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Effects of Soybean Glyceollins and Estradiol in Postmenopausal Female Monkeys

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Abstract: *Glyceollins are a novel class of soybean phytoalexins with potential cancer-protective antiestrogenic effects. The purpose of this study was to evaluate the estrogen-antagonist effects of glyceollin-enriched soy protein on biomarkers for breast cancer risk. Thirty female postmenopausal cynomolgus macaques were randomized to one of three dietary treatments for 3 wk: 1) estradiol (E2, 1 mg/day) + casein/lactalbumin (control); 2) E2 + soy protein isolate (SPI) containing 194 mg/day isoflavonoids; and 3) E2 + glyceollin-enriched soy protein (GLY) containing 189 mg/day isoflavonoids + 134 mg/day glyceollins. Doses are expressed in calorically scaled human equivalents. Mean serum glyceollin concentrations at 4 h postfeeding were 134.2 ± 34.6 nmol/L in the GLY group and negligible in the SPI group ($P = 0.0007$). Breast proliferation was significantly increased in the control group (+237%, $P = 0.01$) but not in the SPI group (+198%, $P = 0.08$) or GLY group (+36%, $P = 0.18$). Gene expression of trefoil factor 1 and progesterone receptor, two markers of estrogen receptor activity in breast epithelium, were also significantly higher in the control ($P < 0.05$ for both) but not in the GLY group. These preliminary findings suggest that soybean glyceollins are natural compounds with potential estrogen-modulating properties in the breast.*

Introduction

Estrogen exposure is an important determinant of breast cancer risk (1,2). Prospective epidemiologic studies demonstrate a strong association between endogenous estrogens and breast cancer risk (3), and recent clinical trials indicate that pharmacologic antiestrogenic agents such as raloxifene may prevent a significant proportion of breast cancer cases (4). Dietary factors that reduce estrogen exposure may thus have an important role in breast cancer prevention.

Isoflavonoids are a class of natural phytochemicals with structural similarities to mammalian estrogens. The major source of dietary isoflavonoids is soy (5,6), which is rich in the glycosylated forms of genistein and daidzein. Regular consumption of soy isoflavonoids has been associated with reduced risk of breast and uterine cancer (7–9), particularly in women with higher estradiol exposure (10,11). Soy isoflavonoids competitively bind estrogen receptors and, in the presence of estradiol, may attenuate estrogenic effects (12–14). This evidence suggests that any potential chemopreventive benefits of dietary soy may relate to the estrogen-antagonist properties of its component phytoestrogens. Nevertheless, the role of specific isoflavonoids and their derivatives in modulating estrogen effects remains poorly understood.

Glyceollins are a class of phytoalexins produced in soybeans under the influence of stressors such as trauma or infection (15). Three major forms of glyceollins have been identified (I–III), all of which are derived from the parent isoflavone daidzein through a series of pterocarpan intermediates (Figure 1). Compared with genistein and daidzein, purified glyceollins show greater inhibition of estradiol effects on proliferation and estrogen receptor signaling in breast cancer cells (16). Glyceollins also have enhanced antagonism toward the estrogen receptor alpha relative to beta and lack any of the estrogen agonist activity of genistein and daidzein seen in low-estrogen conditions (16). These findings suggest that soy protein enriched in glyceollins may have distinct estrogen-modulating properties compared with standard soy protein. To test this idea, we used a postmenopausal primate model to evaluate the short-term effects of glyceollin-enriched soy protein and standard soy protein isolate in combination with estrogen.

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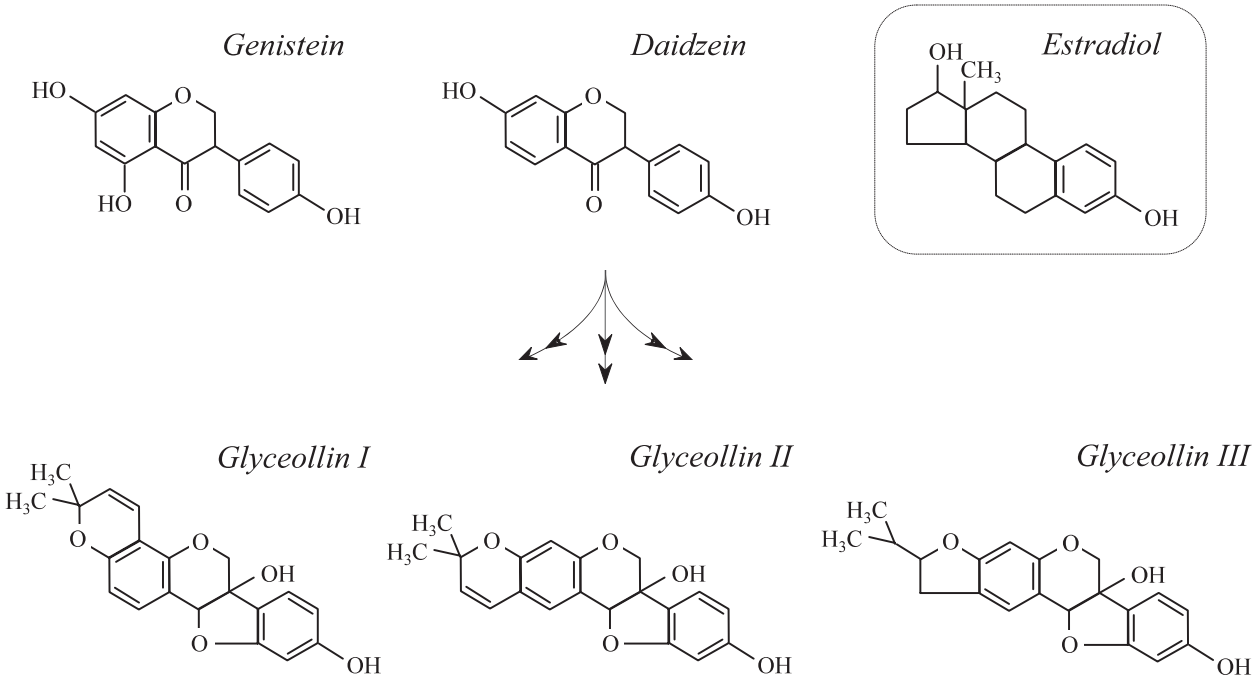

Figure 1. Structural comparison of the three major glyceollins (I–III), the primary soy isoflavones genistein and daidzein, and 17β-estradiol. Glyceollins are phytoalexin compounds produced from daidzein within the soybean in response to stress.

Materials and Methods

Animal Subjects and Diets

We used 30 adult female surgically menopausal cynomolgus macaques *(Macaca fascicularis)* with an average age of 17.8 ± 0.5 years. All animals had been ovariectomized for 4 yr and housed since that time in stable social groups of three to four animals each. These animals were previously enrolled in a randomized Latin-square crossover study evaluating soy isoflavone effects when given with either trace or low-dose oral estradiol (17). In this previous study, each social group of animals received the same experimental treatments but in a different sequence. No significant carryover effects were found for any breast endpoints across the 4-wk washout periods between treatment phases. The estradiol doses used in the previous study (equivalent to 0.09 or 0.5 mg/day in women) were less than those typically prescribed to postmenopausal women for hormone therapy (~1.0 mg/day), and the isoflavone doses (equivalent to 0, 60, 120, or 240 mg/day in women) were within the range of human dietary or supplement exposure. There is no evidence that this level of estrogen or isoflavone exposure alters the subsequent hormonal response of the adult mammary gland.

For the current study, the monkeys all received a control casein/lactalbumin-based diet for 6 wk before the start of the experiment. Animals were then randomized by social group to receive one of three diets containing the following: 1) estradiol (E2, 1 mg/1,800 kcal) + casein/lactalbumin [control (Con), n = 9]; 2) E2 + soy protein isolate (SPI) containing 193.6 mg/1,800 kcal isoflavonoids (n = 11); and 3) E2 + glyceollin-enriched soy protein (GLY) containing 188.5 mg/1,800 kcal isoflavonoids and 134.1 mg/1,800 kcal glyceollins (n = 10). The control diet contained a trace amount of soy protein delivering 6.7 mg/1,800 kcal isoflavonoids. All isoflavonoid doses are expressed in aglycone equivalents. Diets were isocaloric and similar in macronutrients, cholesterol, calcium, and phosphorus (Table 1).

The glyceollin-enriched protein was produced by enzymatic treatment of scarred soybeans (*Glycine max*) to induce conversion of the parent isoflavone daidzein to glyceollins. The beans were then ground, defatted, and incorporated into a fiber concentrate. The GLY supplement contained 959.5 µg of unconjugated glyceollins per gram of product (76.8% glyceollin I, 9.9% glyceollin II, and 13.6% glyceollin III), as determined by high-pressure liquid chromatography (HPLC) and ultraviolet (UV) monitoring (visible spectrophotometry). Glyceollin HPLC analyses were performed on a Waters 600E System Controller combined with a UV-VIS 996 detector. Glyceollins were extracted and homogenized in 0.5 ml 80% EtOH, heated at 50°C for 1 h, cooled, centrifuged at 14,000*g* for 10 min, and filtered. An aliquot (20 µl) of supernatant was directly analyzed by HPLC. Glyceollins were monitored at a wavelength of 285 nm, and separations were carried out using a Vydac Multiring C_{18} (4.6 × 250 mm; 5 µm) reverse-phase column. Elution was carried out at a flow rate of 1.0 ml/min using a standard solvent system. All HPLC analyses were run in triplicate. Relative isoflavonoid content was also measured us-

Table 1. Composition of Experimental Diets Containing Control Casein/Lactalbumin (Control), a Standard Soy Protein Isolate (SPI), or Glyceollin-Enriched Soy Protein (GLY)

Ingredient (per kg dry weight)	Control	SPI	GLY
Protein (g)	206.9	210.9	197.9
Fat (g)	130.7	132.3	133.9
Carbohydrates (g)	311.0	311.0	311.0
Energy (kcal)[a]	3,229.2	3,245.2	3,222.1
Cholesterol (g)	0.85	0.84	0.82
Calcium (g)	3.10	3.05	3.13
Phosphorus (g)	3.07	2.95	3.15
Casein (g)	120.0	22.5	25.0
Lactalbumin (g)	110.0	22.5	25.0
Fiber concentrate (g)[b]	70.4	72.4	—
Glyceollin-enriched fiber concentrate (g)[c]	—	—	250
SPI (g)[d]	—	185	—
Estrace (g)[e]	1.80	1.80	1.80

a: Monkeys were fed 120 kcal/kg body weight/day.
b: FIBRIM 2000® fiber supplement containing 11.4% soy protein with 0.08 mg genistein, 0.07 mg daidzein, and 0.02 mg glycitein per gram of product (in aglycone units).
c: Glyceollin-enriched full fiber concentrate (E100444-84-1) containing soy protein with 0.71 mg genistein, 0.58 mg daidzein, and 0.06 mg glycitein (in aglycone units) and 0.96 mg of glyceollin per gram of product.
d: SPI (1B1.2 UN 30CA) containing 1.12 mg genistein, 0.63 mg daidzein, and 0.07 mg glycitein per gram of soy protein product (in aglycone units).
e: Oral micronized 17β-estradiol tablets delivering 66.7 μg/kg body weight (≈1.0 mg/day in human equivalents).

ing HPLC (by the manufacturer) and reported in aglycone units as 61.5% genistein, 34.6% daidzein, and 3.8% glyceitein for the soy protein isolate and 52.6% genistein, 43.0% daidzein, and 4.4% glyceitein for the glyceollin-enriched protein. To balance the diets, a fiber concentrate (FIBRIM 2000®) was added to the control and SPI diets. This concentrate contained a small amount of soy protein (11.4% by weight) providing 0.17 mg isoflavonoids per gram of product (as measured by HPLC). The soy protein isolate and fiber concentrate were generously provided by Solae, a division of Dupont (St. Louis, MO). The glyceollin-enriched protein was provided through collaborative efforts of Solae; the Southern Regional Research Center, United States Department of Agriculture; and the Tulane University School of Medicine. Estradiol tablets were obtained from Mylan Pharmaceuticals (Morganton, WV).

Animals were fed approximately 120 kcal/kg body weight (BW) once daily. Daily doses of estradiol, isoflavonoids, and glyceollins were scaled to 1,800 kcal of diet (the estimated daily intake for a U.S. woman) to account for differences in metabolic rates between the monkeys and human subjects (18). Monkeys were thus given 66.7 μg of E2/kg BW (all groups); 0.44 mg (Con), 12.91 mg (SPI), or 12.57 mg (GLY) of isoflavonoids/kg BW; and 8.94 mg glyceollins/kg BW (GLY) each day. Of note, the initial SPI and GLY diet formulations lacked adequate palatability, requiring all the animals to be placed on the control group diet (with E2) for 1 wk 14 days into the experiment. All diets were reformulated during this time with sweetened applesauce and fed henceforth for 3 weeks without compliance problems.

All procedures involving these animals were conducted in compliance with state and federal laws, standards of the U.S. Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee (ACUC). The facilities and laboratory animal program of Wake Forest University are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Breast Biopsies

At the beginning and end of the dietary treatment period, the animals were anesthetized with ketamine and buprenorphine for breast biopsy, blood collection, uterine ultrasound, vaginal cytology, and body weight measurement. For the breast biopsy, a 1.5-cm incision was made in a preselected breast quadrant, and a small (~0.4 g) sample of mammary gland was removed. The incision was sutured, and the animals were monitored and given analgesia during recovery following ACUC-approved clinical procedures. The biopsy site was tattooed to prevent later resampling at the same site. Half of the biopsy sample was frozen; the other half was fixed at 4°C in 4% paraformaldehyde for 24 h and then processed for histology using standard procedures.

Serum Isoflavonoid and Glyceollin Analysis

Serum concentrations of soy isoflavonoids (genistein, daidzein, equol) and total glyceollins (I–III) were determined by liquid chromatographic-photodiode array mass spectrometric analysis, using techniques described previously (19). Quantitation was performed by selected reaction monitoring using parent molecules and relevant product ions. Calibration curves for isoflavonoids and glyceollins were constructed using a series of diluted standards with concentrations determined by absorbance readings (ε =10,300 at 285 nm in methanol). Measurements were performed on 4-h and 24-h fasted serum samples collected at the time of biopsy. Se-

rum isoflavonoid concentrations resulting from the casein/lactalbumin-based washout diet (baseline) had been measured previously and found to be negligible.

Immunohistochemistry

Immunostaining procedures were performed on fixed, paraffin-embedded mammary gland tissues using commercially available primary monoclonal antibodies for the proliferation marker Ki67 (Ki67/MIB1, Dako, Carpinteria, CA). Staining methods included antigen retrieval with citrate buffer (pH 6.0), biotinylated rabbit antimouse F_c antibody as a linking reagent, alkaline phosphatase-conjugated streptavidin as the label, and Vector Red as the chromogen (Vector Laboratories, Burlingame, CA). Cell staining was quantified by a computer-assisted counting technique, using a grid filter to select cells for counting and our modified procedure of cell selection, described previously (20). For counting, breast epithelium was subdivided into lobuloalveolar and ductal compartments. Numbers of positively stained cells were measured as a percentage of the total number examined (100 cells for each compartment), and positive cells were scored for staining intensity (1+, 2+, or 3+) to obtain an H-score (Intensity × % + cells) (21). All measurements were blinded to treatment group.

Quantitative Gene Expression

Mammary gland expression of estrogen receptor alpha (*ESR1*) and two estrogen-responsive markers, trefoil factor 1 (*TFF1*) and progesterone receptor (*PGR*), were determined using quantitative real-time polymerase chain reaction (qRT-PCR). Both *TFF1* and *PGR* are upregulated through ESR1-mediated pathways and thus serve as markers of ligand-dependent receptor activation within breast tissue (22,23). Macaque-specific qRT-PCR primer probe sets were generated for *ESR1* (GenBank Accession no. DQ469336), *TFF1* (GenBank Accession no. DQ464113), and the internal control gene GAPDH (GenBank Accession no. DQ464111), and a human ABI Taqman primer probe set was used for *PGR*. Assay specifics have been described previously (24). RNA was extracted from frozen mammary biopsies using Tri Reagent (Molecular Research Center, Cincinnati, OH), quantitated, and reverse-transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City CA). Real-time PCR reactions (20 µl volume) were performed on an Applied Biosystems ABI PRISM® 7000 Sequence Detection System using Taqman Universal Master Mix and associated reagents. The thermocycling protocol involved initial incubations of 2 min at 50° and 10 min at 95° followed by 40 PCR cycles of 95° for 15 s and 60° for 1 min. Relative expression was determined using the ΔΔCt method described in Applied Biosystems, User Bulletin #2 (available online at http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). Baseline and posttreatment samples were run on the same plate. Stock breast tissue was run in duplicate on each plate as an external calibrator. Calculations were performed using ABI Relative Quantification SDS Software version 1.1.

Serum Estradiol

Serum estradiol (E2) concentrations were measured on 24-h fasted serum samples collected at the time of biopsy. Estradiol was quantitated by radioimmunoassay (RIA) using a commercially available kit and protocol from Diagnostic Systems Laboratories (E2, DSL-4800 ultrasensitive; Webster, TX). Serum (0.5 ml) was extracted by adding ethyl ether (4 ml) and vortexing for 5 min. The aqueous layer was frozen in a dry ice/isopropanol bath, and the organic phase was decanted. Extracts were dried and reconstituted with zero-standard serum. Assays were performed in the Clinical Pathology Laboratory at the Comparative Medicine Clinical Research Center, Wake Forest University School of Medicine.

Vaginal Maturation

Vaginal keratinocytes were collected from the anterior vagina with a cotton swab, rolled onto a glass slide, and fixed using a commercial fixative (Spray-cyte, Surgipath Medical Industries, Richmond, IL). Slides were stained using a modified Papanicolau method. Maturation value (MV) was calculated using the following formula: MV = (0.2 × % parabasal cells) + (0.6 × % intermediate cells) + (% of superficial cells).

Uterine Area

Uterine area was determined by transabdominal ultrasound using a Sonosite 180 portable ultrasound machine with a 5.0-MHz linear transducer (Sonosite, Bothell, WA). Images were recorded before and after each treatment phase. Uterine thickness and maximal transverse cross-sectional area were measured on a static representative digital image using public domain software (NIH ImageJ 1.33; available at http://rsbweb.nih.gov/ij/download.html).

Data Analysis

The major statistical outcome was change-from-baseline within each diet group, as determined from a paired *t*-test (baseline vs. treatment) within each diet group. The primary endpoint was breast proliferation. As a pilot investigation, this study was not adequately powered to detect significant intergroup differences for outcome tissue measures. Serum isoflavonoids, glyceollins, and estradiol were evaluated using a general linear model [analysis of variance (ANOVA)]. A general linear model was also used to screen for any significant carryover effects of treatment in the final phase of the previous experiment. For this analysis, baseline and change-from-baseline values for all measures in the current study were evaluated based on prior estradiol dose (0.09 or 0.50 mg/day) and isoflavone dose (0, 60, 120, or 240 mg/day); no significant carryover effects were found for either estradiol or isoflavone treatment (ANOVA $P > 0.1$ for all endpoints).

Among posttreatment breast biopsies, lobular epithelium was absent in two samples (one Con, one GLY) and ductal epithelium in six samples (one Con, two SPI, three GLY), reducing sample size for these particular endpoints. One posttreatment ultrasound image was unmeasurable (Con). All variables were evaluated for their distribution and equality of variances. All data are reported as mean ± standard error. Data were analyzed using the SAS statistical package (version 8; SAS Institute, Cary, NC). A two-tailed significance level of 0.05 was chosen for all comparisons.

Results

Total serum isoflavonoid concentrations were significantly higher in the SPI and GLY groups compared with the control group at 4 h ($P < 0.001$ for both) and 24 h ($P < 0.05$ for both) postfeeding (Table 2). The SPI and GLY groups did not differ in total serum isoflavonoids at either 4 h ($P = 0.59$) or 24 h ($P = 0.73$) postfeeding; individual isoflavonoids were also comparable between the two diets. Total serum isoflavonoids for the SPI and GLY diets at 4 h postfeeding were similar to peak ranges reported in human soy intervention studies (25). Isoflavonoid clearance was approximately 85% between 4-h and 24-h samples. Equol was the predominant circulating isoflavonoid in SPI and GLY groups, comprising 67% (SPI) and 71% (GLY) of total isoflavonoids at 4 h postfeeding.

The mean serum glyceollin concentration (I–III) at 4 h postfeeding was 134.2 ± 34.6 nmol/L in the GLY group (range: 16.7–324.3 nmol/L) and below detectable limits in the SPI group ($P = 0.0007$ compared with GLY). Relative to the major soy isoflavones, mean glyceollin concentrations were 1.21 times that of genistein and 0.60 times that of daidzein. Glyceollin concentrations were undetectable (<1.0 nmol/L) at 24 h postfeeding in the both the SPI and GLY groups.

Body weights and serum estradiol were measured as indicators of dietary intake. Mean body weights were 3.34 ± 0.14, 3.30 ± 0.17, and 3.10 ± 0.14 kg at baseline and 3.15 ± 0.14, 3.19 ± 0.16, and 3.17 ± 0.13 kg at posttreatment for the control, SPI, and GLY groups, respectively. Change in body weight was not significantly different for any of the treatments ($P > 0.1$ for all). Serum E2 concentrations at 24 h postfeeding were 21.3 ± 4.4 pg/ml in the control group, 22.9 ± 4.4 pg/ml in the SPI group, and 29.1 ± 5.6 pg/ml in the GLY group (ANOVA $P = 0.44$). Previous serum E2 values from these animals receiving the baseline diet were all <5.0 pg/ml.

The primary endpoint of this study was breast proliferation, as determined by immunohistochemical expression of the Ki67 (MIB1) antigen. Ki67 expression is an important prognostic indicator in human breast cancer (26) and marker of hormone-associated risk in our model (27,28). Total breast epithelial proliferation was significantly greater following treatment in the control group (+237%, $P = 0.01$) but not in the SPI (+198%, $P = 0.08$) or GLY group (+36%, $P = 0.18$; Table 3). This difference was driven largely by changes in ductal proliferation, which was increased several-fold in the control group but not the GLY group.

No differences in mammary gland histology were detected among treatment groups. Focal atypical ductal hyperplasia was present in one baseline biopsy (Con) and three treatment biopsies (two Con, one SPI). Columnar cell change in mammary ducts was present in three baseline biopsies (one Con, two SPI) and three treatment biopsies (two SPI, one GLY). Mammary lobular enlargement (>30 acinar units) was noted in four baseline samples (one control, two SPI, one GLY) and seven treatment samples (three control, one SPI, three GLY). No neoplastic lesions were identified in any of the biopsies.

We next measured intramammary expression of estrogen receptor alpha (*ESR1*) and two genes driven by *ESR1*, *TFF1* and *PGR*. Breast tissue expression of *TFF1* was low at baseline but markedly increased following treatment in all

Table 2. Serum Isoflavonoid and Glyceollin Concentrations in Animals Fed Diets Containing Control Casein/Lactalbumin (Control), a Standard Soy Protein Isolate (SPI), or Glyceollin-Enriched Soy Protein (GLY)[a]

	Control	SPI	GLY
4 h postfeeding (nmol/L)[b]			
Genistein	20.9 ± 33.0	168.1 ± 29.9[c]	110.6 ± 31.3
Daidzein	26.7 ± 42.9	268.9 ± 38.8[c]	225.2 ± 40.7[c]
Equol	<10	884.3 ± 145.6[c]	816.5 ± 152.7[c]
Glyceollins	ND	<1	134.2 ± 34.6[d]
Total	50.6 ± 197.5	1,322.1 ± 215.0[c]	1,286.5 ± 225.5[c]
24 h postfeeding (nmol/L)[b]			
Genistein	6.7 ± 1.2	<5	<5
Daidzein	10.4 ± 3.1	5.6 ± 2.6	15.3 ± 2.7[d]
Equol	<10	158.3 ± 37.3[c]	171.9 ± 39.1[c]
Glyceollins	ND	<1	<1
Total	<20	167.0 ± 37.9[c]	189.8 ± 39.8[c]

a: Values are mean ± SE with 9–11 animals per group.
b: Quantitation limits for isoflavonoids were 1 nmol/l for glyceollins (I–III), 5 nmol/l for genistein and daidzein, and 10 nmol/l for equol.
c: $P < 0.05$ vs. control group.
d: $P < 0.05$ vs. SPI group.

Table 3. Effects of Diets Containing Control Casein/Lactalbumin (Control), Standard Soy Protein Isolate (SPI), or Glyceollin-Enriched Soy Protein (GLY) on Estrogen-Induced Breast Proliferation[a]

Breast Proliferation (%Ki67 H-score)	Control	SPI	GLY
Lobular epithelium			
Baseline	11.9 ± 5.3	9.5 ± 4.1	15.3 ± 4.2
Treatment	30.4 ± 3.9	23.1 ± 5.2	26.9 ± 5.5
Change (treatment–baseline)	19.3 ± 6.7	13.5 ± 6.9	11.4 ± 4.8
P value (baseline vs. treatment)[b]	0.03	0.07	0.04
Ductal epithelium			
Baseline	0.3 ± 0.3	0.7 ± 0.5	2.6 ± 1.4
Treatment	9.5 ± 4.8	7.3 ± 1.7	2.9 ± 1.4
Change (treatment–baseline)	9.7 ± 5.3	6.4 ± 2.1	0.6 ± 2.4
P value (baseline vs. treatment)[b]	0.12	0.02	0.82
Total			
Baseline	6.1 ± 2.7	5.1 ± 2.2	9.0 ± 2.2
Treatment	19.9 ± 2.9	15.3 ± 3.3	12.6 ± 2.1
Change (treatment–baseline)	14.5 ± 3.9	10.1 ± 5.0	3.2 ± 2.1
P value (baseline vs. treatment)[b]	0.01	0.08	0.18

a: Values represent mean ± SE with 7–11 animals per group.
b: Student's paired *t*-test.

Table 4. Effects of Diets Containing Control Casein/Lactalbumin (Control), Standard Soy Protein Isolate (SPI), or Glyceollin-Enriched Soy Protein (GLY) on Markers of Estrogen Receptor Activity in the Breast[a]

Gene Expression (arbitrary units)[b]	Control	SPI	GLY
TFF1			
Baseline	0.7 ± 0.2	0.6 ± 0.2	1.2 ± 0.3
Treatment	88.0 ± 28.2	66.8 ± 25.9	70.6 ± 35.8
Change (treatment–baseline)	87.3 ± 28.2	66.2 ± 26.0	69.4 ± 35.5
P value (baseline vs. treatment)[c]	0.01	0.03	0.08
PGR			
Baseline	2.7 ± 0.5	3.3 ± 0.8	2.2 ± 0.4
Treatment	14.7 ± 4.9	8.6 ± 3.1	5.3 ± 2.3
Change (treatment–baseline)	12.0 ± 5.0	5.2 ± 2.8	3.0 ± 2.3
P value (baseline vs. treatment)[c]	0.04	0.09	0.22
ESR1			
Baseline	1.0 ± 0.4	0.6 ± 0.3	0.4 ± 0.1
Treatment	0.8 ± 0.2	0.6 ± 0.1	0.9 ± 0.1
Change (treatment–baseline)	–0.1 ± 0.3	0.0 ± 0.3	0.5 ± 0.1
P values (baseline vs. treatment)[c]	0.64	0.98	0.003

a: Values represent mean (± SE) with 9–11 animals per group. Abbreviations are as follows: *ESR1*, estrogen receptor alpha; *PGR*, progesterone receptor; *TFF1*, trefoil factor 1.
b: Gene expression was determined by real-time quantitative polymerase chain reaction and adjusted for the internal control gene *GAPDH*.
c: Student's paired *t*-test.

groups. This change was significant for the control (+125-fold, $P = 0.01$) and SPI groups (+110-fold, $P = 0.03$) but not the GLY group (+58-fold, $P = 0.08$) (Table 4). Progesterone receptor expression, although not as estrogen-sensitive as *TFF1*, followed a similar pattern whereby E2 treatment induced a significant increase in the control group (+4.4-fold, $P = 0.02$) but not in the SPI (+1.6-fold, $P = 0.09$) or the GLY groups (+1.4-fold, $P = 0.22$). These effects were accompanied by a significant increase in *ESR1* gene expression in the GLY group (+1.3-fold, $P = 0.003$) that was not seen in the other groups (Table 4).

Finally, we measured two estrogen-responsive markers in the reproductive tract: uterine size and vaginal maturation. Vaginal maturation increased by 64%, 75%, and 43% in the control, SPI, and GLY groups, respectively ($P \leq 0.001$ for all vs. baseline). Similarly, uterine area increased by 69% in the control and SPI groups and 53% in the GLY group ($P < 0.01$ for all vs. baseline; Table 5).

Discussion

Glyceollins are a unique class of phytoalexin compounds produced in certain legumes as defense molecules. In this study, we evaluated the estrogen-modulating effects of glyceollin-enriched soy protein in a postmenopausal primate model. We found that dietary glyceollins from elicited soy protein are absorbed and present at serum concentrations

Table 5. Effects of Diets Containing Control Casein/Lactalbumin (Control), Standard Soy Protein Isolate (SPI), or Glyceollin-Enriched Soy Protein (GLY) on Reproductive Tract Measures[a]

Tissue Measure	Control	SPI	GLY
Vaginal maturation index			
Index, baseline	51.3 ± 3.6	48.8 ± 5.1	60.0 ± 4.0
Index, treatment	84.2 ± 1.2	85.5 ± 1.2	85.9 ± 2.1
Change (treatment–baseline)	32.9 ± 4.3	36.7 ± 5.9	25.9 ± 5.3
P values (baseline vs. treatment)[c]	<0.0001	0.0001	0.001
Uterine area (cm^2)			
Area, baseline	1.13 ± 0.12	1.04 ± 0.07	1.13 ± 0.09
Area, treatment	1.93 ± 0.18	1.76 ± 0.14	1.73 ± 0.12
Change (treatment–baseline)	0.78 ± 0.18	0.72 ± 0.11	0.60 ± 0.11
P values (baseline vs. treatment)[c]	0.003	<0.0001	0.0004

a: Values are mean ± SE with 8–11 animals per group.
b: Student's paired *t*-test.

comparable to soy isoflavones. When given with estradiol, glyceollin-enriched protein resulted in a modest reduction of estrogenic stimulation in the breast. Similar effects were not seen on more sensitive reproductive tract measures. These findings suggest that glyceollins may have selective estrogen-antagonist properties distinct from soy isoflavonoids.

The attenuating effects of the glyceollin-enriched diet on breast proliferation occurred alongside similar changes in markers of estrogen receptor activity. The *TFF1* and *PGR* genes have near-consensus (*TFF1*) or half-site (*PGR*) estrogen response elements in their respective promoter regions, and their expression is thus driven largely by ligand-bound ESR1 (22,23). In this study, the E2-induced expression of *TFF1* and *PGR* was reduced by 21% and 75%, respectively, in the glyceollin diet compared with the control diet, despite increased *ESR1* expression in the GLY group. This observation, in combination with previous evidence from breast cell culture experiments (16), suggests that dietary glyceollins may competitively bind and inhibit ESR1 in the breast. Further investigation is needed to confirm this finding and evaluate the selectivity of potential ESR1-modulating effects of glyceollins on other body systems. The lack of any detectable uterine effect of the glyceollin-enriched diet may be due to a combination of factors, including the high estrogen sensitivity of the endometrium (relative to the breast), inadequate precision of the ultrasound measurements for the study size, or a variety of tissue-specific factors involved in estrogen signaling (e.g., nuclear receptor coregulator activity).

Outcome measures in the standard soy protein group were generally intermediate between the control and GLY groups. This marginal type of effect is consistent with previous trials in our model. We recently reported that soy isoflavones could attenuate estradiol effects in the breast tissue of postmenopausal macaques, although significant inhibition was seen only at isoflavone levels equivalent to 240 mg/day (17). Typical dietary isoflavone consumption, by contrast, ranges from <5 mg/day in a typical Western diet up to about 60 mg/day in some Asian diets (29). In our longest study, 3 yr of treatment with soy protein containing the human equivalent of 129 mg/day of isoflavones had no estrogen agonist effects in breast or uterine tissue, whereas animals in the highest tertile of serum isoflavones had significantly lower breast density (30). Intervention studies of women (31–36) have generally found minimal to no significant effects of dietary isoflavones on reproductive tissue measures. Overall, this evidence suggests that standard (nonsupplemented) soy protein formulations may have estrogen-modulating effects at high doses or after long-term daily intake but that any such effects are unlikely to be present or detectable at normal dietary levels. We should also note that any mitigating hormonal effects of equol production, which is consistently higher in our model than in human populations, are not known.

This study represents the first attempt to evaluate the bioavailability of glyceollins given within a soy protein matrix. We found glyceollin concentrations to be lower in serum relative to overall isoflavonoids. The glyceollin dose of 134 mg/1,800 kcal represented more than half that of the isoflavonoids in the GLY diet but resulted in concentrations 11.6% that of the total pool of serum isoflavonoids at 4 h after feeding. It is not clear whether this discrepancy is due to lower relative absorption of glyceollins, more rapid clearance, or conversion into unmeasured metabolites. Interestingly, this diet-to-serum ratio was similar to that seen with genistein (99 mg/1,800 kcal in diet, 9.6% of isoflavonoids in serum) but much higher compared with daidzein and its primary metabolite equol (81 mg/1,800 kcal in diet, 90.4% of isoflavonoids in serum). Although the glyceollin dose used in this study is theoretically attainable via dietary means (through elicited soy protein), further studies using purified glyceollin formulations or standardized glyceollin-elicited soy protein are needed to determine specific glyceollin effects and pharmacodynamics.

In this pilot investigation, we evaluated the breast effects of glyceollin-enriched soy protein in combination with estradiol. Our results suggest that glyceollin-enriched soy protein may help buffer breast epithelium against estrogenic effects. These findings indicate a need for further investigation of glyceollins as selective estrogen receptor-modulating dietary compounds.

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