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Characterization of the pharmacologic profile of a standardized soy extract in the ovariectomized rat model of menopause: effects on bone, uterus, and lipid profile

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Abstract

Objective: This study was aimed to assess the effect of a standardized soy extract (SSE, Soyselect) in the ovariectomized rat model of menopause.

Design: Ovariectomized rats were treated for 6 weeks with the soy extract (50 or 100 mg/kg/day − PO), vehicle (distilled water), or 17β-estradiol (0.5 mg/kg/day − PO). Tissue-specific estrogen agonist effects were examined using the endpoints bone mineral density, biochemical parameters of bone turnover, modulation of cytokines involved in the bone remodeling, uterine weight, uterine histology, uterine hormone receptor status, and serum lipid level.

Results: The SSE produced a bone-sparing effect associated with a slowing down in the increased bone turnover observed after ovariectomy (as indicated by measurements of serum osteocalcin levels and excretion ratio of deoxypyridinoline); changes in serum interleukin-6 levels observed after SSE suggested that this bone-sparing effect could be partly attributed to the modulation of osteoclastogenesis induced by interleukin-6. Remarkably, organ weight data and histopathologic analysis did not show any stimulatory activity of the SSE on the uterus. Immunohistochemical analysis showed a significant down-regulation of estrogen receptor-α (ERα) in uterine epithelium after 17β-estradiol treatment, but not after treatment with the SSE; no significant differences among groups were observed in ER-α uterine stromal levels. After treatment with 17β-estradiol, estrogen receptor-β (ER-β) expression was not modulated in the stroma or epithelium, whereas the SSE induced an up-regulation of ER-β stromal expression. Collectively, these results suggest that the lack of stimulatory activity on the uterine epithelium using soy treatment could be due to a negligible stimulatory activity on estrogen receptor-α and/or to the enhanced expression observed in stromal ER-β, the latter being considered as a negative modulator of ERα–mediated uterine proliferation. 17β-estradiol, but not the SSE, down-regulated uterine epithelial progesterone receptor (PR), compared with ovariectomized rats. In the stromal compartment, progesterone receptor expression was fully up-regulated by 17β-estradiol treatment and, to a lesser extent, by SSE treatment. The minor increase in lipid levels induced by ovariectomy was not affected by SSE administration. Finally, the lack of stimulatory activity on uterus was also confirmed in an immature female rat model.

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Conclusions: The results of this study demonstrated that the tested extract has an interesting profile of tissue-specific response, in that it is efficacious in preventing experimental osteoporosis without causing stimulation in uterus at doses that are effective in bone.

Key Words: Soy – Isoflavones – Menopause – Rat – Bone – Uterus.

Hormone therapy (HT) is currently viewed as a strategy for treating climacteric complaints and preventing osteoporosis; however, because of the augmented risks of breast cancer, stroke, and venous thromboembolism, patients look for alternatives.[1] In this context, epidemiological data have suggested that postmenopausal women could find in phytoestrogen-containing foods, such as soy, a promising treatment for the management of menopause-related symptoms and diseases. Phytoestrogens are a broad group of nonsteroidal compounds of different structure that have been shown to bind to estrogen receptors (ERs). There are three main classes of phytoestrogens: isoflavones, coumestans, and lignans; among the isoflavones, genistein and daidzein, are the most investigated. Functionally, phytoestrogens can exert both estrogenic and antiestrogenic effects depending on many factors including their concentration, the concentration of endogenous sex hormones, the relative levels of ERα and ERβ, and the nature of the response elements with which the receptors interact on the estrogen-related genes; phytoestrogens have also been shown to interact with pathways of cellular activity that do not involve ERs.[2] The current research effort is focused on determining whether phytoestrogens provide protection to the systems affected by menopause, without exerting the adverse effects on the breast and uterus encountered with HT regimens. The main claims made for marketed phytoestrogen supplements are a reduction of menopause symptoms, the promotion of cardiovascular health, the promotion of bone health, and an increased breast health. Overall, however, available data are conflicting, in part because of the high variability in the qualitative and quantitative composition of the different soybean products tested. All of the extracts containing phytoestrogens are on the market as food supplements, and because they are not subjected to strict regulatory controls, they vary in quality. Not surprisingly, it has been recently demonstrated that the phytoestrogen content in commercially available tablets varies greatly, and in some cases is totally absent.[3]

To minimize this potential source of bias, a standardized soy extract (SSE) has been prepared and studied. It contains, as main active ingredients, isoflavonoids (as glucosides) and saponins, two classes of compounds endowed with several biologic properties. The extract Soyselect, which is present in commercially available products, has been tested in clinical studies and has been proven to be safe and efficacious for the relief of vasomotor symptoms[4,5] and to have favorable effects on cognitive function, particularly verbal memory, in postmenopausal women.[6] The following experiments were undertaken to characterize the pharmacologic profile of this soy extract in the ovariectomized rat model of menopause, a model resembling the decline in estrogen levels in postmenopausal women, which is at least partially responsible for the increase in osteoporotic fractures and cardiovascular diseases; this model also allows control of some parameters such as those associated with the increased risks for uterine and breast cancer, which are the most serious undesirable side effects of HT.

METHODS

Animals used in these studies were supplied by Harlan Nossan S.r.l. (Correzzana MI, Italy). All animals were housed in a purpose-built facility and allocated in clear solid-bottomed polycarbonate cages. Environmental controls were set at the following levels: temperature 22°C ± 2°C, humidity 50%, 12-hour light/dark cycle. Procedure and facilities followed the requirements of Commission Directive 86/609/EEC concerning the protection of animals used for experimental and other scientific purposes. Italian legislation is defined in the Decreto Legislativo No. 116 of 27 January 1992. The project was approved by the local ethics committee.

Chemicals

The soy extract Soyselect (Indena S.p.A., Milan, Italy) was dissolved in distilled water; formulations were prepared daily, immediately before administration. 17β-Estradiol (Sigma-Aldrich, Milan, Italy) was dissolved in absolute ethanol (20 mg/mL, stock solution) and stored at 4°C, for a maximum of 4 days; a working dilution to 100 μg/mL in peanut oil was made daily, immediately before administration.

Six-week-ovariectomized rat assay: animals and treatment

A total of 60 Sprague-Dawley female rats, aged 3 months, were used. One week after arrival, the animals

were randomized into five groups, each of 12 females, with an average body weight of 237 g. Rats were anesthetized using ketamine (0.67 mL/kg), xylazine (0.17 mL/kg), and acepromazine (0.067 mL/kg) via an intraperitoneal route, and a bilaterally ovariectomy was performed via a dorsal midline incision. Sham-operated rats underwent a sham procedure. Up to the date of surgery, animals received a standard commercial diet (Altromin R, Rieper, Vandoies, Bolzano, Italy) and tap water. Upon recovery from anesthesia, rats were fed a diet containing 0.4% calcium, 0.3% phosphorus, and 3 UI/g diet of vitamin D_3 (Harlan Teklad, Udine, Italy); tap water was substituted by deionized water. All animals were pair-fed to the mean food intake of the sham-operated rats. Body weight was registered twice per week during the study. Ovariectomized animals received the SSE (50 or 100 mg/kg/day −of the extract PO), vehicle (distilled water), or 17β-estradiol (0.5 mg/kg/day − PO). Rats were dosed, by gavage, 6 days a week, for 6 consecutive weeks, starting from the day after ovariectomy. Death was scheduled 6 weeks after surgery. During the last week of treatment, 24-hour urine samples were collected from all animals. At time of death, rats were deeply anesthetized with diethyl ether and venous blood samples were collected from the caudal vena cava. The uterus, liver, and spleen were rapidly removed, free of fat, and weighed. Uteri were fixed in 10% buffered formol saline and subsequently dehydrated and blocked in paraffin. The paraffin block was cut into 3- or 5-μm sections, fixed on coded slides, and processed for light microscopy (hematoxylin/eosin staining) or immunohistochemistry (see below). The hind legs were removed, grossly free of soft tissue and stored at −20°C for later analysis.

Serum assays

Blood samples were centrifuged at 2,000 *g*, at room temperature, for 10 minutes; serum samples were collected and stored at −70°C until analysis. Osteocalcin measurement in serum was performed using the Rat Osteocalcin ELISA Kit (Biomedical Technologies, Stoughton, MA) following the manufacturer's instructions; standards of highly purified rat osteocalcin were used to generate a standard curve in the range of 0.25 to 20 ng/mL. All samples were analyzed in duplicate in the assay. Interelukin-6 levels were determined using a commercial quantitative immunoassay kit for rats (R6000, Research & Diagnostic Systems, Minneapolis, MN). Analyses were performed according to the manufacturer's instructions using rat interleukin-6 standards ranging from 31.2 to 2000 pg/mL for the standard curve. All samples were analyzed in duplicate in the assay. A quantitative determination of serum 17β-estradiol was performed with Coat-A-Count Estradiol (Diagnostic Product Corporation, Los Angeles, CA), a no-extraction, solid-phase ^{125}I radioimmunoassay (calibration curve, 20 to 3,600 pg/mL). The Research Toxicology Center (Pomezia, Rome) performed analytical determinations of total, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol using commercial kits (Sentinel Diagnostic, Milan, Italy) and following standard internal procedures.

Urine assays

Urine samples were collected over a period of 24 hours from all animals and stored at −70°C until analysis. On the day of analysis, samples were thawed and allowed to settle for 15 to 20 minutes. Quantitative measurement of the excretion of deoxypyridinoline was performed using the Metra DPD EIA kit (Quidel Corporation, San Diego, CA), according to the manufacturer's instructions, using deoxypyridinoline standards ranging from 3.0 to 300 nM for the standard curve. Control values were in the range specified in the certificate of analysis supplied with the kit. All samples were analyzed in duplicate in the assay. Results obtained were corrected for urinary concentration by creatinine (see below) and expressed as nanomoles of deoxypyridinoline per millimole creatinine. Urinary creatinine levels were determined by the Metra creatinine assay (Quidel Corporation); creatinine standards in the range of 5 to 40 mM were used to generate a standard curve. Control values were in the range specified in the certificate of analysis supplied with the kit. All samples were analyzed in duplicate in the assay.

Bone density and femoral length

Bone density was determined by Archimedes' principle, as previously described.[7] Briefly, the left femurs were removed at time of death and free of surrounding soft tissue; the length of each femur was measured with a caliper before storing the bones at −20°C for later analysis. Before making the density measurements, femurs were hydrated in distilled water for 1 hour under vacuum to ensure that all the trapped air diffused out of the bone. Femurs were then weighed while submerged in distilled water and again out of water using the Mettler kit ME-33360 (Mettler Toledo, Milan, Italy), and density was calculated in grams per cubic centimeter. All samples were analyzed in duplicate in the assay.

Phytoestrogen analysis

Blood samples were collected in heparinized tubes, centrifuged for 10 minutes at 2,500 *g* at 4°C, and plasma

stored at −20°C until analysis. Levels of daidzein, genistein, and equol were determined in all plasma samples, either as such or after enzymatic hydrolysis with a β-glucuronidase/arylsulfatase mixture (type H-1 from Helix pomatia; Sigma-Aldrich, Milan, Italy), to evaluate the compounds both as free and as total isoflavones. The analytical method previously described by Supko and Phillips[8] was modified by Analist S.r.l. (Trezzano sul Naviglio, Milan, Italy) as follows: plasma samples for the determination of total daidzein, genistein, and equol were prepared by adding n-propyl p-hydroxybenzoate as internal standard, acetate buffer 0.2 M (pH 5), and β-glucuronidase/arylsulfatase solution to 500 μL of plasma aliquots; samples were incubated overnight at 37°C. For analysis of free isoflavones, enzymatic hydrolysis was omitted. For the extraction procedure, methyl ter-butyl ether was added to each sample. Samples were subsequently centrifuged at 3,000 *g* for 10 minutes and the organic layer was then evaporated at 40°C under a stream of nitrogen. The residue was reconstituted with 0.2 mL of mobile phase solution of HPLC method consisting of 63.5% v/v phosphate buffer 0.05 M (pH 5), 12% v/v acetonitrile, and 24.5% v/v methanol. Extracted samples were loaded into a chromatographic column at isocratic conditions using the following chromatographic conditions: column RP 18, 5 mm, Purospher 125 × 3 mm Merck; mobile phase, 63.5% v/v phosphate buffer 0.05 M (pH 5), acetonitrile 12%, methanol 24.5%; rate 0.45 mL/min; inject volume: 10 μL; wavelength 214 nm; retention times: 12, 20, 25 and 30 minutes for daidzein, equol, genistein, and internal standard, respectively. Isoflavone concentrations were quantified by comparing peak areas with standard curves. Instrumentation consisted of LC Waters Alliance 2690 self-injection system, UV-VIS detector Waters 486 (Waters Alliance, Milford, MA), and a Purospher C18 analytical column (Merck, Darmstadt, Germany) 5 μm; 3.0 × 12.5 cm.

Histopathological evaluation

Histopathological evaluation of the uterus was performed on coded slides from 10 to 12 animals in each experimental groups.

Immunohistochemical analysis

The immunohistochemical evaluation of ERα, ERβ, and progesterone receptor (PR) was performed in five to eight uterus samples from each experimental group. Paraffin tissue sections (3 μm) were mounted on poly-l-lysine–coated slides and dried at 37°C overnight; after deparaffinization in xylene and rehydration, the endogenous peroxidase was blocked with 3% H_2O_2 in distilled water for 5 minutes. The antigen retrieval procedure was performed by microwave oven heating, in 1 mM EDTA, pH 8.0 (three times for 5 min) for ERβ and PR, and in 10mM citric acid, pH 6.0 (two times for 4 min) for ERα. To reduce nonspecific binding, sections were incubated with 20% normal goat serum for 30 minutes at room temperature. Cells expressing PR and ERβ were identified after overnight incubation at 4°C by using the polyclonal rabbit anti-PR antibody (C-19 sc-538, Santa Cruz Biotechnology, Rome, Italy) at 1:50 dilution, and the polyclonal rabbit anti-ERβ antibody (06-629, Upstate, Charlottesville, VA) at 1:40 dilution, respectively. Cells expressing ERα were identified after a 1-hour incubation at room temperature by using the polyclonal rabbit anti-ERα antibody (E3565-10, US-BIO, Swampscott, MA) at 1:600 dilution. For PR, ERβ, and ERα, detection was evaluated by a labeled polymer En Vision-rabbit + System-HRP (Dako, Carpenteria, CA) (30 min at room temperature). Diaminobenzidine was used as a chromogen (DAB substrate System, Dako). Positive controls corresponding to rat breast cancer, normal human prostate tissue, and normal human breast tissue (already demonstrated to have positive immunohistochemical staining for ERα, ERβ, and PR, respectively) and negative controls obtained by omission of the primary antibody were run in the assay. The number of positive (brown-stained) cells in a random field of 100 cells was counted. The average number of positive cells was obtained by counting three separate fields per section. The intensity of staining was also evaluated and a receptor score calculated as described by Snijders et al.[9] Immunoreactivity was determined by two independent observers and discrepant cases were reviewed. Immunohistochemical scoring was determined without any knowledge of which group the rats belonged to.

Immature female rat study

Twenty-two-day-old female Sprague-Dawley rats (Harlan, Italy) were treated by oral gavage with vehicle (distilled water), SSE (50 or 100 mg/kg/day of the extract), or 17β-estradiol (0.5 mg/kg/day) for 3 days. Autopsies were performed 24 hours after the final dose; uteri were excised, trimmed free of any fat and adhering nonuterine tissue, and weighed (wet weight). The uterine dry weight was determined by drying uteri at 70°C for 24 hours before reweighing.

Statistical methods

All data were analyzed for homogeneity of variance using Bartelett's test. If the group variance appeared homogenous, a parametric analysis of variance was used, followed by Dunnett's multiple comparison test.

If the variances were heterogeneous, log or reciprocal transformations were made in an attempt to stabilize the variances. If the variances remained heterogeneous nonparametric tests such as the Kruskal-Wallis test, followed by Steel multiple comparison test, were used. Results from histopathological analysis were evaluated by the Fisher exact test for proportion. Statistical analysis was achieved using the Kyplot freeware package (Kyens Lab. Inc., Tokyo, Japan).

RESULTS

Six-week-ovariectomized rat assay

The effects of SSE were evaluated in 3-month-old ovariectomized rats that were dosed for 6 weeks after surgery and compared with ovariectomized rats and sham-operated controls. Tissue-specific estrogen agonist effects were examined using bone mineral density, biochemical parameters of bone turnover, modulation of cytokines involved in the bone remodeling, uterine weight, uterine histology, uterine hormone receptor status, and serum lipid level, as endpoints.

Serum 17β-estradiol levels

Ovariectomized rats had significantly decreased levels of estradiol compared with sham-operated controls (46.4 ± 18.2 and 114.9 ± 69.9 pg/mL, respectively, mean ± SD, $P < 0.05$, n = 8 per group); treatment with 17β-estradiol significantly increased the serum estradiol concentration (179.4 ± 82.8 pg/mL, mean ± SD, $P < 0.001$, n = 8) compared with ovariectomized controls. No changes in 17β-estradiol levels were observed after treatment with 50 or 100 mg/kg/day SSE (53.1 ± 9.0 and 48.2 ± 21.2 pg/mL, respectively, mean ± SD, n = 8 per group) when compared with ovariectomized controls.

Body and organ weights

Group mean body weights at the beginning and at the end of the study are reported in Table 1. Despite pair-feeding, the body weight gain in ovariectomized controls (+ 77 ± 15 g, mean ± SD) was significantly greater ($P < 0.001$) than that of the sham-operated controls (43 ± 9 g, mean ± SD), at the end of the study, whereas the body weight gain in rats treated with 0.5 mg/kg/day 17β-estradiol (28 ± 9 g, mean ± SD) was significantly lower than in the ovariectomized rats ($P < 0.001$). The SSE at both doses tested did not significantly lower the body weight gain of treated animals (63 ± 20 g and 63 ± 19 g in the low- and the high-dose groups, respectively), when compared with ovariectomized controls. No effects on absolute and relative liver and spleen weight were observed in soy-treated rats (data not shown).

TABLE 1. *Body weight and uterus weight after treatment*

Treatment group	Initial body weight (g)	Final body weight (g)	Uterus weight (g)
Sham (n = 12)	237.5 ± 8.3	280.9 ± 10.0[a]	0.51 ± 0.12[a]
OVX (n = 11)	236.4 ± 9.6	313.5 ± 16.0[b]	0.12 ± 0.04[b]
SSE 50 mg/kg/day (n = 11)	236.7 ± 9.1	299.9 ± 23.6[c]	0.19 ± 0.15[b]
SSE 100 mg/kg/day (n = 12)	237.6 ± 9.3	300.5 ± 20.3[c]	0.18 ± 0.10[b]
17β-estradiol 0.5 mg/kg/day (n = 12)	237.6 ± 8.4	266.0 ± 9.3[a]	0.45 ± 0.07[a]

Values expressed as mean ± SD. OVX, ovariectomy; SSE, standardized soy extract.
[a]$P < 0.001$ versus OVX; [b]$P < 0.001$ versus sham; [c]$P < 0.05$ versus sham.

Bone density, femoral length, and biochemical parameters of bone turnover

Table 2 shows bone-related parameters in treatment groups. The femoral density in rats killed 6 weeks after surgery showed that ovariectomized animals had significantly lower bone density than the sham-operated controls ($P < 0.001$). Treatment with SSE dose-dependently suppressed ovariectomy-induced bone loss, and this effect reached statistical significance in rats receiving the high dose ($P < 0.05$ vs ovariectomized rats). As expected, 17β-estradiol also prevented bone loss ($P < 0.001$ vs ovariectomized rats). There were no significant differences in femoral length among the groups (data not shown). Ovariectomy resulted in a significant increase in serum osteocalcin concentration compared with the sham-operated rats ($P < 0.01$), and 17β-estradiol significantly suppressed this increase to the levels of the sham-operated rats ($P < 0.01$ vs ovariectomized rats). A similar effect was observed after treatment with 100 mg/kg/day of the tested extract ($P < 0.05$ vs ovariectomized rats), whereas no changes were detected after treatment with 50 mg/kg/day Because of the limited availability of blood sample volume, the interleukin-6 analysis was performed only on three to six rats per group, and for this reason a statistical analysis was not conducted. Results obtained showed a trend toward increased interleukin-6 serum concentration in ovariectomized animals; treatment with 17β-estradiol or 100 mg/kg/day −SSE considerably reduced the levels of this cytokine in the serum. Urinary excretion ratio of deoxypyridinoline to endogenous creatinine excretion was increased after ovariectomy, although not significantly so. 17β-Estradiol treatment significantly reduced excretion when compared with ovariectomy ($P < 0.05$). Treatment with 100 mg/kg/day −SSE prevented the increase of deoxypyridinoline excretion induced by ovariectomy.

TABLE 2. *Bone-related parameters in rats after treatment*

Treatment group	Bone density (g/cm^3)	Serum osteocalcin (ng/mL)	Serum IL-6 (pg/mL)[a]	Urinary DPD (nM DPD/mM creatinine)
Sham	1.531 ± 0.022[b] (n = 12)	6.6 ± 1.2[c] (n = 8)	151 ± 107 (n = 6)	133.9 ± 52.3 (n = 8)
OVX	1.462 ± 0.034[d] (n = 11)	38.9 ± 20.4[e] (n = 8)	182 ± 100 (n = 6)	157.2 ± 65.9 (n = 8)
SSE 50 mg/kg/day	1.484 ± 0.026[d] (n = 11)	41.3 ± 21.5[d] (n = 6)	169 ± 66 (n = 3)	150.8 ± 77.8 (n = 6)
SSE 100 mg/kg/day	1.496 ± 0.019[f,g] (n = 12)	16.6 ± 3.9[f] (n = 8)	76 ± 32 (n = 6)	126.2 ± 35.1 (n = 8)
17β-estradiol 0.5 mg/kg/day	1.508 ± 0.036[b] (n = 12)	11.0 ± 2.1[c] (n = 8)	39 ± 40 (n = 3)	80.0 ± 49.7[f] (n = 8)

Values expressed as mean ± SD; IL-6, interleukin-6; DPD, deoxypyridinoline; OVX, ovariectomy; SSE, standardized soy extract.
[a]P = Statistical analysis not performed due to the limited number of samples; [b]$P < 0.001$ versus OVX; [c]$P < 0.01$ versus OVX; [d]$P < 0.001$ versus sham; [e]$P < 0.01$ versus sham; [f]$P < 0.05$ versus OVX; [g]$P < 0.05$ versus sham.

Uterus organ weight, histopathological evaluation, and hormonal receptor status

As expected, ovariectomy caused a significant reduction in uterine weight ($P < 0.001$ vs sham) (Table 1). At both doses tested, SSE did not induce any significant change in the uterine weight compared with untreated ovariectomized rats. However, treatment with 0.5 mg/kg/day 17β-estradiol significantly increased the uterus weight ($P < 0.001$ vs ovariectomized rats), increasing it to a level of the sham-operated control. Uterine histopathological analysis showed that the marked atrophy of uterus epithelium due to ovariectomy was not significantly decreased by treatment with SSE (Table 3, Fig. 1); however, stromal edema was observed in the majority of soy-treated rats at both doses tested ($P < 0.001$ vs sham-operated and ovariectomized rats). Treatment with 17β-estradiol induced epithelial hyperplasia and stromal edema in most animals (for both findings, $P < 0.001$ vs ovariectomized and sham-operated rats).

ERα immunoreactivity was confined to the nuclei of endometrial epithelium and stroma (Fig. 2A). In uteri of both sham-operated and ovariectomized rats, a high intensity of ERα immunostaining was seen in the epithelium and stroma, with approximately 65% of stromal nuclei and virtually all epithelial nuclei staining positive; this resulted in very high immunohistochemical receptor scores (Table 4). In the 17β-estradiol–treated rats, the ERα expression significantly declined in the epithelium ($P < 0.05$ vs ovariectomized rats); although no significant changes were observed in the number of cells expressing ERα in this compartment, the intensity of immunostaining was significantly decreased. No significant changes were seen in the stroma after 17β-estradiol administration. Treatment with SSE, at 50 or 100 mg/kg/day, did not significantly affect the expression of ERα in both the epithelial and stromal compartments. Results from ERβ immunostaining in sham-operated rats showed that about 80% and 60% of cells in the epithelium and stroma stained positive (Fig. 2B); however, the intensity was faint, particularly in the stroma, and the final receptor scores was 160 (range 70-180) and 60 (range 50-60) in the two compartments, respectively (Table 4). After ovariectomy, the ERβ expression significantly decreased in the stromal cells ($P < 0.05$ vs sham), whereas no changes were observed in the epithelium. Treatment of animals with 17β-estradiol did not significantly affect the ERβ status of both stromal and epithelial cells when compared with ovariectomized control rats. In contrast to the 17β-estradiol group, administration of SSE to ovariectomized animals resulted in an up–regulation of ERβ expression in the stromal compartment; this effect was not dose-related and statistical significance was achieved in the 50-mg/kg/day group ($P < 0.05$ vs ovariectomized rats). No significant changes were seen in the epithelium after SSE treatment.

As with ERα, PR immunoreactivity was confined to cell nuclei of the endometrium (Fig. 2C). In sham-operated controls, both intensity of PR signal and number of PR-positive cells were low in the epithelium compartment; relatively higher levels of PR expression

TABLE 3. *Endometrial findings in uterus of rats after treatment*

Treatment group	Epithelium: Hyperplasia	Epithelium: Atrophy	Stromal edema
Sham	2/11	0/11[a]	0/11
OVX	0/10	10/10[b]	0/10
SSE 50 mg/kg/day	0/11	10/11[b]	8/11[a,b]
SSE 100 mg/kg/day	1/12	10/12[b]	10/12[a,b]
17β-estradiol 0.5 mg/kg/day	10/12[a,b]	0/12[a]	10/12[a,b]

Data are number of animals with findings/total number of animals. OVX, ovariectomy; SSE, standardized say extract.
[a]$P < 0.001$ versus OVX; [b]$P < 0.001$ versus sham.

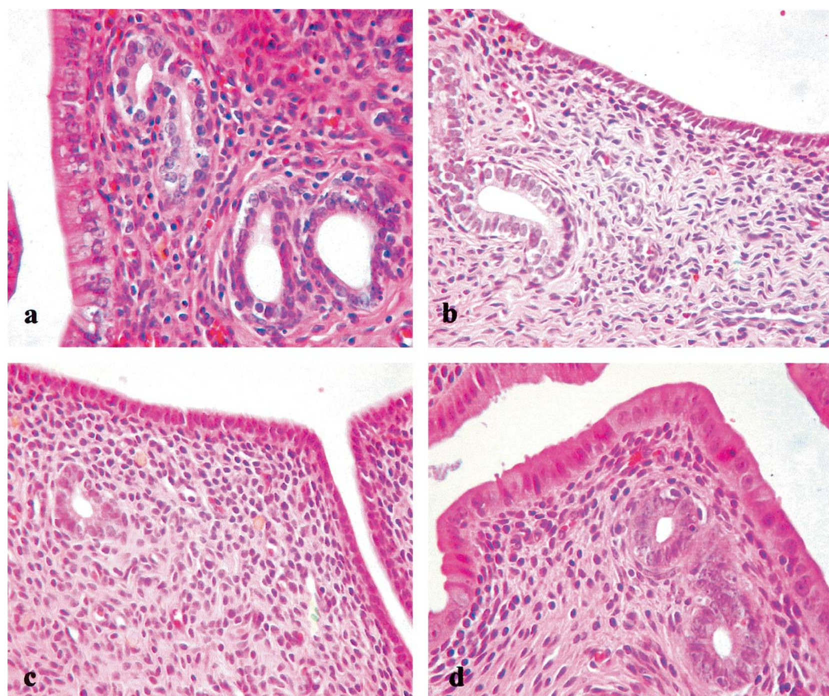

FIG. 1. Hematoxylin/eosin-stained sections of endometrium from sham-operated (**a**), ovariectomized (**b**), SSE-treated (**c**), and 17β-estradiol–treated rats (**d**). A marked atrophy of uterus epithelium was observed in ovariectomized animals and after treatment with SSE. Treatment with 17β-estradiol induced epithelial hyperplasia. Photos were taken at a magnification of ×20.

were found in stromal cells (Table 4). After ovariectomy, the PR expression significantly declined in the stromal cells ($P < 0.05$ vs sham), whereas in the epithelium the PR immunoreactivity was significantly enhanced ($P < 0.05$ vs sham). 17β-Estradiol treatment resulted in opposing changes in PR expression in the epithelial and stromal compartments. Expression of PR in the epithelium was strongly downregulated by 17β-estradiol, when compared with ovariectomized rats, although not significantly so. In contrast to the observations in the epithelium, 17β-estradiol induced intense PR staining in the stromal compartment, the expression levels being significantly higher than both ovariectomized and sham-operated controls ($P < 0.05$ vs sham-operated and ovariectomized rats). Administration of SSE to ovariectomized animals resulted in an up–regulation of PR expression in stromal cells, with levels similar to those observed in sham-operated animals; in the same animals, PR epithelial expression resulted in an ovariectomy-like pattern.

Lipid profile

To evaluate the possible effects on the lipid profile induced by the treatment, serum concentrations of total, HDL, and LDL cholesterol were determined in five or six animals per group. Results obtained showed that SSE did not significantly affect the minor increase in lipid levels induced by ovariectomy (Table 5). A dramatic decrease in total, HDL, and LDL cholesterol levels was observed after treatment with 0.5 mg/kg/day 17β-estradiol ($P < 0.001$ vs ovariectomized and sham-operated rats).

Phytoestrogen plasma levels

HPLC analysis showed a dose-dependent increase in both free and total daidzein and genistein plasma levels of soy-treated rats (Table 6). In the low- and the high-dose groups, unconjugated genistein accounted in plasma for 3.2 ± 2.1% and 3.8 ± 2.8% of the total isoflavone content, respectively, whereas unconjugated daidzein accounted for 4.9 ± 1.6% and 5.4 ± 3.2%. Free equol was not detectable in any animals; total equol concentrations were similar in both dose groups.

Immature female rat assay

In immature female rats, the ovaries do not produce 17β-estradiol; however, the uterus is fully responsive to exogenous estrogen, and hence this model permits a ready measure of agonist or antagonistic actions. In these rats, treatment with 0.5 mg/kg/day 17β-estradiol PO for 3 days significantly increased the uterine wet weight when compared with control treatment ($P < 0.001$) (Table 7). Conversely, SSE administered daily PO did not increase uterine weight compared with control. These results were confirmed when uterine dry weights were compared: 17β-estradiol significantly ($P < 0.001$) increased the uterine dry weight, an effect not elicited by SSE.

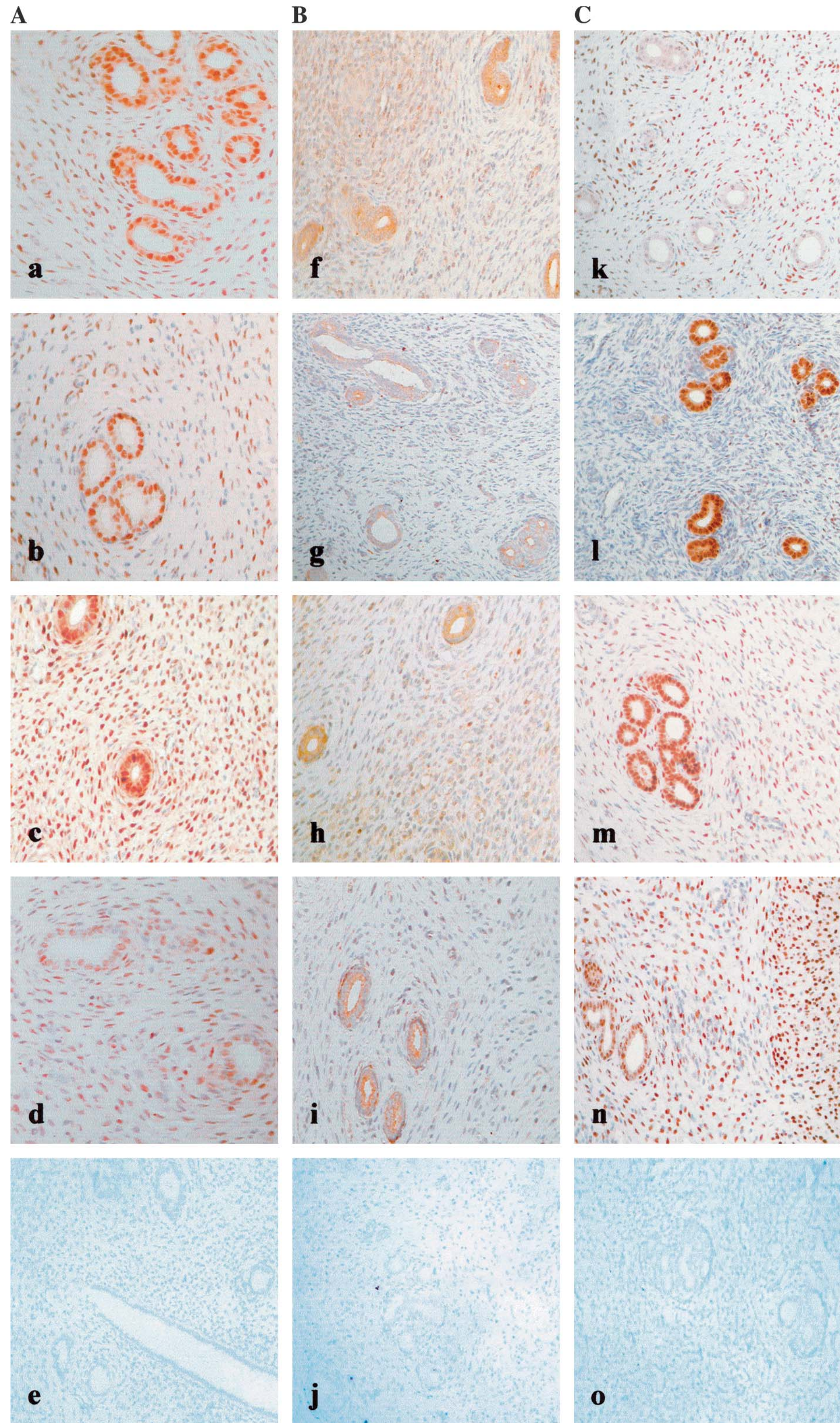

FIG. 2. A: Immunohistochemical localization of ERα in the rat uterus from sham-operated (**a**), ovariectomized (**b**), SSE-treated (**c**), and 17β-estradiol–treated rats (**d**), negative control (**e**). The high immunoreactivity of ERα in epithelium (brown-stained cells) observed in subpanels **a**, **b**, and **c** was significantly decreased in 17β-estradiol–treated rats (**d**). In stroma, nuclear positive immunostaining was present in a similar pattern in all groups. B: Immunohistochemical localization of ERβ in the rat uterus from sham-operated (**f**), ovariectomized (**g**), SSE-treated (**h**), and 17β-estradiol–treated rats (**i**); negative control (**j**). Immunostaining in epithelium (brown-stained cells) was present in a similar pattern in all groups. A very low expression of ERβ was observed in stroma from ovariectomized and 17β-estradiol–treated rats; SSE treatment up-regulated the expression in this compartment. C: Immunohistochemical localization of PR in the rat uterus from sham-operated (**k**), ovariectomized (**l**), SSE-treated (**m**), and 17β-estradiol–treated rats (**n**); negative control (**o**). Ovariectomy significantly increased immunostaining (brown stained cells) in the epithelial compartment and an ovariectomy-like pattern was observed after SSE treatment; less intense immunostaining was seen after 17β-estradiol treatment. Expression of PR was essentially identical in uterine stroma from sham-operated and SSE-treated rats. PR expression was fully up-regulated after 17β-estradiol treatment compared with both ovariectomized and sham-operated rats. Photos were taken at a magnification of ×20.

TABLE 4. *Immunohistochemical ER-α, ER-β, and PR receptor scores in the uterine compartments after treatment*

Treatment group	ER-α		ER-β		PR	
	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma
Sham (n = 8)	300 (180-300)	175 (130-230)	160 (70-180)	60 (50-60)[a]	25 (5-200)[a]	135 (30-165)[a]
OVX (n = 5)	300 (230-300)	180 (135-240)	150 (140-160)	20 (20-20)[b]	250 (80-300)[b]	25 (20-100)[b]
SSE 50 mg/kg/day (n = 8)	300 (190-300)	210 (110-285)	160 (60-160)	45 (30-80)[a]	230 (25-300)[b]	135 (30-200)
SSE 100 mg/kg/day (n = 8)	300 (240-300)	220 (170-300)	160 (120-180)	35 (20-100)	195 (45-300)[b]	115 (50-200)
17β-estradiol 0.5 mg/kg/day (n = 8)	180 (80-250)[a]	175 (30-255)	160 (120-160)	20 (20-20)[c]	100 (10-150)	185 (140-200)[a,b]

Values expressed as median (minimum-maximum). ER, estrogen receptor; PR, progesterone receptor; OVX, ovariectomy; SSE, standardized soy extract.
[a]$P < 0.05$ versus OVX; [b]$P < 0.05$ versus sham; [c]$P < 0.01$ versus sham.

DISCUSSION

Results obtained in this study have demonstrated that the tested soy extract has an interesting profile of tissue-specific response, in that at the assessed doses it is efficacious in preventing experimental osteoporosis without causing stimulation in the uterus and also without modulating the lipid profile. As reviewed by Kalu,[10] the 3-month-old mature ovariectomized rat is an applicable model for early skeletal changes in post-menopausal osteoporosis and, in our study, ovariectomy resulted in a significant osteopenic response at femur sites, as shown by density analysis. In addition, as in postmenopausal women, estrogen deficiency in ovari-ectomized rats produces high-turnover osteoporosis in which both bone resorption and bone formation are increased. As expected, 17β-estradiol prevented the ovariectomy-induced bone loss and reduced the higher rate of bone turnover, an effect extensively demon-strated in several previous reports.[11,12] Notably, after a relatively short treatment period (6 weeks), bone density analysis showed a bone-sparing effect of the soy extract that was associated with a slowing of increased bone turnover. Specifically, in our experimental con-ditions, treatment with 100 mg/kg/day SSE prevented the rise in serum osteocalcin levels (a sensitive marker of bone formation) and, to a lesser extent, the enhanced excretion ratio of deoxypyridinoline (a collagen breakdown product) observed in ovariectomized rats. Overall, our findings are in keeping with the majority of previous preclinical data[13] showing a significant osteoprotective activity of soy/soy isoflavones in the rat model of postmenopausal osteoporosis, where acute ovarian estrogen deficiency leads to rapid and measur-able bone loss. However, Picherit et al[14] showed that daily soybean isoflavone intake in adult ovariectomized rats reduced bone turnover but did not reverse a previously established bone loss, suggesting that ingestion of soybean isoflavones improve bone health in what can be considered a preventive rather than a curative approach toward human postmenopausal osteoporosis. Arjmandi[15] reported that isoflavones may play an important role in protecting bone only in the context of soy protein, a conclusion based on results obtained in a study in which doses of 125 to 250 mg of isoflavone per kilogram diet did not attenuate ovariec-tomy-associated bone loss. Determining the specific components of soy that could be responsible for the bone-sparing effects observed in animal studies is not simple or effortless; nevertheless, there is ample evi-dence of the effectiveness of isoflavones in conserving bone. Actually, numerous in vitro studies with human or animal osteoblast (or osteoblast-like) and osteoclast cell lines have almost consistently shown a direct effect of phytoestrogens and phytoestrogen-related compounds on both cell types.[13]

In the past decade, considerable evidence has been collected that one of the pathways by which estrogen may exert a protective effect on the skeleton may be governing the effect of cytokines on bone remodeling. Estrogen deficiency dramatically alters the dependency of bone cells on several cytokines, including in-terleukin-6, and ovariectomy-induced stimulation of osteoclastogenesis in mice can be prevented by neutralizing antibodies against interleukin-6[16] or

TABLE 5. *Effects on total cholesterol, HDL, and LDL serum levels in rats after treatment*

Treatment group	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Sham (n = 6)	93.5 ± 8.1	40.0 ± 1.9	32.2 ± 4.1
OVX (n = 5)	103.4 ± 8.4	45.0 ± 6.1	37.2 ± 3.6
SSE 50 mg/kg/day (n = 6)	92.3 ± 11.4	38.2 ± 5.0	30.8 ± 6.4
SSE 100 mg/kg/day (n = 6)	98.3 ± 8.5	40.3 ± 3.7	35.5 ± 6.3
17β-estradiol 0.5 mg/kg/day (n = 6)	58.8 ± 11.3[a,b]	19.3 ± 6.0[a,b]	10.3 ± 1.9[a,b]

Values expressed as mean ± SD. OVX, ovariectomy; SSE, standardized say extract.
[a]$P < 0.001$ versus OVX; [b]$P < 0.001$ versus sham.

TABLE 6. *Genistein, daidzein, and equol plasma levels after treament (μM)*

Treatment group	Genistein		Daidzein		Equol	
	Free	Total	Free	Total	Free	Total
SSE 50 mg/kg/day	0.03 ± 0.03	0.91 ± 0.49	0.06 ± 0.04	1.50 ± 0.47	ND	2.26 ± 0.77
SSE 100 mg/kg/day	0.09 ± 0.05	2.65 ± 1.72	0.18 ± 0.11	3.29 ± 1.56	ND	2.30 ± 1.47

Values expressed as mean ± SD; N = 6. SSE, standardized soy extract; ND, not detectable.

the end of interleukin-6 function by gene knockout.[17] Interestingly, treatment of ovariectomized rats with SSE produced changes in serum interleukin-6 levels resembling those observed with 17β-estradiol, suggesting that the bone-sparing effect detected in soy-treated animals could be partly attributed to the modulation of osteoclastogenesis induced by interleukin-6. Chen et al[18] also showed that genistein and daidzein dose dependently reduced the release of interleukin-6 in two human fetal osteoblastic cell lines; however, to our knowledge, the present study is the first to report such an effect in an in vivo experimental model. However, Jenkins et al[19] reported that high soy intake induced an increase in interleukin-6 serum levels in postmenopausal women; although differences in products used and/or dosages could explain these different outcomes, additional studies are needed to elucidate the effect of soy/soy phytoestrogens on the modulation of this cytokine. It should also be pointed out that the low serum interleukin-6 levels obtained in 17β-estradiol–treated rats, although partly supported by the high serum hormone levels, could also be the result of the lower weight gain seen in this group, because adipose tissue has been shown to be an important site of interleukin-6 production.[20] This latter indirect effect seems less relevant in SSE-treated rats, because soy treatment did not significantly reverse the weight gain associated with the ovariectomy in rats.

The different body weight gain observed among experimental groups actually represents a potentially complicating factor for interpretation of bone data, when considering those reports showing that obesity provides partial protection against osteoporosis in the long bones of ovariectomized rats.[21] To date, this protective effect has been explained by a combination of mechanical, hormonal, and biochemical factors.[22] All these findings suggest that different genomic and nongenomic pathways may be involved in the bone-sparing effects of estrogens versus isoflavones.

TABLE 7. *The effects of treament on uterus weight in immature female rats*

Treatment group	Uterus wet weight (mg)	Uterus dry weight (mg)
Sham (n = 6)	39.2 ± 8.1	5.0 ± 2.6
SSE 50 mg/kg/day (n = 6)	42.2 ± 8.6	5.3 ± 1.3
SSE 100 mg/kg/day (n = 6)	38.4 ± 7.7	4.6 ± 1.6
17β-estradiol 0.5 mg/kg/day (n = 6)	126.4 ± 18.4[a]	17.6 ± 2.2[a]

Values expressed as mean ± SD.
[a] $P < 0.001$ versus Control.

Worthy of note is that the levels of circulating total daidzein and genistein in treated rats were in the range of those measured in Asians eating a traditional diet containing soy products.[23-25] In fact, although the mean plasma concentrations of the isoflavones genistein and daidzein reported in the above-mentioned studies were lower than the mean levels in the present study, the extension of range values included our data. Our finding of predominantly conjugated isoflavones in plasma is in keeping with older and more recent studies that show glucuronides to be the major circulating form of all phytoestrogens;[3,26] this, however, does not render them biologically inactive, providing, on the contrary, a constant source to account for the persistent levels of unconjugated isoflavones detected in plasma.

We notably demonstrated that, at the tested doses, the bone protective activity of SSE was not accompanied by an uterotrophic effect, as shown by the organ weight data and by histopathological analysis. Specifically, administration of SSE did not affect the uterine weight in ovariectomized or immature female rats, whereas treatment with 0.5 mg/kg/day −17β-estradiol significantly increased the uterine weight in both experimental models.

In keeping with these findings, the histopathological analysis demonstrated that the uterine epithelium of ovariectomized soy-treated rats was atrophic, and stromal edema was the only finding detected in the majority of animals. On the contrary, evidence of epithelial hyperplasia was shown in all but two rats receiving 17β-estradiol, and this finding was accompanied by stromal edema. Because ER and PR levels are thought to be critical in determining cell responsiveness to steroids, uterine receptor regulation has been extensively evaluated in the present study. Results obtained by immunohistochemical analysis showed, in keeping with previous studies,[27,28] a significant down–regulation of ERα in the uterine epithelium after 17β-estradiol treatment, whereas no similar changes were observed after treatment with SSE. No significant differences

were observed among groups in ERα stromal levels. Because the epithelial proliferation after 17β-estradiol is dependent upon stromal and not epithelial ERα,[28,29] this latter finding suggests that the lack of stimulatory activity on the uterine epithelium with soy treatment (as established by uterine weight and histopathological analysis) could be the result of a negligible stimulatory activity of phytoestrogens on ERα at the doses tested. A decline in uterine ERβ mRNA after estrogen treatment has been reported by other authors.[30,31] In particular, Weihua et al[30] showed by immunofluorescent labeling that the ERβ protein was decreased essentially in the stroma. We did not observe any modulation of ERβ expression after 17β-estradiol in the present study, either in the stroma or in the epithelium; however, because ovariectomy itself caused a marked decrease in stromal ERβ expression, a further decline in response to estrogen might not have been observed. SSE administration did induce an up–regulation of ERβ stromal expression when compared with ovariectomized controls, although this change was not dose related. Considering the proposed role of ERβ as a negative modulator of ERα-mediated uterine proliferation,[30] the absence of an estrogen-like proliferative effect on the uterus of soy-treated animals could also be the result of an enhanced ERβ activity. Our data on estrogen-mediated PR regulation in the rat uterus are in keeping with the previous literature suggesting that PR is differentially regulated by 17β-estradiol in epithelium versus stroma.[32-34] In particular, immunohistochemical analysis of the uterine epithelial PR expression clearly demonstrated a down–regulation after 17β-estradiol treatment, and such a regulation is exactly opposite to that of most other species.[35-39] Differently from 17β-estradiol, rats receiving SSE showed an ovariectomy-like PR epithelial status. The mechanism of estrogen action on the rodent uterine epithelium is not presently clear, even if it has been pointed out that 17β-estradiol stimulates stroma via its ERα to produce a paracrine factor, that, in turn, down–regulates constitutive PR in uterine epithelium.[34] If this is the case, taking into account that there were no significant differences in the expression of stromal ERα between rats treated with 17β-estradiol and soy, the divergent regulation observed could once again be attributed to a lack of significant stimulatory activity by soy phytoestrogens on ERα at the doses tested. In contrast to the epithelial compartment, PR expression in the stromal compartment was fully up-regulated after 17β-estradiol treatment; the effects of SSE treatment on the uterine PR stromal expression were similar, although less pronounced, to those produced by 17β-estradiol.

It has recently been reported that 17β-estradiol can up-regulate uterine stromal PR via both ERα and ERβ, and that these pathways can work independently.[40] Accordingly, the selective stimulatory activity of phytoestrogen on ERβ could account for findings observed in the uterine stroma of soy-treated rats.

Finally, results obtained in the present study did not show any activity of the tested extract in modulating the minor increase in lipid levels observed following ovariectomy. These results are in agreement with data from previous clinical studies on this extract, showing the lack of hypocholesterolemic effects in postmenopausal women.[4,5] Notably, other authors also have reported that phytoestrogen extracts do not influence lipoprotein levels in postmenopausal women.[41,42] A dramatic decrease in cholesterol levels was observed after 17β-estradiol treatment, and these values were lower than those observed in the sham-operated controls. It is possible that this effect was due to the high serum hormone levels detected in treated rats, our data being in keeping with previous reports using the same dose and route for 17β-estradiol;[43,44] however, the influence of the lower weight gain can not be ruled out. Moreover, according to the findings of Lundeen et al,[43] ovariectomized rats treated with 17β-estradiol showed a reduction in both HDL and LDL levels, in contrast to what is observed in humans, where estrogens produce an increase in HDL concentrations.

CONCLUSION

Our data indicate that the tested extract (Soyselect) acts as an estrogen agonist on bone, partially preventing ovariectomy-induced bone loss without exerting stimulatory effects on uterine tissue. In light of the serious human health consequences of osteoporosis and also of HT-related risks, the implication that this soy extract may be used to maintain bone mass in postmenopausal women, without affecting uterine tissue, merits further investigation.

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