

# The influence of phytoestrogens or estrogens on the proliferation of the rat endocervical mucosa

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<http://dx.doi.org/10.1590/1806-9282.66.2.174>

## SUMMARY

**INTRODUCTION:** Although estrogen therapy is widely used against post-menopausal symptoms, it can present adverse effects, including endometrial cancer. Soy isoflavones are considered a possible alternative to estrogen therapy. However, there are still concerns whether isoflavones exert trophic effects on the uterine cervix.

**OBJECTIVES:** To evaluate the histomorphometric and immunohistochemical alterations in the uterine cervix of ovariectomized rats treated with soy isoflavones (Iso).

**METHODS:** Fifteen adult Wistar rats were ovariectomized (Ovx) and divided into three groups: Group I (Ovx), administered with vehicle solution; Group II (OVX-Iso), administered with concentrated extract of Iso (150 mg/kg) by gavage; and Group III (OVX-E2), treated with 17 $\beta$ -estradiol (10  $\mu$ g/kg), subcutaneously. After 30 days of treatments, the uterine cervix was fixed in 10% formaldehyde and processed for paraffin-embedding. Sections were stained with Hematoxylin and eosin for morphological and morphometric studies or subjected to immunohistochemistry for detections of Ki-67 and vascular endothelial growth factor-A (Vegf-A). The data obtained were subjected to statistical analysis ( $p \leq 0.05$ ).

**RESULTS:** We noted an atrophic uterine cervix in G1, whereas it was more voluminous in GII and even more voluminous in GIII. The thickness of the cervical mucosa was significantly higher in GIII, as compared to G1 and GII. The cell proliferation (Ki-67) was significantly elevated in the estradiol and isoflavones treated groups, whereas Vegf-A immunoexpression was significantly higher in GIII, as compared to groups GII and G1.

**CONCLUSIONS:** Soy isoflavones cause less trophic and proliferative effects in the uterine cervix of rats as compared to estrogen.

**KEYWORDS:** Cervix uteri. Isoflavones. Estrogens. Ovariectomy. Rats.

DATE OF SUBMISSION: 17-Jun-2019

DATE OF ACCEPTANCE: 29-Jul-2019

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## INTRODUCTION

Postmenopause is characterized by a pronounced reduction in estrogen levels, which leads to vasomotor symptoms such as hot flashes and night sweats. These conditions can worsen the overall quality of life and, in particular, the quality of sleep by increasing sleep disturbances. Many women may concurrently become depressed, suffer from vaginal dryness, and lose bone mass. All of these changes may have a negative effect on women. In order to mitigate the negative outcomes of hypoestrogenism, hormone therapy is indicated.<sup>1-3</sup> However, there are other negative consequences, including cardiovascular diseases, which appear upon late-onset hormone therapy (over 10 years after menopause) or after 60 years of age.<sup>4</sup> Another concern is the breast cancer risk, which may increase with combined estrogen-progestin therapy.<sup>4,5</sup> Hence, there is a demand for alternative therapies against postmenopausal symptoms.

Soy isoflavones have a chemical structure similar to that of estrogens, making it possible for the former to bind to estrogen receptors, especially the  $\beta$  receptor. Such binding produces biological effects similar to those of estrogens but with less impact on the cardiovascular system and on breast tissue.<sup>6-8</sup> Studies of the genital system have reported that isoflavones may increase the proliferation of both the vaginal<sup>9</sup> and endometrial epithelia.<sup>10</sup> Meanwhile, little is known about the effects of isoflavones on the cervical epithelium.

The cervical epithelium is divided into ectocervical and endocervical epithelia, with a transition region between them.<sup>11</sup> It has been hypothesized that this transition region may be susceptible to develop cervical neoplasia due to human papillomavirus (HPV) infection.<sup>12</sup> Thus, it is important to investigate substances that could show trophic effects in this region. Ford et al.<sup>13</sup> reported trophic effects on the cervix following treatment with genistein, a soy isoflavone, and suggested an estrogenic action by these compounds. Nevertheless, there are still few data on cervical epithelial behavior, mainly on the endocervical epithelium. Thus, in this study, we assessed the effects of a concentrated soy isoflavone extract on the endocervix of ovariectomized rats.

## METHODS

### Animals and study design

This was a randomized controlled experimental study, evaluated and approved by the Research Ethics

Committee of the Universidade Federal de São Paulo – Escola Paulista de Medicina (UNIFESP/EPM); project Number: 0136-12. All the experimental procedures were conducted following national and international guidelines of animal use and care.

The experiment was carried out on 15 EPM-1 Wistar (*Rattus norvegicus albinus*) female rats aged 95 days and weighing  $\pm 227$ g each, provided by the Centro de Desenvolvimento de Modelos Experimentais em Medicina e Biologia-CEDEME, UNIFESP/EPM. The rats were transported to the animal facility of the laboratory of Histology and Structural Biology of UNIFESP/EPM. The animals were kept in cages (45x30x15cm) in an environment with controlled lighting and temperature (constant light/dark cycle of 12/12 hours and room temperature at 22°C) and were given food and water *ad libitum*.

After one week of the adaptation period, vaginal smear tests for five consecutive days were performed in all rats to monitor their estrous cycle. Only rats with regular estrous cycles remained in the experiment. Thereafter, the animals were anesthetized (ketamine, 0.08 mL/100g of body weight; xylazine, 0.04 mL/100g of body weight) and subsequently subjected to bilateral ovariectomy (Ovx).

In order to recover from Ovx surgery and ensure estrogen depletion, a period of twenty-one days after Ovx was adopted; the animals were then randomly divided into three groups, as follows: Group I (Ovx) – administered with 0.1 mL of a vehicle solution of propylene glycol (Sinthy® - São Paulo, Brasil) by gavage; Group II (Ovx-Iso) – administered with 150 mg/kg of a concentrated extract of soy isoflavones, diluted in 0.1 mL of propylene glycol, by gavage. Group III (Ovx-E2) -subcutaneous administration of 10  $\mu$ g/kg of 17 $\beta$ -estradiol (Sigma-Aldrich Chemicals, Oakville, ON, Canada) diluted in corn oil.

The soy extract used in this study was concentrated and enriched with isoflavones (Novasoy®, Archer Daniels Midland, Decatur, IL, EUA) with the following proportions: 40% of total isoflavones (genistein, daidzein, and glycitein at a ratio of 1.3:1:0.3, respectively), 7% to 12% of proteins, 4% of ashes, 6% of humidity, and the remaining 41% was made up of soy phytochemicals. Treatments lasted for 30 consecutive days. No deaths were reported throughout the experimental period.

### Material collection and histological processing

After treatment, the animals were anesthetized with ketamine (0.08 mL/100g of body weight) and

xylazine (0.04/100g of body weight) and euthanized by decapitation using an appropriate rodent guillotine. Subsequently, a laparotomy was performed to visualize the genital organs and collect the cervixes. The cervixes were then fixed in 10% formaldehyde (phosphate buffer, pH 7.2, 0.1M) for 24 hours and subsequently processed for paraffin embedding, which was carried out in such a way to obtain longitudinal sections of the cervical region (Figure 1).

After histological processing, the 4- $\mu$ m longitudinal sections (Minot Leica<sup>®</sup> RM2035 microtome) were stained with hematoxylin and eosin (H.E) for morphological and histomorphometric analyses or subjected to immunohistochemistry. Photomicrographs of the cervical sections were documented using a light microscope (Axiolab Standard 20, Carl Zeiss) coupled with a high-resolution video camera (AxioCam, Carl Zeiss) and subsequently underwent histomorphometric analysis.

#### Histomorphometry of the cervix

The histomorphometric evaluation was carried out with an image capture system, and the resulting images were analyzed using the Axiovision 4.8 REL (Carl Zeiss) software. Measurements were performed with 2.5x objective lenses for assessment of cervical thickness and 10x objective lenses for evaluation of the glandular area.

For each animal, five sections with 80- $\mu$ m intervals between them were made in order to analyze the following parameters: a) epithelial thickness, measurements were taken from the upper margin (luminal

border) of the cervical epithelium outward in eight distinct regions of the endocervix, which were averaged to obtain a mean expressed in  $\mu$ m; b) glandular area, the space occupied by the “glands” was outlined to cover an area up to 500 mm<sup>2</sup> of the cervix in two semi-serial sections. The areas were added up and expressed in mm<sup>2</sup>.

#### Immunohistochemical method

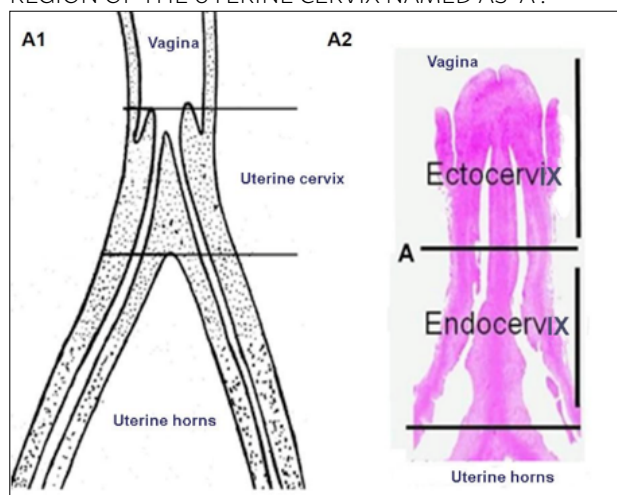
After the sections were deparaffinized and hydrated, endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes. Next, the sections underwent antigen retrieval in a steam cooker with 10-mM sodium citrate buffer solution (pH6.0) for 1 hour. After cooling, the sections were washed with phosphate-buffered saline (PBS) and incubated with 4% bovine serum albumin (BSA) for 20 minutes to block unspecified protein sites. Subsequently, the sections were incubated overnight with rabbit monoclonal primary antibodies (anti-Ki-67 or anti-Vegf-A, Spring, Bioscience, CA, USA) diluted at 1/200 and 1/300, respectively, in a humidity chamber at 4°C. For negative controls, the primary antibodies were replaced by *non-immune serum*. Afterward, the sections were washed in PBS and incubated in a streptavidin-biotinylated secondary antibody (Dako, Denmark) for 30 minutes. After the washes, reactions were revealed with 3,3-diaminobenzidine (DAB) (Dako, Denmark), counterstained with Harris hematoxylin, and mounted using a permanent mounting medium (Entellan<sup>®</sup>). A brownish color in the nuclei for Ki-67 and the cell border region or the cell cytoplasm for Vegf-A was adopted as a standard of positivity.

The slides were examined under a light microscope with a 10x objective lens (Axiolab Standard 20, Carl Zeiss) coupled with a high-resolution video camera (AxioCam, Carl Zeiss). The percentage of Ki67-positive cells was calculated in each section; at least 500 cells per animal were counted, and a mean was calculated for each rat in every experimental group. Meanwhile, the immunoreactivity for Vegf-A was assessed through a semiquantitative scoring system (H) as previously reported<sup>6</sup> and validated in our laboratory by Carbonel et al.<sup>7</sup>. Accordingly, a score of 0 was considered negative immunoreactivity, whereas positive immunoreactivities received scores from 1 to 3 according to the intensity of immunoreactivity.

#### Statistical analysis

The results were expressed as mean  $\pm$  standard deviation (SD). A comparison of the groups was made

**FIGURE 1.** DIAGRAM (A1) AND A LONGITUDINAL HISTOLOGICAL SECTION STAINED WITH H.E (A2) SHOWING THE REGION OF INTEREST. NOTE THAT THE REGION OF INTEREST WAS ESTABLISHED IN THE MID-REGION OF THE UTERINE CERVIX NAMED AS “A”.



with the Kruskal-Wallis test followed by the Dunn test. The level of significance for the rejection of the null hypothesis was  $p \leq 0.05$ . Graph Pad Prism 5.0 (San Diego, CA, USA) software was used for every test.

## RESULTS

### Morphological analysis

The endocervix from GI (Ovx) was covered by cuboidal epithelium with a reduction in crypts lined with the same type of epithelium as in the other two groups. The endocervix from GIII (Ovx-E2) displayed a considerably thicker epithelium of the cervical mucosa than the other two groups. The epithelium itself showed signs of proliferation attested by mitosis and stratification, whereas the lamina propria had abundant crypts. The endocervix of all rats from GII (Ovx-Iso) exhibited greater signs of proliferation than that from GI, and columnar epithelium with a few areas of stratification was noticed. The histological pattern of one rat from GII was similar to that of GI. (Table 1, Figure 2).

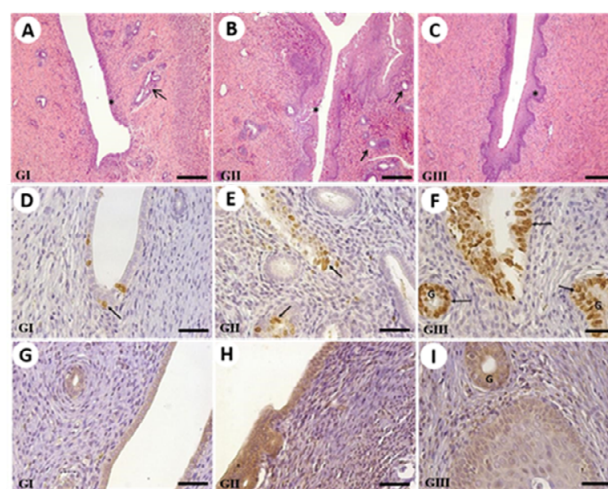
### Histomorphometry of the cervical mucosa

The endocervical mucosa was significantly thicker in GIII (Ovx-E2) than in GI (Ovx) and GII. Meanwhile, the glandular area in GIII (Ovx-E2) was considerably smaller than that in GI (Ovx) and GII (Ovx-Iso). Moreover, the endocervical mucosa in GI and GII presented a similar size. (Table 1, Figure 2).

### Immunohistochemical detection of Ki-67 and Vegf-A

The percentage of Ki-67-positive cells was significantly higher in GII (Ovx-Iso) and GIII (Ovx-E2) than in GI. However, this percentage was more noticeable

**FIGURE 2.** PHOTOMICROGRAPHS OF HISTOLOGICAL SECTIONS OF UTERINE CERVIX PORTIONS OF UTERI OF RATS STAINED WITH H.E (A - C), OR SUBJECTED TO IMMUNOHISTOCHEMISTRY AND COUNTERSTAINED WITH HARRIS' HEMATOXYLIN FOR THE DETECTION OF KI-67 (D - F) AND VEGF-A (G - I). NOTE GREATER THICKNESS OF THE EPITHELIUM OF THE ENDOCERVICAL MUCOSA (ASTERISKS) IN THE GII GROUP (OVX-ISO) (B), WHICH IS MORE EVIDENT IN THE GIII (OVX-E2) (C) AS COMPARED TO GI (OVX) (A). LARGER AREA OCCUPIED BY "GLANDS" (ARROWS) CAN ALSO BE SEEN IN GI (A) AND GII (B) AS COMPARED TO GIII (C). A HIGHER IMMUNOREACTIVITY (ARROWS) TO KI-67 (F) AND VEGF-A (I) CAN ALSO BE NOTICED IN THE GIII GROUP (OVX-E2), WHEN COMPARED TO GI (OVX) (D, G) AND GII (E, H). BARS: 50 $\mu$ M



in GIII. Furthermore, Vegf-A immunoreactivity was significantly greater in GIII (Ovx-E2) than in GII (Ovx-Iso) and GI (Ovx). (Table 1, Figure 2).

## DISCUSSION

The concern about hormone therapy was the consequence of the study published by WHI (Women's Health Initiative) in 2002, in which they reported that combined hormone therapy using estrogens

**TABLE 1.** DATA EXPRESSED AS MEAN  $\pm$  STANDARD DEVIATION. ABBREVIATIONS: TEM = THICKNESS OF THE ENDOCERVICAL MUCOSA; GA = "GLANDULAR" AREA; CE = CERVICAL EPITHELIUM; GE = "GLANDULAR" EPITHELIUM.

Parameters	GROUPS		
	GI (Ovx)	GII (Ovx-Iso)	GIII (Ovx-E2)
TEM	360.30 $\pm$ 11.40	400.40 $\pm$ 23.90*	584.70 $\pm$ 33.80**
GA	28.60 $\pm$ 2.40	24.40 $\pm$ 2.50	17.80 $\pm$ 6.40*
Ki67/CE (%)	1.40 $\pm$ 0.640	4.60 $\pm$ 1.60*	14.80 $\pm$ 1.40**
Ki67/GE (%)	2.46 $\pm$ 1.60	6.12 $\pm$ 2.80*	12.18 $\pm$ 1.16**
Vegf-A (Scores)	1.80 $\pm$ 0.37	2.01 $\pm$ 0.04	2.90 $\pm$ 0.15*

TEM - GIII>GII>GI, \* $p < 0.05$ ; GIII>GI, \*\* $p < 0.01$ ; GA - GII=GI>GIII, \* $p < 0.05$ ; Ki67/CE/GE - GIII>GII>GI, \* $p < 0.05$ ; GIII>GI, \*\* $p < 0.01$ . Vegf-A - GIII>GII=GI, \* $p < 0.05$ .

and progestogens correlated with breast cancer<sup>4</sup>. Therefore, some investigators started looking for options to ameliorate the symptoms of menopause. In fact, isoflavones became an alternative therapy for treating postmenopausal women with symptoms of hypoestrogenism.

Isoflavones are structurally similar to endogenous estrogens because they exhibit a phenolic ring with a hydroxyl radical in carbon<sup>3</sup>. Such a structure grants them a selective binding ability showing a strong affinity for alpha and beta estrogen receptors.<sup>13,14</sup> These compounds have been found to have the same beneficial effects as estrogen therapy against menopausal symptoms but without the side effects<sup>15</sup>. Although the effects of isoflavones are reported to be weaker than those of endogenous estrogens, the impact of isoflavones on the cervix has not been explored so far<sup>16</sup>. In our results, soy isoflavones administration exhibited some trophic effects on the uterine cervical, but it was weaker when compared to estrogen, indicating that isoflavones produced less proliferative effects on the cervix of rats than estrogens.

In fact, we observed a thicker cervical mucosa epithelium in the groups treated with estrogens and isoflavones in contrast to the animals treated with the vehicle. Nonetheless, besides exhibiting the thickest epithelium, the estrogen-treated group showed the highest percentage of Ki-67-positive cells. Ki-67 is a nuclear marker of cell proliferation, and it is expressed in all of the cell cycle phases, except in G0.<sup>7,16-20</sup> Therefore, our results indicate that the thicker cervical mucosa seen in GIII was the result, at least in part, of the increased rate of cell proliferation. On the other hand, it is worth emphasizing that although isoflavones exhibited a trophic effect on the thickness of the cervical mucosa, this effect was significantly smaller than that exerted by estrogens.

## RESUMO

**INTRODUÇÃO:** Embora a terapia estrogênica seja amplamente utilizada contra sintomas pós-menopausais, ela pode apresentar efeitos adversos, incluindo câncer de mama e endometrial. Assim, as isoflavonas da soja são consideradas uma alternativa possível à terapia estrogênica. No entanto, ainda há controvérsias se estes compostos exercem efeitos tróficos significativos no colo do útero.

**OBJETIVOS:** Avaliar as alterações histomorfológicas e imuno-histoquímicas no colo do útero de ratas ovariectomizadas tratadas com isoflavonas da soja (iso).

**MÉTODOS:** Quinze ratas Wistar adultas foram ovariectomizadas bilateralmente (Ovx) e separadas em três grupos: Grupo I (Ovx) - veículo (propilenoglicol); Grupo II (Ovx-Iso) - receberam extrato concentrado de Iso (150 mg/kg) e Grupo III (Ovx-E2) - tratado com 17β-estradiol (10 µg/kg); as soluções foram administradas via gavagem por 30 dias consecutivos. Posteriormente, os colos uterinos foram retirados, fixados em formaldeído a 10% tamponado e processados para inclusão em parafina. Cortes (4 µm) foram coradas com hematoxilina e eosina para estudo morfológico e morfométricos, enquanto outros foram submetidos à imuno-histoquímica para detecção de Ki-67 e do fator de crescimento endotelial vascular-A (Vegf-A). Os dados obtidos foram submetidos à análise estatística (p<0,05).

This suggests that the estrogenic activity of isoflavones is weaker in the endocervical region when compared to estrogens. In fact, isoflavone activity is 500 to 1000 times less potent than that of endogenous estrogens.<sup>21</sup>

Our data suggest that isoflavones exert a proliferative effect on the ectocervix, similar to what has been previously reported about the vaginal epithelium and endocervix. These descriptions are, in turn, similar to what occurs in the cervix.<sup>22</sup> Thus, patients with chronic diseases such as HPV must receive special care to prevent their reactivation, which could be triggered by isoflavone-induced tissue proliferation.<sup>23-25</sup>

## CONCLUSION

In conclusion, our results show that isoflavones have trophic and proliferative effects on rat endocervix; however, these effects are less potent than those produced by estrogens.

## Conflict of interest

The authors declare no conflict of interest in relation to this paper.

## Author Contributions

Conceptualization, 'P.C.F, 'R.S.S., 'A.A.F.C., 'E.C.B., and 'M.J.S.; J.M.S.J Funding acquisition, M.J.S., A.A.F.C., E.C.B., Resources, M.J.S., Execution of experiment, P.C.F., 'R.F-S., G.R.S.S.; A.A.F.C.; Data collection, P.C.F., R.F-S., G.R.S.S.; A.A.F.C.; Data analysis, 'P.C.F, 'R.S.S., 'A.A.F.C., G.R.S.S., R.F-S 'E.C.B, and 'M.J.S.; Writing-original draft, P.C.F., A.A.F.C., R.F-S., Writing-review & editing, R.S.S., A.A.F.C., M.J.S., R.F-S., E.S.C. and 'J.M.S.J; Supervision, M.J.S; Project Administration, P.C.F., and M.J.S.

**RESULTADOS:** Observamos a presença de colo uterino atrófico no G1 (Ovx), sendo este mais volumoso no GII (Ovx+Iso) e ainda mais volumoso no GIII (Ovx+E2). A espessura da mucosa cervical foi significativamente maior no GIII (Ovx-E2), em comparação ao G1 (Ovx) e ao GII (Ovx-Iso). A proliferação celular (Ki-67) foi significativamente mais elevada nos grupos tratados com estradiol e isoflavonas, enquanto a imunexpressão de Vegf-A foi significativamente maior no GIII (Ovx-E2), em comparação ao GII (Ovx-Iso) e ao G1 (Ovx-E2).

**CONCLUSÕES:** As isoflavonas da soja causam menos efeitos tróficos e proliferativos no colo do útero de ratas em comparação ao estrogênio.

**PALAVRAS-CHAVE:** Colo do útero. Isoflavonas. Estrogênios. Ovariectomia. Ratos.

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