β-Sitosterol, β-Sitosterol Glucoside, and a Mixture of β-Sitosterol and β-Sitosterol Glucoside Modulate the Growth of Estrogen-Responsive Breast Cancer Cells In Vitro and in Ovariectomized Athymic Mice[1]

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ABSTRACT We hypothesized that the phytosterols β-sitosterol (BSS), β-sitosterol glucoside (BSSG), and Modu-care (MC; BSS:BSSG = 99:1) could modulate the growth of estrogen-dependent human breast cancer cells in vitro and in vivo. The present study evaluated the estrogenic and antiestrogenic effects of BSS, BSSG, and MC (0.001 to 150 μmol/L) on the proliferation of Michigan Cancer Foundation 7 (MCF-7) cells in vitro. Both BSS (>1 μmol/L) and MC (>50 μmol/L) increased MCF-7 cell proliferation. Treatment with 150 μmol/L of BSS and MC increased cell growth by 2.4 and 1.5 times, respectively, compared to the negative control (NC) group. However, BSSG had no effect at the concentrations tested. The effects of dietary BSS, BSSG, and MC on the growth of MCF-7 cells implanted in ovariectomized athymic mice were also evaluated. Estrogenic effects of the phytosterols were evaluated in the NC, BSS, BSSG, and MC treatment groups, and antiestrogenic effects were evaluated in the 17β-estradiol (E_2), E_2 + BSS, E_2 + BSSG, and E_2 + MC treatment groups. Mice were treated with dietary BSS (9.8 g/kg AIN93G diet), BSSG (0.2 g/kg diet), or MC (10.0 g/kg diet) for 11 wk. Dietary BSS, BSSG, and MC did not stimulate MCF-7 tumor growth. However, dietary BSS, BSSG, and MC reduced E_2-induced MCF-7 tumor growth by 38.9% ($P < 0.05$), 31.6% ($P = 0.08$), and 42.13% ($P < 0.05$), respectively. The dietary phytosterols lowered serum E_2 levels by 35.1, 30.2, and 36.5% in the E_2 + BSS, E_2 + BSSG, and E_2 + MC groups, respectively ($P < 0.05$), compared to that of the E_2 treatment group. Estrogen-responsive pS2 mRNA expression in tumors did not differ among groups, but expression of the antiapoptotic marker B-cell lymphoma/leukemia-2 (bcl-2) in tumors from the E_2 + MC group was downregulated, compared to that of the E_2 treatment group. In summary, BSS and MC stimulated MCF-7 cell growth in vitro. Although BSSG comprises only 1% of MC, BSSG made MC less estrogenic than BSS alone in vitro. However, dietary BSS and MC protected against E_2-stimulated MCF-7 tumor growth and lowered circulating E_2 levels. J. Nutr. 134: 1145–1151, 2004.

KEY WORDS: • *β-sitosterol* • *β-sitosterol glucoside* • *breast cancer* • *MCF-7 cells*

More than 100 different types of phytosterols (plant sterols) have been identified (1). β-Sitosterol (BSS),[3] stigmasterol, and campesterol are the most abundant in plants. Most phytosterols have 1 or 2 carbon–carbon double bonds. Plant stanols (saturated phytosterols without double bonds) are present in small amounts in many plants. Phytosterols can be converted to phytostanols by chemical dehydrogenation (1). Phytosterols also occur as conjugate forms in which the 3β-OH group is esterified to a fatty acid or a hydroxycinnamic acid, or glycosylated with a glucose or a 6-fatty-acyl hexose (1). Phytosterols are bound to the fibers of the plant and are similar in chemical structure to cholesterol except for the C_{24} position on the sterol side chain. Some studies suggest that phytosterols affect the reproductive system in animals, and in particular, that they have estrogenic effects (2,3). BSS is also a weak agonist for estrogen receptors (ERs) α and β, and preferentially binds to ERβ (4). Phytosterols are potential environmental endocrine disruptors in animals. Paper pulp mill effluents containing phytosterols alter sex steroid levels and reproductive organ size in fish (5). Studies report that BSS accumulates in animals in laboratory tests (6,7) and alters sex steroid levels in humans (8), animals (9,10), and fish (11,12).

The biochemical effects of cholesterol differ from those of phytosterols. Phytosterols are more hydrophobic than cholesterol and have greater affinity to the micelles involved in fat digestion, and they can displace intestinal cholesterol from the

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[3] Abbreviations used: BCS, bovine calf serum; bcl-2, B-cell lymphoma/leukemia-2 gene; BSS, β-sitosterol; BSSG, β-sitosterol glucoside; ER, estrogen receptor; ERE, estrogen response element; E_2, 17β-estradiol; 4OHTAM, 4-hydroxytamoxifen; 6-FAM, 6-carboxyfluoresein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase of human; LNCaP, lymph node cancer cell of the prostate; MDA-MB-231, MD Anderson Center MB-231; NC, negative control; MC, Modu-care; MCF-7, Michigan Cancer Foundation 7; MEM, minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pS2, presenilin 2; TAMRA, 6-carboxytetramethylrhodamine.

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micelles, reducing intestinal cholesterol absorption (13). The major phytosterol sources in the human diet are vegetable oils, cereals, fruits, and vegetables (14). In humans, <10% of the total dietary BSS consumed is absorbed in the intestine (15), whereas ~45 to 54% of the total intake of cholesterol is absorbed (16). In rats, ~4% of BSS is absorbed, and 1% of sitostanol is absorbed (17). Human dietary intake ranges from 40 to 400 mg/d (8,18–20). In Western diets, phytosterol intake is low, ~80 mg/d (8). BSS and β-sitosterol glycoside (BSSG) are the major phytosterols in higher plants. The phytosterols are very stable, and intense processes (such as boiling, neutralization, bleaching, and deodorization) do not affect the phytosterol content of vegetables and fruits (21,22). Dietary phytosterols reduce cholesterol absorption and plasma cholesterol levels and prevent cardiovascular events (23–25). However, due to poor solubility and bioavailability of phytosterols, the serum cholesterol–lowering effect of phytosterols is not consistent, and high dosages (up to 25 to 50 g/d) are required for efficacy (1). The addition of sterols in esterified form to food products or dietary supplements further lowers serum cholesterol, especially LDL-cholesterol (26,27). Commercial cholesterol-lowering spreads and supplements are enriched with sterols and sterol esters, for which the recommended dose is 300 mg/d (19,28).

In addition to their cholesterol-lowering effect, BSS and BSSG have anti-inflammatory, antipyretic (29,30), antineoplastic, immune-modulating (31), and blood sugar–controlling effects (32). Increased dietary intake of fruits and vegetables or supplements modulates the immune system in such cases as chronic viral infection, stress-induced immune suppression, tuberculosis, allergy, cancer, and rheumatoid arthritis and other autoimmune conditions (30,31). BSS and a BSS + BSSG mixture are common dietary supplements used by older adults to enhance immune function.

Postmenopausal women who have or are at high risk for estrogen-dependent breast cancer may consume high levels of dietary or supplemental phytosterol to lower their cholesterol levels or enhance their immune functioning. If these dietary phytosterols have estrogenic potential, they may affect the growth of breast tumors. The present study evaluated the estrogenic and antiestrogenic effects of the phytosterols BSS, BSSG, and MC (a dietary supplement) on the growth of Michigan Cancer Foundation 7 (MCF-7) human breast cancer cells in vitro and in ovariectomized athymic mice.

MATERIALS AND METHODS

Materials. Supplies of BSS, BSSG, and Moducare (MC; BSS:BSSG = 99:1) (>99.9% purity, checked by HPLC) were gifts from IMAGINutrition. Minimal essential medium (MEM; without gentamicin, with glutamine) and phenol red–free MEM were purchased from the Media Facility, University of Illinois at Urbana-Champaign. Bovine calf serum (BCS) was purchased from Hyclone. Penicillin/streptomycin and trypsin/EDTA were purchased from Invitrogen. The laboratory animal diet and dietary components were purchased from Dyets. Reagents for RT-PCR analysis were purchased from PE Applied Biosystems, Synthegen, and Invitrogen. The Double Antibody Estradiol RIA kit was purchased from Diagnostic Products.

Human MCF-7 carcinoma cells. The MCF-7 cells were isolated from a postmenopausal woman with estrogen-dependent metabolic infiltrating ductal carcinomas (33). Human MCF-7 carcinoma cells are the most widely used strain for the study of estrogen-dependent human breast cancer. Estrogens stimulate MCF-7 cell growth. The MCF-7 cells were maintained in MEM with Eagle's salts with 1 mmol/L pyruvate, 2 mmol/L glutamine, 5% heat-inactivated BCS, 1% penicillin/streptomycin and 1 nmol/L 17β-estradiol (E_2).

In vitro assay of MCF-7 cell proliferation. The effects of BSS, BSSG, and MC on MCF-7 cell proliferation were evaluated by a modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (34). The estrogenic activity of BSS, BSSG, and MC (0.001 to 150 μmol/L) was evaluated by measuring the phytosterol-induced changes in MCF-7 cell growth. The antiestrogenic activity of the phytosterols was evaluated by measuring the phytosterol-induced changes in E_2 (1 nmol/L)-induced MCF-7 cell growth. The effect of the phytosterols on the inhibitory effect of 4-hydroxytamoxifen (4OHTAM; 10 μmol/L) on E_2 (1 nmol/L)-induced MCF-7 cell growth was also evaluated. The MCF-7 cells (1.5×10^4) were seeded in 1 mL of estrogen-free culture media on a 24-well polystyrene culture plate. After 24 h, the cells in each well were washed with 1 mL of PBS and treated with BSS, BSSG, or MC (0.001 to 150 μmol/L) every 48 h. After 5 d of treatment, the cells were treated with MTT for 5 h and treated with 10% SDS (in 0.01 mol/L HCl) for 12 to 18 h. Optical density was measured at 570 nm and normalized to the number of cells, based on a standard curve. The cell proliferation assay was repeated 5 times.

Athymic nude mice. Ovariectomized female athymic BALB/c (nude) mice were purchased from Charles River Laboratories and acclimated for 1 wk. The mice were ovariectomized at 21 d of age by the vendor, then allowed to recover for 7 d.

Insertion of E_2 pellet. At 28 d of age, an E_2 pellet containing 1 mg of E_2 and 19 mg of cholesterol was placed subcutaneously behind the neck of each mouse ($n = 73$) to induce rapid MCF-7 tumor growth (35).

Implantation of MCF-7 cells. At 3 d after insertion of the E_2 pellet (i.e., at 31 d of age), MCF-7 cells were prepared as described by Ju et al. (36). The cells were adjusted to a concentration of 1×10^5 cells per 40 μL of Matrigel (Collaborative Biomedical Products), and 40 μL per site was injected into each of the 4 flanks of the athymic mice.

Diet. The AIN93G diet (37), with lard as fat (to eliminate an additional phytosterol source from the diet), was used as a control diet. For the dietary phytosterol treatments, BSS (9.8 g/kg diet), BSSG (0.2 g/kg diet), or MC (10.0 g/kg diet) was added to the AIN93G diet. The supplementation levels selected were based on previously published references (38–41).

Estrogenic or antiestrogenic effects of dietary phytosterols on tumor growth. At 7 wk after injection of the MCF-7 cells, the mean tumor cross-sectional area reached 33.3 mm^2 in all mice, and the E_2 pellets were removed. To evaluate the estrogenic potential of the phytosterols, the mice were separated into 4 groups: negative control (NC; $n = 9$), BSS ($n = 10$), BSSG ($n = 9$), and MC ($n = 9$). To evaluate the antiestrogenic effect of the phytosterols on E_2-induced tumor growth, the mice were separated into 4 groups: 1:47 E_2 (1:47 = E_2:cholesterol; $n = 9$), 1:47 E_2 + BSS ($n = 9$), 1:47 E_2 + BSSG ($n = 9$), and 1:47 E_2 + MC ($n = 9$). An E_2 implant consisting of ~3 mg of 1:47 E_2 in a silastic tube (1.5 cm × 0.1 cm i.d. × 0.06 cm wall) was placed subcutaneously in each mouse in the 1:47 E_2, 1:47 E_2 + BSS, 1:47 E_2 + BSSG, and 1:47 E_2 + MC groups. The 1:47 E_2 silastic implant generated a circulating E_2 level of ~100 to 150 pmol/L (unpublished data), which is similar to the serum E_2 concentration in postmenopausal women (42). Mice in the NC and 1:47 E_2 groups were fed the AIN93G diet, and mice in the BSS, BSSG, MC, 1:47 E_2 + BSS, 1:47 E_2 + BSSG, and 1:47 E_2 + MC groups were fed AIN93G containing BSS (9.8 g/kg), BSSG (0.2 g/kg), or MC (10.0 g/kg). During the dietary treatment, tumor growth and body weight were measured weekly, and tumor surface area was calculated using the following formula: length/2 × width/2 × π (43). Food intake was measured throughout the study. Uterine weight was measured at the end of the study.

RNA preparation and RT-PCR analysis of changes in gene expression. The mRNA expressions of presenilin 2 (pS2), an estrogen-responsive gene marker, and B-cell lymphoma/leukemia-2 gene (bcl-2), an antiapoptotic marker, were analyzed by RT-PCR. Tumors with approximately the mean surface area of tumors from mice in the NC (3 tumors), 1:47 E_2 (6 tumors), 1:47 E_2 + BSS (6 tumors), 1:47 E_2 + BSSG (6 tumors), and 1:47 E_2 + MC (6 tumors) groups were used for mRNA analysis. Mice in the NC, BSS, BSSG, and MC

groups did not have enough tumors for RT-PCR analysis. Samples of RNA were prepared from frozen tumor (~200 mg) as described by Ju et al. (44). Samples of cDNA were generated using 10 ng of RNA and TaqMan Reverse Transcription Reagents (PE Applied Biosystems). The pS2 and bcl-2 primers and fluorescence 6-carboxyfluorescein (6-FAM)/6-carboxytetramethylrhodamine (TAMRA)-labeled probes were designed using Primer and Probe Design Express (PE Applied Biosystems) (pS2 forward: 5′-TCCCCTGGTGCTTCTATCCTAA-3′; pS2 reverse: 5′-CGTCAGGATGCAGGCAGAT-3′; pS2 probe: 6-FAM-5′-ACCATCGACGTCCCTCCAGAAGAGG-3′-TAMRA; bcl-2 forward: 5′-ACTTCTCCCGCCGCTACC-3′; bcl-2 reverse: 5′-CAGTTCACCCCGTCCCTGA-3′; bcl-2 probe: 6-FAM-5′-ACT-TCGCCGAGATGTCCAGCCAGC-3′). The human glyceraldehyde-3-phosphate dehydrogenase of human (GAPDH) primers and a fluorescence (6-FAM/TAMRA)-labeled probe (User Bulletin 2, PE Applied Biosystems) were used as a control. The PCR and analysis of PCR products were performed using the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). Data were analyzed using a comparative threshold cycle method (User Bulletin, PE Applied Biosystems). One sample was analyzed in triplicate in separate tubes to permit quantification of target genes normalized to a control, GAPDH.

Plasma E_2 level. The plasma E_2 level of the mice was measured using a Double Antibody Estradiol RIA kit and a company protocol (Diagnostic Products). A plasma sample (100 μL) was used for RIA. Controls included E_2 and a plasma blank, plus a known amount of E_2 (for recovery). The sensitivity of this RIA is 5 ng/L (~18 pmol/L), and the interassay CV was 2–5%.

Statistics. Results are presented as means ± SEM. Data from the in vitro cellular proliferation assays, tumor area and uterine weight at the end of the study (at wk 18), food intake during the study, RT-PCR analysis, and serum concentration of E_2 were analyzed by one-way or repeated-measures ANOVA according to the characteristics of the data set, using SAS statistical software. When the overall treatment *F*-ratio was significant ($P < 0.05$), the differences between treatment means were tested by Fisher's least significant differences test. Values of $P < 0.05$ were considered significant. All statistical analysis was done using SAS statistical software (SAS Institute, 1985).

RESULTS

Estrogenic effects of BSS, BSSG, and MC on the growth of MCF-7 cells in vitro. BSS increased MCF-7 cell proliferation in a dose dependent manner at concentrations ranging from 1 to 150 μmol/L (80 to 240% greater than the MCF-7 control; **Fig. 1***A*). The glucoside form of BSS, BSSG, did not affect the growth of MCF-7 cells at the concentrations tested (Fig. 1*B*). Moducare increased MCF-7 cell proliferation at concentrations ranging from 50 to 150 μmol/L (80 to 150% greater than control; Fig. 1C). This differed from our predictions. We expected no difference in effect on cell proliferation between BSS and MC, because MC is a mixture of 99% BSS plus 1% BSSG. These data show that the BSS + BSSG mixture is less estrogenic than BSS alone.

Antiestrogenic effects of BSS, BSSG, and MC on the growth of MCF-7 cells in vitro. The E_2 (1 nmol/L) treatment increased MCF-7 cell numbers by 250% compared with the NC group (data not shown). BSS, BSSG, and MC had no antiestrogenic effect on the E_2 (1 nmol/L)-induced MCF-7 cell proliferation in vitro (data not shown). The phytosterols did not affect the stimulatory effect of E_2 on the growth of MCF-7 cells. The effects of the E_2 and E_2 + phytosterol treatments did not differ. The 4OHTAM (10 μmol/L) treatment completely blocked the E_2-stimulated MCF-7 cell proliferation. The phytosterols did not affect the inhibitory effect of 4OHTAM on E_2-induced MCF-7 cell growth. The effects of the E_2 + TAM and E_2 + TAM + phytosterol treatments did not differ (data not shown).

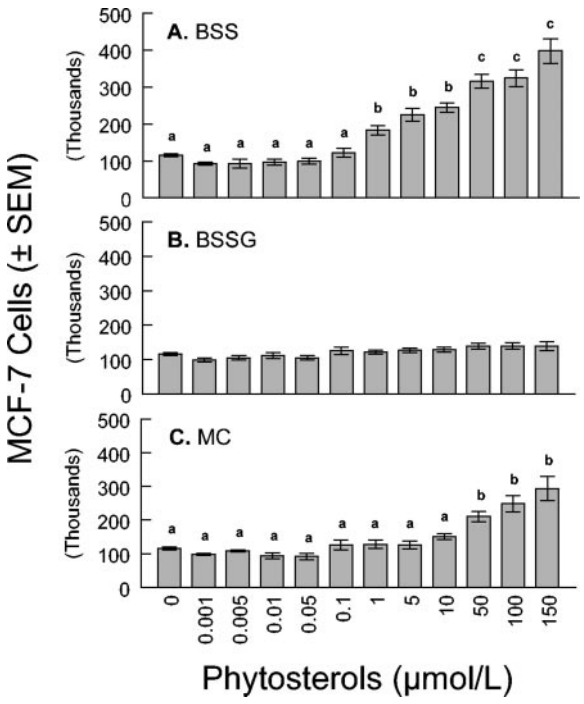

FIGURE 1 The effect of the phytosterols BSS (*A*), BSSG (*B*), and MC (*C*) on the growth of estrogen-dependent MCF-7 cells. The MCF-7 cells were treated with the phytosterols at concentrations ranging from 0.001 to 150 μmol/L. Values are means ± SEM, $n = 15$. Bars with different letters differ, $P < 0.05$.

Estrogenic effects of BSS, BSSG, and MC on the growth of MCF-7 tumors implanted in ovariectomized athymic mice. Dietary BSS, BSSG, and MC did not affect tumor growth. The tumors in mice in all phytosterol treatment groups regressed after removal of the E_2 pellet at wk 7. At wk 18, the mean tumor sizes of the NC, BSS, BSSG, and MC groups were 4.5 ± 0.5 mm^2, 5.0 ± 0.4 mm^2, 4.5 ± 0.4 mm^2, and 4.6 ± 0.6 mm^2, respectively. The mean tumor size did not differ between the NC and phytosterol groups (data not shown).

Antiestrogenic effects of BSS, BSSG, and MC on the growth of MCF-7 tumors implanted in ovariectomized athymic mice. When the mean tumor size of the 1:47 E_2 group reached 128.6 mm^2 at wk 18, the mice were killed due to the tumor burden. At wk 18, the mean tumor sizes of the 1:47 E_2 + BSS, 1:47 E_2 + BSSG, and 1:47 E_2 + MC groups were 78.6 mm^2, 88.0 mm^2, and 74.5 mm^2, respectively (**Fig. 2**). Dietary BSS, BSSG, and MC reduced 1:47 E_2-induced tumor growth by 38.9% ($P < 0.05$), 31.6% ($P = 0.08$), and 42.13% ($P < 0.05$), respectively.

Effect of the phytosterols on pS2 expression. The implanted 1:47 E_2 upregulated the mRNA expression of pS2, an estrogen-responsive gene, in tumors by 9.9 times compared with MCF-7 control cells ($P < 0.05$; **Fig. 3***A*). However, the effects of the various dietary phytosterol treatments in the presence of 1:47 E_2 did not differ from one another.

Effect of the phytosterols on bcl-2 expression. The implanted 1:47 E_2 upregulated the mRNA expression of bcl-2, an antiapoptotic marker, in tumors by 1.1 times compared with MCF-7 control cells ($P < 0.05$; Fig. 3*B*). Dietary BSS and BSSG treatments in the presence of 1:47 E_2 did not affect bcl-2 expression in tumors, but dietary MC in the presence of 1:47 E_2 downregulated bcl-2 expression by 38% ($P < 0.05$), compared with mice in the 1:47 E_2 group.

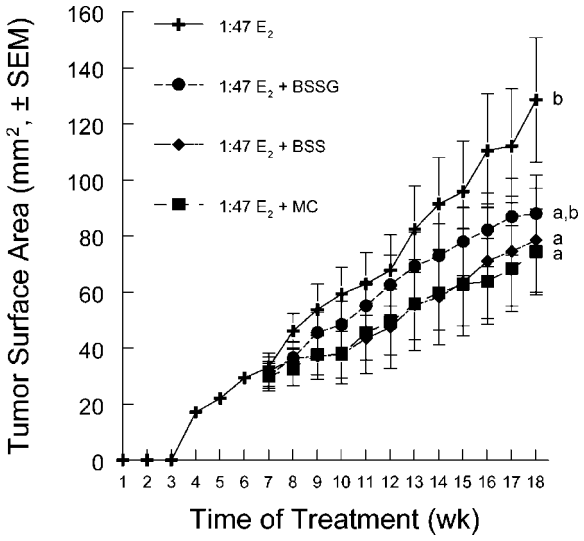

FIGURE 2 Antiestrogenic effect of dietary phytosterols on breast tumor growth in female ovariectomized athymic mice. Pellets of E_2 (1 mg) were implanted in athymic mice. The mice were then injected with 1×10^5 MCF-7 cells/site in 4 locations, and the tumors reached a mean cross-sectional area of 33.3 mm^2 at wk 7. The E_2 pellets were then removed, and 1:47 E_2 (~3 mg) implants were placed subcutaneously. The mice were separated into 4 groups: 1:47 E_2 (9 mice; $n = 34$ tumors), 1:47 E_2 + BSS (9.8 g/kg diet; 9 mice; $n = 34$ tumors), 1:47 E_2 + BSSG (0.2 g/kg diet; 9 mice; $n = 36$ tumors), and 1:47 E_2 + MC (10.0 g/kg diet; 9 mice; $n = 33$ tumors). At wk 7, treatment with the experimental diets began. Tumors were then measured weekly for 11 wk. Data are mean cross-sectional tumor areas (mm^2) ± SEM for all tumors in each group. Means at wk 18 without a common letter differ, $P < 0.05$.

Plasma E_2 level. The mean E_2 concentration in the NC group was 32.5 pmol/L (**Fig. 4**). Dietary phytosterols did not affect the serum E_2 level. Implanted 1:47 E_2 increased the mean E_2 level to 123 pmol/L. Dietary phytosterols lowered the serum E_2 level in the 1:47 E_2 + BSS group by 35.1%, in the 1:47 E_2 + BSSG group by 30.2%, and in the 1:47 E_2 + MC group by 36.5%, compared to the level in the 1:47 E_2 group ($P < 0.05$).

Body weight, food intake, and uterine weight. Body weight did not differ among the groups (data not shown), and the food intake of all treated mice did not differ from that of the NC group (data not shown). Dietary phytosterols did not affect uterine weight. Uterine weight was 25.7 ± 3.9 mg in the NC group, 28.7 ± 3.6 mg in the BSS group, 32.6 ± 4.0 mg in the BSSG group, and 32.3 ± 6.8 mg in the MC group, and did not differ between the NC and phytosterol-fed groups. Implanted E_2 markedly increased uterine weight, but uterine weight did not differ between the 1:47 E_2 and the 1:47 E_2 + phytosterol groups, suggesting that dietary phytosterols at the selected dosages had no effect on uterine size. Uterine weight was 143.9 ± 9.8 mg in the 1:47 E_2 group, 141.2 ± 18.9 mg in the 1:47 E_2 + BSS group, 140.3 ± 14.3 mg in the 1:47 E_2 + BSSG group, and 152.4 ± 20.8 mg in the 1:47 E_2 + MC group.

DISCUSSION

Serum cholesterol reduction and immune system modulation are established biological effects of phytosterols. Epidemiological studies report protective effects of a diet high in phytosterols against colon (45), prostate (46), ovarian (47), stomach (48) and breast (49) cancers.

The present study evaluated the effects of phytosterols on the growth of estrogen-dependent human breast cancer cells in vitro and in vivo. BSS (≥1 μmol/L) stimulated the growth of MCF-7 cells in vitro (Fig. 1A), consistent with previously published results (11). BSSG did not affect cultured MCF-7 cell growth at the concentrations used (Fig. 1*B*). Moducare stimulated MCF-7 cell proliferation at concentrations of at least 50 μmol/L (Fig. 1C). Although MC contains only 1% BSSG, the presence of BSSG in MC made MC less estrogenic than BSS. The reason for the lower induction of cellular proliferation by MC is unclear. The phytosterols had no antiestrogenic effect and did not interact with 4OHTAM to affect E_2-induced MCF-7 cell growth (data not shown). The differing estrogenic responses of BSS and MC suggest that BSSG plays an important role in the estrogenic activity of these phytosterols, involving unknown mechanisms. In a separate study, we evaluated the effect of stigmasterol, another common phytosterol found in plants, on the growth of MCF-7 cells in vitro. Unlike BSS, stigmasterol did not affect MCF-7 cell proliferation at concentrations from 0.001 to 100 μmol/L (unpublished data), suggesting that each phytosterol has a different estrogenic potential.

Serum phytosterol levels vary in humans. A dietary phytosterol intake of 200 to 240 mg (~130 to 160 mg of BSS) appears to lower cholesterol levels (50) and produce serum phytosterol levels of 0.03 to 0.17 mg/L (~0.07 to 0.41 μmol/L) (24,51). Dietary supplements are reported to increase serum

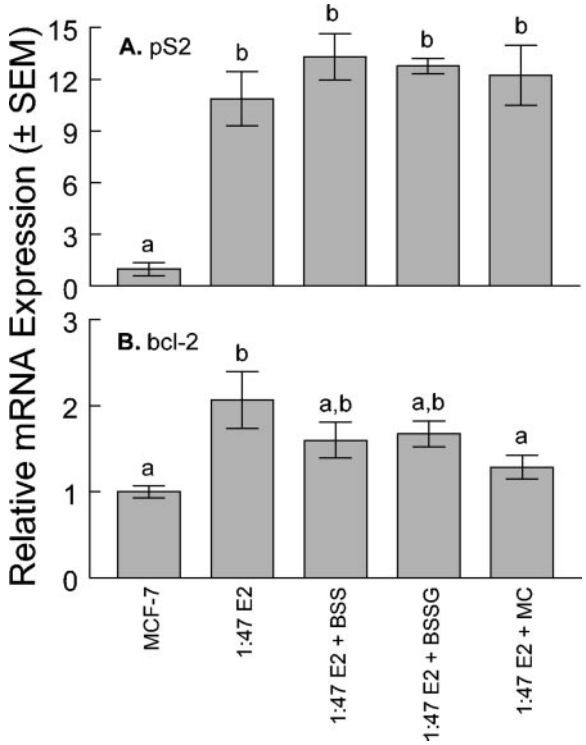

FIGURE 3 Relative pS2 (*A*) and bcl-2 (*B*) mRNA levels in tumors from mice in the control (MCF-7), 1:47 E_2, 1:47 E_2 + BSS, 1:47 E_2 + BSSG, and 1:47 E_2 + MC groups. Six tumors per treatment group were analyzed. (Tumors from mice in the BSS, BSSG, and MC groups were not evaluated, because there was no difference among the treatments and the number of tumors per group was insufficient for analysis). Levels of mRNA expression were evaluated by RT-PCR analysis; the *y*-axis presents the relative mRNA level; GAPDH was used as a standard. Values are means ± SEM, $n = 6$. Bars without a common letter differ, $P < 0.05$.

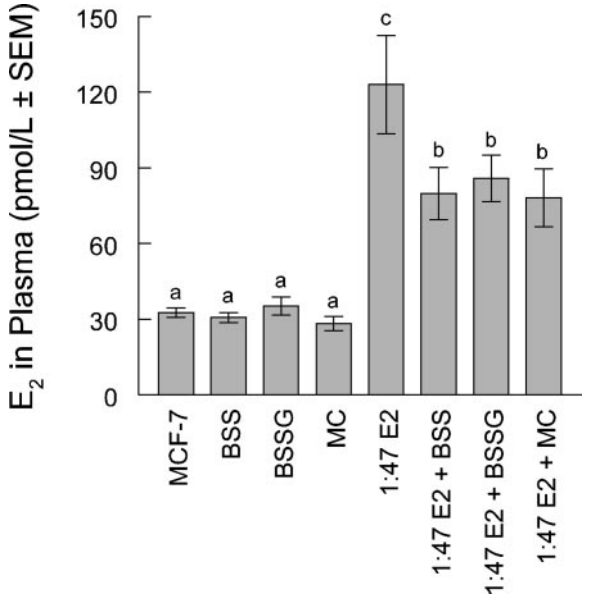

FIGURE 4 Plasma E_2 concentrations in mice in the control (MCF-7), BSS, BSSG, MC, 1:47 E_2, 1:47 E_2 + BSS, 1:47 E_2 + BSSG, and 1:47 E_2 + MC groups. Values are means ± SEM, $n = 6$. Bars with different letters differ, $P < 0.05$.

phytosterol levels by 200% (52). Other studies report a wider range of serum free phytosterol levels [1 to 17 mg/L (~2.5 to 41 μmol/L, <1 to 10% of total sterol) and up to 480 mg/L (~1.2 mmol/L) in sitosterolemic subjects] (53–56). The present in vitro experiment therefore suggests that dietary phytosterol intake sufficient to lower serum cholesterol may not have an estrogenic effect on the growth of estrogen-dependent breast cancer cells, but consumption of a diet high in phytosterols or dietary supplements may increase blood phytosterols enough to modulate the growth of estrogen-dependent breast cancers.

The effects of dietary intake of the phytosterols BSS (9.8 g/kg diet), BSSG (0.2 g/kg), and MC (10.0 g/kg) on the growth of estrogen-responsive breast tumors were evaluated using a xenograft model. Dietary BSS, BSSG, and MC did not stimulate the growth of MCF-7 tumors implanted in athymic mice at the concentrations used (data not shown). However, dietary BSS and MC both markedly reduced 1:47 E_2-induced MCF-7 tumors in mice (Fig. 2). Although BSSG did not significantly reduce tumor size ($P = 0.08$), it is important to note that 0.2 g BSSG/kg diet—a much lower intake than that of BSS (10 g/kg diet)—reduced 1:47 E_2-induced tumor size by 31.6%, suggesting potential benefits of BSSG for individuals with estrogen-dependent breast cancer.

The expression of pS2 and bcl-2 in tumors was evaluated to determine whether the tumor reduction caused by 1:47 E_2 + phytosterols involves an ER-mediated mechanism. Both pS2 and bcl-2 are well-characterized ER–downstream regulated gene markers that require the activation of estrogen response elements (EREs) (57,58). Dietary BSS and BSSG in the presence of E_2 did not affect pS2 or bcl-2 expression. However, dietary MC downregulated bcl-2 expression but did not affect pS2 expression (Fig. 4), suggesting that the tumor reduction caused by 1:47 E_2 + MC (Fig. 2) may involve a non–ER-mediated apoptotic mechanism. It is possible that dietary MC activates different ERE-containing promoters independent of ER. The mechanism by which dietary phytosterols protect against estrogen-induced tumor growth is unclear. There are several possible mechanisms, including an increase in the percentage of unsaturated fatty acids and a change in membrane composition (59,60), a decrease in fluidity (61,62), an increase in apoptosis (41,63,64), and the inhibition of lipid oxidation (65). The inhibition of lipid peroxidation by phytosterols may be caused by membrane stabilization that might be associated with decreased plasma membrane fluidity in cancer cells. There is evidence that phytosterols inhibit the growth of estrogen-independent breast cancer MD Anderson Cancer Center (MDA)-MB-231 (MDA-MB-231) cells and androgen-dependent prostate cancer lymphnode cancer cell of the prostate (LNCaP) cells by initiating apoptosis. β-Sitosterol (16 μmol/L) stimulates apoptosis in MDA-MB-231 cells (63) and LNCaP cells (41,64) in vitro. Phytosterols reduce the metastasis of MDA-MB-231 (66) and LNCaP (41) tumors implanted in severe combined immuno-deficient (SCID) mice.

In the present study, dietary phytosterols lowered the plasma E_2 level by 35.3% in the 1:47 E_2 + BSS group, 30.2% in the 1:47 E_2 + BSSG group, and 36.5% in the 1:47 E_2 + MC group, compared to the level in the 1:47 E_2 group. The mechanism responsible is unclear. One possibility is that the dietary phytosterols modulate the oxidative metabolism of E_2 (67,68) by enhancing C2- or C4-hydroxylation rather than 16-α-hydroxylation, generating hydroxy- and methoxy-estrogen metabolites. The hydroxy-estrogen metabolites are estrogenic, but the circulating concentration of the metabolites is low, and they are excreted faster than E_2 (69), and the methoxy-estrogen metabolites may induce apoptosis by a non–ER-mediated mechanism (70). It is also important to note that the dietary BSSG concentration diet was much lower than that of BSS or MC. It is possible that the apoptotic effects of phytosterols in combination with changes in membrane fluidity may play a role in the mechanism of protection against E_2-stimulated breast tumor growth.

In summary, the present study demonstrated that BSS and MC (a mixture of BSS + BSSG) stimulated the growth of MCF-7 cells in vitro. However, dietary BSS and MC reduced E_2-induced MCF-7 tumor growth in ovariectomized athymic nude mice. Although the phytosterols lowered plasma E_2 levels in the E_2 + phytosterol groups, dietary phytosterols did not affect estrogen-responsive pS2 expression. The regression of estrogen-stimulated breast tumor growth induced by MC may involve a non–ER-mediated mechanism. These data show that high dietary or supplemental consumption of phytosterols may be beneficial for women with breast cancer. Continuing research on the effects of phytosterols and their glucoside forms is required to provide information regarding short- and long-term effects, interactions with prescription drugs, and the mechanisms of prevention of breast cancer by dietary manipulation and dietary supplements.

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