Genistein studies in rats: potential for breast cancer prevention and reproductive and developmental toxicity\textsuperscript{1–3}

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**ABSTRACT**

Asian women and men who consume a traditional diet high in soy products have low incidences of breast and prostate cancers, respectively. Yet Asians who immigrate to the United States and adopt a Western diet lose this protection. We investigated the potential of genistein, a component of soy, to protect against breast cancer and to cause reproductive and developmental toxicity. Our study showed that injections of genistein in rats during the prepubertal period resulted in a 50% reduction of chemically induced mammary tumorigenesis. Studies in mammary whole mounts revealed that prepubertal genistein exposure resulted in fewer terminal end buds and more lobules type II. Cell proliferation in the terminal end buds of adult rats treated prepubertally with genistein was less than that in animals treated with the vehicle (dimethyl sulfoxide). Reproductive and developmental toxicity studies did not find significant alterations to fertility, number of male and female offspring, body weight, anogenital distance, vaginal opening, testes descent, estrus cycle, or follicular development. We concluded that pharmacologic doses of genistein given to immature rats enhance mammary gland differentiation, resulting in a significantly less proliferative gland that is not as susceptible to mammary cancer. We speculate that breast cancer protection in Asian women consuming traditional soy-containing diets is, in part, derived from early exposure to genistein-containing soy. We believe that early programming events are essential for cancer protection benefits. *Am J Clin Nutr* 1998;68(suppl):1400S–5S.

**KEY WORDS**

Soy, genistein, mammary cancer prevention, reproduction, development, toxicology

**INTRODUCTION**

Soy products containing phytoestrogens have received much attention as dietary components to promote better health. Epidemiologic reports have associated soy products with a reduced incidence of breast and prostate cancers, cardiovascular disease, and osteoporosis and lower total cholesterol. Soy has been hypothesized to play a role in reducing the risk of breast cancer because populations ingesting high amounts of isoflavones through soy consumption have low cancer rates. Hence, there is a great deal of interest from scientists, soy producers, pharmaceutical companies, and lay people in having soy products and their active components approved and made available for human consumption.

One of the chemical components of soy is genistein. Reports of genistein’s ability to inhibit tyrosine kinases (1), angiogenesis (2), topoisomerase II (3), diacylglycerol synthesis (4), lipid peroxidation (5), and platelet-activating factor and epidermal growth factor–induced expression of c-fos (6), as well as genistein’s antioxidant properties (7–9), support the possibility that genistein may have anticancer effects. It was recently shown that an immunoconjugate composed of genistein linked to an antibody specific for the B cell leukemia ED19 receptor was >99% effective at eliminating leukemia cells in an in vitro system (10). Genistein has also been shown to induce differentiation of cells in vitro (11–16). In vivo, we showed (17–19) that genistein given to immature rats caused mammary gland differentiation. Although some of the epidemiologic data support these health claims, the potential of soy and its products to cause toxicity must be considered, especially if exposure occurs during early, critical periods of development and at high concentrations, as might occur with the marketing of soy concentrate and pure chemicals. Therefore, the National Institutes of Health, the Environmental Protection Agency, the Food and Drug Administration, soy producers, and pharmaceutical companies have cosponsored several meetings and workshops on the potential benefits and adverse effects of phytoestrogens (20–22; Dietary Phytoestrogens: Cause or Prevention? Sponsored by the National Cancer Institute, Herndon, VA, 1994; Third International Conference on Phytoestrogens, Little Rock, AR, December 3–6, 1995). From a mechanistic standpoint, the estrogenic nature of genistein, a phytoestrogen component of soy, has raised the most concern.

**MAMMARY GLAND DEVELOPMENT AND CANCER**

**Mammary cancer prevention**

We were the first to show that exposure to purified genistein could protect against chemically induced mammary cancer (17–19). In these studies, we used high doses of genistein to enhance...
the possibility of a protective effect. As seen in Figure 1, injections of 500 mg genistein/g body wt to rats on days 16, 18, and 20 postpartum suppressed dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis. The DMBA was administered by gavage at 80 μg/g body wt on day 50 postpartum. There were 50% fewer tumors in animals treated with genistein and DMBA than in animals treated with the vehicle, dimethylsulfoxide (DMSO) and DMBA (P < 0.001). In the DMBA rodent mammary cancer model, this is considered a protective effect against chemically induced mammary cancer.

**Mammary gland differentiation**

Investigations into genistein’s cellular mechanism of action led us to the evaluation of terminal ductal structures in the mammary glands of 50-d-old rats treated prepubertally with genistein or the vehicle, DMSO (19). This was the age at which the animals received the carcinogen in the tumorigenesis study. Analysis of mammary whole mounts of the abdominal glands revealed that female rats injected prepubertally with genistein (as opposed to the vehicle only) had fewer terminal end buds and more lobules type II (Figure 2). Because terminal end buds are the least mature terminal ductal structures and the ones most susceptible to carcinogens (23–25), a reduction in these numbers can account for the reduced incidence of mammary cancer. An increase in lobules, structures that are more mature and less susceptible to chemical carcinogens, suggests that genistein enhances gland differentiation.

**Cell proliferation**

By using bromodeoxyuridine incorporation and immunohistochemistry, we investigated cell proliferation in the mammary glands (19). We found fewer cells proliferating in the terminal end buds in genistein-treated animals than in vehicle-treated animals (Figure 3). On the other hand, there was more cell proliferation in lobules type II of genistein-treated animals than in control-treated animals. However, the latter constituted a small proliferative compartment. Lobules type II of genistein-treated rats were 10% as proliferative as terminal end buds of DMSO treated rats.

**Relevance of rat model to humans**

There are similarities between human and rodent-model gland development. Although most developmental changes occur during the perinatal period, mammary tissue continues to undergo maturation even during adulthood in both species. At birth and during the first week postpartum in rats, the mammary gland is composed of a single main lactiferous duct that branches into 3–5 secondary ducts (23–25). During the second week, further sprouting of ducts occurs up to the sixth generation. This sprouting of ducts causes a marked increase in the density of terminal end buds, which reach their maximum during puberty. Terminal end buds are the growing fringe of the mammary gland with lateral buds branching dichotomously to form structures more proximal to the nipple. A portion of the terminal end buds differ
differentiates in response to each estrous cycle, giving rise to alveolar buds that comprise lobules.

Comparative studies of the development of the human breast and the pathogenesis of breast cancer validate the rat-to-human extrapolations (23–26). The breasts of premenarchal human females contain both terminal end buds and lobules type I, or virginal lobules, that are undifferentiated terminal ductal structures. The latter progress to lobules type II and then to lobules type III in women during hormonal stimulation or pregnancy. A fourth type of lobule, type IV, is the most differentiated. The latter is not found in nulliparous postpubertal women but is found in pregnant women. Women who experience a full-term pregnancy early in life are 4-fold less likely to develop breast cancer than pregnant women. Women who experience a full-term pregnancy not found in nulliparous postpubertal women but is found in offspring from the third breeding were evaluated for number of male and female offspring, body weights, anogenital distance, vaginal opening, and reproductive tract (30, 31). Estrogen therapy has also been correlated with an increased incidence of endometrial cancer (32). Accordingly, we investigated the potential of this genistein–cancer prevention protocol to cause reproductive and developmental toxicity. Prepubertal female Sprague-Dawley CD rats were injected with 500 μg genistein/g body wt or an equivalent volume of dimethyl sulfoxide (DMSO) on days 16, 18, and 20 postpartum (20 females per group). When these animals were 9 wk old, they were mated, 2 females (1 genistein-treated and 1 DMSO-treated female) per breeder male for 3 wk. The males had not received genistein or DMSO. Analyses were performed for number of dams giving birth, number of male and female offspring, and body weights. The dams were separated from the offspring and immediately rebred to a different male. The continuous breeding study was carried out for 3 breedings of each female. The offspring from the third breeding were evaluated for number of male and female offspring, body weights, anogenital distance, vaginal opening, testes descent, estrus cycle, and follicular development. The fertility study revealed a slight decrease in the percentage of DMSO-treated and genistein-treated dams producing offspring with each mating (Table 1). Statistical analysis using the SIGMA STAT computer program (Jandel Scientific, San Rafael, CA) did not reveal any significant differences between treated and control groups. (Differences were considered significant at P < 0.05.)

As seen in Figure 4, the numbers of male and female offspring from the third breeding of prepubertal females were not significantly different between the 2 treatment groups. This was also true of the first and second breedings. Likewise, the body weights of these 2-d-old animals were not significantly different between the 2 treatment groups (Figure 5). Although anogenital distances were significantly shorter in 2-d-old females than in 2-d-old males (P < 0.001), there was no significant effect on anogenital distance within each sex from the dams’ prepubertal genistein or DMSO exposure.

Sexual maturity was investigated by determining the average age of testicular descent and vaginal opening in offspring from the third breeding of dams exposed prepubertally to genistein. There were no significant differences between genistein-treated and DMSO-treated groups of the same sex (Figure 6). Evaluation of the estrus cycle (33) in offspring of female rats exposed

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>First mating</th>
<th>Second mating</th>
<th>Third mating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide</td>
<td>20 (100%)</td>
<td>19 (95%)</td>
<td>19 (95%)</td>
</tr>
<tr>
<td>Genistein</td>
<td>19 (95%)</td>
<td>18 (90%)</td>
<td>16 (80%)</td>
</tr>
</tbody>
</table>

There were no significant differences between genistein- and dimethyl sulfoxide-treated groups.

**REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY**

Exposure to estrogen during early, critical periods of development has been associated with alterations to the endocrine system and reproductive tract (30, 31). Estrogen therapy has also been correlated with an increased incidence of endometrial cancer (32). Accordingly, we investigated the potential of this genistein–cancer prevention protocol to cause reproductive and developmental toxicity. Prepubertal female Sprague-Dawley CD rats were injected with 500 μg genistein/g body wt or an equivalent volume of dimethyl sulfoxide (DMSO) on days 16, 18, and 20 postpartum (20 females per group). When these animals were 9 wk old, they were mated, 2 females (1 genistein-treated and 1 DMSO-treated female) per breeder male for 3 wk. The males had not received genistein or DMSO. Analyses were performed for number of dams giving birth, number of male and female offspring, and body weights. The dams were separated from the offspring and immediately rebred to a different male. The continuous breeding study was carried out for 3 breedings of each female. The offspring from the third breeding were evaluated for number of male and female offspring, body weights, anogenital distance, vaginal opening, testes descent, estrus cycle, and follicular development. The fertility study revealed a slight decrease in the percentage of DMSO-treated and genistein-treated dams producing offspring with each mating (Table 1). Statistical analysis using the SIGMA STAT computer program (Jandel Scientific, San Rafael, CA) did not reveal any significant differences between treated and control groups. (Differences were considered significant at P < 0.05.)

As seen in Figure 4, the numbers of male and female offspring from the third breeding of prepubertal females were not significantly different between the 2 treatment groups. This was also true of the first and second breedings. Likewise, the body weights of these 2-d-old animals were not significantly different between the 2 treatment groups (Figure 5). Although anogenital distances were significantly shorter in 2-d-old females than in 2-d-old males (P < 0.001), there was no significant effect on anogenital distance within each sex from the dams’ prepubertal genistein or DMSO exposure.

Sexual maturity was investigated by determining the average age of testicular descent and vaginal opening in offspring from the third breeding of dams exposed prepubertally to genistein. There were no significant differences between genistein-treated and DMSO-treated groups of the same sex (Figure 6). Evaluation of the estrus cycle (33) in offspring of female rats exposed

**FIGURE 4.** Average number of male and female offspring per litter born from the third breeding of female rats treated prepubertally with genistein (n = 16) or dimethyl sulfoxide (DMSO; n = 19). x ± SEM. There were no significant differences between genistein- and DMSO-treated groups of the same sex.

**FIGURE 5.** Body weights and anogenital distances of 2-d-old male and female offspring from the third breeding of female rats treated prepubertally with genistein (n = 16) or dimethyl sulfoxide (DMSO; n = 19). x ± SEM. There were no significant differences between genistein- and DMSO-treated groups of the same sex.

**FIGURE 6.** Average number of male and female offspring per litter born from the third breeding of female rats treated prepubertally with genistein (n = 16) or dimethyl sulfoxide (DMSO; n = 19). x ± SEM. There were no significant differences between genistein- and DMSO-treated groups of the same sex.
between genistein- and DMSO-treated groups of the same sex.

After the third breedings, the multiparous dams were necropsied. Body and ovarian weights of females prepubertally treated with genistein were not significantly different from those of females prepubertally treated with DMSO (Table 2). However, the uterine weights of multiparous female rats exposed prepubertally to genistein were significantly less than those of the DMSO-treated rats. We found this result to be consistent with our earlier observation of reduced uterine weights in 50-d-old virgin female rats exposed prepubertally to genistein. In these rats we also found that progesterone and estrogen concentrations were slightly, but not significantly, lower. This may help explain the slight, but not significant, decrease in the percentage of dams producing litters (Table 1).

Accordingly, we investigated the ovaries of these multiparous dams. Histomorphologic analysis (34, 35) showed that rats treated with genistein had slightly, but not significantly, more primordial and growing normal follicles and antral normal and antral atretic follicles than did vehicle-treated rats. The numbers of corpora lutea were not significantly different between groups.

From the third breedings, we retained 8 males per group (all from different litters) until they were 56 d old. The male offspring from genistein-treated dams were found to have body, dorsolateral prostate, ventral prostate, and epididymal weights that were slightly, but not significantly, different from those of vehicle-treated animals. No further studies were carried out in the males.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body w</th>
<th>Ovarian w</th>
<th>Uterine w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide</td>
<td>217 ± 6</td>
<td>142 ± 7</td>
<td>447 ± 28</td>
</tr>
<tr>
<td>Genistein</td>
<td>217 ± 5</td>
<td>132 ± 5</td>
<td>433 ± 25</td>
</tr>
</tbody>
</table>

\(^1\bar{x} ± \text{SEM}. \text{ There were no significant differences between genistein- and dimethyl sulfoxide–treated groups.}\)

**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body w</th>
<th>Ovarian w</th>
<th>Uterine w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide</td>
<td>347 ± 6</td>
<td>201 ± 7</td>
<td>553 ± 23</td>
</tr>
<tr>
<td>Genistein</td>
<td>348 ± 8</td>
<td>205 ± 9</td>
<td>464 ± 31^2</td>
</tr>
</tbody>
</table>

\(^2\bar{x} ± \text{SEM}. \text{ Significantly different from dimethyl sulfoxide–treated group; } P < 0.05.\)

**CONCLUSIONS AND FUTURE DIRECTIONS**

We have shown that treatment of immature female rats with pharmacologic doses of genistein enhanced mammary gland differentiation and protected against chemically induced mammary cancer. At the time of carcinogen exposure, cellular proliferation was reduced in mammary terminal end buds of genistein-treated females compared with vehicle-treated females. This may have occurred as a consequence of programming effects on the cells in the terminal ductal structures of the mammary gland. Programming sets the pattern of how the host responds to biochemical effectors (36–38). We speculate that these developmental modifications are driven by genistein acting via the estrogen receptor and growth factor mechanisms. Accordingly, we hypothesize that breast cancer protection in Asian women consuming traditional soy-containing diets is, in part, derived from preadolescent exposure to soy-containing genistein. We believe that early programming events are essential for the full cancer-protection benefits.

Developmental studies in the offspring of female rats treated during the prepubertal period with high doses of genistein did not reveal any significant alterations in fertility, number of male and female offspring, body weight, anogenital distance, vaginal opening, testes descent, estrus cycle, or follicular development. We surmise that genistein is too weak an estrogen to cause endocrine and reproductive tract alterations when exposure occurs prepubertally.

Future research is necessary to determine whether dietary
Folicular analysis in multiparous female rats injected prepubertally with genistein (n = 18) or dimethyl sulfoxide (DMSO) (n = 13) and subjected to 3 breeding cycles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Primordial</th>
<th>Growing</th>
<th>Growing</th>
<th>Antral</th>
<th>Antral</th>
<th>Corpora</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>60 ± 5</td>
<td>44 ± 6</td>
<td>1 ± 5</td>
<td>30 ± 4</td>
<td>8 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>Genistein</td>
<td>71 ± 6</td>
<td>67 ± 4</td>
<td>5 ± 1</td>
<td>40 ± 3</td>
<td>18 ± 5</td>
<td>43 ± 2</td>
</tr>
</tbody>
</table>

\( \text{SEM} \). There were no significant differences between genistein- and DMSO-treated groups.

genistein or soy consumed prepubertally can protect against mammary cancer. It is also possible that low doses (physiologic concentrations) of genistein during a more sensitive period of mammary gland development (perinatal) could exert this programming effect without any adverse effect on the endocrine and reproductive systems. Because high concentrations of genistein cause only slight alterations in the reproductive tract and fertility, it is plausible that the most sensitive mechanism by which genistein acts in vivo is not via the estrogen receptor, but by regulating signal transduction (39). In the prostate of Lobund-Wistar rats, we observed that genistein in the diet can result in the inhibition of tyrosine phosphorylation at tissue concentrations that approximate those of plasma concentrations in Asians consuming a traditional diet high in soy products (40).

REFERENCES