Genistein Alters the Ontogeny of Mammary Gland Development and Protects Against Chemically-Induced Mammary Cancer in Rats (44245)

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Abstract. **Breast cancer is the most common cancer in US females and is the second leading cause of cancer death among women. By contrast, Asian women consuming a traditional diet high in soy products have a relatively low incidence of breast cancer. Asians who emigrate to the United States and adopt a Western diet lose this protection. Soy-based diets are high in phytoestrogens, and one of these components is genistein. Using the dimethylbenz(a)anthracene (DMBA) mammary cancer rodent model, we have investigated the breast cancer protective potential of genistein. Our results demonstrate that neonatal and prepubertal genistein treatments altered the ontogeny of the mammary gland and rendered the adult animals less susceptible to chemically-induced mammary cancer. Neonatal genistein treatment did not significantly alter the rate of formation and persistence of DMBA-DNA adducts in the mammary gland. While high concentrations of genistein during the neonatal period caused adverse effects on ovarian follicular development, prepubertal genistein treatment did not appear to be toxic in either the female reproductive tract or the endocrine system.**

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Asian women and men consuming a traditional diet high in soy products have a low incidence of breast and prostate cancers, respectively (1, 2). Yet, Asians who emigrate to the United States and adopt a Western diet lose this protection. Soy-based diets are high in phytochemicals, and analysis of dietary constituents indicates that isoflavones are among normal constituents of human urine from subjects consuming large amounts of whole-grain products and vegetables high in soy products (tofu, soy flour, soy milk, tempeh, etc.) (2). Genistein, an isoflavonic phytoestrogen, is found in soy products as a β-glucoside. Intestinal microflora are capable of hydrolyzing the β-glucoside, genistin, to genistein. Genistein, a planar molecule with an aromatic A-ring, has a second oxygen atom 11.5 Å from the oxygen in the A-ring and a molecular weight similar to those of the steroidal estrogens (Fig. 1).

Genistein has been shown to exhibit estrogenic properties in estrogen receptor binding assays (3, 4), cell cultures (5, 6), and uterine weight assays (7–9); and to inhibit topoisomerase II activity (10), platelet-activating factor/EGF-induced expression of c-fos (11), diacylglycerol synthesis (12) and tyrosine kinases (13). It inhibits both microsomal lipid peroxidation (14) and angiogenesis (15). Genistein exhibits antioxidant properties (16–18). It has been reported to induce differentiation of human myeloid leukemia cells (19), human melanoma cells (20), mouse leukemia cells (21), mouse embryonal carcinoma cells (22), and rat pheochromocytoma cells (23). In human promyelotic HL-60 and erythroid K-562 leukemia cells, genistein has been shown to induce cell differentiation and inhibit cell multiplication in

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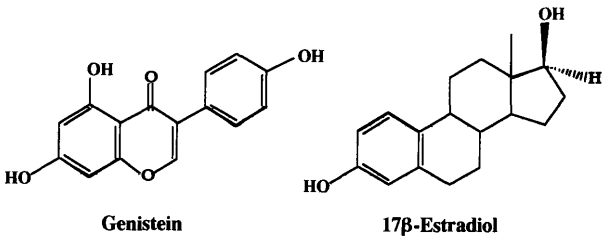

Genistein 17β-Estradiol

Figure 1. Chemical structures for genistein and 17-β estradiol.

a dose-dependent manner (24). While most of these mechanistic data have been derived from *in vitro* studies, it was recently demonstrated that an immunoconjugate composed of genistein, linked to an antibody specific for the B-cell leukemia ED19 receptor was greater than 99% effective at eliminating leukemia cells in an *in vivo* system (25).

In 1990, Barnes *et al.* (26) demonstrated that rats, fed soy-based diets beginning at puberty, developed a lower number of DMBA-induced mammary tumors. At about the same time, we observed that neonatal exposure to diethylstilbestrol reduced the incidence and multiplicity of spontaneously developing mammary tumors (27, 28). Despite the fact that this estrogen suppressed the development of mammary tumors in rats, we were not ready to advocate diethylstilbestrol treatment against breast cancer due to the potential for reproductive tract toxicity (29–32). On the other hand, to our knowledge, there have been no reports of toxicity in humans resulting from ingestion of soy products. Would one of these components of soy, such as genistein, be capable of exerting a chemopreventive effect against mammary cancer? And if so, what would the mechanism of action be and would there be a potential for toxicity in other organs?

Chemoprevention

Female Sprague-Dawley CD rats were treated *via* subcutaneous injections on days 2, 4, and 6 postpartum (neonatally) with 5 mg genistein/rat (19/group) or an equivalent volume of the vehicle, dimethylsulfoxide (DMSO) (25/group). In the second study, female rats were injected on days 16, 18, and 20 postpartum (prepubertally) with 500 μg genistein/g body weight (25 rats/group) or an equivalent volume of DMSO (27/group). Animals treated with genistein, as compared to animals receiving DMSO, developed a lower incidence of dimethybenz(a)anthracene (DMBA)-induced mammary tumors (Fig. 2). The numbers of tumors/animal were reduced by approximately one-half in both studies. Female rats exposed neonatally to genistein, but not prepubertally to genistein, had significantly increased mean time to tumor detection as compared to vehicle-treated animals (124 ± 33 days and 87 ± 37 days, respectively). Ninety-four percent of the mammary tumors evaluated for histopathology were adenocarcinomas (34, 35).

Body and Uterine Weights

There were no significant effects on body weights at all ages investigated (2 days–230 days postpartum) from the neonatal or prepubertal genistein treatments. However, neonatal genistein treatment did result in decreased uterine weights at 21 days and 50 days postpartum (Fig. 3) (34). On the other hand, prepubertal genistein treatment resulted in larger uterine weights at 22 days, an effect that did not persist at 50 days postpartum (35). The effect of genistein given either neonatally or prepubertally to rats, on the uterus is similar to that of estrogen. Neonatal estrogen treatment has been shown to reduce uterine weights, whereas estrogen given to rats prepubertally or later in life increased uterine weights (36, 37). The neonatal estrogen effect is permanent and occurs as a consequence of altered imprinting mechanisms *via* the hypothalamic-pituitary axis (36–41).

Mammary Gland Size

Neonatal and prepubertal genistein treatments resulted in increased mammary gland sizes in 21- to 22-day-old

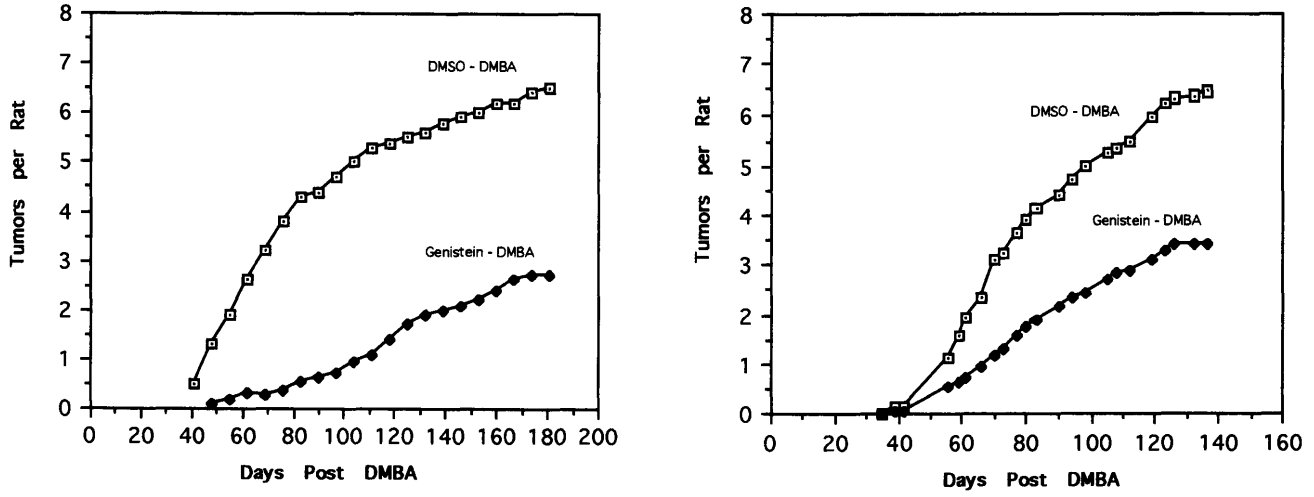

Figure 2. Ontogeny of palpable mammary tumors. Rats were treated neonatally with 5 mg genistein/pup or 20 μl of the vehicle, DMSO (left), or prepubertally with 500 μg genistein/g body weight (right) or an equivalent volume of DMSO. DMBA (80 μg/g body weight) was administered on Day 50 postpartum. (Modified from Refs 34 and 35. Used by permission of Oxford University Press.)

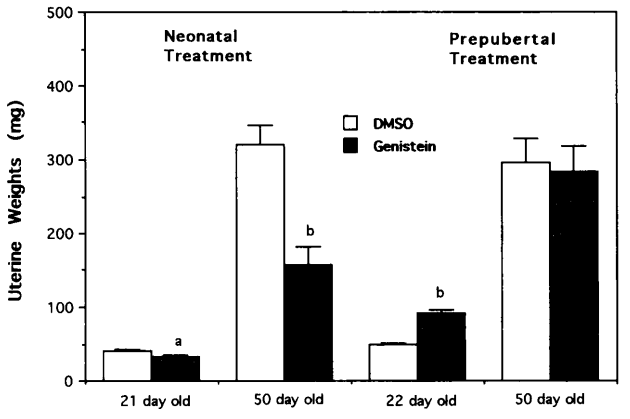

Figure 3. Uterine weights in 21- to 22-day-old and 50-day-old rats injected neonatally or prepubertally with genistein. $^{a}P < 0.05$; $^{b}P < 0.01$ as compared to respective age-matched controls (DMSO Treatment).

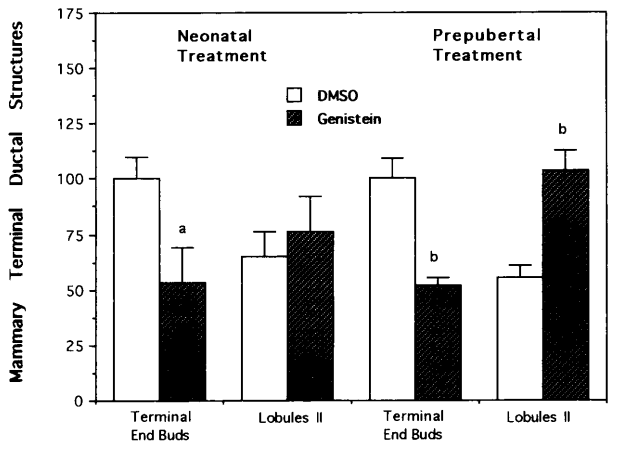

Figure 5. Terminal ductal structures in mammary glands of 50-day-old female rats treated neonatally or prepubertally with genistein. Values for terminal end buds from DMSO-treated rats were set at 100. Numbers represent terminal end buds or lobules II in the outer fringe of the abdominal mammary gland. Numbers represent mean ± SEM. $^{a}P < 0.05$; $^{b}P < 0.001$ as compared to respective age-matched controls (DMSO Treatment).

female rats, but in 50-day-old rats gland sizes were similar to those in vehicle-treated animals (Fig. 4). It appears that at high concentrations, genistein initially promotes mammary gland growth, but eventually glands from control animals "catch-up" by 50 days of age, probably because endogenous estrogen (from puberty on) stimulates the control gland for further growth.

Mammary Gland Development

In mammary glands of 50-day-old vehicle-treated female rats, there were approximately 40% more terminal end buds than lobules II (Fig. 5). Neonatal genistein treatment resulted in reduced numbers of terminal end buds (47%); no significant effect was observed on lobules II. Prepubertal genistein treatment also resulted in reduced numbers of terminal end buds (48%), but it increased the number of lobules II (92%). In both treatments, genistein given prior to puberty enhanced mammary gland maturation and reduced the number of terminal end buds. Terminal end buds are the least mature of the terminal ductal structures and are the most susceptible to carcinogenesis (42–44). On the other hand, lobules are the more differentiated terminal ductal structures of the mammary gland and are least susceptible to chemical carcinogens. We believe that the state of differentiation plays a significant role in chemoprevention.

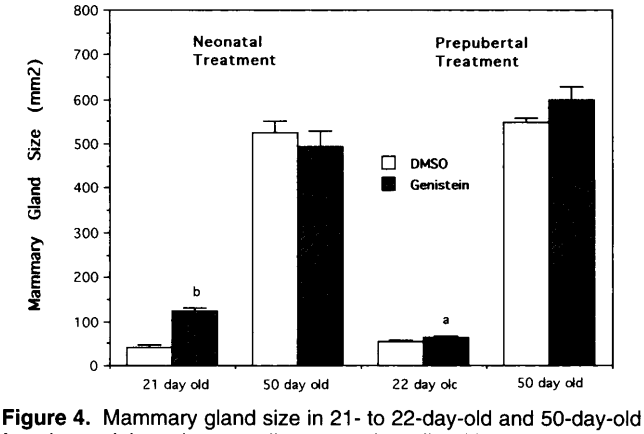

Figure 4. Mammary gland size in 21- to 22-day-old and 50-day-old female rats injected neonatally or prepubertally with genistein. Numbers represent mean ± SEM. $^{a}P < 0.05$; $^{b}P < 0.001$ as compared to respective age-matched controls (DMSO Treatment).

Cell Proliferation

Neonatal- and prepubertal-genistein treatments resulted in mammary glands of 50-day-old female rats having terminal end buds that were less proliferative than those from vehicle-treated animals (Fig. 6). Lobules II from neonatally genistein-treated rats were also less proliferative while lobules II from prepubertally genistein-treated rats were more proliferative. Overall, mammary glands from genistein-treated female rats had smaller proliferative compartments. A decrease in total number of undifferentiated terminal end bud cells in S-phase, which are considered to be maximally

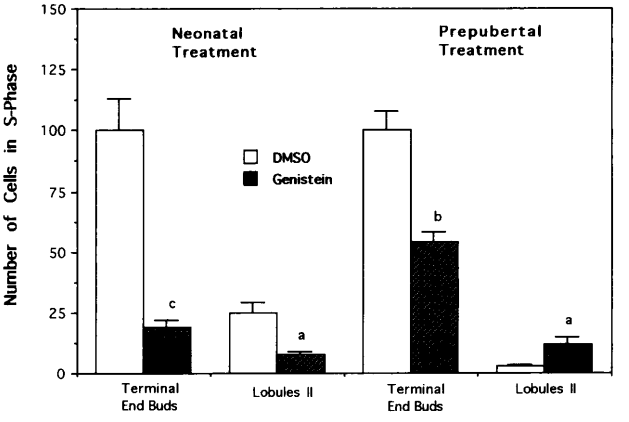

Figure 6. Cells in S-phase in terminal ductal structures of mammary glands from 50-day-old female rats treated neonatally or prepubertally with genistein. Values for terminal end buds from DMSO-treated rats were set at 100. Numbers represent mean ± SEM. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$ as compared to respective age-matched controls (DMSO Treatment).

sensitive to carcinogenic transformation (42–48), may be an explanation for a lower rate of mammary tumorigenesis.

DMBA-DNA Adducts

We measured the rate of formation and persistence of DMBA-DNA adducts in mammary glands of adult rats treated with DMBA. Fifty-day-old female rats treated neonatally with DMSO or genistein were gavaged with 80 μg DMBA/g body weight. Animals were subsequently sacrificed after a lapse of 2, 4, 6, 14, or 24 hr. The 4th (abdominal) gland was removed, frozen in liquid nitrogen, and stored at −70°C until processed. The glands were crushed under liquid nitrogen, and the DNA was isolated by phenol/chloroform extraction after proteinase K digestion (49). All samples were characterized with 260/280 ratios of 1.8 or greater. Ten μg DNA were enzymatically digested to 3′-mononucleotides at 37°C for 3.5 hr by micrococcal nuclease and spleen phosphodiesterase. This mixture was incubated with nuclease P1 at 37°C for 1 hr to remove normal nucleotides and to enrich modified nucleotides. The latter were converted to ^{32}P-labeled deoxyribonucleoside 3′,5-biphosphates by T4 polynucleotide kinase-catalyzed transfer of [^{32}P]phosphate from [γ-^{32}P] ATP (200 μCi) at 37°C for 1 hr. The adducts were analyzed by the nuclease P1-enhanced ^{32}P-postlabeling technique (50–52).

Ten different adducts were observed, 3 of which appeared as early as 2 hr after administration of DMBA (Fig. 7; adducts 1, 2, and 3). The predominant adducts during the course of this study were numbers 2, 3, 4, and 7. All of the adducts persisted for the 24 hr of the study. Figure 8 illustrates the formation and persistence of one of these DMBA-DNA adducts, number 4. Overall, there was no significant change to the levels of all of these adducts as a consequence of neonatal genistein treatment, therefore leading us to conclude that the protective effect of early genistein exposure is not due to early initiation events. Giri and Lu came to the same conclusion in mice pretreated with genistein, and later with DMBA (53).

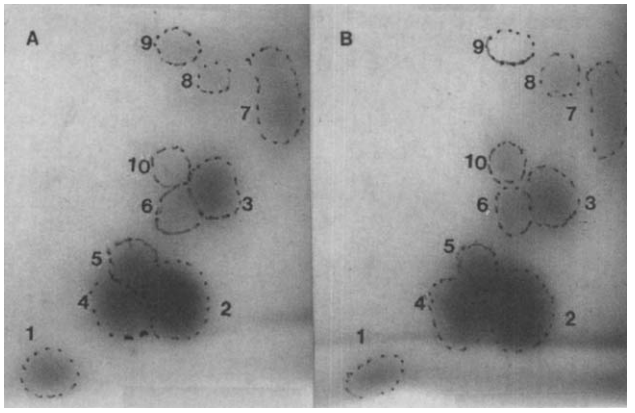

Figure 7. Autoradiograms of ^{32}P-postlabeling analysis of mammary DNA of female rats treated neonatally with (A) DMSO and (B) genistein, followed by DMBA at Day 50 postpartum (A) and (B). These autoradiograms are from mammary glands of rats sacrificed 24 hr after gavaging with 80 μg DMBA/g body weight. Labeled DNA adducts were separated by chromatography on PEI-cellulose sheets and visualized by screen-enhanced autoradiography using Kodak XAR-5 film for 24 hr at −70°C.

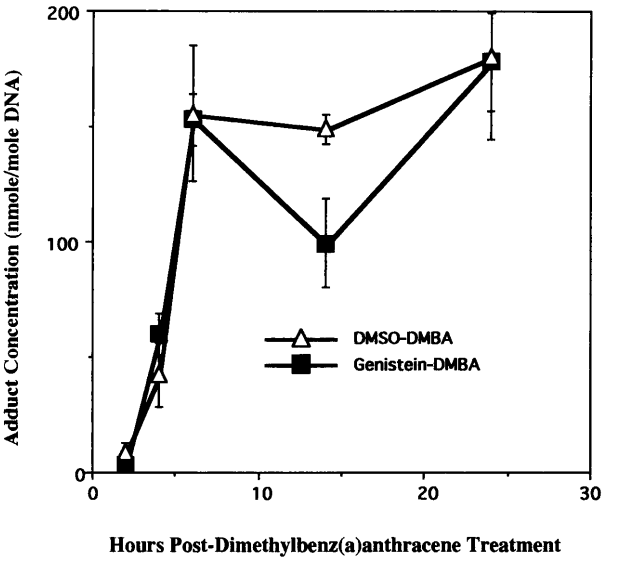

Figure 8. Rate of formation and persistence of carcinogen-DNA adducts in mammary glands of female rats treated neonatally with genistein or DMSO and at Day 50 with DMBA. This chart illustrates the appearance and persistence of DMBA-DNA adduct number 4 (from Fig. 7).

Estrus Cycle

Female rats treated with vehicle spent approximately 50% of their time in the diestrous phase of the estrous cycle (Table I). Slightly less than 25% of the time was spent equally in proestrous and estrous phases. However, animals treated neonatally or prepubertally with genistein spent more time in estrus and less time in diestrus. Both groups of genistein-treated females did cycle, but their cycles averaged 5 days in length as opposed to 4 days for controls.

The effects of genistein on the estrous cycle in rats are comparable with the menstrual cycle of women on a diet supplemented with soy protein. Soy protein given daily for one month to premenopausal women significantly increased follicular phase length by an average of 2.5 days (54). Mitotic rate for breast tissue was almost 4-fold greater during the luteal phase than during the follicular phase (55, 56). Menstrual cycle length is also longer in Asian women than in Western women (55, 57), and Asian women have a lower incidence of breast cancer (58). This may be due in part to ingestion of soy products containing genistein. A retrospective assessment of cycle length also revealed shorter cycle lengths for breast cancer patients compared with control subjects (57).

Ovarian Follicular Development and Sex Steroids

Histomorphological evaluation of the ovaries from 50-day-old female rats revealed that neonatal genistein, but not prepubertal genistein treatment reduced the number of corpora lutea and increased numbers of antral atretic and grow-

Table I. Estrous Cycle in Female Rats Treated Neonatally or Prepubertally with Genistein

Treatment (number/group)	Percentage of time spent in each phase of estrus			
	Proestrus	Estrus	Metestrus	Diestrus
Neonatal DMSO (16)	24 ± 4	23 ± 4	5 ± 2	48 ± 5
Neonatal Genistein (16)	22 ± 5	43 ± 5	6 ± 2	29 ± 4
Prepubertal DMSO (8)	22 ± 4	23 ± 3	0	55 ± 2
Prepubertal Genistein (8)	17 ± 4	36 ± 4	1 ± 1	46 ± 3

Note. Female Sprague-Dawley CD rats were treated neonatally or prepubertally with genistein or DMSO. Daily vaginal smears (Days 43–50 postpartum) were analyzed. Values represent mean ± SEM. (Modified from Refs 34 and 35. Used by permission of Oxford University Press.)

Table II. Follicular Analysis in Female Rats Treated Neonatally or Prepubertally with Genistein

Treatment	Numbers of follicular structures					
	Primordial normal	Growing normal	Growing atretic	Antral normal	Antral atretic	Corpora lutea
Neonatal DMSO	113 ± 15	109 ± 10	12 ± 1	67 ± 6	29 ± 2	41 ± 2
Neonatal Genistein	162 ± 25	131 ± 8	57 ± 12[a]	57 ± 5	139 ± 13[b]	10 ± 2[b]
Prepubertal DMSO	155 ± 19	108 ± 8	14 ± 2	48 ± 6	31 ± 5	42 ± 4
Prepubertal Genistein	137 ± 21	93 ± 11	13 ± 1	46 ± 6	31 ± 4	38 ± 4

Note. Female Sprague-Dawley CD rats were injected neonatally or prepubertally with genistein or DMSO. Ovarian from 50-day-old females were prepared for histopathological evaluation. Values represent the mean ± SEM from eight animals/group. [a] $P < 0.05$; [b] $P < 0.001$ as compared to respective DMSO-controls. (Modified from Ref 35. Used by permission of Oxford University Press.)

ing atretic follicles (Table II). This resulted in a decrease in circulating progesterone concentration (but not 17β-estradiol concentration) in 50-day-old female rats exposed neonatally to genistein (Table III). Prepubertal genistein treatment did not significantly affect circulating progesterone or estrogen concentrations (34).

Circulating genistein (aglucones and conjugates) in 50-day-old females treated with 5 mg genistein/rat on Days 2, 4, and 6 postpartum was undetectable. Genistein levels were not measured in young rats treated neonatally with genistein. Circulating genistein concentrations in 21- and 50-day-old female rats treated on days 16, 18, and 20 postpartum with 500 μg genistein/g body weight were 4.2 ± 0.6 μ*M* and 102 ± 30 n*M*, respectively. The high circulating genistein concentration 24 hr after the last of 3 injections points to the potential of genistein to stimulate mammary gland growth, cellular differentiation, and uterine weights. However, circulating genistein concentrations diminished with time. Since circulating genistein concentrations were low or nonexistent in adult animals, this suggests that the chemoprevention occurred due to early biochemical events that caused permanent developmental modifications on the mammary gland.

Summary

We have demonstrated that exposure to genistein during early critical periods of postnatal life in the rat can suppress the development of chemically-induced mammary cancer. We conclude that the cellular mechanism of action by which genistein elicits this chemoprevention is *via* alteration of early mammary gland maturation. More specifically, we suggest that genistein caused gland differentiation

Table III. Serum Estradiol 17-β and Progesterone Concentrations in 50-Day-Old Female Rats Treated Neonatally or Prepubertally with Genistein

Treatment (number/group)	Estradiol-17β (pg/ml)	Progesterone (ng/ml)
Neonatal DMSO (16)	43.2 ± 13.1	25.3 ± 6.0
Neonatal Genistein (16)	46.8 ± 6.6	4.8 ± 0.9[a]
Prepubertal DMSO (8)	43.6 ± 11.2	26.4 ± 3.6
Prepubertal Genistein (8)	35.0 ± 12.6	19.7 ± 3.1

Note. Values represent the mean ± SEM. [a] $P < 0.05$ as compared to respective DMSO-Controls. (Modified from Ref 35. Used by permission of Oxford University Press.)

by "driving" terminal end buds to mature into lobules. The former are more susceptible to chemically-induced carcinogenesis and the latter are less susceptible terminal ductal structures to chemical carcinogens (42–44). A reduction in total mammary gland cell proliferation may also contribute to the chemoprevention. From our experiments on the rate of appearance and persistence of DMBA-DNA adducts in the mammary glands of rats treated neonatally with genistein, we conclude that early initiation events (carcinogen-DNA damage) do not explain the protective effect of genistein. Therefore, promotion/progression events are more likely to be altered from early genistein exposure and account for the protective effect.

These animal studies also indicate the potential of genistein to cause toxicity to the female reproductive tract and endocrine system, especially injections at high concentrations during the neonatal period. In later developmental periods genistein may be safe, even at high concentrations. Furthermore, we point out that the genistein dose given in these experiments resulted in circulating genistein concen-

trations after the third injection that were approximately 16-fold higher than those in humans on a traditional soy diet (59).

The ability of genistein to enhance mammary gland differentiation in the prepubertal female rat closely parallels that of gland maturation in the human female. The breast of the premenarchal human female contains many undifferentiated terminal ductal structures (46). Eventually, these terminal ductal structures mature to more differentiated lobules during pregnancy. Women who experience a full-term pregnancy early in life have a 2-fold less likelihood of developing breast cancer than women who never become pregnant (60). Likewise, in rats, early exposure to estrogenic hormone mimics also enhances mammary gland differentiation and renders protection against mammary cancer (61–63). As evidenced by our results, genistein can accomplish this protective effect in the rat-DMBA model. A similar process may be ongoing in human females partaking of a traditional Asian diet high in soy.

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