

## Pharmacologic, but Not Dietary, Genistein Supports Endometriosis in a Rat Model

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Endometriosis is a disease in which uterine tissue proliferates in extrauterine sites. Using a surgical model to simulate endometriosis, we explored the potential for the phytoestrogen genistein, by injection and diet, to sustain endometriosis in rats. Uterine tissue was attached to intestinal mesentery of 8-week-old Sprague Dawley rats. After 3 weeks, the rats were ovariectomized and the implants measured. Following 3 weeks of daily injections or exposure to dietary genistein, animals were necropsied and implants located and measured. Injections of genistein (50 and 16.6  $\mu\text{g/g}$  BW) or estrone (1  $\mu\text{g}/\text{rat}$ ) sustained the implants; injection of sesame oil (vehicle for estrone), dimethylsulfoxide (DMSO; vehicle for genistein), or genistein at 5.0  $\mu\text{g/g}$  BW did not sustain implants. Dietary genistein (250 or 1000 mg genistein/kg AIN-76A diet) did not support the implants. In ovary-intact rats exposed to 250 mg genistein/kg AIN-76A diet, implant size was not altered, compared to control-fed animals. To assess estrogenic actions of genistein, we measured uterine estrogen receptor alpha (ER- $\alpha$ ) and progesterone receptor (PR) isoforms A and B by Western blot analyses. Injections of estrone or genistein (50 or 16.6  $\mu\text{g/g}$  BW) significantly reduced uterine ER- $\alpha$  compared to vehicle-treated animals. PR (B) was significantly increased by all injected doses of genistein or estrone and by the higher dietary dose (1000 mg genistein/kg AIN-76A). PR (A) was significantly increased by injected doses of genistein (16.6 and 5.0  $\mu\text{g/g}$  BW). We conclude that pharmacologic injections, but not dietary physiological concentrations of genistein, support surgically induced endometriosis in rats. Our results suggest a critical role for ER modulation and genistein bioavailability in the maintenance of the implants.

**Key Words:** genistein; endometriosis; estrogen-; progesterone-receptors; uterus; rat.

Endometriosis is a disease in which endometrial tissue proliferates outside of the uterine cavity. In premenopausal women, its incidence is as high as 10% (Wheeler, 1989), and in women with pelvic pain or infertility, the estimated incidence reaches 60% (Koninckx *et al.*, 1991). Although the exact cause of the disease remains largely unknown, it has been hypothesized that retrograde menstruation may play a role in its etiology (Sampson, 1927) by allowing endometrial cells to

escape the uterine cavity through the fallopian tubes. Since retrograde menstruation is a normal process occurring in most women (Halme *et al.*, 1984), other elements are involved in disease progression. Factors which have been suggested to contribute to the pathology or progression of endometriosis include genetic susceptibility (Kennedy, 1998), immune system defects, inappropriate expression of matrix proteinases (reviewed by Giudice *et al.*, 1998), steroid-converting enzymes (Bulun *et al.*, 1999), and angiogenic factors (Shifren *et al.*, 1996). Endometriosis is also dependent upon ovarian steroid hormones (Dizerega *et al.*, 1980; Olive and Schwartz, 1993), and most therapies for the disease involve suppression of their synthesis or function.

Due to the dependence of endometriosis upon ovarian hormones, an increasing amount of attention has been given to the investigation of potential links between incidence or severity and exposure to chemicals that may affect the endocrine or immune systems. In humans, increased incidence of endometriosis has been associated with exposure to 2,3,7,8-TCDD (Mayani *et al.*, 1997) or polychlorinated biphenyls (PCBs) (Koninckx *et al.*, 1994) via the environment. Studies in animal models simulating endometriosis have indicated that one potential contributory mechanism for such compounds in the pathogenesis of endometriosis may be antiestrogenicity (Cummings *et al.*, 1996). Likewise, environmental chemicals that are weakly estrogenic, such as methoxychlor, have been shown to support surgically induced endometriosis in rodents (Cummings and Metcalf, 1995). Given the multitude of environmental compounds with the ability to modulate the endocrine or immune systems, simultaneous exposure represents the potential for synergistic action.

Another potential source of exposure to estrogenic compounds is the diet. Genistein is an isoflavonic phytoestrogen found in high concentrations in soy products, legumes, and grains. The traditional Asian diet, high in soy products, has been associated with health benefits such as prevention of hormone-dependent cancers and coronary heart disease (reviewed by Adlercreutz, 1990). Despite the numerous health advantages of a diet high in soy, a limited number of epidemiologic studies have suggested that Asian women have a higher incidence of endometriosis than other ethnic groups

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(Arumugam and Templeton, 1990; Miyazawa, 1976; Sangi-Hagheykar and Poindexter, 1995). However, the possible role of a soy diet in the disease process of endometriosis has never been explored.

Although soy has many components, it is believed that genistein is responsible for a majority of the biological consequences. Structurally similar to estradiol-17- $\beta$ , genistein is capable of eliciting numerous biological responses that mimic those of estradiol. It has affinity for the estrogen receptors alpha (Mathieson and Kitts, 1980; Shutt and Cox, 1972) and beta (Kuiper *et al.*, 1998) in receptor binding assays, induces a uterotrophic response in animals (Bickoff *et al.*, 1962; Cheng *et al.*, 1953; Folman and Pope, 1966), and increases uterine *c-fos* mRNA transcripts *in vivo* (Santell *et al.*, 1997). Although cell culture studies and animal models have provided a wealth of information as to the mechanisms of action of genistein in estrogen-responsive tissues, the biological effects of a soy diet in humans are difficult to measure. Therefore, we have employed a model that simulates endometriosis in rats via implantation of uterine tissue into the peritoneal cavity (Vernon and Wilson, 1985). Following surgery, rats were exposed to genistein, by injection or through the diet, to determine whether genistein could support the implanted tissue.

Given the hormone-dependent nature of endometriosis, alterations in ovarian steroid hormone production induced by phytoestrogen exposure have the potential to either stimulate or inhibit the growth of endometriotic lesions. Genistein and other isoflavones have been shown to induce changes in circulating estradiol in humans (Cassidy *et al.*, 1994; Lu *et al.*, 1996; Nagata *et al.*, 1998; Petrakis *et al.*, 1996) and rats (Cotroneo *et al.*, 2001); possibly through alterations in steroid-converting enzymes such as aromatase (Adlercreutz *et al.*, 1992; Kao *et al.*, 1998; Wang *et al.*, 1994), 17 $\beta$ -hydroxysteroid oxidoreductases (Makela *et al.*, 1995; 1998) and 5 $\alpha$ -reductase (Weber *et al.*, 1999). A role for steroid-metabolizing enzymes in the pathogenesis of endometriosis has been suggested, as recent studies have identified aberrations in the expression of aromatase and type 2 17 $\beta$ -hydroxysteroid reductase in human endometriotic lesions (reviewed by Bulun *et al.*, 1999). Because of the multifactorial nature of endometriosis and the numerous mechanisms by which genistein could affect lesion growth, we also investigated the potential for genistein to affect lesion growth via ovarian steroidogenesis and/or steroid receptor modulation.

## MATERIALS AND METHODS

**Implant surgery.** These studies were approved by the University of Alabama at Birmingham Animal Use Committee. Adult female Sprague-Dawley CD rats (Charles River, Raleigh, NC) were used for all experiments. Rats were housed in a temperature-controlled environment with a 12-h light/dark cycle and given food and water *ad libitum*. At the age of 9 weeks, endometriosis was induced using a surgical procedure similar to that of Vernon and Wilson (1985). Under ketamine/xylazine anesthesia, an abdominal incision was made through which a small piece of uterine tissue was removed. The tissue was cut

into 3-mm squares. Using Prolene 4-0 nylon sutures (Ethicon, Somerville, NJ), two squares per rat were sutured directly onto blood vessels within the intestinal mesentery.

**Location of implants/ovariectomy.** Three weeks following implant surgery, the rats were bilaterally ovariectomized (Experiments 1 and 2) or left ovary-intact (Experiment 3). At this time, implants were located and assessed for viability by visual inspection; viable implants appeared as fluid-filled, spherical structures. In Experiment 3, where net growth of the implants was to be determined, two different colored sutures were used per rat, in order to facilitate their identification at the conclusion of the experiment. The diameter of each implant was measured using calipers. Following surgery, the rats were exposed to genistein via injections or diet.

**Injections (Experiment 1).** After ovariectomy, the rats were fed a diet devoid of phytoestrogens (AIN-76A, Harlan-Teklad, Madison, WI) until the conclusion of the experiment. Subcutaneous injections of the following were given in the nape of the neck: estrone (1  $\mu$ g/rat) (Sigma Chemical Co., St. Louis, MO), genistein (50  $\mu$ g/g BW), genistein (16.6  $\mu$ g/g BW), genistein (5.0  $\mu$ g/g BW). Sesame oil (Sigma Chemical Co.) served as the vehicle for estrone; DMSO (Sigma Chemical Co.) was used for genistein. The dose response for genistein was based on preliminary experiments in our laboratory in which 50  $\mu$ g/g BW produced a uterotrophic response in ovariectomized rats. Cummings (1993) reported that 1  $\mu$ g estrone treatment resulted in a more reproducible biological response in the uteri of rats than 17 $\beta$ -estradiol and that this treatment supported surgically induced endometriosis (Cummings and Metcalf, 1995), thus serving as a positive control. Daily injections were given for 3 weeks.

**Dietary genistein (Experiments 2 and 3).** Following ovariectomy (Experiment 2) or exploratory surgery to locate and measure implants (Experiment 3), rats were fed one of the following diets for a 3-week period: 250 mg genistein/kg AIN-76A diet, 1000 mg genistein/kg AIN-76A, or AIN-76A alone (controls). Ovary-intact rats (Experiment 3) were fed control diet (AIN-76A) or 250 mg genistein/kg AIN-76A. Animals remained on the diets for 3 weeks.

**Necropsy.** At the age of 15 weeks, all rats were necropsied. Ovary-intact rats were sacrificed in the estrous phase of the estrous cycle, as determined by vaginal cytology. Implants were located, measured, and assessed visually for viability. Implants presenting as fluid-filled vesicles were deemed viable. Uteri were removed and frozen in liquid nitrogen for Western blot analysis for estrogen receptor- $\alpha$  and progesterone receptors. Serum samples were stored at  $-20^{\circ}\text{C}$  until analyses for circulating estradiol, progesterone, and genistein were conducted.

**Body weight analyses.** Body weights were recorded at the time of implant surgery, at ovariectomy/exploratory surgery, and at necropsy. Percent body weight gain from implant surgery to necropsy was calculated for each animal and averaged for each treatment group and compared.

**Western blot analyses.** A more detailed description of the protocol may be found elsewhere (Brown *et al.*, 1998). Uteri were homogenized in a buffer containing the following: 1% Triton-X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, and protease/phosphatase inhibitors. Equal amounts of protein were electrophoresed and transferred to nitrocellulose membranes. The membranes were blocked and incubated overnight with anti-ER- $\alpha$  (NHKDD) or anti-PR (Neomarkers, Fremont, CA) at 1:1000 and 1:250, respectively. Following washes with TBS-T, pH 7.5, the membranes were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase. Proteins were detected using chemiluminescence (Pierce, Rockford, IL). The relative intensity of the bands was measured using a scanner (Hewlett Packard Scan Jet 4p, Boise, ID).

**Serum estradiol-17- $\beta$  and progesterone.** RIA analyses were done by the laboratory of Dr. Larry Boots, University of Alabama at Birmingham, using commercially available kits (Pantex, Santa Monica, CA). Assays were performed according to the manufacturers' instructions.

**Serum genistein.** Total (unconjugated and conjugated forms) and free (unconjugated) genistein concentrations were measured using high perfor-

**TABLE 1**  
**Implant Survival in Ovariectomized Rats**

	No. of rats	Rats with surviving implants (%)
Experiment 1 (injections)		
Sesame oil (vehicle for estrone)	6	0
DMSO (vehicle for genistein)	14	0
Estrone (1.0 $\mu\text{g}/\text{rat}$ )	7	100
Genistein (5.0 $\mu\text{g}/\text{g}$ BW)	10	0
Genistein (16.6 $\mu\text{g}/\text{g}$ BW)	8	100
Genistein (50 $\mu\text{g}/\text{g}$ BW)	7	100
Experiment 2 (dietary)		
AIN-76A	17	0
Genistein (250 mg/kg AIN-76A)	12	0
Genistein (1000 mg/kg AIN-76A)	11	0

*Note.* Surgically induced endometriotic implants (two/rat) were inspected visually and assessed for viability at necropsy following 3 weeks of daily injections (Experiment 1), or dietary exposure (Experiment 2) to genistein. Viable implants were spherical, fluid-filled structures; nonsurviving implants were necrotic in appearance.

mance liquid chromatography coupled with mass spectrometry (HPLC-MS) (Coward *et al.*, 1996), with a lower limit of detection of 10 pM.

*Statistical analyses.* Mean values from serum steroid hormone analyses were calculated and compared using Student's *t*-test (Sigma Stat, Jandel Scientific, San Rafael, CA). For Western blot analyses, mean densitometric values for each treatment were compared to controls using one-way ANOVA (Sigma Stat, Jandel Scientific). Average body weight and percent body weight gain were also compared using one-way ANOVA. In Experiment 3, implant growth was compared for the two groups using Fisher's Exact test, (SAS/STAT, Cary, NC). Average growth or shrinkage of the implants was assessed using Wilcoxon Scores test (SAS/STAT).

## RESULTS

### Implant Survival

Genistein, when given by injection (Experiment 1), supported the growth of the implanted tissue in a dose-responsive manner (Table 1); the lowest effective dose was 16.6  $\mu\text{g}/\text{g}$  BW. Estrone injections also supported the implants. Injection of either vehicle, or the lowest dose of genistein (5.0  $\mu\text{g}/\text{g}$  BW) did not sustain the implants. Because the implants in the control group were nonviable, comparative analyses regarding

the size of the implants were not conducted. Treatment of ovariectomized rats (Experiment 2) with dietary genistein failed to sustain the implants at either dose (250 or 1000 mg genistein/kg AIN-76A). Ovariectomized animals fed the control diet (AIN-76A) did not have surviving implants. In ovary-intact animals (Experiment 3), control-fed rats as well as animals receiving 250 mg genistein/kg AIN-76A had 100% implant survival. Dietary genistein treatment did not result in any significant differences in the relative percentages of implants that increased, decreased, or stayed the same size when compared to controls (Table 2). The treatment had no effect on the degree to which the implants grew or shrank; average size increase and decrease were not statistically different between the control and genistein-fed groups.

### Uterine:Body Weight Ratio

Rats injected (Experiment 1) with estrone or genistein (50  $\mu\text{g}/\text{g}$  BW and 16.6  $\mu\text{g}/\text{g}$  BW doses) daily for 3 weeks had significantly increased uterine:body weight ratios (Fig. 1), compared to vehicle-treated ovariectomized rats. The increase in uterine weight represented tissue growth, not simply fluid imbibition, as previous studies have shown that multiple injections of genistein increase uterine size and cellularity (Brown and Lamartiniere, 2000). No treatment-related effects were observed in average body weights at the conclusion of the experiment. Average percent body weight gain following injections of 5.0 and 16.6  $\mu\text{g}$  genistein was  $46 \pm 3\%$  and  $41 \pm 3\%$ , respectively. These values were not statistically different from those of vehicle-treated animals ( $44 \pm 1\%$ ); however, percent body weight gain was significantly reduced ( $p < 0.001$ ) by treatment with estrone ( $32 \pm 1\%$ ) or 50  $\mu\text{g}$  genistein ( $19 \pm 2\%$ ). Injection with the lowest dose of genistein (5.0  $\mu\text{g}/\text{g}$  BW) induced a slight uterotrophic response; however, the difference did not reach statistical significance. In Experiment 2, ovariectomized rats receiving dietary genistein (1000 mg genistein/kg AIN-76A) had significantly increased uterine:body weight ratios (Fig. 2), compared to animals fed the control diet. The lower dietary dose, 250 mg genistein/kg AIN-76A, was not significantly uterotrophic. In ovary-intact rats (Experiment 3), treatment with 250 mg genistein/kg AIN-76A did not result in a uterotrophic response when compared

**TABLE 2**  
**Uterine Implants in Ovary-Intact Rats (Experiment 3)**

Diet	No growth	Increased growth	Decreased growth	Average size increase (mm)	Average size decrease (mm)
AIN-76A diet	16.7%	58.3%	25.0%	$1.2 \pm 0.3$	$3.2 \pm 0.5$
250 mg genistein/kg AIN-76A	18.8%	64.5%	16.7%	$1.6 \pm 0.2$	$3.5 \pm 0.6$

*Note.* Rats (10/group) were implanted with two pieces of uterine tissue. Each rat had at least one surviving implant at the end of the experiment. Implant size was recorded 3 weeks after surgical induction of endometriosis, and again at necropsy following a 3-week exposure to dietary genistein. Implant growth was determined by subtraction. Nonsurviving implants occurred with similar frequency in both groups and were excluded from size analysis. No statistical differences were revealed for implant growth/shrinkage (Fisher's Exact test), or average size increase/decrease (Wilcoxon Scores test).

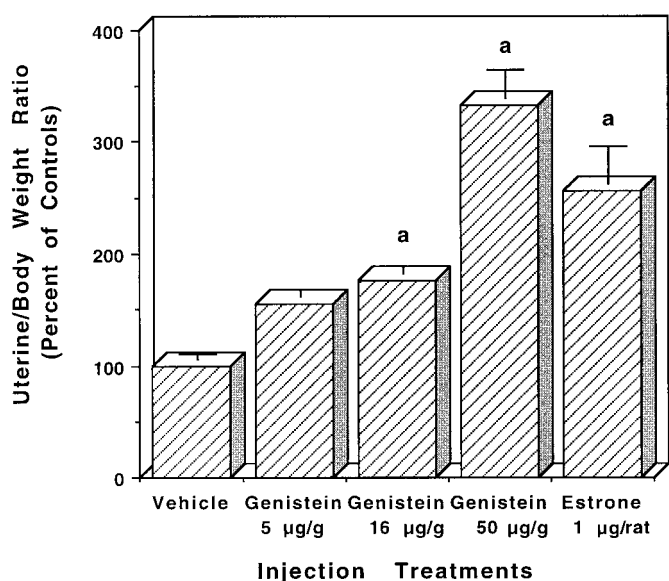


FIG. 1. Uterine to body weight ratio in ovariectomized rats injected with 5.0, 16.6, or 50.0 µg genistein/g BW or 1 µg estrone/rat; ( $n = 10, 8, 7,$  and  $7,$  respectively). Uterine weight was divided by body weight and the mean for the controls was set to 100; values for each treatment group were expressed as a percentage of the controls. As both vehicles, DMSO and sesame oil, produced a similar response, the data were combined,  $n = 20$ . The experiments were conducted in two parts and data were normalized in order to compare all groups together.  $a = p < 0.001$  compared to vehicle-treated animals.

to controls (data not shown). None of the dietary treatments resulted in significant alterations to final body weight or percent body weight gain when compared to controls (data not shown).

#### Uterine ER- $\alpha$ and PR

Since injection with the vehicle did not sustain the implants in ovariectomized rats, receptor expression was measured in the uterus as an indirect indicator of events occurring within the implanted tissue. Experiment 1, daily injections of estrone or genistein for 3 weeks at the two higher doses, resulted in significantly decreased expression of ER- $\alpha$  protein in the uterus when compared to animals injected with the vehicle (Fig. 3). The lowest dose of genistein did not significantly alter ER- $\alpha$  protein. All injected doses of genistein, as well as estrone, significantly increased expression of the 116 kDa isoform of the progesterone receptor (B form) when compared to controls (Fig. 4). However, only the low and medium doses of genistein significantly increased the 81 kDa PR isoform, the A form. Treatment with the high dose of genistein or estrone only slightly increased the PR A form when compared to controls.

In Experiment 2, ER- $\alpha$  decreased in a slight, but not statistically significant, dose-responsive manner following genistein treatment for 3 weeks (data not shown). However, the 1000 mg genistein/kg dose caused a significant increase in both the A and B forms of the progesterone receptor (Fig. 5). In ovary-

intact rats (Experiment 3), dietary genistein (250 mg/kg) did not produce any changes in ER- $\alpha$  or PR when compared to controls (data not shown).

#### Serum Estradiol and Progesterone Concentrations

In Experiment 3 (ovary-intact rats), circulating estradiol concentrations were  $16.7 \pm 5.2$  pg/ml in control-fed animals and  $15.4 \pm 2.8$  pg/ml in animals fed 250 mg genistein/kg diet. Progesterone concentrations were  $14.1 \pm 2.1$  ng/ml and  $15.6 \pm 2.9$  ng/ml for control-fed and genistein-fed animals, respectively. There were no statistically significant differences in the values of the genistein-treated rats when compared to controls.

#### Serum Genistein Concentrations

Free (unconjugated) and total (conjugated and unconjugated forms) serum genistein concentrations were determined from ovariectomized rats injected (Experiment 1) or fed genistein (Experiment 2) (Table 3). For rats given injections, serum genistein concentrations exhibited a dose-responsive relationship. However, the percent free genistein was similar for the 16.6 and 50 µg/g BW doses. The lower dietary dose (250 mg genistein/kg AIN-76A) resulted in total genistein concentrations that were approximately one-half that of the higher dose (1000 mg genistein/kg AIN-76A); the percent free genistein showed a similar relationship.

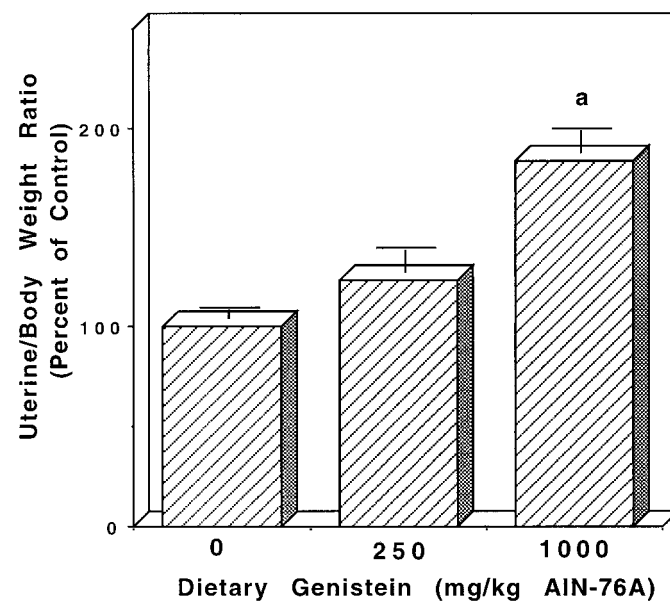


FIG. 2. Uterine to body weight ratio in ovariectomized rats fed 250 mg genistein/kg AIN-76A ( $n = 12$ ), 1000 mg genistein/kg AIN-76A ( $n = 12$ ), or AIN-76A alone ( $n = 17$ ). Uterine weight was divided by body weight and the mean for the controls was set to 100; values for each treatment group were expressed as a percentage of the controls. The experiments were conducted in two parts and data were normalized in order to compare all groups together.  $a = p < 0.001$  compared to controls.

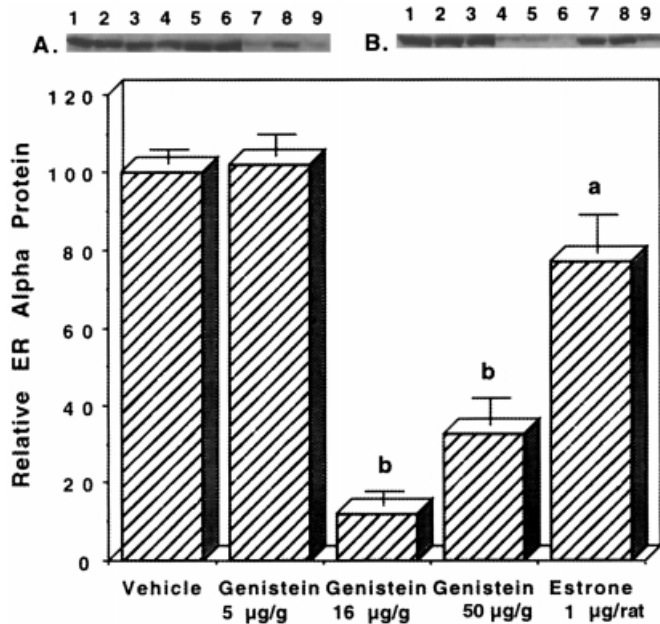


FIG. 3. Uterine ER- $\alpha$  protein in ovarioectomized rats injected with 5.0, 16.6, or 50.0  $\mu\text{g}$  genistein/g BW or 1  $\mu\text{g}$  estrone/rat; ( $n = 8, 8, 8,$  and  $7,$  respectively). Uterine extracts were subjected to western blot analysis using an antibody to ER- $\alpha$ . Photograph: panel A represents uterine ER- $\alpha$  in rats injected with vehicle (lanes 1-3), 5  $\mu\text{g}$  genistein/g BW (lanes 4-6), or 16.6  $\mu\text{g}$  genistein/g BW (lanes 7-9); panel B represents uterine ER- $\alpha$  in rats injected with vehicle (lanes 1-3), or 50  $\mu\text{g}$  genistein/g BW (lanes 4-6), or 1  $\mu\text{g}$  estrone/rat BW (lanes 7-9). Densitometric values were reported as a percentage of the controls  $\pm$  SEM. As both vehicles, DMSO and sesame oil, produced a similar response, the data were combined,  $n = 16$ . The experiments were conducted in two parts and data were normalized in order to compare all groups together.  $a = p < 0.05$ ;  $b = p < 0.001$  compared to vehicle-treated animals.

## DISCUSSION

In the ovarioectomized rat, genistein treatment successfully supported surgically induced endometriosis only when given by injection; the minimum effective dose that sustained endometriosis was 16.6  $\mu\text{g}/\text{g}$  BW. This dose was also the minimum required to induce a significantly uterotrophic response and to reduce the expression of uterine ER- $\alpha$ . A reduction in uterine ER- $\alpha$  protein and mRNA was demonstrated in response to administration of exogenous estrogen to ovarioectomized rats (Zhou *et al.*, 1993). The correlation between these events suggests that genistein achieved its action in a manner analogous to estradiol, through the classical estrogen receptor pathway. As further evidence of estrogen agonism by genistein, we measured expression of PR, an estrogen-inducible protein in the uterus (Kraus and Katzenellenbogen, 1993; Manni *et al.*, 1981). Injected genistein and estrone resulted in increased uterine PR expression at all doses. However, the increase in the A form was not statistically significant following treatment with the 50  $\mu\text{g}/\text{kg}$  dose of genistein and estrone. Because weight loss due to dietary restriction in rats can result in reduced uterine PR expression (O'Connor *et al.*, 1996), we

examined body weights in these two treatments as a potential explanation. Although there were no significant differences in average body weights taken at necropsy between treated animals and controls, the percent body weight gain from the beginning of the experiment to the end was significantly decreased by estrone and 50  $\mu\text{g}$  genistein compared to controls, thus explaining the effects on PR. These data suggest that measurement of the B isoform of PR is a good indicator of estrogenic action, while the A isoform may be a useful marker to detect potential uterine toxicity.

Human exposure to genistein occurs via the diet, not through injections. Therefore, we administered dietary genistein to rats with surgically induced endometriosis to more closely simulate a human situation. Dietary administration of genistein to ovariectomized rats did not support surgically induced endometriosis in ovariectomized rats. Based on blood genistein concentrations of Asian men (Adlercreutz *et al.*, 1993) and women (Morton *et al.*, 1999) of 276 nM, the 250 mg genistein/kg AIN-76A dose resulted in total genistein concentrations that were approximately 4-fold higher. The higher dose, 1000 mg genistein/kg AIN-76A, resulted in serum genistein concentrations of 2  $\mu\text{M}$ , approximately 8-fold higher than those of Asians eating a traditional diet high in soy. This dose was significantly uterotrophic and induced PR protein expression, indicative of estrogenic action. However, ER- $\alpha$  protein was only slightly reduced. This observation is supported by the

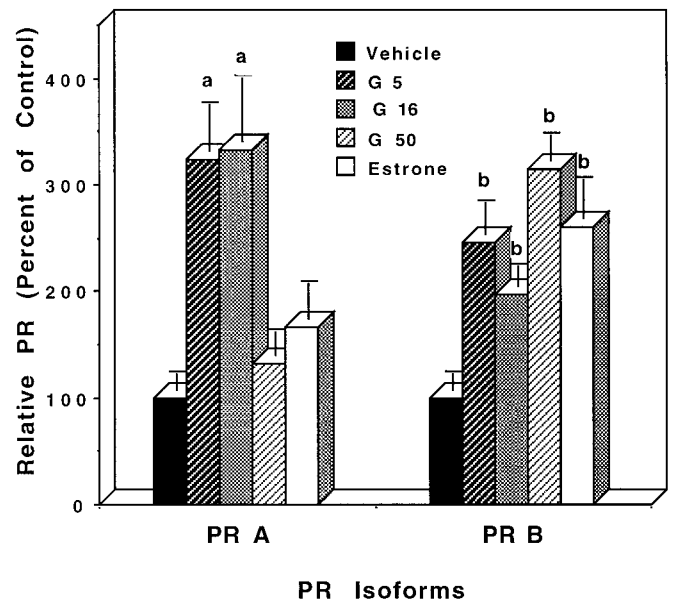


FIG. 4. Uterine PR proteins in ovarioectomized rats injected with 5.0 (G 5), 16.6 (G 16), or 50.0 (G 50)  $\mu\text{g}/\text{g}$  BW genistein or estrone (1  $\mu\text{g}/\text{rat}$ ); ( $n = 8, 8, 8,$  and  $7,$  respectively). Uterine extracts were subjected to Western blot analysis using an antibody to PR (A and B isoforms). Densitometric values were reported as a percentage of the controls  $\pm$  SEM. As both vehicles, DMSO and sesame oil, produced a similar response, the data were combined,  $n = 16$ . The experiments were conducted in two parts and data were normalized in order to compare all groups together.  $a = p < 0.05$ ;  $b = p < 0.001$  compared to vehicle-treated animals.

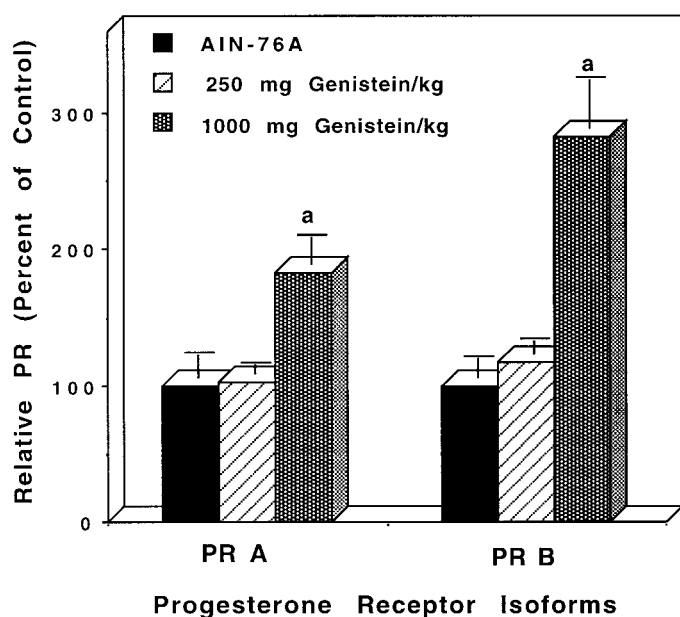


FIG. 5. Uterine PR proteins in ovariectomized rats fed 250 mg genistein/kg AIN-76A ( $n = 12$ ), 1000 mg genistein/kg AIN-76A ( $n = 12$ ), or AIN-76A alone ( $n = 17$ ). Uterine extracts were subjected to Western blot analysis using an antibody to PR (A and B isoforms). Densitometric values were reported as a percentage of the controls  $\pm$  SEM. The experiments were conducted in two parts and data were normalized in order to compare all groups together.  $a = p < 0.001$  compared to controls.

suggestion that PR is a more sensitive uterine biomarker for estrogenic action than ER- $\alpha$  (Carthew *et al.*, 1999).

The lack of viable implants in the dietary study may be explained by bioavailability. For example, the 16.6  $\mu\text{g/g}$  BW injected dose and the 250 mg dietary dose result in similar total serum genistein concentrations, 1380 nM and 1115 nM, respectively. Based on average daily food consumption of 15 g of feed per 300 g rat, rats consuming the 250 mg/kg diet are exposed to approximately 16  $\mu\text{g/g}$  BW genistein per day. However, injections of 16.6  $\mu\text{g}$  genistein/g BW resulted in four

times as much free genistein as that of the animals in the dietary study. When administered via the diet, the fraction of free genistein circulating in the blood is reduced by the processes of absorption through the digestive tract, binding to plasma proteins, conjugation by sulfation or glucuronidation, and subsequent excretion through the urine and feces.

The advantage of using ovariectomized rats in this endometriosis model is the absence of ovarian hormones, which may confound steroid receptor analyses. However, the use of ovary-intact rats more closely simulated a physiologic situation. With an intact pituitary/hypothalamic/ovarian signaling axis, this experiment allowed for the potential to detect implant growth effects related to ovarian steroidogenesis.

In previous studies, we observed significantly increased serum estradiol and decreased progesterone following pharmacologic injections of 500  $\mu\text{g}$  genistein/g BW to prepubertal female rats (Cotroneo *et al.*, 2001). However, in the current study, which used a more physiologically relevant dose and mode of administration, genistein did not significantly alter either hormone level when compared to controls. Likewise, uterine weight, steroid receptor expression, and implant growth were not affected by dietary genistein. Because no growth effects were observed, further biochemical analyses on the implants were not performed.

We conclude that pharmacologic injections of genistein supported surgically induced endometriosis in ovariectomized rats. The action of genistein on uterine steroid hormone receptors indicated estrogen agonism. When given in the diet, evidence of estrogenic activity by genistein was observed only at a high dose, which resulted in a uterotrophic response and increased PR expression. This observation is in accordance with the work of our lab (Fritz *et al.*, 1998) and others (Casanova *et al.*, 1999; Flynn *et al.*, 2000; Santell *et al.*, 1997), indicating that physiological doses of dietary genistein do not produce significant estrogen agonism or toxicity. Although the endometriosis model used in these studies provided valuable mechanistic data, its limitations in extrapolation to humans

TABLE 3  
Serum Genistein Concentrations

	$n$	Total genistein (nM)	Free genistein (nM)	Percent free genistein
Experiment 1 (injections)				
Vehicle	4	4 $\pm$ 2	Not done	Not applicable
Genistein (5.0 $\mu\text{g/g}$ BW)	5	450 $\pm$ 180	Not done	Not applicable
Genistein (16.6 $\mu\text{g/g}$ BW)	5	1380 $\pm$ 250	662 $\pm$ 94	48
Genistein (50.0 $\mu\text{g/g}$ BW)	5	5090 $\pm$ 700	2243 $\pm$ 477	44
Experiment 2 (dietary)				
AIN-76A	2	49 $\pm$ 23	Not done	Not applicable
Genistein (250 mg/kg AIN-76A)	8	1115 $\pm$ 552	138 $\pm$ 9	12
Genistein (1000 mg/kg AIN-76A)	7	2031 $\pm$ 271	466 $\pm$ 35	23

Note. Serum genistein concentrations were measured by HPLC-MS from ovariectomized rats injected daily or fed genistein for 3 weeks. In Experiments 1 and 2, blood was collected 16–18 h after the last injection and 6 h after the end of the dark cycle, respectively.

underscore the need for studies that evaluate the effects of exposure to soy or genistein on endometriosis in humans.

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