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Tofupill lacks peripheral estrogen-like actions in the rat reproductive tract

Martha V. Oropeza [a], Sandra Orozco [b], Héctor Ponce [c], María G. Campos [a,*]

[a] *Medical Research Unit in Pharmacology, National Medical Center SXXI, IMSS, Mexico City, Mexico*
[b] *Medical Research Unit in Neurological Diseases, Specialties Hospital, National Medical Center SXXI, IMSS, Mexico City, Mexico*
[c] *Universidad Autonoma del Estado de Hidalgo, Campus Ciudad Sahagun, Mexico*

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Abstract

Objective: The objective of this study was to evaluate the estrogenic effect of phytoestrogens contained in a commercial food supplement (Tofupill) on the reproductive tract of ovariectomized rats.
Methods: Food supplement (3.4 or 10.2 mg/kg) and conjugated equine estrogens (CEE, 31 or 100 μg/kg) were orally administered, daily during 14 days to ovariectomized rats. At the end of treatment, the following determinations were done: dry and wet uterine weight, vaginal epithelium condition, and uterine serotonin-induced contractile response. A group treated with 17β-estradiol was included as control for serotonin-induced contractile response.
Results: Food supplement did not display clear estrogenic effects on vaginal epithelium, uterine weight or myometrial sensitivity to serotonin, whereas high doses of conjugated equine estrogens showed estrogenic action.
Conclusions: The present data showed that Tofupill displayed a lower estrogenic effect than conjugated equine estrogens, which are one of the most commonly used hormone replacement therapy for postmenopausal women. However, further studies are needed to evaluate the risk associated to the use of Tofupill as an alternative to hormone replacement therapy for postmenopausal women.

Keywords: Tofupill; Phytoestrogens; Uterus; Vaginal epithelium; Serotonin; Contractile response; Smooth muscle

1. Introduction

Phytoestrogens are plant-derived compounds with structural and functional similarities to estrogens [1]. They appear to have estrogen-like effects in humans [2]. There are three main classes of phytoestrogens: isoflavones, coumestanes and lignanes that are found mainly in soybeans, clover, or alfalfa sprouts, green tea, and oilseeds. Several studies have suggested that diets rich in phytoestrogens protect against breast cancer, prostate cancer, colon cancer, cardiovascular disease, and osteoporosis [3].

In premenopausal women, dietary phytoestrogens increase follicular phase length and/or delay menstruation and significantly suppress midcycle surges of luteinizing and follicle-stimulating hormones [4]. Japanese women are reported to have a low frequency of hot flashes compared with postmenopausal Western women, in part attributed to their high phytoestrogen consumption [5]. Other studies have shown that some dietary phytoestrogens may produce mild estrogenic effects in postmenopausal women, for instance, estrogen-like effects on vaginal cytology and reductions in hot flashes [6–8]. From these findings, phytoestrogens have been proposed as an alternative to estrogen replacement therapy. However, findings are inconsistent. Despite the well-documented effect of estrogens to enhance leptin production, even high levels of isoflavone consumption do not alter leptin concentrations in women [9]. Likewise, there

* Corresponding author. Tel.: +52 55 5627 6900x21368; fax: +52 55 5761 0952.
E-mail address: mariagcampos@yahoo.com (M.G. Campos).

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are intra-assay response variations in some studies, and there is no clear correlation between estrogenic changes in vaginal cytology and the effect on hot flashes. These differences in response may depend on the population studied, soy products consumed, duration of exposure, and variability in response to phytoestrogen supplementation in postmenopausal women [10].

Studies with animals have shown that coumestrol (one of the most potent phytoestrogens), administered parenterally at a dose of 200 μg/day for 3 days exhibited dose-dependent uterotrophic activity, revealed by uterine weights similar to those produced by physiological doses of estradiol [11]. In rats, Picherit et al. observed that genistein and daidzein might bind to estrogen receptors in the rat uterus homogenate, and induce a weak estrogenic effect on in vitro contractile response to either oxytocin or luprostiol [12]. On the other hand, when soybean estrogens were administered to surgically postmenopausal macaques the vaginal cytological pattern was similar to that of postmenopausal controls without treatment [13]. In both, animals and postmenopausal women studies, findings were inconclusive. One fact to take into account when analyzing phytoestrogen actions is biological potency. The majority of these compounds lack steroidal structure and are less potent than synthetic estrogens (10^{-3} M versus 10^{-5} M, respectively), and their effects vary among species, routes of administration, and end points [14].

In spite of inconclusive experimental data, a food supplement, named Tofupill, was developed and commercialized as an alternative to estrogen replacement therapy for postmenopausal women. In Tofupill formulation, each capsule contains 34 mg of phytoestrogens extracted from soybean and flaxseeds. To our knowledge, there are only scarce evaluations of the actions of Tofupill on the female reproductive tract.

The myometrial sensitivity to serotonin is a well-characterized estrogen-dependent response. It has been shown that estrogens promote specific serotonin-induced myometrial contractility, which is related to an estrogen-dependent increase in the number of uterine serotonin receptors [15–18]. Indeed, in the absence of an adequate estrogenic stimulus, the myometrium is not sensitive to serotonin [19]. Based on these results, we included the serotonin-induced contractile response as an index of estrogenic effects on myometrium.

The aim of the present study was to evaluate the peripheral estrogenic effect of Tofupill on the following estrogen-dependent characteristics of female reproductive tract: uterine weight, keratinization of the vaginal epithelium, and sensitivity of uterine smooth muscle to serotonin-induced contractile response.

Since postmenopausal women are potential consumers of Tofupill, we decided to test the effects of Tofupill on the reproductive tract of a postmenopausal model, the adult bilaterally ovariectomized rat.

2. Materials and methods

2.1. Animals

Adult female Sprague–Dawley rats, 200–250 g body weight, 10–12 weeks old, bred in the center for laboratory animals production and maintenance (National Medical Center SXXI, IMSS), with free access to food and water, and housed in groups of 4–5 per cage under a 12:12 h light:dark schedule, were used. The rats were bilaterally ovariectomized under xylacine (20 mg/kg body weight) and ketamine (45 mg/kg body weight) anesthesia.

2.2. Treatments

After a recovery period of 2 weeks, rats were randomly assigned to one of the following groups of 14 day treatment:

Group	Treatment	Dose	*n*
I	Conjugated equine estrogens, low dose	10.4 μg/kg	5
II	Tofupill, low dose	3.4 mg/kg	5
III	Vehicle, low dose	1.0 mL	6
IV	Conjugated equine estrogens, high dose	31.2 μg/kg	6
V	Tofupill, high dose	10.2 mg/kg	6
VI	Vehicle, high dose	1.5 mL	5
VII	17β-Estradiol	40.0 μg/kg	4

Low dose was calculated on the body weight basis as three times the dose recommended to postmenopausal women, whereas high dose was calculated as three times the low dose. Conjugated equine estrogens (CEE) used were the commercial presentation of commonly used estrogen replacement therapy in postmenopausal women and served as positive control. The groups III and VI that received only the vehicle were included as negative controls for the low- and high-dose treatments, respectively. The group VII was included as a positive control for the serotonin-induced contractile response.

Drugs were prepared from the commercial presentation of conjugated equine estrogens (Premarin 0.625 mg, Wyeth, Mexico) or the food supplement (Tofupill MR, Suave y Fácil, Mexico); tablet or capsule content was pulverized and suspended in the necessary volume of methyl cellulose 0.5%, to administer the corresponding dosage in a maximum volume of 1.0 mL/day (low dose) or 1.5 mL/day (high dose) to each rat. During 14 days, CEE, phytoestrogens, or vehicle were orally administered to rats through a silastic gastric probe every 24 h. Estradiol 17β (10 μg/day/0.1 mL) was prepared in corn oil and injected subcutaneously 48 and 24 h prior experiments.

2.3. Estrogenic activity

Uterine weight. Before performing contractile activity assays, the dissected uterus was placed on a filter paper to eliminate liquid excess and to record wet weight. At the end of contractile assays, tested segments and remainder of the uter-

ine tissue from each rat was dried in a laboratory oven and dry weight was recorded.

Vaginal epithelium. Daily vaginal smears were taken to observe the estrogenic effect on vaginal epithelium. Vaginal smears were observed fresh and were subsequently fixed in absolute ethanol and stained with hematoxilin-eosin.

In ovariectomized rats, the vaginal smear showed an atrophic pattern consisting of leukocytes, mucus and scarce nucleated epithelial cells. When estrogens were administered to ovariectomized rats, the vaginal smear consisted mainly of exfoliated cornified cells [20].

2.4. Uterine smooth muscle sensitivity to serotonin

The day after the last day of treatment, vaginal smear was recorded, the rat was killed by decapitation, and the uterus was dissected. Rings approximately 5–7 mm long were dissected from cervical segment of each uterine horn. Uterine cervical segments were placed longitudinally in a 5 mL organ bath containing Krebs–Ringer-bicarbonate (KRB) solution with the following composition (mM): NaCl, 120; KCl, 4.6; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.5; $NaHCO_3$, 20; and glucose, 11. KRB solution pH 7.4 was maintained at 37 °C and gassed continuously with a mixture of 95% O_2–5% CO_2. Each uterine segment was placed under optimum resting force of 1 g and allowed to equilibrate for 1 h before exposure to drugs; during this equilibration period tissues were washed with fresh KRB every 10 min. After equilibration, uterine rings were bathed in a depolarizing solution (60 mM KCl), prepared by equimolar substitution of NaCl for KCl. This solution produces uterine smooth muscle contractile response that was considered a maximal standard response. Contractile responses were recorded isometrically with a FT03 Grass tension transducer connected to a Grass model 7B polygraph. After two consecutive similar responses to 60 mM KCl, uterine segments were exposed to different concentrations of serotonin in a non-cumulative manner (10^{-8} to 10^{-4} M). Each serotonin concentration remained in the tissue bath for 10 min.

2.5. Analysis of data

Differences in uterine weight between treatments were evaluated with one-way analysis of variance (ANOVA) followed by Bonferroni test. $P < 0.05$ was considered statistically significant. Values reported are means ± standard error (S.E.M.).

3. Results

3.1. Effects on uterine weight

Wet weight of uterus from rats treated with either low or high-dose CEE was higher than that from Tofupill-treated ($P < 0.05$ and 0.01, respectively) or non-treated ovariectomized controls ($P < 0.01$) (Fig. 1). Only uterus from the CEE-treated rats showed the classical hyperemic appearance due to estrogenic stimulation.

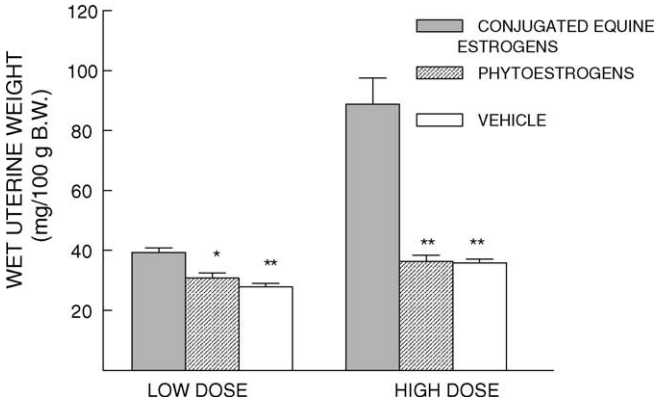

Fig. 1. Wet uterine weight of uterus from ovariectomized rats treated per os with conjugated equine estrogens: low dose = 10.4 μg/kg, high dose = 31.2 μg/kg; phytoestrogens: low dose = 3.4 mg/kg, high dose = 10.2 mg/kg; or vehicle: low dose = 1 mL, high dose = 1.5 mL. Bars represent mean of 5–6 animals, T-lines represent S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with conjugated equine estrogens.

Similar values of dry weight were recorded for all of the low-dose treatments. On the contrary, dry weight of high-dose CEE-treated rats was higher than those of either high-dose Tofupill-treated rats ($P < 0.01$) or non-treated ovariectomized controls ($P < 0.05$) (Fig. 2). Also, the uterine weight (wet and dry) from Tofupill-treated rats was similar to that of the ovariectomized controls (Figs. 1 and 2).

3.2. Effects on vaginal epithelium

Vaginal epithelium from rats treated with low doses of either CEE or Tofupill was similar to that of non-treated ovariectomized controls. In the case of high-dose treatments, only CEE-induced keratinization of vaginal epithelium as

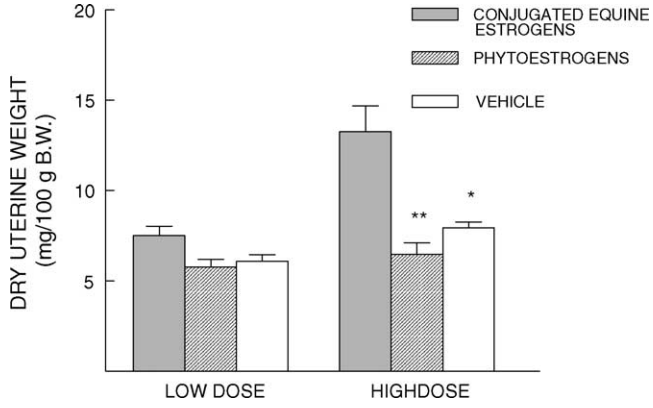

Fig. 2. Dry uterine weight of uterus from rats treated p.o. with conjugated equine estrogens: low dose = 10.4 μg/kg, high dose = 31.2 μg/kg; phytoestrogens: low dose = 3.4 mg/kg, high dose = 10.2 mg/kg; or vehicle: low dose = 1 mL, high dose = 1.5 mL. Bars represent mean of 5–6 animals, T-lines represent S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared with conjugated equine estrogens.

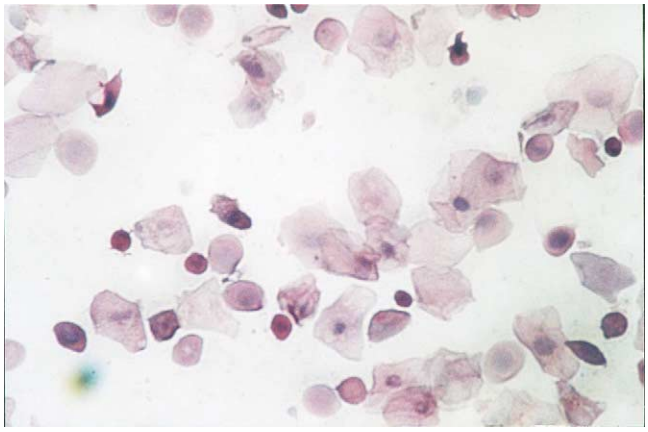

Fig. 3. Vaginal smear from high-dose conjugated equine estrogen-treated rat, cornified and nucleated epithelial cells are observed (hematoxilin-eosin, 40×).

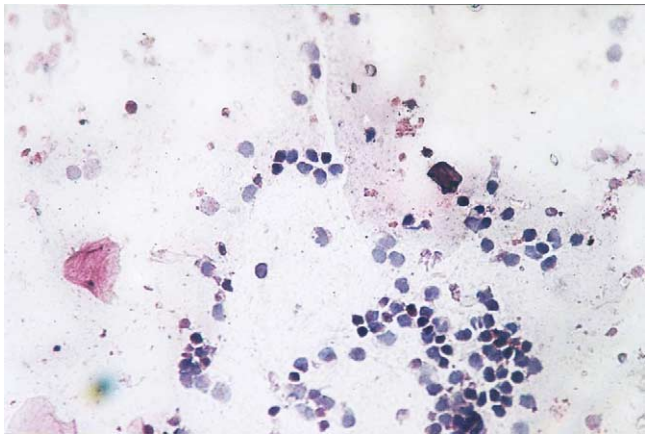

Fig. 4. Vaginal smear from high-dose phytoestrogen treated rat. Small, nucleated cells are observed (hematoxilin-eosin, 40×).

evidenced by abundance of cornified cells and presence of some epithelial nucleated cells (Fig. 3). Vaginal epithelium of Tofupill-treated rats displayed presence of leukocytes, mucus, and small-nucleated cells (Fig. 4). On the other hand,

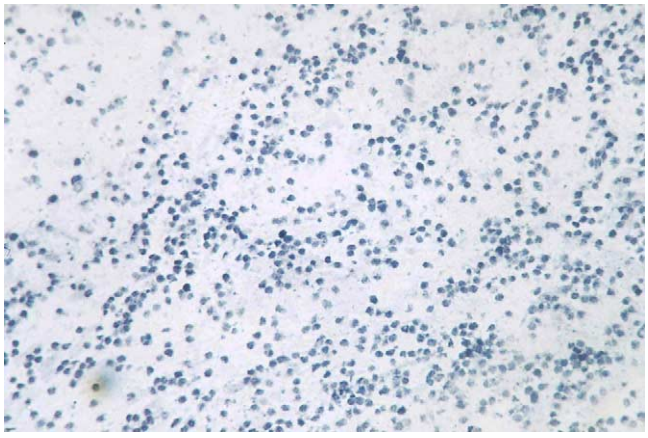

Fig. 5. Vaginal smear from ovariectomized, non-treated rat. A great number of leukocytes are observed (hematoxilin-eosin, 40×).

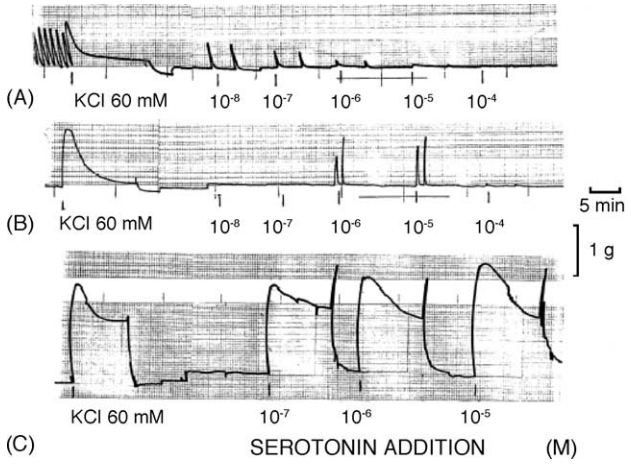

Fig. 6. Poligraphic traces of contractile activity of uterine segments from ovariectomized rats treated with high-dose Tofupill (A), high-dose conjugated equine estrogens (B) or 17β-estradiol, 10 μg/rat, 48 and 24 h prior sacrifice (C).

only leukocytes were observed in vaginal epithelium of control rats (Fig. 5).

3.3. Contractile activity assays

In uterus from rats treated with either conjugated estrogens or Tofupill, or in vehicle-treated ovariectomized controls (data not shown), serotonin did not induce contractile activity. Only rats treated with 17β-estradiol displayed a clear serotonin-induced contractile response (Fig. 6).

4. Discussion

The present study showed that in our model treatment with Tofupill did not display either uterotrophic effect or uterine smooth muscle sensitization to serotonin, but it induced the presence of small-nucleated cells in the vaginal epithelium. This is not a clear estrogenic action but may be indicative of either early or mild estrogenic stimulation. Taken together, these findings suggest that phytoestrogens in Tofupill lack clear peripheral estrogenic actions on female reproductive tract of ovariectomized rats. However, the present data do not rule out the possibility that phytoestrogens contained in Tofupill might control hot flashes in postmenopausal women.

In contrast with present findings, a food supplement of phytoestrogens induced total maturation of vaginal epithelium in postmenopausal women [6]. However, in postmenopausal macaques soybean estrogens administered during 6 months in the diet did not display estrogenic effect on vaginal cytology [13]. These discordances might be due to species differences. In support of this notion, previous studies have reported eight residue differences in the deduced amino acid sequence of hormone-binding domains in comparing human to rodent estrogen receptors [21,22];

therefore, estrogenicity assays performed in laboratory rodents might not invariably reflect the activity of the same compounds on human estrogen receptors.

The different estrogenic potency of phytoestrogens in relation to estrogens should be considered. Flavonoids are 10^3 to 10^4-fold less potent agonists than 17β-estradiol, and micromolar concentrations are required to generate estrogenic activity [2]. In the present study, we focused on estrogenic actions of Tofupill as indicated for hormone replacement therapy; thus, low doses were adjusted from those recommended for postmenopausal women.

Regarding duration of treatment, in the present study daily oral doses were administered during 14 days, which was longer than the time of administration in other studies. Whitten et al. observed that a 4 day oral treatment with coumestrol augmented uterine growth response to estradiol in immature rats [11,23]. In premenopausal women, soy protein given daily during 30 days (one menstrual cycle) was sufficient to exert effects on the menstrual cycle [4]. Rat estrous cycle lasts 4 days; thus, the treatment schedule used in the present study was equivalent to more than three estrous cycles, which seems adequate in view of the studies previously mentioned.

Other studies are in accord with present findings, for instance: soybean estrogens did not demonstrate estrogenic activity on uterine weight of rats in a 2 month treatment schedule [24]. Similarly, soybean estrogens administered during 3 years did not exert estrogenic actions on vaginal epithelium of ovariectomized macaques [13].

Nevertheless, it is noteworthy that Tofupill lacks estrogenic effects on the female reproductive tract, whereas it has been reported that it diminishes hot flashes in postmenopausal women [5–8]. A possible explanation could be that phytoestrogens contained in Tofupill acted as selective estrogen receptor modulators (SERM) [25], and could diminish hot flashes without exerting any effect on uterus or vaginal epithelium. Studies on the binding of Tofupill phytoestrogens to estrogen receptors (α and β) and the participation of transcriptional systems are required to elucidate this notion, and will be the subject of future studies.

On the other hand, a surprising finding was the lack of effect of CEE on the uterus sensitivity to serotonin. CEE is a combination of water-soluble sodium salts of sulfated esters of several estrogens that may be significantly less potent than 17β-estradiol itself. Thus, the relative potency of CEE as compared with 17β-estradiol as well as route of administration might explain this feature. The present results also suggest that both uterotrophic effect and keratinization of vaginal epithelium are more sensitive indicators of estrogenic effects than the uterine smooth muscle sensitivity to serotonin in rats.

The present data showed that Tofupill administered orally did not induce clear estrogenic effects on uterine weight, vaginal epithelium or serotonin-induced contractile activity of rat uterus. Because of the limitations of this descriptive approach, additional studies are needed to evaluate the risk associated to the use of Tofupill as an alternative for hormone replacement therapy for postmenopausal women.

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