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Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat

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Asian women consuming a traditional diet high in soy have a low incidence of breast cancer, yet when they emigrate to the USA the second but not the first generation lose this protection. Accordingly, we hypothesized that early exposure to genistein, a major component of soy, could have a permanent protective effect against breast cancer. Sprague–Dawley CD rats were exposed to genistein from conception to day 21 post-partum in the diet at concentrations of 0, 25 and 250 mg genistein/kg AIN-76A diet. At day 50 post-partum, all animals were treated with 80 mg dimethylbenz[*a*]anthracene/kg body wt to induce mammary cancers. Dietary genistein resulted in dose-dependent protection against development of mammary tumors (fewer tumors per rat). Analysis of mammary whole mounts showed that 21- and 50-day-old female rats had fewer terminal end buds, terminal ductal structures that were undifferentiated and were most susceptible to carcinogenesis. Bromodeoxyuridine incorporation studies revealed that dietary perinatal genistein resulted in a smaller proliferative compartment for terminal end buds. In rats fed the high genistein dose (250 mg/kg diet) total genistein concentrations in the serum and milk of dams 7 days post-partum were 418 ± 198 and 137 pmol/ml, respectively. Total genistein concentrations in stomach milk, serum and mammary glands of 7-day-old offspring were 4439 ± 1109 and 726 pmol/ml and 440 ± 129 pmol/g, respectively. Total genistein concentrations in the serum and mammary glands of 21-day-old offspring were 1810 ± 135 pmol/ml and 370±36 pmol/g, respectively. Dietary perinatal genistein did not cause significant toxicity in F_0 and F_1 females. We conclude that genistein in the diet at 'physiological levels' enhances cell differentiation, resulting in programming of mammary gland cells for reduced susceptibility to mammary cancer, with no observed toxicity to the reproductive tract of F_1 females.

Introduction

In 1998 it was estimated that there will be 178 000 new cases of breast cancer and 43 000 deaths in the USA (1). These new cases would account for 32% of all newly diagnosed cancers among women, which is the most for any malignancy. Breast cancer is the second leading cause of cancer deaths, second only to lung cancer (2). It is believed that certain lifestyle factors can reduce the risk of breast cancer (3). Some investigators have hypothesized that an Asian diet, which is typically high in soy content, may be one factor that explains the lower incidence of breast cancer in those countries compared with other countries on a diet that lacks soy as a common component (4–7). The influence of diet is evident when it is considered that Asian women consuming a traditional diet high in soy have a lower incidence of breast cancer but when they emigrate to the USA the second, but not the first, generation lose this protection (8).

The primary isoflavone component of soybeans associated with chemoprevention is genistein. While two-thirds of the genistein is present in the form of glycosidic conjugates, this can be rapidly modified by intestinal bacteria to unconjugated genistein, which closely resembles steroid hormones in its structure and function (9,10). Isoflavones are also metabolized via the enterohepatic circulation, which allows for conjugation in the liver. Modification of isoflavones in the body takes place in animals and man. Studies have shown that genistein can be found in low micromolar concentrations in mammals, with Asians having the highest concentrations (10).

Genistein has been reported to have weak estrogenic and anti-estrogenic properties, to be an antioxidant, to inhibit protein tyrosine kinase, topoisomerase II and angiogenesis and to induce cell differentiation (reviewed in ref. 11). Some of the characteristics of genistein are evident at physiological levels and the variety of actions explain why it is a potent modulator of cell growth in both normal and cancerous cells.

We have previously shown that pharmacological injections of genistein during the neonatal and prepubertal periods are capable of reducing the risk of chemically induced mammary cancer in adult rats (11–15). This study investigates the potential of dietary genistein during perinatal life to protect against mammary cancer. The dietary genistein doses were selected on the basis of a previous report that male Lobund–Wistar rats fed 25 mg genistein/kg diet resulted in total genistein concentrations of 252 pmol/ml in the serum (16). This was comparable with the total genistein concentration (276 pmol/ml) in the blood of Asian men eating a traditional diet high in soy (17). A dose one order of magnitude higher was also selected for the purpose of investigating the potential toxicity of dietary genistein and for a bioavailability dose–response study. We also investigate genistein's cellular mechanism of action and its potential for toxicity when exposure takes place perinatally.

Abbreviations: BrdU, bromodeoxyuridine; DMBA, dimethylbenz[*a*]anthracene; HPLC-MS, high pressure liquid chromatography–mass spectrometry.

Materials and methods

Animals

This study was approved by the University of Alabama at Birmingham Animal Use Committee. Seven-week-old female Sprague–Dawley CD rats were obtained from Charles River Breeding Laboratories (Raleigh, NC) and were housed in a climate controlled room with a 12 h light/12 h dark cycle in the UAB Animal Resources Facility. Animals were fed powdered AIN-76A diet (Harlan Teklad, Madison, WI), supplemented with 0, 25 or 250 mg genistein/

kg diet. Genistein was chemically synthesized (Roche, Basel, Switzerland) and analyzed by HPLC (98.5% pure, 1.5% methanol). The AIN-76A diet was chosen because it was determined to be free of any phytoestrogens (limit of detection 5 nM). Animals were given free access to diet and water. At 9 weeks of age the females were bred (2 females/male) for 2 weeks. The males were fed Prolab 3000 rodent diet (Agway, Syracuse, NY) until breeding, at which time they were placed on the same diet as the females. Offspring were sexed at birth and litters reduced so that each dam had 10 offspring (4–6 females/dam). At day 21 post-partum, all offspring were weaned and fed AIN-76A pellets (without genistein) for the remainder of the experiment.

Tumor induction

At day 50 post-partum, female offspring were gavaged with 80 mg dimethylbenz[*a*]anthracene (DMBA)/kg body wt (in sesame oil). Animals were palpated twice a week in order to record the presence, location, size and date of detection for all tumors. Animals were killed when tumor diameter reached 2.5 cm, when the animals became moribund or when they reached 200 days of age. At the time of necropsy all tumors were removed and weighed. A 1–2 mm section was taken from each tumor $\geqslant$1 cm in diameter and was preserved in 4% paraformaldehyde. The section was dehydrated and blocked in paraffin. The paraffin block was cut into 5 μm sections, fixed on slides and stained using hematoxylin and eosin. Coded slides were evaluated histopathologically by Dr Roger Thompson, a board certified veterinary pathologist.

Milk collection

Milk was obtained from lactating female rats in a similar fashion to the procedure described by Schaudies *et al.* (18). On days 7 and 21 post-partum, the dams were separated from their offspring 4–6 h prior to milking. Dams were anesthetized by i.m. injection with xylazine/ketamine (Phoenix Scientific, St Joseph, MO) followed by i.p. injection of 8.4 U oxytocin (Sigma, St Louis, MO) suspended in 100 μl normal saline (Baxter Laboratories, Morton Grove, IL). Glass medicine droppers were used to create a light suction on each nipple. Samples of 1.0–1.5 ml milk were collected from each dam and were immediately frozen in liquid nitrogen and stored at –85°C until analyzed.

To collect stomach milk from the offspring, 7-day-old rats were killed using CO_2 asphyxiation. The stomach, including part of the esophagus and duodenum, was removed and frozen in liquid nitrogen. The stomach lining was removed from the frozen milk and discarded. The remaining milk 'pellet' was then analyzed for genistein concentration.

Genistein analysis

Serum concentrations of genistein and its metabolites were analyzed by HPLC–multiple reaction ion monitoring mass spectrometry (19). Tissue samples were digested overnight with 0.1 mg proteinase K/ml (Sigma) in 10 mM Tris buffer, pH 8.0, containing 100 mM NaCl, 25 mM EDTA, 0.5% SDS. The SDS was removed by centrifugation after addition of saturated KCl. Proteinase K was removed by passing the samples over Sep-pak C_{18} cartridges (Waters, Milford, MA). After a water wash, the genistein was eluted with methanol. To determine total genistein concentrations (free genistein and metabolites), samples were incubated with β-glucuronidase/sulfatase (type H-1 from *Helix pomatia*; Sigma) after adjusting the pH to 5.0 with ammonium acetate buffer. Genistein was recovered by diethyl ether extraction. Serum and milk samples were extracted with hexane to remove lipids, followed by incubation with the β-glucuronidase/sulfatase enzymes and extraction with diethyl ether. For analysis of free genistein, enzymatic hydrolysis was omitted. Extracted samples were evaporated under nitrogen and redissolved in 80% aqueous methanol prior to analysis by high pressure liquid chromatography–mass spectrometry (HPLC-MS). Samples were spiked with biochanin A, 4-methylumbelliferone and phenolphthalein glucuronide (Sigma) as internal standards. A genistein standard curve was run each day.

Extracted samples were analyzed under isocratic conditions (30% acetonitrile in 10 mM ammonium acetate) on a Hewlett Packard 1050 HPLC apparatus using a 100×4.6 mm i.d. Aquapore C8 reversed phase column. Samples were introduced into the Pe-Sciex API III triple quadrupole mass spectrometer via the Heated Nebulizer Atmospheric Pressure Chemical Ionization Interface in negative mode. Multiple reaction monitoring was carried out by selection of parent molecular ions and specific daughter ions formed by collision with argon/10% nitrogen gas. Genistein and metabolite concentrations were quantified by comparison of peak areas with standard curves. The limit of detection was 5 nM.

Mammary gland differentiation

Whole mounts of mammary glands were prepared as previously described (14,20). Mammary glands were removed at the time of death and spread on a microscope slide. For 21-day-old animals, the slides were then placed in neutral buffered formalin for 8 h, followed by acetone for 4 h to remove fat. The glands were placed in 70% alcohol for 30 min and then rehydrated in water for 15 min. The glands were stained using alum carmine for 8 h. After staining, the slides were dehydrated in an increasing gradient of alcohol concentrations from 35 to 100%. The slides were placed in xylene and then compressed between two glass slides for 24 h. The glands were allowed to expand for 8 h and were then mounted using glass coverslips and Permount (Fisher Scientific, Atlanta, GA) for preservation. For 50-day-old animals, the procedure was the same except that the time in formalin, acetone and alum carmine was overnight to compensate for increased gland size.

Glands were analyzed for the quantity of terminal end buds, terminal ducts, lobules type I and lobules type II as previously described (14,20). Terminal end buds were characterized as elongated ductal structures that contained three to six epithelial cell layers and were >100 μm in diameter. Terminal ducts had one to three epithelial cell layers and were <100 μm in diameter. Lobules type I were identified as having five to ten alveolar buds while lobules type II had 10–20 alveolar buds.

Cell proliferation

Bromodeoxyuridine (BrdU) incorporation was used as an index of cell proliferation. Two hours prior to death, animals were injected i.p. with 100 mg BrdU/kg body wt (dissolved in dimethyl sulfoxide) (14). Glands were removed and fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin blocks, cut into 5 μm sections and placed on SuperfrostPlus microscope slides (Fisher Scientific). On the following day, the sections were placed in xylene to remove the paraffin and rehydrated using a gradient of alcohols of decreasing concentration. The slides were then placed in 3.5 N HCl for 20 min and then trypsinized in 0.01% trypsin solution in phosphate-buffered saline. The slides were placed in a solution of 3% aqueous H_2O_2 to quench endogenous peroxidase activity. Sections were blocked using 10 or 5% horse serum for 21- and 50-day-old animals, respectively. The sections were incubated at 37°C with anti-BrdU primary antibody (Dako, Carpenteria, CA) at 1:200 dilution for 30 min, followed by a 1:250 concentration of a biotinylated horse anti-mouse secondary antibody (Vector, Burlingame, CA) for 20 min at room temperature. Detection was performed using streptavidin (Signet, Dedham, MA) and color was developed using 3,3′-diaminobenzidine tetrahydrochloride (Signet) for 14 min. Gills no. 2 hematoxylin (Sigma) was used as a counterstain and then the slides were preserved using Permount (Fisher Scientific). Labeling index [(no. cells incorporating BrdU/total no. cells counted)×100] was determined in terminal end buds, terminal ducts, lobules type I and lobules type II using a Nikon light microscope and a Sony video camera and monitor.

Statistics

Data from the tumorigenesis study were analyzed using the mathematical model proposed by Kokoska *et al.* (21). Standard analysis was conducted using the Wilcoxon rank sum test and Fisher's exact test. The Armitage test, as suggested by NCI guidelines (22), was used as an alternative perspective to the results obtained via the Kokoska approach. The analysis strategy followed for the mathematical modeling consisted of three parts. First, the distributional characteristics of the data were examined. Using the goodness-of-fit test suggested by Freedman *et al.* (23), the Poisson distribution was selected as the most appropriate model for no. tumors/animal and the Weibull distribution was selected to describe the distribution of tumor appearance times. Second, the parameters associated with the model were estimated. The third step was to test the overall experimental effect of a change in the number of induced tumors and/or the time of tumor appearance (global test). All other data were analyzed by one way analysis of variance (ANOVA) using the SigmaStat computer program (Jandel Scientific, San Rafael, CA).

Other analyses

Serum testosterone concentrations were determined using an in-house radio-immunoassay procedure (24). Analysis of estrus phases and evaluation of follicular development were carried out as previously described (14). Histomorphological evaluation of the female reproductive tract was carried out by Dr William Rodgers, a board certified pathologist (Department of Pathology) on coded slides.

Results

Tumor induction

Female offspring exposed perinatally to genistein in the diet and to DMBA on day 50 post-partum developed fewer tumors per rat than those fed the control diet and treated with DMBA. Animals receiving the highest concentration of dietary genistein developed the lowest number of mammary tumors per rat (Figure 1; 4.4 ± 0.6 tumors/animal). This was a 50% reduction in the number of mammary tumors formed in control animals not receiving any genistein (8.8 ± 0.8 tumors/rat, $P < 0.001$).

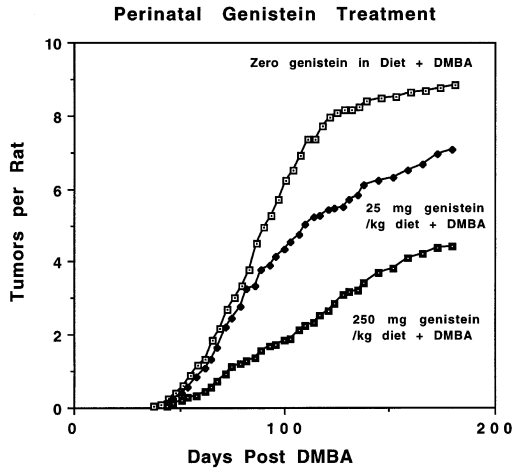

Fig. 1. Ontogeny of palpable mammary tumors in female Sprague–Dawley CD rats exposed perinatally to genistein in the diet from conception until 21 days post-partum. After weaning, the offspring were fed AIN-76A diet only. On day 50 post-partum all animals were treated with 80 mg DMBA/kg body wt.

Table I. Body and uterine weights and mammary gland size for female rats exposed perinatally to genistein in the diet

Age and treatment	Body weight (g)	Uterine weight (mg)	Mammary gland size (mm^2)
21 days, 0 mg genistein/kg diet	43 ± 2	22 ± 2	31 ± 4
21 days, 25 mg genistein/kg diet	42 ± 2	20 ± 1	32 ± 6
21 days, 250 mg genistein/kg diet	45 ± 2	25 ± 1	30 ± 2
50 days, 0 mg genistein/kg diet	209 ± 6	389 ± 25	468 ± 10
50 days, 25 mg genistein/kg diet	204 ± 5	416 ± 27	548 ± 29
50 days, 250 mg genistein/kg diet	203 ± 4	394 ± 24	511 ± 33

Female offspring of Sprague–Dawley CD rats were exposed to genistein in AIN-76A diet from conception until 21 days post-partum. Thereafter, all animals were fed AIN-76A diet only. Values represent means ± SEM.

Animals that received the low genistein concentration in the diet (25 mg/kg) developed 20% fewer tumors per rat (7.1 ± 0.8) compared with animals that did not receive any genistein in the diet. There was no significant influence of dietary genistein on the latency of tumor development for any of the concentrations tested. All animals with tumors had at least one adenocarcinoma. These results demonstrate that perinatal genistein in the diet significantly reduced the number of DMBA-induced mammary tumors in female rats.

Body and uterine weights and mammary gland size

Perinatal genistein in the diet at both concentrations did not significantly alter offspring body and uterine weights or mammary gland size at 21 or at 50 days of age (Table I).

Mammary gland differentiation

The primary mammary terminal ductal structures found in the 21-day-old control female rats were terminal ducts, terminal end buds and lobules type I, which accounted for 62, 24 and 14% of the structures, respectively (Table II). In the 50-day-old animals, the types of terminal ductal structures in decreasing quantity were the terminal end buds (36%), lobules type I (25%), lobules type II (20%) and terminal ducts (19%).

The high but not the low concentration of genistein in the diet resulted in significantly fewer terminal end buds and terminal ducts in 21-day-old rats. At day 50, there were still significantly fewer terminal end buds in animals exposed to genistein, compared with control animals. Exposure to the high and low genistein doses also resulted in significantly fewer lobules type I, but had no significant effect on lobules type II in 50-day-old animals.

Cell proliferation

The DNA labeling indices in the mammary terminal end buds and terminal ducts were ~20% for 21- and 50-day-old animals (Table III). In animals that were 50 days of age, the labeling indices in lobules type I and lobules type II were 3–4%. Perinatal genistein exposure did not significantly alter the labeling index in any of the terminal ductal structures at 21 or 50 days of age.

Genistein concentrations

Free (aglycone) and total genistein (free and conjugated genistein) concentrations were measured in the blood and milk of lactating dams and in the stomach milk, blood and mammary glands of offspring exposed to genistein in the diet. In lactating females there was an excellent correlation (10-fold) between total genistein concentration in the serum (40 ± 19 and 418 ± 198 pmol/ml) and the genistein feed concentration (25 and 250 mg/kg, respectively) (Table IV). In serum of lactating dams fed the high genistein diet, the free genistein concentration was only 2% of the total genistein concentration. In the milk of lactating dams fed the low and high dose genistein diets, total genistein concentrations were 67 ± 10 and 137 ± 34 pmol/ml, respectively. Free genistein concentration in milk was measured only for lactating dams fed the high genistein diet and was found to be 57% of the total genistein in the dam's milk.

In the stomach milk of 7-day-old offspring we found very high concentrations of genistein (Table IV). The total genistein concentrations from stomach milk of 7-day-old offspring exposed to the low and high genistein diets were 490 ± 62 and 4439 ± 1109 pmol/ml, respectively. Of those, the free genistein concentrations comprised 97 and 78% of the total genistein concentrations in the stomach milk. The total genistein concentrations in the serum were 86 and 726 pmol/ml, only 18 and 16% of those found in the respective stomach milk. In the 7-day-old offspring serum the free genistein concentrations were 14 and 19% of the total serum genistein concentrations. In the 7-day-old female rat mammary gland the total and free genistein concentrations were 440 ± 129 and 318 ± 56 pmol/g, respectively. The free genistein concentration in the mammary gland was 72% that of the total genistein concentration.

We also analyzed the genistein concentrations in serum and the mammary glands of 21-day-old rats. In the serum of rats fed the high genistein diet, the total genistein concentration was 2.5-fold higher (1810 versus 726 pmol/ml) in 21- than in 7-day-old rats. The free genistein concentration in the serum was not much higher in 21- (120 ± 14 pmol/ml) than in 7-day-old (103 pmol/ml) rats. In the mammary glands of 21-day-old rats fed the high genistein diet the total and free genistein concentrations were not significantly different from those measured from mammary glands of 7-day-old rats provided with the same diets, but the total genistein concentration in serum of 21-day-old offspring was 2.5-fold higher than in 7-day-old animals.

Toxicology studies

Virgin female rats fed genistein in the diet from 2 weeks prior to and after the start of breeding with proven studs had slightly

Table II. Terminal ductal structures in mammary glands of female rats exposed perinatally to genistein in the diet

Age and treatment	Terminal end buds	Terminal ducts	Lobules type I	Lobules type II
21 days, 0 mg genistein/kg diet	40 ± 1	105 ± 7	23 ± 4	0
21 days, 25 mg genistein/kg diet	48 ± 8	118 ± 15	30 ± 3	0
21 days, 250 mg genistein/kg diet	19 ± 1[c]	54 ± 7[b]	21 ± 4	0
50 days, 0 mg genistein/kg diet	65 ± 6	35 ± 5	45 ± 4	36 ± 4
50 days, 25 mg genistein/kg diet	46 ± 10	45 ± 2	28 ± 3[a]	42 ± 8
50 days, 250 mg genistein/kg diet	31 ± 4[b]	45 ± 5	15 ± 3[c]	34 ± 6

Female offspring of Sprague–Dawley CD rats were exposed to genistein in AIN-76A diet from conception until 21 days post-partum. Thereafter, all animals were fed AIN-76A diet only. Values for terminal ductal structures represent means ± SEM.
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$ compared with animals of the same age that did not receive genistein in the diet.

Table III. Cell proliferation in mammary terminal ductal structures of rats exposed perinatally to genistein in the diet

Age and treatment	Terminal end buds	Terminal ducts	Lobules type I	Lobules type II
21 days, 0 mg genistein/kg diet	21.4 ± 3.1	22.9 ± 4.4	[a]	[a]
21 days, 25 mg genistein/kg diet	18.0 ± 2.5	13.8 ± 2.4	[a]	[a]
21 days, 250 mg genistein/kg diet	20.7 ± 2.0	20.7 ± 3.1	[a]	[a]
50 days, 0 mg genistein/kg diet	17.4 ± 1.6	21.1 ± 2.0	4.1 ± 0.8	3.0 ± 0.8
50 days, 25 mg genistein/kg diet	19.3 ± 1.8	20.4 ± 2.6	4.1 ± 1.4	1.9 ± 0.6
50 days, 250 mg genistein/kg diet	20.8 ± 1.4	26.6 ± 1.5	4.9 ± 1.2	3.0 ± 1.0

Female offspring of Sprague–Dawley CD rats were exposed to genistein in AIN-76A diet from conception until 21 days post-partum. Thereafter, all animals were fed AIN-76A diet only. Two hours prior to death the animals were injected i.p. with 100 mg BrdU/kg body wt. Labeling index was determined in three terminal end buds, terminal ducts, lobules I and lobules II from 7 rats/treatment. Values represent means ± SEM.
[a]Insufficient number to analyze.

Table IV. Genistein concentrations in dams and offspring exposed to genistein in the diet

	Genistein in the diet (mg/kg)	Serum free genistein	Serum total genistein	Milk free genistein[a]	Milk total genistein[a]	Mammary gland free genistein	Mammary gland total genistein
Lactating rats	0	6 ± 3	6 ± 3	[b]	[b]	[b]	[b]
	25	9 ± 3	40 ± 19	[b]	67 ± 10	[b]	[b]
	250	7 ± 3	418 ± 198	78 ± 13	137 ± 34	[b]	[b]
Offspring (7 days)	0	9[c]	9[c]	[b]	9 ± 2	[b]	0
	25	16[c]	86[c]	473 ± 94	490 ± 62	[b]	0
	250	103[c]	726[c]	3454 ± 298	4439 ± 1109	318 ± 56	440 ± 129
Offspring (21 days)	0	6 ± 1	6 ± 1	[b]	[b]	[b]	[b]
	25	18 ± 5	54 ± 6	[b]	[b]	[b]	0
	250	120 ± 14	1810 ± 135	[b]	[b]	304 ± 13	370 ± 36

Sprague–Dawley CD rats were exposed from 2 weeks prior to breeding to day 21 post-partum to genistein via the diet. Genistein concentrations in the serum and milk are expressed as pmol/ml and as pmol/g in the mammary glands. Values represent a minimum of five samples per group.
[a]Milk was collected from maternal nipples or from the stomach of 7-day-old offspring. Genistein concentrations were measured by HPLC-MS.
[b]Not determined.
[c]Serum samples represent pooled samples from littermates.

but not significantly reduced numbers of litters [77 (44/57), 86 (25/29) and 88% (35/40) for animals fed 250, 25 and 0 mg genistein/kg diet, respectively]. The number of female offspring was slightly but not significantly increased in litters exposed prenatally to the high, but not to the low, genistein dose in the diet as compared with those receiving no genistein in the diet (Figure 2). The number of male offspring were similar for all treatments. There were no treatment effects on ano-genital distances (Figure 3) and for time of testes descent and vaginal opening (Figure 4) for male and female offspring, respectively. Perinatal genistein in the diet did not significantly alter percent of time spent in each phase of the estrous cycle (Table V). Adult female offspring exposed perinatally to increasing concentrations of genistein in the diet had slightly but not significantly increased numbers of primordial normal follicles and slightly but not significantly decreased numbers of corpora lutea as compared with animals not receiving genistein in the diet (Table VI). Histomorphological analysis of 50- and 100-day-old female offspring exposed perinatally to genistein did not reveal any significant alterations to the vaginal, uterine and ovarian tissues.

Discussion

Chemoprevention

Perinatal genistein in the diet at concentrations of 25 and 250 mg/kg diet resulted in protection against DMBA-induced mammary cancer in a dose-dependent manner. Rats receiving the highest concentration of genistein in the diet developed half as many mammary tumors per animal than those that

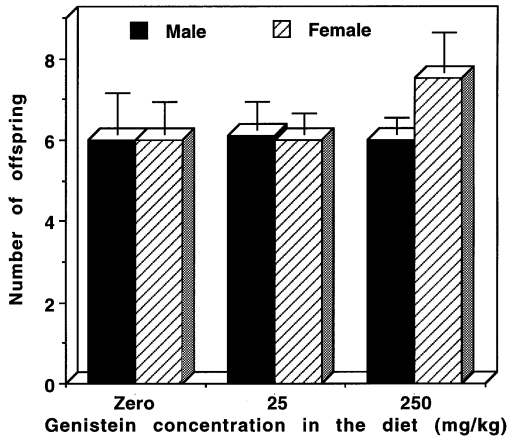

Fig. 2. Number of offspring born from female Sprague–Dawley CD rats fed AIN-76A diet ± genistein in the diet from 2 weeks prior to breeding. Number of offspring were quantitated on day 1 post-partum.

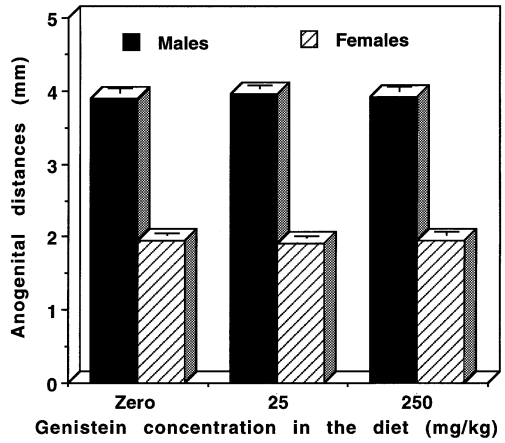

Fig. 3. Ano-genital distances of offspring born to female Sprague–Dawley CD rats fed AIN-76A diet ± genistein in the diet from 2 weeks prior to breeding. Ano-genital distances were measured in 1-day-old rats.

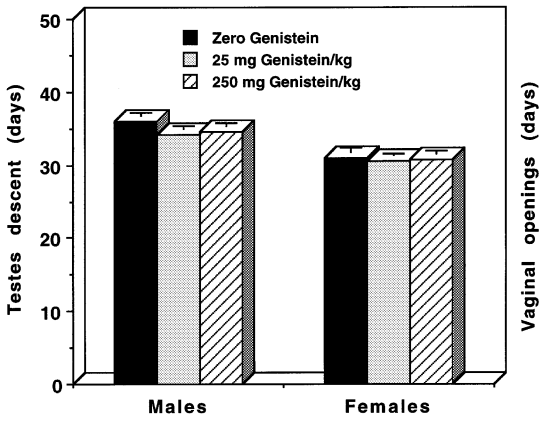

Fig. 4. Testes descent and vaginal opening in Sprague–Dawley CD rats exposed to genistein in the diet from conception to day 21 post-partum. Thereafter, all animals were fed AIN-76A diet only. Values represent means ± SEM.

did not receive any genistein in the diet. Also, the lower concentration resulted in significantly fewer tumors per rat (20% less than in those not receiving genistein in the diet). The chemopreventive effect of perinatal dietary genistein is similar to our previous reports of neonatal and prepubertal genistein injections rendering a protective effect against chemically induced mammary cancer (11–15).

Table V. Estrous cycle in rats exposed perinatally to genistein in the diet

	Percentage of time spent in each phase of the estrus cycle			
Treatment	Proestrus	Estrus	Metestrus	Diestrus
0 mg genistein/kg diet	31 ± 4	26 ± 4	2 ± 1	41 ± 2
25 mg genistein/kg diet	31 ± 3	24 ± 2	2 ± 1	43 ± 2
250 mg genistein/kg diet	35 ± 3	23 ± 2	1 ± 1	41 ± 3

Estrous cycle was evaluated in rats exposed to genistein from conception to day 21 post-partum. Thereafter, all animals were fed AIN-76A diet only. Daily vaginal smears (days 41–50 post-partum) were analyzed. Values represent means ± SEM.

Body and uterine weights and mammary gland size

Genistein given perinatally in the diet at these concentrations did not cause any alterations on body and uterine weights or mammary gland size in 21- or 50-day-old animals. Since carcinogen exposure (at day 50 post-partum) did not occur until after exposure to genistein was completed (day 21 post-partum) and sufficient time had passed to allow for genistein in the animals to decline, it is likely that the effects of genistein in the prevention of chemically induced mammary cancer is not a result of direct genistein action during tumor formation and development, but rather is a result of the events that result following early genistein administration and manifest later in adulthood.

Mammary gland differentiation

The effects of genistein in the diet on differentiation of the mammary gland was evident by the number and distribution of the types of mammary terminal ductal structures. In 21-day-old female rats there were significantly fewer terminal end buds and terminal ducts in animals exposed to the high concentration of dietary genistein. By day 50 post-partum, the number of terminal end buds following administration of the high genistein dose in the diet remained lower than in the control group. The group receiving the lower genistein concentration in the diet had slightly but not significantly fewer terminal end buds at 50 days post-partum. The terminal end buds are the least differentiated of the terminal ductal structures and are reported to be the most susceptible of the mammary structures to carcinogenesis (20). Reduction in the number of these terminal ductal structures correlates with animals developing fewer mammary tumors (11–15,20). Another important aspect of development is the ratio of lobules to terminal end buds (ratio of most differentiated to least differentiated terminal ductal structures) (20). This ratio increased from 0.55 to 0.91 to 1.00 for mammary glands from 50 day rats exposed to 0, 25 and 250 mg genistein/kg AIN-76A diet. An increase in mammary gland differentiation and reduction in the number of the most vulnerable structures to carcinogenesis could explain the mechanism of the protective effects attributed to genistein in the diet.

Mammary gland cell proliferation

The DNA labeling index was 5- to 7-fold higher in mammary terminal end buds and terminal ducts (20%) than in lobules (3–4%), demonstrating more active cellular proliferation in the more undifferentiated terminal ductal structures, a property that probably contributes to cancer susceptibility (20). Perinatal

Table VI. Follicular analysis in female rats exposed perinatally to genistein in the diet

Genistein concentration	Number of follicular structures					
	Primordial normal	Growing normal	Growing atretic	Antral normal	Antral atretic	Corpora lutea
0 (mg/kg AIN-76A diet)	98 ± 8	3 ± 1	94 ± 6	13 ± 4	63 ± 6	36 ± 3
25 (mg/kg AIN-76A diet)	113 ± 23	5 ± 2	85 ± 14	18 ± 4	74 ± 12	34 ± 3
250 (mg/kg AIN-76A diet)	128 ± 19	3 ± 1	100 ± 5	12 ± 2	67 ± 7	31 ± 2

Follicular analysis was evaluated in rats exposed to genistein from conception to day 21 post-partum. Thereafter, all animals were fed AIN-76A diet only. Each group had eight 50-day-old females. Values represent means ± SEM.

genistein exposure via the diet did not significantly alter the labeling index in any of the terminal ductal structures in the 21- and 50-day-old rats. However, the proliferative compartment (labeling index×no. of specific structures), was decreased in a dose-responsive manner. The total numbers of proliferating cells in terminal end buds were 1131 (17.4×65), 888 (19.3×46) and 645 (20.8×31) for animals fed 0, 25 and 250 mg genistein/kg AIN-76A diet. These 22 and 43% decreases in cell proliferation in terminal end buds of the mammary gland may also contribute to the protective effect of dietary genistein. These results are in agreement with our previous report (14).

Bioavailability

The results revealed a dose–response effect for the two doses used, i.e. a 10-fold difference in feed concentrations yielded a 10-fold difference in total genistein concentration in the serum of lactating female rats. However, the dietary genistein fed to these female rats yielded significantly lower serum genistein concentrations as compared with the male rats in our previous study. The high and low doses (250 and 25 mg genistein/kg AIN-76A diets) in lactating female Sprague–Dawley CD rats yielded total genistein concentrations in the serum of 418 ± 198 and 40 ± 19 pmol/ml, while the male Lobund–Wistar rats yielded total genistein concentrations of 1094 ± 322 and 252 ± 57 pmol/ml in the serum (16). This is 2.6- to 6.3-fold less in the females than in the males. This could be due to sex-, lactation- and/or species-related differences. Nevertheless, in the present experiment both concentrations were capable of yielding a chemopreventive effect.

In a short-term dietary study in adult ovariectomized Sprague–Dawley rats that received 750 mg genistein/kg diet, Santell *et al*. reported serum total and free genistein concentrations of 2200 and 400 pmol/ml, respectively (25). Assuming a linear dose–response effect, this would be comparable with the values we observed in the 7-day-old rats (Table IV) and approximately twice as high as we observed in lactating females. Similar to our results, they also reported that a diet containing 375 or 750 mg genistein/kg did not alter uterine weights in intact female rats.

The concentration of total genistein in the serum of 7-day-old animals nursing on dams fed genistein in the diet was 86 and 726 pmol/ml for the low and high genistein diets, respectively. These blood concentrations 'frame' the 276 pmol total genistein/ml concentration found in Asians on a traditional diet that is high in soy content (17). Setchell *et al*. have reported the mean plasma concentration of total genistein in infants fed soy-based formula to be 684 ng/ml (2500 pmol/ml) (26). In the DMBA–rat model we observed mammary cancer prevention with one-thirtieth and one-third of that concentration. Hence, we have been able to achieve a protective effect against chemically induced mammary cancer in rats from short-term exposure (perinatal) to lower concentrations of genistein than can be achieved in infants consuming a high soy-based formula.

One of the most dramatic results of this work is the efficiency of transfer and compartmentalization of genistein from the dam's milk to the stomach milk of the neonate. The total genistein concentration was 11- to 12-fold higher in the stomach milk of neonates as compared with the mother's milk. In the dam's milk, 43% of the genistein was conjugated, while in the 7-day-old offspring's stomach milk only 3–22% of the genistein was conjugated. This implies that it is mostly free genistein that is transferred from the dam to the offspring or that conjugated genistein is capable of being hydrolyzed to free genistein in the offspring's stomach. In human mothers' milk almost all the isoflavones are reported to be present as conjugates (27). In the rat the primary conjugates are glucuronides, sulfates and glucuronide sulfates (unpublished data).

There appears to be restricted genistein bioavailability from the offspring's stomach milk to the blood. In 7-day-old offspring, the total and free genistein concentrations in the blood were 6- and 30-fold less than in the stomach milk. Of the genistein found in the blood of the 7-day-old offspring, 81–86% was conjugated, demonstrating the neonate's capability to conjugate genistein, but to a lesser extent than lactating dams, which conjugated 98% of the genistein. Bioavailability of genistein to the mammary gland is evident by detection of relatively high genistein concentrations. With the high dose, the total genistein concentration in mammary glands of 7-day-old female rats was 440 pmol/ml, 72% of that in the form of free genistein. It is also interesting that the total genistein concentrations in mammary glands of 7- and 21-day-old rats were comparable with the total genistein concentration in serum of the dams.

By day 14 post-partum, the offspring began eating the powdered diet. Hence, the genistein concentrations obtained from 21-day-old offspring reflect direct genistein ingestion and genistein from the dam's milk. While the free genistein concentrations in the serum of animals exposed to the high genistein dose were similar in 21- and 7-day-old offspring, the total genistein concentration in 21-day-old offspring exposed to the same diet was 4-fold higher than in 7-day-old offspring. In the mammary glands, free and total genistein concentrations were similar in the 21- and 7-day-old offspring, indicating that 2 weeks of development did not alter genistein bioavailability to the mammary gland.

Toxicology

Laboratory studies whereby injections of high doses of genistein given to neonatal rats have been shown to result in alterations to the hypothalamic–pituitary–gonadal axis (28–30). However, no comprehensive toxicity studies have been reported with dietary genistein during the perinatal period. Our studies were designed with the hypothesis that the perinatal period of development was the most sensitive for exposure to an estrogenic chemical. To determine if genistein would adversely effect fertility in F_0 females, we initiated dietary genistein exposure 2 weeks prior to initiation of mating. To eliminate the potential of the male being affected, they were maintained on standard laboratory rodent feed until they were placed with the females. In this study we made no attempt to determine if dietary genistein adversely affected the F_0 males. One day after birth, we determined the number of male and female offspring and weighed them. We found no significant alterations in percent of litters produced, in numbers of male and female offspring, their body weights and respective anogenital distances from exposure to genistein in the diet. Likewise, perinatal dietary genistein exposure did not significantly alter the time of testes descent and vaginal opening or the percent of time spent in the phases of the estrous cycle or of follicular development. This is in contrast to our previous reports of high doses of genistein (500 mg/kg body wt) injected into neonates resulting in precocious vaginal opening and fewer corpora lutea and more growing and antral atretic follicles in adult rats (13). We conclude that route of administration (dietary versus injection) and/or concentration of genistein may play a significant role in bioavailability and potential for toxicity. Because of the reported prenatal toxicities of diethylstilbestrol on the female reproductive tract (vaginal adenocarcinoma) (31,32), we also investigated this for genistein. Histomorphological analysis of 50- and 100-day-old female offspring exposed perinatally to genistein did not reveal any significant alterations to vaginal, uterine or ovarian tissues. This is consistent with our previous histomorphological evaluations of the female reproductive tract of rats injected prepubertally with high doses of genistein (15). Future toxicology studies should include multigenerational exposure to males and females.

Summary

Our laboratory research supports the epidemiological data that Asian women consuming a diet high in soy are less susceptible to mammary cancer (7,8). We demonstrate that this protection can be achieved by only perinatal exposure to genistein in the diet. This suggests a programming effect on the target tissue resulting in lifetime protection against mammary cancer. This may be similar to the first generation of Asian women migrating to the USA being protected against breast cancer from early exposure to soy (8).

We do not yet know if this protective mechanism occurs as a consequence of prenatal genistein exposure only or postnatal genistein exposure only or if the complete perinatal period is necessary. Early short-term exposure to 'physiological' concentrations of genistein in the diet during the perinatal period did result in short-term altered mammary gland differentiation that in turn resulted in a reduced number of the most susceptible terminal end buds in young adult females, which is the proposed cellular mechanism of action. The differentiation appears to program the mammary cells to be resistant to future biochemical insult.

The genistein concentrations in rat serum were comparable with those found in Asians on a traditional diet high in soy (17). The circulating genistein concentrations in neonatal rats were considerably less than blood genistein concentrations reported for infants consuming a high soy-based formula (26), suggesting that this protective mechanism could be achieved in infants. We have also demonstrated that perinatal dietary administration of genistein at concentrations up to 250 mg/kg diet did not result in toxic effects on F_0 female fertility and on development of the F_1 female reproductive tract in the rat.

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