterotrophic effect of dietary genistein.** Genistein administered in the diet to ovariectomized adult rats in Experiment 1 at 150, 375 and 750  $\mu\text{g/g}$  produced a dose-dependent increase in both uterine wet and dry weights (Table 1). In rats fed genistein at 375 and 750  $\mu\text{g/g}$ , significantly greater uterine wet weights and dry weights than those in the control group were measured. Rats fed 750  $\mu\text{g/g}$  genistein had uterine weights similar to those of rats fed 1.0  $\mu\text{g/g}$  estradiol.

In Experiment 2, the potential of genistein to antagonize



**FIGURE 1** Competitive binding analysis of genistein and estradiol using rat uterine cytosol. Genistein or 17,  $\beta$ -estradiol and  $^3\text{H}$ -estradiol were incubated with rat uterine cytosol for 2 h. Following incubation, free compounds were removed with dextran-coated charcoal and aliquots counted in a Beckman LS-100 scintillation counter. Each point represents the mean of two replicates. Values are expressed as a percentage of the total radioactivity.

the uterotrophic effect of 1.0  $\mu\text{g/g}$  17, $\beta$ -estradiol was evaluated by comparing uterine weights of the 17, $\beta$ -estradiol treated group with those of the groups receiving 17, $\beta$ -estradiol plus genistein. Uterine weights in the base-line group (rats killed at the start of dietary treatment, 14 d after ovariectomy) indicated that substantial regression had occurred (Table 2) when compared with intact rats at the same age used in Experiment 1 (Table 1). Rats receiving genistein and estradiol at all doses had significantly greater uterine weights than those in the control and base-line groups (Table 2). Genistein at 150, 375 or 750  $\mu\text{g/g}$  did not inhibit the uterotrophic effect of concurrently administered 17, $\beta$ -estradiol (Table 2).

In Experiment 3, the effect of genistein on the development of the uterus of intact immature rats was evaluated. Ten rats

**TABLE 1**

*Effect of dietary genistein and estradiol on uterine weight in ovariectomized mature rats<sup>1</sup>*

Treatment	n	Uterine wet weight	Uterine dry weight
mg			
Control <sup>2</sup>	8	76.5 $\pm$ 3.2 <sup>a</sup>	20.7 $\pm$ 1.1 <sup>a</sup>
Intact <sup>3</sup>	6	386.6 $\pm$ 41.1 <sup>e</sup>	73.7 $\pm$ 6.5 <sup>d</sup>
Estradiol, $\mu\text{g/g}$			
0.5	6	122.1 $\pm$ 6.6 <sup>b</sup>	17.6 $\pm$ 1.7 <sup>a</sup>
1.0	6	194.8 $\pm$ 8.8 <sup>c</sup>	40.4 $\pm$ 6.0 <sup>c</sup>
1.5	6	255.0 $\pm$ 8.8 <sup>d</sup>	54.0 $\pm$ 2.2 <sup>d</sup>
Genistein, $\mu\text{g/g}$			
150	6	92.4 $\pm$ 2.6 <sup>a</sup>	28.0 $\pm$ 2.8 <sup>b</sup>
375	6	135.6 $\pm$ 9.8 <sup>b</sup>	30.0 $\pm$ 3.6 <sup>bc</sup>
750	6	189.3 $\pm$ 26.6 <sup>c</sup>	39.0 $\pm$ 5.6 <sup>bc</sup>

<sup>1</sup> Values are means  $\pm$  SEM. The experimental groups included a negative control (control), positive control (intact), genistein 150, 375 and 750  $\mu\text{g/g}$ , estradiol 0.5, 1.0 and 1.5  $\mu\text{g/g}$ . Values in a column with different superscripts are significantly different ( $P < 0.05$ ). ANOVA was performed on log-transformed data followed by multiple means comparison using the least significant difference method.

<sup>2</sup> Controls were ovariectomized, nontreated rats.

<sup>3</sup> Intact rats were not ovariectomized.

TABLE 2

Effect of dietary genistein and estradiol on uterine weight and plasma prolactin in adult ovariectomized rats<sup>1</sup>

Treatment	n	Uterine weight	Plasma prolactin
		mg	μg/L
Baseline <sup>2</sup>	10	130.7 ± 5.5 <sup>b</sup>	—
Control <sup>4</sup>	6	96.5 ± 3.9 <sup>a</sup>	5.68 ± 1.05 <sup>a</sup>
Gen 750 μg/g	6	343.6 ± 24.3 <sup>d</sup>	12.0 ± 2.73 <sup>b</sup>
E <sub>2</sub> 1.0 μg/g	6	220.1 ± 17.3 <sup>c</sup>	16.0 ± 2.53 <sup>b</sup>
E <sub>2</sub> 1.0 + Gen 150	6	241.3 ± 24.9 <sup>c</sup>	—
E <sub>2</sub> 1.0 + Gen 375	6	312.4 ± 13.4 <sup>d</sup>	16.7 ± 1.52 <sup>b</sup>
E <sub>2</sub> 1.0 + Gen 750	6	305.6 ± 24.3 <sup>d</sup>	18.8 ± 2.33 <sup>b</sup>

<sup>1</sup> Values are means ± SEM. The experimental groups included an ovariectomized control, baseline, genistein (Gen) 750 μg/g, estradiol (E<sub>2</sub>) 1 μg/g, E<sub>2</sub> 1 μg/g + 150 μg/g Gen, E<sub>2</sub> 1 μg/g + 375 μg/g Gen, and E<sub>2</sub> 1 μg/g + 750 μg/g Gen. Values in a column with different superscripts are significantly different ( $P < 0.05$ ). ANOVA was performed on log-transformed uterine weight data followed by multiple means comparison using the least significant difference method.

<sup>2</sup> Baseline rats were killed at the beginning of dietary treatment.

<sup>3</sup> Plasma prolactin was not determined in the baseline and E<sub>2</sub> + Gen 150 treated rats. Uterine weights in these groups did not differ.

<sup>4</sup> Control rats were ovariectomized, nontreated.

were killed at the beginning of the study to obtain base-line data for uterine weights. Baseline uterine weights were similar to the uterine weights of the ovariectomized rats observed in Experiments 1 and 2, thereby confirming the immaturity of the rats in this study. Dietary genistein administered at either 375 or 750 μg/g for 14 d had no effect on the development of the uterus as indicated by uterine weight, relative to the control, intact rats (Table 3).

**Induction of uterine c-fos.** Uterine tissue from ovariectomized rats administered 750 μg/g dietary genistein or 1.0 μg/g 17,β-estradiol and untreated controls were analyzed for the presence of c-fos mRNA (Fig. 2). Gels were stained with ethidium bromide to confirm equal loading of RNA and to assess the integrity of the RNA (data not shown). Both dietary genistein and estradiol induced the expression of c-fos mRNA relative to that of the untreated control rats.

**Mammatrophic effect of dietary genistein.** In Experiment 2, the mammatrophic effects of dietary genistein were evaluated in ovariectomized rats by analyzing the following two

TABLE 3

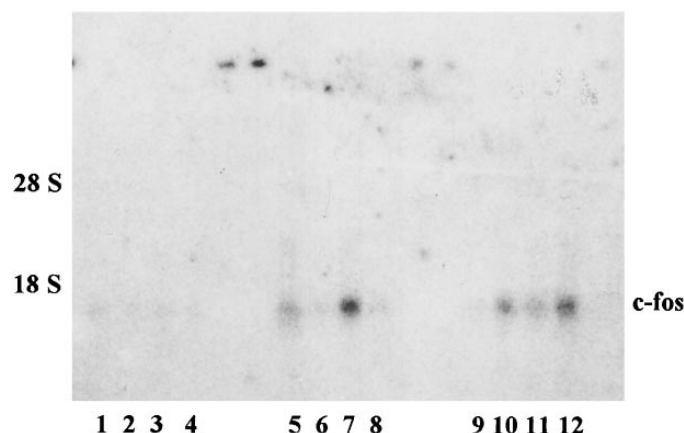
Effect of dietary genistein on uterine weight of intact immature rats<sup>1</sup>

Treatment	n	Uterine weight
		mg
Baseline <sup>2</sup>	8	99.1 ± 14.2 <sup>a</sup>
Control <sup>3</sup>	8	230.7 ± 17.9 <sup>b</sup>
Genistein 375 μg/g	8	243.2 ± 10.8 <sup>b</sup>
Genistein 750 μg/g	8	234.4 ± 13.8 <sup>b</sup>

<sup>1</sup> Values are means ± SEM. The experimental groups included baseline, genistein 375 μg/g, genistein 750 μg/g and control. Values in a column with different superscripts are significantly different ( $P < 0.05$ ). ANOVA was performed on log-transformed data followed by multiple means comparison using the least significant difference method.

<sup>2</sup> Baseline rats were killed at the start of dietary treatment.

<sup>3</sup> Control rats were not treated.



**FIGURE 2** Effect of dietary genistein and estradiol on the uterine expression of c-fos in ovariectomized mature rats. Total RNA was prepared from four rats randomly selected from each of the following treatment groups: lanes 1–4 control, 5–8 750 μg/g genistein and 9–12 1.0 μg/g 17, β-estradiol. Ten micrograms of total RNA was electrophoresed on a 1.2% denaturing agarose gel, transferred to nylon and hybridized with random primed rat c-fos DNA probe labeled with <sup>32</sup>P. The film was exposed for 3 d.

criteria: 1) lobulo-alveolar structure and 2) ductal structure including side branching and infiltration of ducts into the fat pad of the mammary gland. Dietary treatment of ovariectomized rats for 21 d with 750 μg/g genistein prevented mammary gland regression, seen primarily in lobulo-alveolar structure, relative to that of the ovariectomized, untreated control rats (Table 4 and Fig. 3). Average lobulo-alveolar development in the 17,β-estradiol-treated rats did not differ from controls. Average ductal development did not differ for the genistein- or estradiol-treated groups compared with the controls. Lobulo-alveolar development was significantly greater for the groups receiving 750 μg/g genistein or genistein 750 μg/g + 17,β-estradiol 1.0 μg/g, compared with the control group. The potential of genistein to antagonize the mammatrophic effect of estradiol was also evaluated. Coadministration of dietary genistein at 150, 375 or 750 μg/g, with 1.0 μg/g 17,β-estradiol, did not result in lower mammary scores compared with rats receiving 17,β-estradiol alone (Table 4).

In Experiment 3, the effect of genistein on the mammary gland in immature rats was evaluated. Ten rats were killed at the beginning of the study to obtain base-line data for mammary gland development. Dietary genistein had no effect on the development of the mammary gland, as assessed by lobulo-alveolar and ductal development, relative to the control untreated intact rats (data not shown).

**Plasma prolactin analysis.** The effect of dietary genistein on plasma prolactin in ovariectomized rats was determined in Experiment 2. Plasma prolactin was significantly higher in the dietary genistein- and estradiol-treated rats relative to the control group (Table 2). Genistein coadministered with estradiol did not stimulate or inhibit the effects of estradiol.

**Serum genistein analysis.** The serum concentration of genistein was assessed in four rats from Experiment 2 to determine the concentration present in rats fed 750 μg/g genistein. Total genistein (conjugated plus free) concentration was  $2.2 \pm 0.01$  μmol/L and the free concentration of genistein was  $0.4 \pm 0.03$  μmol/L. Efficiency of recovery was determined by quantifying recovery of a genistein spike from control serum. Average recoveries were  $74 \pm 1.68\%$ .

TABLE 4

*Effect of dietary genistein and estradiol on the mammary gland of ovariectomized adult rats<sup>1</sup>*

Treatment	n	Mean raw score		Mean rank score	
		Lob/av <sup>2</sup>	Duct	Lob/av	Duct
Baseline <sup>3</sup>	10	2.27	2.89	35.65 <sup>c</sup>	34.35 <sup>c</sup>
Control <sup>4</sup>	6	1.38	2.29	11.75 <sup>ab</sup>	22.83 <sup>abc</sup>
Gen 750 $\mu\text{g/g}$	6	3.42	2.50	34.42 <sup>c</sup>	27.08 <sup>bc</sup>
E <sub>2</sub> 1.0 $\mu\text{g/g}$	6	0.96	1.63	7.83 <sup>a</sup>	11.83 <sup>a</sup>
E <sub>2</sub> 1.0 + Gen 150	6	1.86	1.92	16.58 <sup>ab</sup>	13.42 <sup>a</sup>
E <sub>2</sub> 1.0 + Gen 375	6	1.83	1.72	18.75 <sup>b</sup>	19.17 <sup>ab</sup>
E <sub>2</sub> 1.0 + Gen 750	6	3.21	2.63	31.25 <sup>c</sup>	28.58 <sup>bc</sup>

<sup>1</sup> The experimental groups included an ovariectomized control, base-line, genistein (Gen) 750  $\mu\text{g/g}$ , estradiol (E<sub>2</sub>) 1  $\mu\text{g/g}$ , E<sub>2</sub> 1  $\mu\text{g/g}$  + 150  $\mu\text{g/g}$  Gen, E<sub>2</sub> 1  $\mu\text{g/g}$  + 375  $\mu\text{g/g}$  Gen, and E<sub>2</sub> 1  $\mu\text{g/g}$  + 750  $\mu\text{g/g}$  Gen. Values in a column with different superscripts are significantly different ( $P < 0.05$ ). Data were ranked and analyzed with Kruskal-Wallis nonparametric ANOVA. Raw mean and rank mean scores are included in the table. When a significant treatment effect was found, the rank means were compared with the least significant difference method.

<sup>2</sup> Lob/Av = lobular/aveolar.

<sup>3</sup> Baseline rats were killed at the start of dietary treatment.

<sup>4</sup> Control rats were ovariectomized, nontreated.

## DISCUSSION

**Competitive binding analysis and induction of c-fos.** Competitive binding analysis demonstrated that the relative binding affinity of genistein for the rat uterine estrogen receptor (ER) was  $\sim 0.01$  that of estradiol. The binding of a compound to a receptor does not necessarily result in the production of a complex capable of initiating the biological response; therefore, additional studies were undertaken to ascertain whether dietary administration of genistein would induce the expression of an estrogen-responsive gene, c-fos (Weisz and Rosales 1990), in an estrogen-responsive tissue. Uterine expression of c-fos was induced in ovariectomized rats following the dietary administration of genistein or 17 $\beta$ -estradiol. The variable expression of c-fos may be due to several factors, including the following: 1) the timing of food consumption, 2) variability in food consumption, 3) metabolism of genistein, and 4) the short half-life of c-fos mRNA (Greenburg and Ziff 1984). The induction of c-fos by genistein suggests that genistein is acting through the ER, similar to estradiol, and is capable of forming an active complex with the ER in uterine tissue.

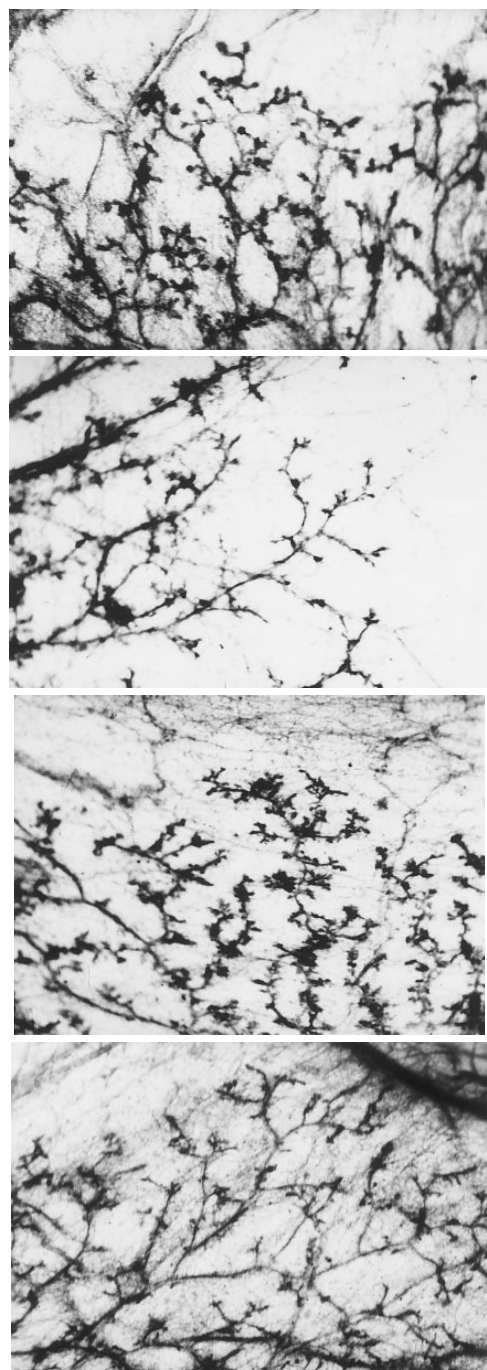
**Uterotrophic effects of dietary genistein.** Phytoestrogens have long been recognized for their uterotrophic activity in a variety of animal species. These effects range from temporary to permanent infertility (reviewed by Adams 1989). In the present study, there was a dose-dependent increase in uterine weight with effects seen at a dietary dose of genistein as low as 375  $\mu\text{g/g}$  diet, suggesting that genistein acts in the uterus in a manner similar to that of estradiol. That is, genistein binds to the ER, and the ligand:receptor complex induces the expression of estrogen-responsive genes which ultimately result in increased uterine mass.

Genistein competes with estradiol binding to the ER and has shown estrogenic effects in estrogen-responsive tissues. Many antiestrogens, including tamoxifen, possess agonistic properties when administered in low amounts; however, in higher concentrations, they are antagonists (Martinez-Campos et al. 1986). As a weak agonist, genistein also has the potential to antagonize the effects of estradiol. Effects of antagonistic properties have been reported in mice coadministered subcutaneous injections of genistein and estrone. Folman and Pope (1966) inhibited the uterotrophic effect induced by subcutaneous injections in mice of 0.4  $\mu\text{g}$  estrone (total dose) by administering concurrent subcutaneous injections of either 800 or 1600  $\mu\text{g}$  genistein (total dose) twice daily over a 3-d period.

In our study, the coadministration of 150, 375 or 750  $\mu\text{g/g}$  genistein with 1.0  $\mu\text{g/g}$  17 $\beta$ -estradiol did not inhibit the effects of estradiol on uterine weight. In addition, dietary genistein did not affect the development of the uterus, when administered to immature rats, as assessed by uterine weight during maturation of the organ. In the studies reported here, genistein was administered in the diet to rats, whereas in the study by Folman and Pope (1966), genistein was administered subcutaneously to mice. The dose of genistein administered in our studies, relative to the dose of estradiol, was much lower than that of Folman and Pope. Furthermore, variability in species as well as strain in response to compounds with estrogenic activity is well documented (reviewed by Adams 1989, Farmakalidis and Murphy 1984). The amount of genistein absorbed from the gut is currently unknown. All of these variables could account for the different results obtained in this study compared with that of Folman and Pope.

**Effects of dietary genistein on the mammary gland and plasma prolactin.** Dietary genistein consistently elicits an estrogenic response in the uterus of ovariectomized and immature rodents; however, the effect of dietary genistein on the mammary gland, another estrogen-responsive tissue, has not been assessed. Increased differentiation of the mammary gland has been observed in prepubertal rats following the subcutaneous injection of 5 mg genistein per rat on d 2, 4 and 6 postpartum (Lamartiniere et al. 1995). The model employed in these studies utilized 60-d-old rats and assessed the ability of dietary genistein and/or estradiol to inhibit mammary gland regression postovariectomy.

Development and maintenance of the mammary gland in rats are controlled by many factors including estrogen, progesterone, growth hormone and prolactin (reviewed by Topper and Freeman 1980). Estrogen acts directly at the mammary gland by inducing gene transcription and the subsequent translation of many proteins, including the progesterone receptor required for progesterone action. Estrogen also acts indirectly through the induced synthesis and release of prolactin from the anterior pituitary gland which then exerts its mitogenic effects on the mammary gland. Removal of endogenous estrogen results in regression of the mammary gland, particularly the lobulo-alveolar structures. Mammary gland regression at 35 d postovariectomy in untreated control rats was greater than that in the untreated base-line rats measured at 14 d postovariectomy (the start of dietary treatment). Dietary gen-



**FIGURE 3** Photographs of the mammary glands from rats treated with dietary genistein and estradiol. Mammary glands were dissected from the rats and stained with alum-carmin. Photographs were taken through a dissecting microscope under 43X magnification. Photograph a is from the base-line group (killed at the start of dietary treatments), b from the control group (ovariectomized nontreated), c from the genistein 750  $\mu\text{g/g}$  treatment group, and d from the 1.0  $\mu\text{g/g}$  estradiol treatment group.

istein prevented regression of the mammary gland compared with the untreated controls. Our goal in this set of experiments was to evaluate whether the concurrent administration of genistein at different doses would inhibit the effects of estradiol in preventing regression of the mammary gland. Orally administered estradiol (1  $\mu\text{g/g}$ ) was effective in increasing uterine weight and plasma prolactin; however, it was ineffective in the

mammary gland. The reason dietary estradiol exerts estrogenic effects in the uterus and not in the mammary gland is unclear. The ability of genistein to inhibit the effects of estradiol in the mammary gland could not be determined with the dose of estradiol used in this study.

It is unlikely that genistein alone is directly responsible for the effects observed in the mammary gland. Maintenance of the mammary gland in rats is also dependent on prolactin. Prolactin synthesis in the anterior pituitary gland is under tonic inhibition by dopamine produced in the hypothalamus (Jones and Naftolin 1990). Estrogen is thought to decrease the activity of tyrosine hydroxylase, thereby decreasing the concentration of dopamine in the hypothalamus and pituitary (Jones and Naftolin 1990), resulting in increased plasma prolactin. Genistein or estradiol administered in the diet resulted in increased plasma prolactin compared with the ovariectomized control animals. The increase in plasma prolactin in this study suggests that dietary genistein functions in the hypothalamus and pituitary gland in a manner analogous to that of estradiol, leading to the synthesis and release of prolactin from the anterior pituitary gland.

Estradiol increased uterine weight and plasma prolactin yet did not affect regression of the mammary gland. This suggests that dietary estradiol (1.0  $\mu\text{g/g}$ ) is estrogenic in the uterus and on the hypothalamic/pituitary axis. However, this dose was not sufficient to affect the mammary gland.

**Serum genistein analysis.** Our results indicate that dietary genistein can elicit uterotrophic effects at a dose as low as 375  $\mu\text{g/g}$ , mammatrophic effects at 750  $\mu\text{g/g}$  and effects upon the hypothalamic/pituitary axis at 750  $\mu\text{g/g}$ . The plasma concentration of genistein in rats fed 750  $\mu\text{g/g}$  genistein was 2.2  $\mu\text{mol/L}$  (conjugated plus free). This concentration was sufficient to elicit estrogenic effects in ovariectomized rodents. The intestinal metabolism, absorption, and half-life of dietary genistein, all of which could affect its bioactivity, are currently unknown. Genistein is present in soy products at concentrations as high as 1500  $\mu\text{g/g}$  (Eldridge 1982). Women who consumed soy milk powder daily, which contained 227 mg genistein, had plasma genistein concentrations (conjugated plus free) of up to 6.0  $\mu\text{mol/L}$  (Xu et al. 1995).

At present, there are no human studies in which the biological effects of pure genistein have been assessed and only a few which have evaluated the effects of a diet supplemented with soy. Published studies in which postmenopausal women consumed diets supplemented with soy have yielded conflicting results. Wilcox et al. (1990) showed that soy supplementation produced changes in the uterus similar to those produced by estrogen. However, other studies have failed to show effects different from controls by supplementing soy in the diet of postmenopausal women (Baird et al. 1995).

In the studies described here, genistein did not inhibit the effects of estrogen in either intact or estrogen-fed ovariectomized rats. However, in ovariectomized rats, genistein stimulated the growth of estrogen-responsive tissues, particularly the mammary gland. Perhaps in premenopausal women, genistein would have little, if any, estrogenic activity, whereas in postmenopausal women, the effects would be more pronounced. This raises some concern with regard to mammary tumorigenesis, which initially requires estrogen, because these studies have demonstrated that dietary genistein has estrogenic effects in the mammary gland of ovariectomized rodents. Further research is required in this area to clarify the effects of dietary soy, particularly in postmenopausal women in whom circulating estradiol is low.

Genistein is receiving much attention as a potential chemopreventive/therapeutic agent in the treatment and or preven-

tion of various cancers. The results of these studies demonstrate the need for additional experiments on the biological effects of dietary genistein, particularly in tumor models, before any dietary recommendations can be made or supported.

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