

Dietary genistein results in larger MNU-induced, estrogen-dependent mammary tumors following ovariectomy of Sprague–Dawley rats

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Due to the estrogenic properties of soy-derived isoflavones, many postmenopausal women are using these compounds as a natural alternative to hormone replacement therapy (HRT). How isoflavones impact breast cancer in postmenopausal women is important, because a majority of breast cancer cases occur in this age group. Chemical induction of mammary tumors in female rats has been used to determine that exposure of the mammary gland to soy isoflavones prior to tumor induction is protective against tumor formation. Here we investigate the effect of dietary genistein on mammary tumors that have already formed. The study was designed to determine the action of dietary genistein in a low endogenous estrogen environment as is observed in postmenopausal women. Animals were ovariectomized (OVX) after mammary tumor development and were then placed into one of three treatment groups: positive-control (OVX+ estradiol implant), genistein (OVX+ 750 p.p.m. genistein) and negative-control (OVX alone). Tumors were distinguished as malignant or benign by histopathological examination and were further characterized as either estrogen-dependent or estrogen-independent using immunohistochemistry to identify the presence of both estrogen receptor (ER) α and the progesterone receptor (PR). Genistein at 750 p.p.m. increased the weight of estrogen-dependent adenocarcinomas in ovariectomized rats compared with the negative-control animals. Genistein treatment also resulted in a higher percentage of proliferative cells in tumors and increased uterine weights when compared with negative-control animals. Collectively, these effects are probably due to the estrogenic activity of genistein. Plasma genistein concentrations in animals fed the isoflavone-containing diet were at physiological levels relevant to human exposure. Estradiol concentrations in ovariectomized animals not receiving an estradiol supplement were similar to those observed in postmenopausal women. The data suggest that in an endogenous estrogen environment similar to that of a postmenopausal woman, dietary genistein can stimulate the growth of

a mammary carcinogen MNU-induced estrogen-dependent mammary tumors.

Introduction

The use of dietary supplements for therapeutic purposes has increased in popularity over the past several years. One area of interest has been the use of dietary estrogens, derived from plants, as a means to relieve the symptoms of menopause in older women. The products are marketed as a natural alternative to hormone replacement therapy (HRT). There is often a negative connotation associated with the use of traditional HRT. For various reasons, the most common being the fear of developing breast cancer, many women are reluctant to take HRT (1–3). Among postmenopausal women there is concern regarding the development of breast cancer, and this group is at the greatest risk, with 75% of new breast cancer cases being diagnosed in postmenopausal women (over 50 years of age) (4). While many postmenopausal women may benefit from its use, only 35–40% choose to begin taking HRT and of those only 15% continue using HRT for an extended period of time (5,6). Additionally, recent controversy with HRT use has increased the use of dietary estrogens such as the isoflavones, as substitutes for HRT (7).

Many studies have been conducted to elucidate the beneficial and detrimental biological effects of the isoflavones present in soy. Isoflavones act as estrogen agonists by binding to the estrogen receptor and generating estrogen-induced responses (8,9). Genistein, the isoflavone most studied, stimulates the growth of estrogen-dependent human breast cancer (MCF-7) cells *in vitro* and *in vivo* (9). Genistin, the glycoside form of genistein, also stimulates growth of these tumors (10). This was an important discovery because genistin is the predominant form of genistein present in soy. Other research has demonstrated that soy protein, containing genistein in increasing concentrations, stimulates MCF-7 cells in a dose-dependent manner (11). The model involved in these experiments utilized transplantation of MCF-7 cells into ovariectomized female athymic mice that are implanted with an estradiol pellet. Once tumors have developed, the estradiol pellets are removed and the tumors from mice on various dietary treatments can be evaluated. This is the most well defined animal model for evaluating the effects of chemicals on estrogen-dependent breast cancer and has been used in the development of hormonal therapies for breast cancer treatment. The MCF-7 breast tumor cell line was derived from one individual whom had estrogen-dependent breast cancer, and the use of this cell line allows for human tumors to be examined both *in vitro* and *in vivo*. However, human breast cancer tumors are not all the same with regards to their histopathological characteristics. While the findings in the transplant model are critical in showing that a dietary estrogen can stimulate growth of

Abbreviations: BrdU, bromo-deoxyuridine; ER α , estrogen receptor α ; HRT, hormone replacement therapy; MNU, 1-methyl-1-nitrosourea; p.p.m., parts per million; PR, progesterone receptor.

estrogen-dependent human breast tumors, it is important to evaluate this response in other models.

Another animal model widely used to study breast cancer is a Sprague–Dawley rat model in which mammary carcinomas are chemically induced. The model was first developed by Huggins and Yang (12) and has been modified and further characterized for specific chemical carcinogens such as 1-methyl-1-nitrosourea (MNU) (13). One advantage of this model is that chemical carcinogens induce diverse mammary tumors that differ in estrogen dependency, type and location of formation in the mammary gland, which allows for observation of the effect a compound has on different types of tumors (14). The mammary tumors induced by MNU are similar in terms of both hormone responsiveness and histology to those of human mammary carcinomas (15). Utilizing the MNU-induced tumor model provides investigators with the opportunity to study mammary tumors that have histopathological characteristics of human breast tumors. The model has been used to examine the effects of genistein at the initiation phase of breast cancer, when a tumor is first developing. Fritz *et al.* (16) demonstrated that when genistein (25 and 250 mg/kg diet) was fed from conception to day 21 postpartum prior to treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA), a chemical carcinogen, a reduction in number of tumors per rat was observed. The authors attributed the protective effect of genistein to earlier mammary cell differentiation in rats given genistein prepubertally. Another study has examined consumption of dietary soy protein after administration of MNU. The authors of this paper did not observe any protective or stimulative effects on tumors in this model (17). However, two variables make the research presented here unique. First, the animals in the study conducted by Cohen *et al.* (17) were left intact with normal circulating estradiol. It is our hypothesis that genistein has greater biological activity and efficacy as a dietary estrogen in conditions of low circulating endogenous estradiol such as in prepubertal girls and postmenopausal women. In the project reported here, the animals were ovariectomized after tumors had sufficient time to develop, creating circulating estradiol levels similar to that of postmenopausal women. Therefore, we were able to examine the activity of dietary genistein in the presence of plasma estradiol concentrations relevant to those observed in postmenopausal women. A second component of this research that separates it from other studies evaluating the effects of isoflavones on breast cancer is that each tumor was characterized for its estrogen-dependence. The objective of this study was to determine how genistein affected the size of estrogen-dependent tumors in the mammary gland, but the nature of the diversity of tumors in the model allows for evaluating both estrogen-dependent and estrogen-independent tumors simultaneously. It was hypothesized that genistein would stimulate

the growth of chemically induced, estrogen-dependent tumors in an environment where the endogenous estrogen was low.

Materials and methods

Effects of consumption of genistein on uterine weight and growth of estrogen-dependent, chemically induced tumors

Animal model and study design. Ninety female Sprague–Dawley rats were obtained at 20 days of age. A protocol developed by Thompson *et al.* (18) that has been well defined and evaluated was used to induce mammary tumor development. At 21 days of age, all rats were treated with 75 mg MNU/kg body wt (Figure 1). The MNU (Midwest Research Institute NCI Chemical Repository, Kansas City, MO) was freshly prepared and dissolved in physiological saline containing 0.05% acetic acid and then administered through i.p. injection. At this stage, all animals were fed semi-purified American Institute of Nutrition 93 growth (AIN 93G) diet, with corn oil substituted for soy oil, until time of individual treatments. This diet was used as a control diet for all animals until they reached study eligibility and were randomly placed into one of the treatment groups. The AIN-93G diet has been demonstrated to meet all of the requirements for growth and maintenance for rodents (19). Palpable tumors began to appear 6 weeks after carcinogen exposure. Tumors were allowed to develop until they were of acceptable size (≥ 80 mm²) and number (one to four tumors). When each rat met these requirements they were deemed eligible for the study and were randomly placed into one of three treatment groups. Rats in the first group (positive-control) were ovariectomized and implanted with a silastic implant containing 17 β -estradiol (Sigma, St Louis, MO). As described previously by Clinton *et al.* (20), the silastic implants (5 mm in length, 1.57 mm i.d., 3.18 mm o.d., Dow Corning, Midland, MI) were placed subcutaneously on the back of the rats following ovariectomy. The implants were designed (21) to deliver and maintain serum estrogen concentrations within a physiological range (22). The positive-control group remained on control diet throughout the study. The other two groups were ovariectomized, and one remained on control diet alone (negative-control) while the other group was fed control diet supplemented with genistein at 750 p.p.m. The rationale for doing this is that consumption of 750 p.p.m. genistein by rodents results in a blood concentration of 0.5–5 μ M (23), which is a similar level to that of humans consuming a soy diet (24). Five rats were selected to serve as intact controls. The purpose of this group was to collect tumors from a group of animals that had no treatment to determine if mammary tumors that developed were characteristic of the MNU model. After 90 days of treatment, each rat was killed and tissues collected (Figure 1). Palpable tumors formed in the animals after the ovaries were removed that were undetectable prior to the procedure. These tumors appeared in all three treatment groups and were not included in the final analysis. Tumors that formed before ovariectomy were excised and weighed for total mass. Each tumor was then fixed in formalin to be characterized as described below. Ninety days after ovariectomy, some tumors regressed to the point that they could not be recovered at necropsy. For these tumors, 0 g was recorded for their weight to take into account their complete regression. Uterine tissue was also removed for measurement of wet uterine weight. Blood was taken via cardiac puncture and used to determine plasma genistein and estradiol concentrations.

Chemically induced tumor classification

Tumor characterization. In MNU-induced mammary cancer experiments, both benign and malignant mammary tumors are produced. Mammary cancers are defined to include both adenocarcinomas and papillary carcinomas. Benign tumors are defined to include fibroadenomas, fibromas and adenomas. Mammary cysts are not included (18). Histopathology, as determined by a certified pathologist, was utilized for initial classification of each tumor as either benign or malignant. Due to the lack of tissue remaining at necropsy, tumors that completely regressed following ovariectomy were not characterized for

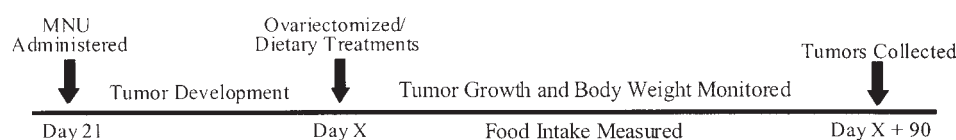


Fig. 1. Time line of mammary tumor study. MNU was used to induce mammary tumors at 35 days of age in female Sprague–Dawley rats. Once each animal met study criteria (one to four tumors, ~ 80 mm² each) then it was ovariectomized and placed into one of three treatment groups. Groups included: positive-control (OVX+ estradiol implant), genistein (OVX+ 750 p.p.m genistein) and negative control (OVX alone). Tumor growth and body weight were measured twice weekly and food intake was calculated several times throughout the study. After 90 days of treatment, the rats were euthanized and tissues collected.

malignancy status. However, >95% of mammary tumors that form in rats following MNU exposure at the dose used in this study have been demonstrated to be malignant (18). Chemical induction of mammary tumors using MNU has also been demonstrated to produce both estrogen-dependent and estrogen-independent tumors, with the majority being estrogen-dependent (25). As a result, tumors identified as malignant were further characterized as either estrogen-dependent or estrogen-independent. To do this, immunohistochemical stains were performed on the tumors, utilizing primary antibodies against the estrogen receptor (ER) α and progesterone receptor (PR) proteins. Tumors identified as positive for ER α and PR were considered estrogen-dependent. Tumors were also evaluated for their relative rate of cellular proliferation using immunohistochemistry staining against bromo-deoxyuridine (BrdU) incorporation into the DNA. The following sections discuss the specific methodology used to complete the tumor characterization.

ER α and PR immunohistochemistry staining. When the tumors were excised, they were immediately fixed in 10% buffered formalin for 24 h and stored in 70% ethanol. Then 3–4 μ m sections were cut from each tumor and mounted on slides for staining. To identify the presence of ER α and the PR, a slightly modified immunohistochemistry protocol described previously by Chou *et al.* (26) was used. Slides were deparaffinized and rehydrated using a series of ethanol solutions. Following a 5 min wash in water, slides were placed into warmed antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 20 min, removed, allowed to cool to room temperature for 20 min and then washed for 5 min in phosphate buffered saline (PBS). At this point, tissues were rimmed with a grease pencil and enough Immunopure peroxidase suppressor (DAKO, Carpinteria, CA) was added to each section to fully cover the tissue. This solution was left on the slide for 30 min and then removed with a series of three PBS washes. Slides were then incubated in a blocking solution (Pierce Superblock in TBS, Pierce, Rockford, IL). After this step, the primary antibody against either ER α (anti-estrogen receptor monoclonal antibody 1D5, DAKO) or PR (polyclonal rabbit anti-human progesterone receptor immunogen, DAKO) was prepared by diluting them to their final concentrations of 1:30 and 1:100, respectively, in antibody diluents with background reducing components (DAKO). Sections were incubated with the primary antibodies at 4°C overnight. Slides were then washed three times in fresh PBS. Sections were then incubated with secondary antibodies from a Universal DAKO LSAB2 kit and labeled with a streptavidin peroxidase solution from the kit (DAKO). The slides were incubated with the biotinylated secondary antibodies for 1 h at room temperature and the streptavidin peroxidase solution was also added for 1 h at room temperature. After each of these steps, the sections were again washed in PBS. For color development, the sections were treated with a 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) solution containing 0.02% hydrogen peroxide. The DAB solution was freshly prepared just before use and color change in the sections was observed. Following a series of washes in water and PBS, the tissues were counterstained with Mayer's hematoxylin (Sigma) for 1 min. Sections were then dehydrated, cleaned in xylene and coverslipped using permount (Fischer Scientific, Pittsburgh, PA). Positively stained cells appeared brown while negative cells were blue. Tissue sections with weak or undetectable brown stain were considered negative for either ER α or PR. Tissue sections from rat uteri were used as a positive-control for the analysis.

Tumor cell proliferation measurement

BrdU immunohistochemistry. Cellular proliferation in tumors was determined using immunohistochemistry. BrdU incorporation into cellular DNA was used as an indicator of cells that are actively proliferating. Four hours prior to killing the animals, each rat was injected intraperitoneally with 50 mg BrdU/kg body wt. Tumors were then fixed and processed as described previously (10,11). The protocol for the BrdU staining is a modification of the one this laboratory has used for identifying proliferative cells in xenografted tumors in athymic mice (10). Briefly, slides were first deparaffinized and rehydrated. Then, they were submerged in 10% H₂O₂ to block endogenous peroxidase activity. Sections were microwaved in a citrate buffer solution for 20 min to increase permeability of the nuclear membrane. Next, the anti-BrdU, primary antibody (Amersham, Biosciences, Piscataway, NJ) was added to each section and allowed to incubate at 37°C for 90 min. After a series of washes in PBS the secondary antibody, goat anti-mouse peroxidase conjugated antibody (Sigma), was applied for 1 h. Again following a series of PBS washes, DAB was added to each section and observed for a change in color. Once this occurred, the compound was quickly removed and each slide was counterstained with hematoxylin (Shandon, Pittsburgh, PA). The slides were then dehydrated and coverslipped. Due to the variation in tumor area among treatment groups, a line was drawn down the center of each tumor and positive and negative cells touching the line were counted. Final data of proliferative cells are expressed as the percentage of positively stained cells per 100 total cells counted. In the positive-control and genistein groups in excess of 8000 total cells were counted. Due to a lower number of collected tumors, ~1000 cells were counted

in the negative-control group. A value of zero percentage proliferation was assigned to tumors that completely regressed in each group.

Plasma analysis

Plasma genistein analysis. Total genistein content in rat plasma was determined using LC/ES-MS/MS following enzymatic deconjugation. The protocol used has been validated previously (27). For each sample, 10 μ l of plasma was diluted with 90 μ l of 25 mM citrate buffer (pH 5.0) and mixed with a 100 μ l of acetonitrile to eliminate protein binding. Then the tubes were centrifuged. Samples were enzymatically deconjugated using H. pomatia glucuronidase and sulfatase (Sigma S3009, 100 μ g in 0.9 ml of 25 mM citrate, pH 5.0) at 37°C for 2 h. Enzymatic hydrolysis of phenolphthalein glucuronide to phenolphthalein (Sigma) was performed under the same conditions to verify enzyme activity. Further sample cleanup was accomplished using parallel off-line solid phase extraction (SPE) in 96-well plates (Isolute ENV+, 25 mg, Jones Chromatography, Lakewood, CO). Samples were applied to the equilibrated SPE cartridges, washed with 1 ml of 30% methanol in water, and then eluted with two 0.5 ml aliquots of acetonitrile. The extracts were evaporated to dryness using a heated centrifugal concentrator (SpeedVac, Savant Co., Farmingham, NY), reconstituted in 50% aqueous methanol, and then injected onto the LC/ES-MS/MS system (Quattro Ultima, Micromass, Manchester, UK) for detection using multiple reaction monitoring (MRM). The internal standard used in this study was 5,7,3',4'-d₄-genistein obtained from Cambridge Isotope Laboratories (Andover, MA). Genistein was quantified using isotope dilution MS by monitoring the MRM transitions for labeled/unlabeled genistein at m/z 275 > 219/271 > 215. Quality control procedures included concurrent analysis of isoflavone-fortified rat plasma, blank rat plasma, and a mixture of labeled and unlabeled standards interspersed throughout each sample set. Data are presented as μ M genistein in the plasma.

Plasma estradiol analysis. Plasma levels of estradiol were determined by radioimmunoassay (RIA) using a Coat-A-Count kit from Diagnostic Products Corporation (Los Angeles, CA). The kit had a sensitivity of 20 pg/ml, which is sufficient to reveal differences between plasma levels of estradiol in ovariectomized and estradiol implanted animals. Plasma from each rat included in this experiment was run together in a single assay. Briefly, 100 μ l of plasma sample was added to tubes coated with antibodies against estradiol (derived from rabbit). Approximately 30 min later, 1 ml of ¹²⁵I-labeled estradiol (0.035 μ Ci) was added to each tube and allowed to incubate with plasma for 3 h at room temperature. Unbound estradiol was eluted by emptying and air-drying tubes before counting in a gamma-counter using COBRA > II 5002/5003 (OS2), SW ver. 1.10 software (Packard Instrument Company). Circulating estradiol values (pg/ml) were derived from a standard curve run at the same time as the plasma samples. A series of standards were run in conjunction with unknown plasma samples. First, standard curves were determined by assaying a range of calibrated solutions of estradiol provided by the kit, with concentrations including 0, 10, 20, 50, 150, 500 and 1800 pg/ml. Secondly, non-specific binding was assessed by adding ¹²⁵I-labeled estradiol to non-coated, antibody-free tubes and eluting the solution after the allotted time. Thirdly, total counts were determined by counting total radioactivity without eluting solution from test tubes. Finally, single concentration standards were generated by dissolving a known concentration of estradiol (40 pg/ml) in serum obtained from Sigma-Aldrich (St Louis, MO). These were assayed at the same time as the unknown plasma samples and positioned just before and after the plasma samples to control for changes in assay kinetics from the beginning to the end of the run. All samples were run in duplicate. The intra-assay coefficient of variance was 10.99%. Inter-assay variance is commonly ~7%. Final data are presented as nM estradiol in the plasma.

Statistical analysis

Data from the tumor weight measurements were analyzed using an independent *t*-test. Differences were considered significant at a *P*-value of <0.05. Data from uteri weights, cellular proliferation, plasma estradiol analysis and plasma genistein analysis were analyzed according to a completely randomized design with a one-way analysis of treatment. If the overall treatment *F*-ratio was significant (*P* < 0.05), the differences between treatment means were tested with Fisher's least significant difference test. Error bars on all graphs represent the standard error of the mean. All statistical analysis was done using the SAS program (SAS, Cary, NC, 1985).

Results

Effect of genistein on MNU-induced mammary tumors in ovariectomized rats

Mammary carcinomas appeared in the rats at different time intervals following the exposure of the rats to MNU at 35 days

Table I. Determination of estrogen and progesterone receptor status in MNU-induced malignant tumors removed from ovariectomized Sprague–Dawley rats^a

Treatment	Number of animals	Total number of malignant tumors observed (number of tumors at final collection ^b)				
			ER+	ER–	PR+	PR–
Negative control	21	35 (6)	4	2	4	2
Genistein	26	37 (35)	32	3	33	2
Positive-control	26	34 (32)	31	1	31	1

^aFor each treatment, excised tumors were characterized as either malignant or benign. Adenocarcinomas were then further characterized utilizing immunohistochemistry for the estrogen and progesterone receptors. The data were used to distinguish which tumors were estrogen responsive.

^bTumor size was monitored throughout the 90 day treatment period in all groups. By the end of 90 days of treatment, some tumors had regressed to the point that they were no longer detectable and could not be collected for further analysis. The majority of the tumors in the negative-control group had regressed in the absence of estrogen. The number in parentheses reflects the actual number of tumors collected. Malignancy status of fully regressed tumors was not determined.

of age. Rats were placed into one of the three treatment groups when they met the predefined requirements (one to four tumors, $\geq 80 \text{ mm}^2$). Rats were then ovariectomized and treated animals were administered estradiol (positive-control) or dietary genistein. Negative controls were fed AIN 93G diet. All animals were on the study for 90 days. At the beginning of the 90 days of treatment 21, 26 and 26 animals were included into the negative-control, genistein and positive-control groups, respectively (Table I). These rats collectively provided 35, 37 and 34 adenocarcinomas to be monitored in the negative-control, genistein and positive-control groups, respectively. Other mammary tumors were collected from the animals, but through histopathological characterization these tissues were classified as non-malignant (data not shown). Non-mammary cancerous lesions were also observed in the animals. However, these cancers were few in number and appeared in all of the treatment groups (data not shown). Therefore, no conclusions were drawn regarding how genistein influenced the development of non-mammary cancers induced by MNU. After ovariectomy, tumors initially regressed in all groups. Tumors in the negative-control rats regressed to the point that they were no longer detectable, and at necropsy only six malignant tumors were recovered for tissue analysis (Table I). By the end of the study, 35 malignant mammary tumors were collected and identified from the genistein group and 32 were recovered in the positive-control (E_2 treated) group. Tumors classified as malignant were then further characterized for their expression of ER α and the PR by immunohistochemistry staining. In the negative-control group, four of the six tumors were positive for ER α and PR while two were negative for the receptors. In the genistein-fed animals, three of 35 adenocarcinomas were identified as ER-negative while only one tumor was ER-negative in the positive-control group (Table I).

After 90 days of treatment, each animal was killed and tumors excised and weighed. Since the focus of our study was on estrogen-dependent tumors, only malignant tissues that were later identified as estrogen-dependent (ER/PR positive) were included in the data set. Dietary genistein resulted in significantly ($P < 0.05$) larger estrogen-dependent adenocarcinomas when compared with tumors from the negative-control

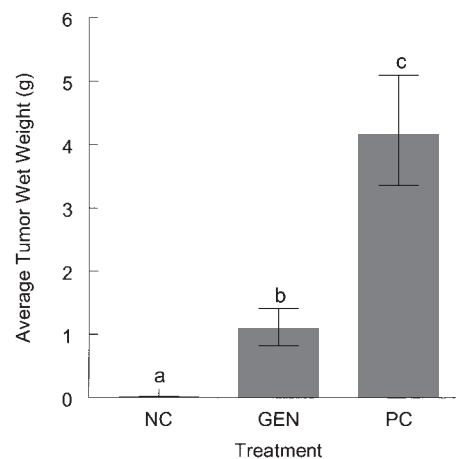


Fig. 2. Effect of genistein on MNU-induced mammary tumor wet weight in ovariectomized rats. Mammary tumors were chemically induced in Sprague–Dawley rats and then allowed to fully develop. The animals were then ovariectomized and placed into one of three treatment groups. Groups included: positive-control (OVX+ estradiol implant, PC) ($n = 32$), genistein (OVX+ 750 p.p.m. genistein, GEN) ($n = 35$), and negative-control (OVX alone, NC) ($n = 35$). Animals were left on treatment for 90 days and then killed for tissue collection. Data are expressed as average wet weight in grams for each treatment. Bars with different letters are significantly different ($P < 0.05$).

animals. The average wet weight of the tumors in the genistein-fed animals was 1.1 g compared with 0.02 g in the negative-control group (Figure 2). The weight of the tumors in the estradiol-treated rats was also significantly ($P < 0.01$) higher than those collected from the genistein-fed animals with the average weight being 4.2 g. It should also be noted that food intake was measured several times throughout the study and that the positive-control rats consumed $\sim 20\%$ less food (average 24 h food intake) when compared with negative-control and genistein-fed animals. Similar reduction in food intake has been observed in other studies where rats were treated with the type of estradiol implants utilized in this study (20). However, no significant difference was observed between the negative-control and genistein-fed rats (data not shown).

Effect of genistein on uterine weight in ovariectomized rats

Uterine tissue was collected from each animal to evaluate the estrogenic environment in the animals, as estrogen supplementation following ovariectomy has been shown to slow/stop the uterotrophic process caused by the procedure. In this study, silastic implants delivering 17β -estradiol resulted in significantly ($P < 0.01$) larger uteri when compared with either the negative-control or genistein-fed rats. The average wet weight of the uterine tissue removed from the positive-control rats was 0.38 g (Figure 3). The uterus tissues collected from the animals fed genistein were also significantly ($P < 0.01$) larger than those collected from the negative-control animals, with an average uterus weight of 0.17 g compared with 0.10 g in the negative-controls.

Cellular proliferation in adenocarcinomas excised from animals consuming genistein

Cellular incorporation of BrdU was utilized as an indicator of cellular proliferation in MNU-induced adenocarcinomas. Cells that stained positive after immunohistochemical analysis were considered actively proliferating cells. Both proliferating and non-proliferating cells were counted and final values were

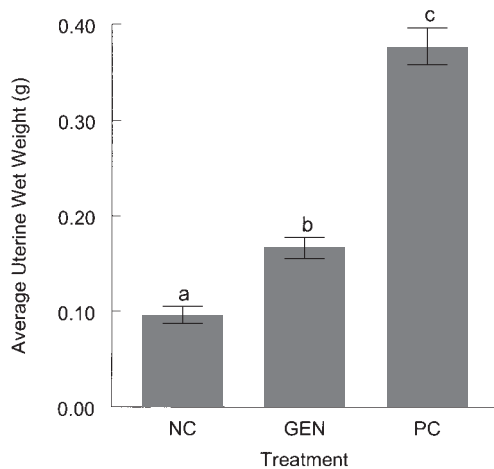


Fig. 3. Effect of genistein on uterine weight in ovariectomized rats. The rats were ovariectomized and placed into one of three treatment groups. Groups included: positive-control (OVX+ estradiol implant, PC) ($n = 25$), genistein (OVX+ 750 p.p.m. genistein, GEN) ($n = 26$) and negative-control (OVX alone, NC) ($n = 21$). Animals were left on treatment for 90 days and then killed for tissue collection. Data are expressed as average wet weight in grams for each treatment. Bars with different letters are significantly different ($P < 0.05$).

expressed as percentage of proliferating cells. The percentage of proliferating cells for the negative-control group was 0.03%. Positive-control animals receiving estrogen supplementation had a significantly ($P < 0.01$) higher percentage of cellular proliferation when compared with the negative-control group with a value of 5.2% proliferation. The animals consuming genistein had proliferation of 3.4%, which was significantly ($P < 0.05$) higher than that observed in the negative-controls. Although tumors in the estradiol-treated group were larger relative to those in the genistein-fed group, cellular proliferation in the genistein-treated animals was not significantly different than that of the positive-control group (Figure 4).

Plasma concentrations of 17 β -estradiol and genistein

The concentrations of both 17 β -estradiol and genistein were measured in randomly selected animals (Table II). The average concentration of 17 β -estradiol in the positive-control rats was 0.14 nM, which was significantly ($P < 0.01$) higher than either the negative-control or genistein-fed animals (Table II). The concentrations of 17 β -estradiol in the negative-control and genistein groups were 0.02 and 0.05 nM, respectively. The higher concentration of estradiol in the genistein-fed animals did reach significance ($P < 0.01$) when compared with the plasma of the negative-control rats. The average genistein concentration in the plasma of the genistein treatment group was 3.41 μ M, which was significantly ($P < 0.01$) higher than either the negative- or positive-control animals, with plasma concentrations of 0.04 and 0.01 μ M, respectively (Table II).

Discussion

The objective of this study was to evaluate the effect of dietary genistein at dosages relevant to human exposure on the growth of MNU-induced, estrogen-dependent mammary carcinomas in ovariectomized rats. It was observed that after ovariectomy, a number of tumors in each of the treatment groups regressed to a size where they were undetectable at the point of necropsy.

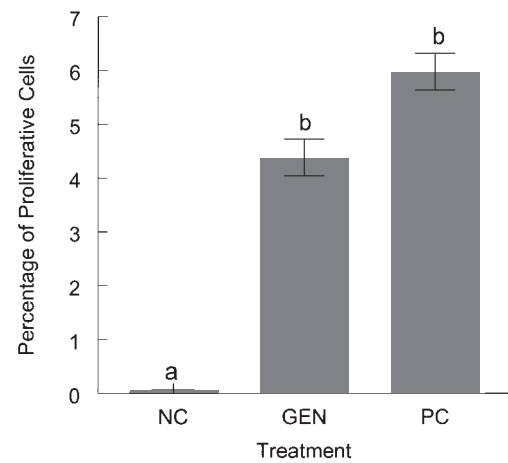


Fig. 4. Effect of genistein on the cellular proliferation within MNU-induced mammary tumors. Tumors were removed from the rats and collected for immunohistochemical analysis. Incorporation of BrdU into cellular DNA was utilized as a marker of cellular proliferation. Immunohistochemistry was utilized to stain for cells containing BrdU. Positively staining as well as background cells were counted that touched a line drawn down the center of the tissue. In the positive-control (PC) and genistein (GEN) groups in excess of 8000 total cells were counted whereas ~1000 cells were counted in the negative-control (NC) group due to a lower number of collected tissues. Cell counts from each group were then combined and averaged for that group. The data are presented as a percentage of actively proliferating cells. Bars with different letters are significantly different ($P < 0.05$).

Table II. Measurements of the concentrations of 17 β -estradiol and genistein in the plasma of ovariectomized Sprague–Dawley rats^a

Treatment	<i>n</i>	Total genistein (μ M \pm SEM)	<i>n</i>	17 β -Estradiol (nM \pm SEM)
Negative-control	9	0.04 \pm 0.02	9	0.02 \pm 0.005
Genistein	14	3.41 \pm 0.43	9	0.05 \pm 0.006
Positive-control	10	0.01 \pm 0.001	17	0.14 \pm 0.004

^aRats were ovariectomized (OVX) and then put into one of three treatment groups: 1, Negative-control—OVX+ no further manipulation; 2, Genistein—OVX+ fed 750 p.p.m. genistein in the diet; 3, Positive-control—OVX+ implanted with 100% estradiol silastic implant.

This was most evident in the negative-control animals in which 29 tumors fully regressed after ovariectomy, demonstrating that the absence of a source of estrogen resulted in the complete regression of these tumors. This has been reported previously in this model, and in those studies, any tumor that regressed after the point of ovariectomy was classified as estrogen-dependent. Of interest in this study, is the fact that dietary treatment with genistein resulted in a greater number of tumors that were still present and collected 90 days after the rats were ovariectomized. Only two tumors regressed to the point they could not be recovered in the genistein group, which is the same number that fully regressed in the estradiol-treated animals. Not only did genistein prevent tumor regression, but it also stimulated the growth of tumors resulting in an average wet weight of the tumors, after 90 days of treatment, that was statistically greater than the negative-control animals. These data are further supported by the fact that genistein stimulated cellular proliferation within estrogen-dependent tumors that was similar to treatment with estradiol. Similar stimulation of estrogen-dependent breast cancer cells *in vivo* by genistein and estradiol has also been observed in athymic mice transplanted with MCF-7 cells (9,10,28). It is probable that the effect of

genistein on the size of the chemically induced tumors is the result of the estrogenic activity of the compound. This is supported by the fact that genistein in the diet caused the uterine tissue of the ovariectomized rats to be larger than that measured in the negative-control animals. It is generally believed that the presence of estrogen after ovariectomy of an animal will reduce the uterotrophic effect of the procedure. It can be concluded that a greater uterine weight in genistein-fed animals is evidence of estrogen agonistic activity. Therefore, the data presented here suggest that genistein has estrogenic action in both mammary and uterine tissues of the rat.

One of the advantages of evaluating the effect of compounds on tumor development and growth in the chemically induced mammary tumor model is that both estrogen-dependent and estrogen-independent tumors can be examined (15). The focus of this study was to evaluate only estrogen-dependent tumors. Utilizing immunohistochemistry to identify the presence of both ER α and the PR, we found that the vast majority of tumors collected at the end of the study were estrogen-dependent (ER/PR positive). There were not a sufficient number of estrogen-independent tumors to be able to evaluate the effects of genistein on these mammary tumors. It was reported previously that dietary treatment with 0.7% soy extract, resulted in a reduction in the number of DMBA-induced tumors that were identified as ER α and PR positive when compared with control animals (29). The data suggest that isoflavone consumption may result in the formation of a greater number of estrogen-independent mammary tumors. Few estrogen-independent tumors were observed in the genistein-fed animals in this study. The difference in findings may be related to when the animals were exposed to the isoflavones. In the Gallo *et al.* (29) study, animals were placed on diets containing soy extract at weaning and tumors were induced at 50 days of age. In the study presented here, animals were not fed genistein until after tumor formation. These studies collectively suggest that the exposure of rats to the isoflavones prior to chemical induction of mammary tumors may impact the type of tumors that develop (estrogen-dependent/independent), while treatment after tumor development has no effect on ER α and PR expression.

The chemically induced mammary tumor model has been used extensively to investigate how soy and its isoflavones affect breast cancer development. The ability of genistein to inhibit mammary tumor development in rats has been evaluated. In these studies, the authors reported that rats injected with 5 mg of genistein on days 2, 4 and 6 postpartum (30) or 500 mg/kg body wt of genistein on days 16, 18 and 20 postpartum (31) had a lower incidence of chemically induced mammary tumors. Also, rats fed genistein at doses of 25 and 250 mg/kg diet from conception to day 21 postpartum had fewer DMBA-induced tumors (16). The authors of these studies hypothesize that protection against tumor development is the result of earlier cellular proliferation and differentiation in the mammary gland resulting in the tissue being less susceptible to damage by chemical carcinogen. Genistein acts via an ER-based mechanism that results in the increase of cellular proliferation and differentiation within the mammary gland (32). Collectively, the data suggest that prepubertal exposure to genistein is protective against chemically induced mammary tumor formation. This finding is not unique to the estrogenic isoflavones. Prepubertal exposure of rats to either estradiol (33,34) or diethylstilbesterol (35) has been shown to reduce

the number of adenocarcinomas formed after carcinogen exposure. Others have investigated the interaction of soy isoflavones with breast cancer development. Injecting pregnant rats with genistein resulted in increased mammary tumor incidence in the offspring of the animals (36). Furthermore, starting to feed isoflavone-containing diets at weaning, near the point of or after chemical induction of mammary tumors, has no effect on the incidence of tumor development (17,37). Left unexplored was how the diet may impact tumor growth if the animals were not exposed to genistein until after the tumors had fully developed. In this study, genistein treatment was not started until the tumors were completely established and dietary genistein resulted in larger tumors when compared to negative-controls. Therefore, it appears that the timing of exposure to the isoflavones is critical to how they impact tumor development and growth. *In utero* exposure may be detrimental; resulting in the formation of a greater number of tumors, while exposure prepubertally appears to be protective against formation of chemically induced mammary tumors. Dietary genistein exposure at the point of weaning and near or on the time of carcinogen exposure appears to have no effect on mammary tumor development. However, we report here that consumption of genistein after the tumors are fully formed results in growth of the mammary adenocarcinomas. This is important as this experimental design is relevant to breast cancer patients consuming genistein.

While the timing of exposure to genistein may play a critical role in how the compound affects chemically induced mammary tumors in rats, another important aspect is the endogenous estrogen environment. As mentioned, early exposure (prepubertal) of the mammary gland to estrogenic compounds protects against the development of mammary carcinomas, and it has been described that in the case of genistein that this is due to an ER-based mechanism (32). It is probable that these compounds are able to modulate cellular activity stimulating cellular proliferation and differentiation because the endogenous estrogen in these prepubertal animals is very low. Plasma estrogen concentrations in postmenopausal women are in the range of 100–200 pM (38). In such an estrogen environment, it is reasonable to think that dietary consumption of genistein will contribute substantial estrogenic action. This would explain the observation that genistein in this study increased tumor size, stimulated tumor cell proliferation and increased uterine weight compared to negative-controls that had low endogenous estrogen. The plasma concentrations of 17 β -estradiol in the ovariectomized animals not receiving the estradiol implants were 0.02 and 0.05 nM for the negative-control and genistein groups, respectively. These values are beneath the range of what has been measured in postmenopausal women. This may also explain why other studies in which isoflavones have been fed throughout the entirety of the study have not reported a difference in the growth of tumors in animals fed the isoflavones compared with controls (17,37). In these studies, the animals were left intact. It is probable that the addition of a weak ER agonist, such as genistein, did not significantly impact the growth of estrogen-dependent tumors, as there was already a substantial amount of endogenous estradiol present. It has been reported previously and confirmed in this study, that if mammary tumors are chemically induced and the rats are subsequently ovariectomized the tumors can be maintained and stimulated by treating the animals with exogenous estradiol (39). Therefore, it can be concluded that genistein is acting as an ER agonist and is also able to

maintain/stimulate the chemically induced, estrogen-dependent mammary tumors.

When evaluating the effect of soy isoflavones on breast cancer, it is important to take into account both the timing of exposure and the endogenous estrogen environment. We report that if genistein is fed following formation of chemically induced mammary tumors and the rat is ovariectomized creating a low endogenous estrogen environment, then it will stimulate the tumors resulting in final tumors that are larger than those in negative-control animals. This has clinical relevance to postmenopausal women with estrogen-dependent breast cancer, because the tumors formed in the mammary of the rat following chemical-induction with MNU are similar in their histopathology and hormonal status to those that arise in women. Additionally, these animals have plasma estradiol concentrations that are similar to those reported in postmenopausal women. Therefore, the present study and other data demonstrating that dietary isoflavones stimulate the growth of MCF-7 cells transplanted in athymic mice (9–11,28), collectively raise the concern that consumption of dietary genistein by a postmenopausal woman with estrogen-dependent breast cancer may present an increased risk to these women.

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References

- Garton, M., Reid, D. and Rennie, E. (1995) The climacteric, osteoporosis and hormone replacement therapy; views of women aged 45–49. *Maturitas*, **21**, 7–15.
- Vihtamaki, T., Savilahti, R. and Tuimala, R. (1999) Why do postmenopausal women discontinue hormone replacement therapy? *Maturitas*, **33**, 99–105.
- MacLennan, A., Lester, S. and Moore, V. (2001) Oral estrogen replacement therapy versus placebo for hot flushes: a systematic review. *Climacteric*, **4**, 58–74.
- American Cancer Society (2001) Breast Cancer Cancer Facts and Figures, 2001–2002.
- Brett, K.M. and Madans, J.H. (1997) Use of postmenopausal hormone replacement therapy: estimates from a nationally representative cohort study. *Am. J. Epidemiol.*, **145**, 536–545.
- Keating, N.L., Cleary, P.D., Rossi, A.S., Zaslavsky, A.M. and Ayanian, J.Z. (1999) Use of hormone replacement therapy by postmenopausal women in the United States. *Ann. Intern. Med.*, **130**, 545–553.
- Writing Group for the Women's Health Initiative Investigators (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the women's health initiative randomized controlled trial. *J. Am. Med. Assoc.*, **288**, 321–333.
- Martin, P.M., Horwitz, K.B., Ryan, D.S. and McGuire, W.L. (1978) Hypoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology*, **103**, 1860–1867.
- Hsieh, C.Y., Santell, R.C. and Helferich, W.G. (1998) Genistein enhances estrogen-dependent breast cancer cell growth *in vitro* and *in vivo*. *Cancer Res.*, **58**, 3833–3838.
- Allred, C.D., Ju, Y.H., Allred, K.F., Chang, J. and Helferich, W.G. (2001) Dietary genistin stimulates growth of estrogen-dependent breast cancer similar to that observed with genistein. *Carcinogenesis*, **22**, 1667–1673.
- Allred, C.D., Allred, K.F., Ju, Y.H., Virant, S.M. and Helferich, W.G. (2001) Soy diets containing varying amounts of genistein stimulate growth of estrogen-dependent tumors in a dose dependent manner. *Cancer Res.*, **61**, 5045–5050.
- Huggins, C. and Yang, N.C. (1962) Induction and extinction of mammary cancer. *Science*, **137**, 257–262.
- Gullino, P.M., Pettigrew, H.M. and Grantham, F.H. (1975) *N*-Nitrosomethylurea as a mammary gland carcinogen in rats. *J. Natl Cancer Inst.*, **54**, 401–414.
- Rivera, E.S., Andrade, N., Martin, G., Melito, G., Cricco, G., Mohamad, N., Davio, C., Caro, R. and Bergoc, R.M. (1994) Induction of mammary tumors in rat by intraperitoneal injection of NMU: histopathology and estral cycle influence. *Cancer Lett.*, **86**, 223–228.
- Russo, J., Gusterson, B.A., Rogers, A.E., Russo, I.H., Wellings, S.R. and Zwieten, V. (1990) Comparative study of human and rat mammary tumorigenesis. *Lab. Invest.*, **62**, 244–278.
- Fritz, W.A., Coward, L., Wang, J. and Lamartiniere, C.A. (1998) Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. *Carcinogenesis*, **19**, 2151–2158.
- Cohen, L.A., Zhao, Z., Pittman, B. and Scimeca, J.A. (2000) Effect of intact and isoflavone-depleted soy protein on NMU-induced rat mammary tumorigenesis. *Carcinogenesis*, **21**, 929–935.
- Thompson, H.J., Adlukha, H. and Singh, M. (1992) Effect of carcinogen dose and age at administration on induction of mammary carcinogenesis by 1-methyl-1-nitrosourea. *Carcinogenesis*, **13**, 1535–1539.
- Reeves, P.G., Nielsen, F.H. and Fahey, G.C., Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of AIN-76A rodent diet. *J. Nutr.*, **123**, 1939–1951.
- Clinton, S.K., Li, P.S., Mulloy, A.L., Imrey, P.B., Handkumar, S. and Visek, W.J. (1995) The combined effects of dietary fat and estrogen on survival, 7,12-dimethylbenz(a)anthracene-induced breast cancer and prolactin metabolism in rats. *J. Nutr.*, **125**, 1192–1204.
- Legan, S.J., Coon, G.A. and Karsch, F.J. (1975) Role of estrogen as initiator of daily LH surges in the ovariectomized rat. *Endocrinology*, **96**, 50–56.
- Kao, K.J. and Ramirez, V.D. (1979) Induction of pituitary and mammary tumors in male, 'fale' and female rats by either DMBA, estradiol implant or combined treatment. *Proc. Soc. Exp. Bio. Med.*, **160**, 296–301.
- Santell, R.C., Kieu, N. and Helferich, W.G. (2000) Genistein inhibits growth of estrogen-independent human breast cancer cell in culture but not in athymic mice. *J. Nutr.*, **130**, 1665–1669.
- Xu, X., Wang, H.J., Murphy, P.A. and Hendrich, S. (1994) Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J. Nutr.*, **124**, 825–832.
- Rose, D.P., Pruitt, B., Stauber, P., Erturk, E. and Bryan, G.T. (1980) Influence of dosage schedule on the biological characteristics of *N*-nitrosomethylurea-induced rat mammary tumors. *Cancer Res.*, **40**, 235–239.
- Chou, Y., Guzman, R.C., Swanson, S.M., Yang, J., Lui, H.M., Wu, V. and Nandi, S. (1999) Induction of mammary carcinomas by *N*-methyl-*N*-nitrosourea in ovariectomized rats treated with epidermal growth factor. *Carcinogenesis*, **20**, 677–684.
- Twaddle, N.C., Churchwell, M.I. and Doerge, D.R. (2002) High throughput quantification of soy isoflavones in human and mouse plasma using LC with electrospray-MS and MS/MS detection. *J. Chromatogr. B*, **777**, 137–143.
- Ju, Y.H., Allred, C.D., Allred, K.F., Karko, K.L., Doerge, D.R. and Helferich, W.G. (2001) Physiological concentrations of dietary genistein stimulate dose-dependently growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted into athymic nude mice. *J. Nutr.*, **131**, 2957–2962.
- Gallo, D., Giacomelli, S., Cantelmo, F., Zannoni, G.F., Ferrendina, G., Fruscella, E., Riva, A., Morazzoni, P., Bombardelli, E., Mancuso, S. and Scambia, G. (2001) Chemoprevention of DMBA-induced mammary cancer in rats by dietary soy. *Breast Cancer Res. Treat.*, **69**, 153–164.
- Lamartiniere, C.A., Moore, J.B., Brown, N.M., Thompson, R., Hardin, M.J. and Barnes, S. (1995) Genistein suppresses mammary cancer in rats. *Carcinogenesis*, **16**, 2833–2840.
- Murrill, W.B., Brown, N.M., Zhang, J.-X., Manziolillo, P.A. and Lamartiniere, C.A. (1996) Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis*, **17**, 1451–1457.
- Cotroneo, M.S., Wang, J., Fritz, W.A., Eltoum, E. and Lamartiniere, C.A. (2002) Genistein action in the prepubertal mammary gland in a chemoprevention model. *Carcinogenesis*, **23**, 1467–1474.
- Grubbs, C.J., Farnell, D.R., Hill, D.L. and McDonough, K.C. (1985) Chemoprevention of *N*-nitroso-*N*-methylurea-induced mammary cancers by pretreatment with 17 β -estradiol and progesterone. *J. Natl Cancer Inst.*, **74**, 927–931.
- Shellabarger, C.J. and Soo, V.A. (1973) Effects of neonatally administered sex steroids on 7,12-dimethylbenz(a)anthracene-induced mammary neoplasia in rats. *Cancer Res.*, **33**, 1567–1569.
- Lamartiniere, C.A. and Holland, M.B. (1992) Neonatal diethylstilbestrol prevents spontaneously developing mammary tumors. In Li, J.J., Nandi, S.A. and Li, S.A. (eds), *Hormonal Carcinogenesis*. Springer Verlag, Berlin, pp. 305–308.

36. Hilakivi-Clarke, L., Cho, E., Onojafe, I., Raygada, M. and Clarke, R. (1999) Maternal exposure to genistein during pregnancy increases carcinogen-induced mammary tumorigenesis in female rat offspring. *Oncol. Rep.*, **6**, 1089–1095.
37. Constantinou, A.I., Lantvit, D., Hawthorne, M., Xu, X., van Breemen, R.B. and Pezzuto, J.M. (2001) Chemopreventive effects of soy protein and purified soy isoflavones on DMBA-induced mammary tumors in female Sprague–Dawley rats. *Nutr. Cancer*, **41**, 75–81.
38. Lonning, P.E., Geisler, J., Johannessen, D.C. and Ekse, D. (1997) Plasma estrogen suppression with aromatase inhibitors evaluated by a novel, sensitive assay for estrone sulphate. *J. Steroid Biochem. Mol. Biol.*, **61**, 255–260.
39. Li, S., Martel, C., Dauvois, S., Belanger, A. and Labrie, F. (1994) Effect of estrone on the growth of 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in the rat: a model of postmenopausal breast cancer. *Endocrinology*, **134**, 1352–1357.

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