ORIGINAL ARTICLE

Effects of high-dose isoflavones on rat uterus

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Summary

Objective: To evaluate the effects of high-dose isoflavones on the uterus of castrated adult rats. **Methods:** Adult, ovariectomized virgin rats (n = 40) were treated by gavage during 30 consecutive days with vehicle (propylene glycol, group GCtrl) or different doses of genistein: 42 (group GES42), 125 (GES125), or 250 (GES250) μg/g body weight per day. Animals were killed, weighed, vaginal and uterine samples were taken for cytologic evaluation, and serum levels of 17 β-estradiol and progesterone were determined. The middle third of the uterine horns was dissected, fixed in 10% formaldehyde and processed for paraffin inclusion; 5-μm thick sections were obtained and stained with HE for further histological study under light microscopy. The endometrial morphology and area, number and area of glands, and number of eosinophils in the lamina propria were analyzed. ANOVA and the Tukey-Kramer test were used for statistical analyses. **Results:** Uterine weight, endometrial glandular area, and number of glands and eosinophils were all higher in GES250 > G125 than in the other groups (GES250 > GES125 > GES42 = GCtrl; $p < 0.05$). Morphological data showed signs of endometrial proliferation upon treatment with genistein, especially in animals in GES125 and GES250 compared to other groups. In all animals in GES250, signs of uterine squamous metaplasia were observed. **Conclusion:** A short treatment period with high daily doses of isoflavones can promote endometrial squamous metaplasia in ovariectomized rats.

Keywords: Isoflavones; ovariectomy; uterus; metaplasia; rats.

INTRODUCTION

Atrophy of the female genital tract secondary to hypoestrogenism, usually associated with vaginal or urinary symptoms, such as desiccation, pruritus, discomfort, dispaurenia, dysuria, and urinary urgency, is common after menopause[1]. Besides, hypoestrogenism can cause different organic changes that can lead to the development of vasomotor symptoms, cardiovascular diseases, osteoporosis, cognitive dysfunction, loss of bone mass, and urogenital changes[2].

Hormone replacement therapy (HRT) is the classical treatment for relief of postmenopausal vasomotor symptoms, especially hot flashes and urogenital atrophy. However, due to possible adverse effects on the breasts and uterus, i.e., higher rate of breast cancer and endometrial hyperplasia, investigators have been searching for other therapeutic alternatives[3,4].

Over the last few years, isoflavones became an alternative to HRT because they are considered safer, they are estrogen agonists, and have weak intrinsic activity[5]. Isoflavones are the most studied phytotherapeutic agents, being found mainly in soy, and their main active elements include genistein, daidzein, biochanin A, and formononetin[6]. Genistein is the most active one and is found in large quantities in soy[7].

However, despite several clinical assays having investigated its efficacy, most of them were unable to demonstrate its action on vaginal epithelium nor that it counteracts vasomotor symptoms[8,9]. Chiechi et al.[10] reported that post-menopausal isoflavones for long periods have trophic effects on the vaginal epithelium. The authors attributed this fact to prolonged exposure to dietary phytoestrogens. On the other hand, Ferrari[11] reported that high-dose isoflavones, genistein in particular, can be used to treat hot flashes in post-menopausal women, with a good safety profile.

Epidemiological studies showed that phytoestrogens are associated with a reduction in the risk of estrogen-dependent cancer[12,13]. *In vitro* studies, especially with genistein, have demonstrated induction of apoptosis in cells with estrogen receptors in breast and cervical tumors[14].

Several *in vivo* studies, especially in rats, have demonstrated a trophic action of isoflavones on the female genital tract. However, the response of this system to high doses was not properly assessed[15-17]. The objective of the present study was to investigate the actions of high-dose isoflavones on endometrial morphology in rats.

METHODS

This is an experimental, prospective, double-blind, randomized study with adult virgin female rats (*Rattus norvegicus albinus*), weighing approximately 250 g, furnished by the Experimentation Model Development Center (CEDEME) of Universidade Federal de São Paulo – Escola Paulista de Medicina (UNIFESP – EPM). This study was approved by the Ethics on Research Committee of UNIFESP/EPM (protocol #0749/07).

DRUG

The soy extract used in this study had a total of 42.6% of isoflavones. Approximately 36% were genistein, 62%, daidzein, and 2%, glycitein (including isoflavones isoforms). Four per cent of the remaining extract 57 was composed of proteins (Zhonshan Road, Dalian, China).

ANIMALS

Forty virgin adult female rats, approximately 90 days old, weighing a mean of 250 g, of the Wistar EPM-1 lineage (*Rattus norvegicus albinus*) were used. Animals were initially taken to the Histology and Structural Biology Department Bioterium, being confined in plastic cages with metal grates at 22°C under artificial light (Philips fluorescent light bulbs – mode daylight with potency of 40 W) with daylight-dark periods of 12 hours each. Animals underwent colpocytologic tests for 21 consecutive days, and those in normal estrous cycle underwent bilateral ovariectomy.

Twenty-eight days after the ovariectomy, animals were randomly divided into four groups: GCtrl (control) – received propylene glycol; GES42 – received 42 μg/g per day of genistein; GES125 – received 125 μg/g per day of genistein; and GES250 – received 250 μg/g per day of genistein.

The drug was administered daily during a 30-day period diluted in 1.0 mL of propylene glycol and administered by gavage at the beginning of the daylight period, around 7 a.m.

In the last week, colpocytologic exams were performed daily and stained by the Harris-Shorr method to assess stimulation of the phytohormone. For this purpose, a cotton swab (cotonette®) embedded in saline was introduced in the vaginal ostium. Material from the vaginal lumen was collected and placed on a glass slide, which was immediately immersed in ethanol and ether solution (1:1) for 10 minutes. At the end of this period, the material was stained by the Shorr-Harris method.

SAMPLE COLLECTING PROCEDURES

Thirty days after the onset of drug administration, all animals were weighed on semi-analytic scales and anesthetized with xylazine (20 mg/kg) and ketamine (100 mg/kg) intraperitoneally. Soon after, blood was drawn by cardiac puncture and the serum was separated and stored at -20°C until determination of 17β-estradiol (E_2) and progesterone (P_4).

After a longitudinal incision of the abdomen, the uterus was removed. Afterwards, animals were euthanized with deepening of the anesthetic plane. Immediately after removal, the uterus was dissected and the adipose tissue

was removed. The material was then weighed on a precision scale (Mettler-Toledo®), its middle third was removed and immersed in 10% formaldehyde for 24 hours. Tissue samples were dehydrated in increasing concentrations of ethylic alcohol and diaphanized in xylene. Samples were, then, processed for paraffin inclusion. Transversal samples were obtained perpendicular to the greater axis of the uterine horn and stained by hematoxylin-eosin (HE).

Hormone analysis

Serum levels of 17β-estradiol (E_2) and progesterone (P_4) were determined. After the blood was drawn, it was centrifuged at 4°C (1500 × g) for 10 minutes and the supernatant was frozen at -20°C until it was analyzed by radioimmunoassay (RIA) using the double-antibody technique in precoated tubes (ICN Biomedical Inc., Costa Mera, CA, USA) according to manufacturer's recommendations. Assays were performed in duplicate. Samples were processed on the same day. The mean detection limit for E_2 and P_4 was 0.1 pg/mL and 0.1 ng/mL, respectively.

Histological analysis

Morphologic assessment was performed with light microscopy using a Carl Zeiss® microscope with objective lenses of 10 × magnification and ocular lenses of 10 and 40 × magnification. Mophometric assessment was performed by image digitalization with the Carl Zeiss® Axio-Vision software 4.1. The area occupied by the endometrium and glands in each histological slide from each animal slide was evaluated.

The areas to be evaluated were determined using a mouse and calculated automatically by the Carl Zeiss® AxioVision software 4.1. This procedure was performed to measure the areas occupied by the endometrium and endometrial glands in each histological slide of the uterus of each rat. The number of eosinophils was assessed by counting four fields, one in each quadrant of each histological slide, in a 500-μm² area in each quadrant. Four histological slides from each animal were evaluated.

Statistical analysis

The data underwent analysis of variance (ANOVA), which was complemented by the Tukey-Kramer test for multiple comparisons. The level of significance of a null hypothesis was 5% ($p < 0.05$).

Results

Weight

Rats treated with isoflavones had lower body weight; however, this data was not significantly different among the groups analyzed. On the other hand, the weight of the uterus was increased in GES250 and GES150 compared to the others (GES250 > GES125 > GES42 = GCtrl, $p < 0.05$) (Table 1).

Hormone levels and colpocytology

Serum levels of 17β-estradiol (E_2) and progesterone (P_4) among the different groups did not show variations (Table 1). Among animals in the CGtrl and GES42 groups, vaginal cytology showed very few cells, some of which were phagocytes (Figures 1A and B). In GES125 and GES250, the test showed large amounts of polyhedral, anucleated, acidophilic cells from the superficial layers (Figures 1C and D).

Morphology

On morphologic analysis the uterus of animals in the control group (GCtrl) and GES42 had basically the same aspect, i.e., slim and atrophic with few endometrial glands.

Table 1 – Body weight, uterine weight, serum levels of 17ß-estradiol, progesterone, and endometrial morphometry, as well as the presence of squamous metaplasia in rats treated with different doses of genistein

	Study groups			
	GCtrl	GES42	GES125	GES250
Body weight (g)	238.5 ± 24.4	227.3 ± 33.9	214.2 ± 22.1	217.4 ± 23.9
Uterine weight (g)	2.7 ± 0.5[a]	3.2 ± 0.8[a]	6.2 ± 1.1[b]	9.5 ± 1.3[c]
Estradiol (pg/mL)	2.5 ± 0.4	3.0 ± 1.1	2.8 ± 1.3	3.4 ± 1.6
Progesterone (ng/mL)	1.4 ± 0.3	1.6 ± 0.9	1.9 ± 0.8	1.7 ± 1.5
Squamous metaplasia	0/10	0/10	0/10	10/10
Endometrium				
Area x 10^5 (μm²)	3.75 ± 1.49[a]	4.39 ± 1.10[a]	11.03 ± 4.64[b]	23.11 ± 5.67[c]
Number of endometrial glands/area (nr./10^4μm²)	7.40 ± 3.33[a]	6.23 ± 3.47[a]	10.30 ± 2.21[b]	13.2 ± 3.21[b]
Area of endometrial glands (10^4μm²)	0.32 ± 0.12[a]	0.35 ± 0.11[a]	0.77 ± 0.21[b]	1.77 ± 0.42[c]
Eosinophils (nr./500μm²)	22.50 ± 8.06[a]	25.83 ± 5.21[a]	54.90 ± 14.19[b]	85.60 ± 11.12[c]

Superscript letters indicate statistical comparison: c > b > a, $p < 0.05$. G, group; Ctrl, control; ES42 = 42 μg of genistein; ES125 = 125 μg of genistein; ES250 = 250 μg of genistein.

The endometrium was covered by a simple cubic epithelium and by squamous epithelium in some areas. The lamina propria contained countless cells with round, markedly stained nuclei and very little cytoplasm (Figures 2A and B). In GES125 and GES250, enlarged uteri with thicker layers were observed. In GES125, uteri were covered by a simple prismatic epithelium. Lamina propria contained cells with large, clear nuclei and pronounced nucleoli. A large concentration of endometrial glands constituted by large cubic cells was observed. In the endometrial stroma, we noted countless eosinophils (Figure 2C). In GES250, the uterus was even larger than in other groups, with a viscous clear fluid, and it was covered by simple prismatic epithelium and, in some areas, non-keratinized stratified squamous epithelium, indicating the presence of squamous metaplasia (Figure 2D). In most animals (n = 8), prominences were observed on the base of the superficial epithelium in the direction of the lamina propria (Figure 2D). Endometrial glands were dilated and constituted by large cuboidal cells, and in some areas by non-keratinized stratified squamous epithelium, indicating squamous metaplasia (Figure 2E). In the lamina propria, a large concentration of eosinophils was observed, some infiltrating the superficial or glandular epithelium (Figures 2D and E).

HISTOMORPHOMETRY

Morphometric analysis of the endometrium demonstrated greater endometrial area, greater number and area occupied by endometrial glands, as well as greater number of eosinophils in the GES250 then in the other groups (GES250 > GES125 > GES42 = GCtrl; $p < 0.05$) (Table 1).
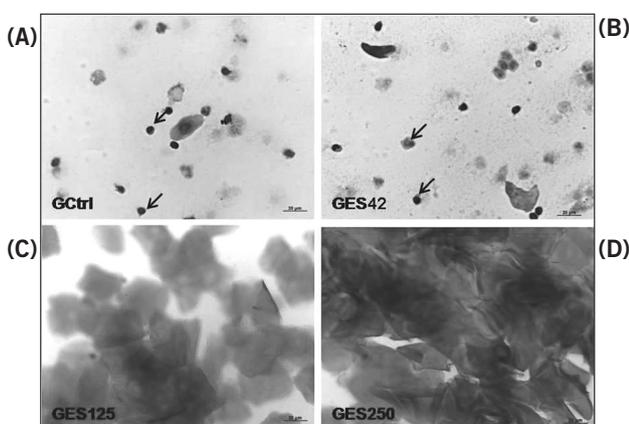

Figure 1 – Microphotographs showing colpocytology of ovariectomized rats and/or treated with different concentrations of genistein (G, group; Ctrl, control; ES42 = 42 μg of genistein; ES125 = 125 μg of genistein; ES250 = 250 μg of genistein). In A and B, note the scarcity of cells from desquamation of the vaginal epithelium and countless leucocytes (arrows). In C and D, observe anucleated acidophil epithelial cells from more superficial layers of the vaginal epithelium, and in D, large cell concentration. Harris-Shorr, 400X. Bar = 20 μm.

DISCUSSION

After publication of the WHI (Women's Health Initiative) study, there was a considerable increase in prescription of isoflavones and other drugs, since the aforementioned clinical assay demonstrated that menopausal hormonal therapy with continuous estrogens and medroxyprogesterone was associated with higher risk of breast cancer and thromboembolism[18]. Since then, isoflavones became an interesting therapeutic alternative in postmenopausal women with symptoms of hypoestrogenism.

However, the clinical impact of using isoflavones is still controversial. In regard to its effects on the breasts, Wood et al.[19] observed it has an anti-estrogenic effect while other authors observed estrogenic effects. As for the endometrium, Unfer et al.[20] reported greater risk of endometrial hyperplasia in long-term users.

To investigate the effects of high-dose of isoflavones in the uterus of ovariectomized rats, we performed the present study. For such, we used rats after 28 days post-ovariectomy. This period corresponds to the hypoestrogenism state in postmenopausal females[21].

Soy extract was administered in this study as source of isoflavones. The dose was determined based on previous study on the subject where, using the same extract in rats, the dose-effect curve regarding the colpocytologic exam and weight of the uterus was determined[22]. The doses selected were based on the following parameters: one dose
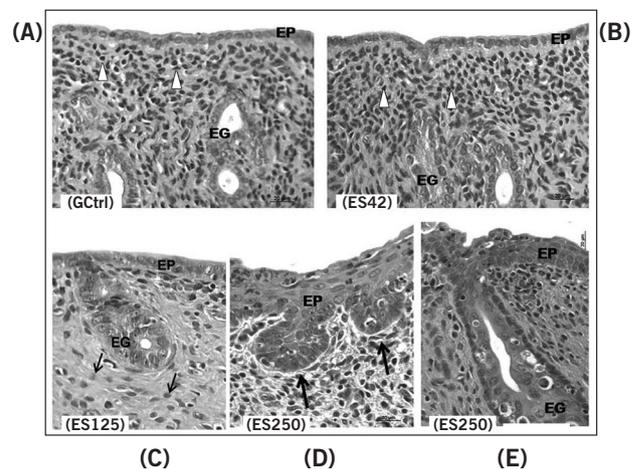

Figure 2 – Microphotographs showing endometrial areas from ovariectomized rats and/or treated with different concentrations of genistein (G, group; Ctrl, control; ES42 = 42 μg of genistein; ES125 = 125 μg of genistein; ES250 = 250 μg of genistein). In A and B, note the endometrium covered by superficial (EP) and glandular (EG) simple cylindrical epithelium with lamina propria with large concentration of cells with heterochromatic nuclei (arrow head). In C, note superficial (RP) and glandular (EG) epithelium with large euchromatic nuclei (arrows). In D and E, observe superficial (EP) and glandular (EG) non-keratinized stratified squamous epithelium (EP). In D, note superficial epithelial sprouting into the lamina propria (arrows). Harris-Shorr, 400X. Bar = 20 μm.

that did not change the weight of the uterus neither the colpocytologic exam (42 µg/g); and one smaller dose capable of changing the weight of the uterus and keratinization of the vaginal epithelium (125 µg/g). A dose twice as high as the latter was considered a high-dose[22].

As expected in our experiment, an increase in the weight of the uterus was observed in animals in GES125 and GES250 compared to the control and GES42. These data suggest that the doses of isoflavones used affected the uterus promoting dose-dependent trophic changes. Those findings were reported by Ishimi et al.[23], Picherit et al.[24], and Uesugi et al.[25] who also noticed a dose-dependent trophic effect on the uterus when evaluating the effects of genistein on prevention of bone mass in castrated rats.

Hormone levels (17β-estradiol and progesterone) were low and significant changes were not observed among the different groups. However, colpocytopathological aspects showed that 125 and 250 µg/g of isoflavones had dose-dependent proliferative and differentiating actions on vaginal epithelium, since the colpocytologic exam showed large concentration of anucleated acidophil cells.

Studies have demonstrated that prolonged exposure of rats to diets containing isoflavones increases uterine sensitivity to those substances, an effect that is mediated by nuclear estrogen-specific receptors. The mechanism of action of isoflavones on target tissue results in estrogen and anti-estrogen effects, among others, depending on the type and concentration of the stimulated receptor on tissues and their concentration in the body[26]. Currently, isoflavones are considered natural selective estrogen receptor modulators (SERMs)[6,27].

Isoflavones have greater affinity for β estrogen receptors (ERβ)[28], which are less frequent in the endometrium and breasts than α receptors (ERα)[29]. Its activity is 500-1000 times weaker than endogenous estrogens, but isoflavones can produce the same levels of estradiol bioactivity, as long as they are used in concentrations high enough to achieve the maximum response. That indicates that estrogen receptors and receptor-isoflavonoid complex are functionally equivalent[30]. Note that Möller et al.[31] reported that the chronic ingestion of isoflavones increase dramatically the receptivity of β estrogen receptors in rat uterus. Apart from the classical mechanism of hormone action (genomic), one should consider the presence of estrogen receptors on the cellular membrane (GPR30/GPRE), as well as β receptors (monomers) bound to the internal surface of the cell membrane. Those present non-genomic immediate effects[32].

Our results showed uterine hypertrophy with endometrial epithelial squamous metaplasia, both on superficial and glandular epithelium, in all animals treated with high-doses of isoflavones (250 µg/kg). These data suggest that this dose of isoflavones has endometrial proliferative effects. In fact, experimental studies with different doses

of isoflavones reported different actions on endometrial tissue[32-34]. Thus, the minimum dose usually used in humans does not show any morphologic changes. On the other hand, high-doses like those used in the GES250 group caused metaplastic changes in rat endometrium.

Studies with 395 postmenopausal women who received 70 mg/kg of isoflavones for three months showed relative endometrial safety without any mitogenic effects, being considered safe both to the endometrium and the breasts. However, there is a lack of data on long-term endometrial effects.

Sacks et al.[35] suggested that the use of isoflavones supplements both in foods and in pills is not recommended. On the other hand, the actions of several soy products considered beneficial for cardiovascular health are based on their high contents of polyunsaturated fats, fibers, vitamins, minerals, and low contents of saturated fats and not on their genistein content.

Conclusion

Our results lead to the conclusion that administration of soy extract to ovariectomized rats at a dose of 125 µg/g of genistein had uterotrophic effects. Those include increased endometrial area, and number of glands and eosinophils. High-doses (250 µg/g of genistein) triggered the development of squamous metaplasia in all animals, in addition to the uterotrophic effects.

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