Clinical Pharmacology & Biopharmaceutics

Helmy et al., Clinic Pharmacol Biopharmaceut 2014, S2
DOI: 10.4172/2167-065X.S2-001

Research Article Open Access

Estrogenic Effect of Soy Phytoestrogens on the Uterus of Ovariectomized Female Rats

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Abstract

Objectives: Phytoestrogens exert variety of actions involving many target tissues. The effects of dietary phytoestrogens, as hormonal replacement therapy (HRT), on levels estradiol receptor alpha (ERα), estradiol receptor beta (ERβ) and vascular endothelial growth factor (VEGF) in uterine tissues of ovariectomized female rats were studied.

Methods: Twenty mature ovariectomized female albino rats weighed (130-150 gm) were divided into two groups (G1) control group (n=10) fed on a casein based ration (phytoestrogens free) and (G2) treated group (n=10) received high phytoestrogens diet. Daily food intake (FI) and body weight gain (BWG) were recorded. After 30 days of treatment all rats were sacrificed and relative uterine weight for each female was recorded. Histological and immunohistochemical studies for ERα, ERβ and VEGF expression were performed on uteri for both groups.

Results: Dietary phytoestrogens significantly (P<0.01) decreased daily FI (18.91 ± 0.47 g versus 22.60 ± 0.50 g and BWG (1 ± 0.07 g versus 1.46 ± 0.10 g). Phytoestrogens-fed group revealed highly significant (P<0.01) increase in the relative uterine weight than control with value (0.48 ± 0.01 g and 0.35 ± 0.01 g, respectively). Hyperplasia in the luminal epithelium and glandular acini, connective tissue edema, as well as newly formed blood vessels was observed in G2. Dietary phytoestrogens significantly (P<0.05) increased the expression of uterine ERβ and VEGF in G2 than in G1 while uterine ERα expression showed non-significant changes.

Conclusions: The current results suggest that dietary phytoestrogens induce a proliferative effect in ovariectomized rats' uteri which is mediated by estradiol receptors expression especially ERβ. Also dietary phytoestrogens up regulate VEGF expression that seems to be accompanied by the changes in estrogen receptors expression, and stimulate angiogenesis and hyper permeability in blood vessels that makes soy phytoestrogens may be used as a natural HRT in case of reduction of endogenous estrogens especially after ovariectomy or during ovarian hormonal dysfunction. However the usage of these compounds should be concerned as it may predispose uterine neoplasia.

Keywords: Phytoestrogens; Uterus; Histology; Immunohistochemistry

Introduction

Estrogen plays an important role in growth, differentiation and function of many target tissues, including tissues of the female and male reproductive system [1]. Obstetricians and gynecologists recognized the fundamental importance of estrogen in the pathogenesis of multiple disorders of female reproductive tract, including endometriosis, endometrial cancer and pelvic floor dysfunction. Estrogen was believed to act through 2 estrogen receptor species alpha and beta (ERα and ERβ) [2]. These two receptors species may interact by forming homodimers and heterodimers to alter tissue response to estrogens and selective esrtrogen receptor modulators (SERMs) [3,4] followed by activation of target gene transcription [5].

Phytoestrogens are plant based estrogenic compounds, which are considered natural SERMs due to their ability to induce agonistic and antagonistic effects they are increasingly being researched for management of hormonal and reproductive pathologies [6,7]. Phytoestrogens vary considerably in terms of structure, estrogenic potency, and availability in common food sources such as soybeans, cereals, and sprouts [7]. The three most common types of phytoestrogens produced by plants are isoflavones, lignans, and coumestans. The major isoflavone phytoestrogens are genistein and daidzein [8,9]. The importance of genistein and daidzein is aroused from that they are present in virtually all natural-ingredient rodent diets that use soy as a source of protein [7]. Genistein and many of phytoestrogens have been shown to bind more strongly to ERβ than ERα [6,10-13].

There has been great interest in the potential beneficial and adverse effects of isoflavones from soy, as demonstrated by the thousands of scientific publications on this subject in the past decade. Most phytoestrogens are abundant in soy products, which are used as the major protein source in all natural- ingredients, commercially available, rodent diets (that range from approximately 200 to 600 µg/g). Therefore, animals ingesting these diets are continually exposed to endocrine active compounds [14,15].

Ovaries gradually produce estrogen in the period up to menopause, then blood level of estrogen declines after menopause [16] which can cause distressing symptoms with increased incidence of osteoporosis, cardiovascular disease [17]. Isoflavones are being increasingly used as

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Received December 26, 2013; **Accepted** February 10, 2014; **Published** February 12, 2014

Citation: Helmy SA, Emarah HA, Abdelrazek HMA (2014) Estrogenic Effect of Soy Phytoestrogens on the Uterus of Ovariectomized Female Rats. Clinic Pharmacol Biopharmaceut S2: 001. doi:10.4172/2167-065X.S2-001

an alternative or complement of hormonal replacement therapy (HRT) in postmenopausal women [18,19] especially in cases of long-term administration [18]. The effect of isoflavones depends on the level of endogenous estradiol, since isoflavones and estradiol are competing for their binding on ERs. In a state of high levels of endogenous estrogens as in the follicular phase of the estrous cycle, isoflavones may obstruct full estrogen activity by occupying a part of the ERs. On the other hand, in a state with low levels of endogenous estrogens as after ovariectomy or menopause, the estrogen activity of isoflavones may become manifest [20-22].

Responses of the reproductive tract of ovariectomized rodents, which include changes in gene expression, cellular hypertrophy and DNA synthesis, and vascular changes, have been used extensively to evaluate test compounds for estrogenic activity. Regulation of vascular permeability and blood vessel growth in mammalian female reproductive tract are associated with changes in gene expression of several angiogenic factors, including vascular endothelial growth factor (VEGF) [23] or referred as vascular permeability factor (VPF) or VEGF/VPF [24], VEGF expression is induced by estrogen in the uterus of many species, including humans [25,26]. The peak induction of VEGF expression in the rat uterus occurs within 1 to 3 hr after treatment with female sex steroid hormones [27-29]. So far expression of VEGF with relation to ER was not fully described, and the available data on the possible estrogenic effect of dietary phytoestrogens and regulation of expression of uterine VEGF is rather scarce. Therefore this study was undertaken to provide an insight about the effect of dietary phytoestrogens as natural estrogen replacer on the expression of uterine of ERα, ERβ and VEGF of ovariectomized female albino rats.

Materials and Methods

Animals

Mature (12–13 week-old) Albino female rats weighing 130-150 g were obtained from National Research Center, Dokki, Giza, Egypt. Rats were allowed to acclimate for one week prior to the initiation of experiment. They were maintained at control diet and tap water that were allowed *ad libitum*.

Ovariectomy

Animals were ovariectomized (OVX) 1 week after arrival according to Lasota and Danowska-Klonowska [30] under effect of diethyl ether inhalation anesthesia. Rats were given amoxicillin 10 mg/kg orally for 3 successive days after ovariectomy, given control diet & water *ad libitum* and maintained on natural day light cycle. All animal studies were performed under Animal Care and Use protocols procedures approved by Faculty of Veterinary Medicine, Suez Canal University Committee.

Experimental design and sampling

Three weeks after ovariectomy, the ovariectomized female rats 130-150 g were divided randomly into two groups: Group I (G1), control group, n=10, they were fed on a casein based diet and Group II (G2), received high phytoestrogens diet, n = 10. All diets were formulated to fulfill all the nutritional requirements of adult rat (Table 1) according to NRC [31] and were offered for 30 days.

Daily food intake and body weight gain were recorded. At the end of experiment the overiectomized females were weighed then sacrificed under the effect of light diethyl ether anesthesia. Uteri were, removed after dissection from fat and weighed. The relative uterine weight (RUW) was obtained as follow: RUW= (uterine weight/ body weight) × 100.

Ingredients	Control (G1) %	High Pytoestrogen (G2) %
Yellow Corn	40.59	35.04
Corn Gluten	15.00	-
Soybean*	-	26.41
Casein	5.00	5.00
Sucrose	22.43	22.32
Starch	7.63	4.16
Cellulose	1.30	0.17
Corn Oil	5.00	-
Soybean Oil	-	5.00
Ground Limestone	1.02	1.04
Dicalcium Phosphate	0.34	-
Common Salt	0.13	0.13
Premix	0.30	0.30
Methionine	0.30	0.43
Lysine	0.26	-
Tryptophan	0.70	-
Total	100.00	100.00

*Soybean was autoclaved at 110°C for 30 minutes according to [33] to inactivate trypsin inhibitor, tannins, saponins, phytate, protease inhibitors, lectins and goitrogens

Table 1: Composition of experimental diets for control group (G1) and high phytoestrogens group (G2).

Histology

Uteri were kept in 10% neutral buffer formalin for 24 hours for fixation. Then dehydrated in a serial of ascending gradient of ethyl alcohol (70%, 80%, 95%, and 100%). The samples were finally embedded in paraffin wax. Serial sections of 5 µm were stained with hematoxylin and eosin then examined by microscopy [32].

Immunohistochemistry

The paraffin embedded uteri, fixed in formalin saline 10%, were cut into 5 µm sections and mounted on positively charged slides for ERα, ERβ and VEGF immunohistochemistry. Sections were dewaxed, rehydrated and autoclaved at 120°C for 10 minutes in 10 Mm citrate buffer (pH 6) for ERα and ERβ and 1 Mm EDTA for VEGF**.** After washing with PBS endogenous peroxidase was blocked using 0.3% hydrogen peroxide in methanol (15 minutes). Slides were washed in PBS again and blocking was performed by adding blocking buffer and incubated for 30 minutes at room temperature. Primary antibody for ERα, (Cat. No. MS-750-R7, Thermo Scientific Co., UK), ERβ (Cat. No. RB- 10658-R7, Thermo Scientific Co., UK) and VEGF (catalog MA1-16629. Thermo Scientific Co., UK) was added after dilution by PBS (1:100, 1:10 and 1:50 respectively) and incubated for 30 minutes. The slides were washed three times for 3 minutes each with PBS. Biotinylated polyvalent secondary antibody (Cat. No. 32230, Thermo Scientific Co., UK) was applied to tissue sections and co-incubated for 30 minutes. The slides were washed three times for 3 minutes each with wash buffer. The reaction was visualized by adding Metal Enhanced DAB Substrate Working Solution to the tissue and incubated 10 minutes. The slides were washed two times for 3 minutes each with wash buffer. Counterstaining was performed by adding adequate amount of hematoxylin stain to the slide to cover the entire tissue surface [34]. For quantitative analysis, the intensity of immunoreactive parts was used as a criterion of cellular activity after substracting background noise. Measurement was done using an image analyzer (Image J program). From each slide of both experimental groups, 9 fields were randomly selected. The total field and immunohistochemial (IHC) stained areas were calculated then the %IHC stained area calculated as follow:

%IHC stained area = (IHC stained area)/(Total area) × 100%.

Statistical analysis

All data in the present study were expressed as mean ± SE. they were subjected to student T test using SPSS® software (Statistical Package for Social science, version 17.01, IIIinois, USA). The probability criterion for significance was P> 0.05 and P<0.01 for high significance.

Results

Food intake, body weight and relative uterine weight

The results obtained from the current study and presented in Table 2 revealed that, the high dietary phytoestrogens group (G2) showed highly significant (P<0.01) reduction in daily FI (18.91 ± 0.47 g versus 22.60 ± 0.50 g in G1) and BWG (1 ± 0.07 g versus 1.46 ± 0.10 g in G1). Phytoestrogens-fed group revealed highly significant (P<0.01) increase in the relative uterine weight with value 0.48 ± 0.01 g than control 0.35 ± 0.01 g.

Histological findings

The histological examination of uteri of both groups revealed normal thickness and normal tissue architecture in all layer of the uterus in G1 (Figures 1a and 1b) while there was an increase in the thickness of the uterine wall and the uterine lumen become more branched in high dietary phytoestrogens group (Figure 1c). The thickness in G2 increased due to hyperplasia in the lining and the glandular epithelium, connective tissue edema and increase the blood supply (Figures 1d, 1e & 1f). Newly formed blood vessels were observed in G2 (Figure 1f).

Immunohistochemistry

Phytoestrogens fed group (G2) showed non-significant increase in ERα expression when compared with control group (Figure 2). The lining and the glandular epithelium (Figure 3c) showed low intensity of ERα expression in (G2), while the myometrium and the surface epithelium (Figure 3d) showed slightly increase but no significant changes in the intensity of ERα expression where observed when compared with control group (Figures 3a and 3b).

The expression levels of uterine ERβ and VEGF were significantly (P<0.05) increased in G2 than in G1 (Figure 4,5). The expression of ERβ was significantly increased in G2. The intensity varied from low in the lining and the glandular epithelium (Figure 3g) and high in the myometrium and surface epithelium (Figure 3h) than G1 (Figure 3e,f).

Expression of VEGF was significantly increased in G2 especially in the myometrium, perimetrium and around the blood vessels (Figure 3j) than that of G1 (Figure 3i).

Discussion

The current study was conducted to evaluate the effect of dietary soy phytoestrogens as HRT on uterine weight and uterine gene expression of ERα, ERβ and VEGF in ovariectomized female albino rats.

Item	*Control (G1)*	*High phytoestrogens (G2)*
Food intake (FI)/g	22.596 ± 0.50	18.909 ± 0.47**
Body weight gain (BWG)/g	1.46 ± 0.1	1.00 ± 0.07**
Relative uterine weight (RUW)/g	0.35 ± 0.01	0.48 ± 0.01**

**Means significantly high at P ≤ 0.01

Table 2: The effect of dietary phytoestrogens on food intake/ g, body weight gain/g and relative uterine weight/g for control and high phytoestrogens group.

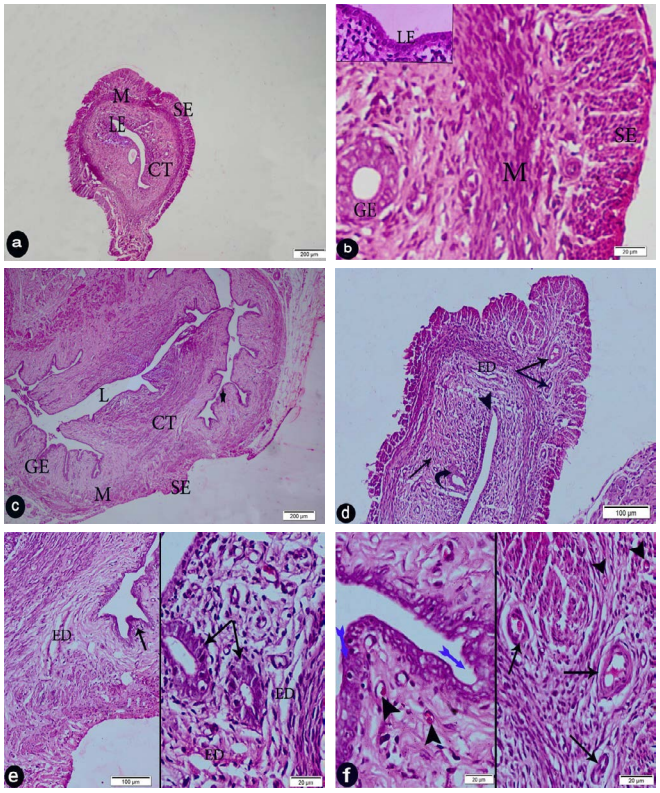

Figure 1: Photomicrographs of sections in uterus of ovariectomized female albino rats represent Figure a,b: section in control rat showing normal lining epithelium (LE), glandular epithelium (GE), connective tissue (CT), tunica muscularis (M) and surface epithelium (SE). Figure c: section in phytoesterogens treated rats showing branched lumen (L), lining epithelium (star), glandular epithelium (GE), tunica muscularis (M) and surface epithelium (SE). Figure d: section in phytoesterogens treated rats showing hyperplasia in the lining epithelium (head arrow), and the glandular epithelium (curved arrow), connective tissue edema (ED) and increase the blood supply (straight arrow). Figure e: section in phytoesterogens treated rats showing hyperplasia in the glandular epithelium (arrow) and connective tissue edema (ED). Figure f: section in phytoesterogens treated rats showing hyperplasia in the lining & glandular epithelium (blue arrow), increase the blood supply (black arrow) and newly formed blood vessels (head arrow). (All the figures H&E stain).

The study revealed that the ovariectomized female rats fed high dietary soy phytoestrogens showed decrease in the BWG and FI with higher significance (p<0.01) than control. These results are in agreement with those reported by [35-43]. While they disagreed with those of [44,45]. Reduction in food intake may be due to the appetite repressing action of estrogen [46] as dietary phytostrogens decrease food intake and hence decrease body weight. Also the increased locomotors activity observed in the current study in G2 which may be due to preferential use of lipids as fuel source [42,47]. The decrease implies that the estrogenic hormone action of phytoestrogens is beneficial to body fat regulation and influences hypothalamic neuropeptide Y (NPY) levels which regulates feeding behavior [35,39].

The increased uterine weight after isoflavones administration has been well documented and is dose dependent [48-50]. In the current experiment the increased RUW in G2, supports the uterotrophic effect of phytoestrogens that has been confirmed in a variety of animal species [51-55]. This may support the concept that the isoflavones contained in the tested diet is capable of promoting estrogenic activity and reversing the effect of ovariectomy.

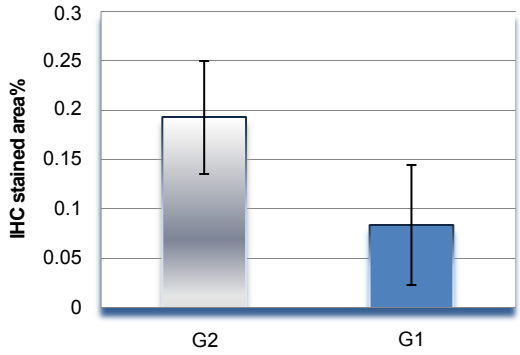

Figure 2: Effect of dietary soy phytoestrogens on IHC stained area % of ERα expression in ovariectomized female albino rats.

Estrogen action is exerted in target tissues via binding to one of the two estradiol receptors (ERα or ERβ) each of which is encoded by unique genes. Estradiol receptors act as dimers to regulate transcriptional activation [56]. Receptor levels and dynamics influence target tissue responsiveness to steroid hormones and other estrogenic compounds; thus, there are great interest in understanding how the estrogen receptor is regulated in both genomic and non-genomic estrogen-responsive tissues. Both estradiol receptors subtypes act differently in the uterine tissue, where ERα is necessary for maturation, paracrine and autocrine mitotic activation and function of uterine tissues. ERα knockout mice showed premature uterine appearance manifested by reduced number of endometrial glands and altered organization of the stromal, myometrial and epithelial layers [57], as well as, these females appeared unfertile [58]. In contrast, ERβ is thus apparently required for normal development of the female reproductive tract where, the ERβ knockout mice uteri are indistinguishable, and show normal organization and development of the stromal, myometrial and epithelial layers, as well as glandular structures [57] and they are either infertile or exhibit variable degrees of subfertility [58]. The current study demonstrates that the given dose of soybeans phytoestrogens does not alter the expression level of ERα mRNA level in ovariectomized rats' uterus while ERβ mRNA expression showed a significant $P<0.05$ increase in phytoestrogens fed rats (G2) than control one (G1). These findings implied that modification of isoflavones on estrogen receptor mRNA expression are able to elicit an estrogenic response in the uterus of ovariectomized rats which confirmed by presence of ERβ immunostaining characteristic of estrogen exposure in estrogen responsive- target organs (uterus). The presence of ERβ may play a role in modulating and regulation of the effect of ERα [59]. Uterine weight gain, increase the height of luminal epithelium, uterine edema, hyperplasia of luminal epithelium and glandular aceni in addition to increase in the blood supply of the uterus all suggesting that soy phytoestrogens act in the uterus in a manner similar to that of estradiol, that is, may be through binding to the ER, and the ligand-receptor complex that induce the expression of estrogen-responsive genes which ultimately result in increased uterine mass. These results agree with Francisco et al. [60]. Immunohistochemical analysis of estradiol receptors indicates the preferential affinity of genestein for ERβ [20,61]. The estrogenicity of isoflavones can be existed by the molecular similarity of them with estradiol and their transcriptional properties via estradiol receptor [52,62-65]. The results indicating non-significant change in ERα, in this study, with evidence of hyperplesia of luminal epithelium and glandular aceni could be explained by the down regulating effect of phytoestrogens on endogenous estrogen (adrenal) level [66-69], where, endogenous estrogen is more selective to ERα than phytoestrogens. Moreover, the uterotropic effect mediated by ERα may be due to contribution of other endogenous factors rather than ERα as well as, modulating effect of ERβ on ERα [59].

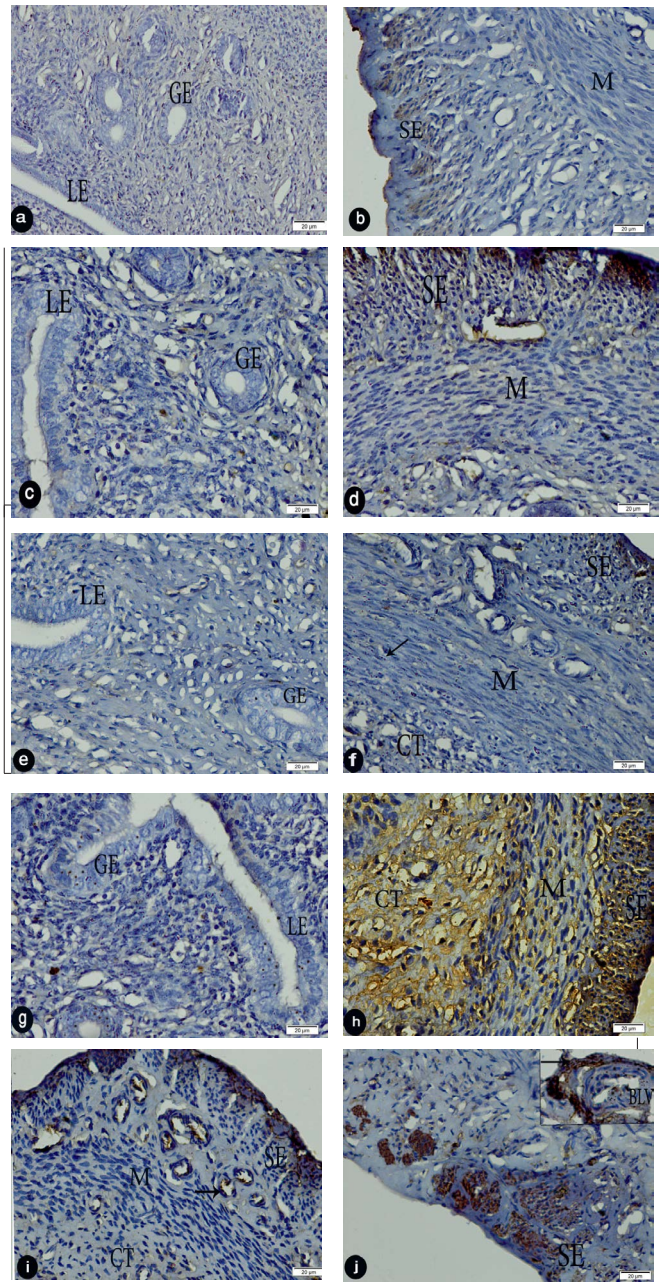

Figure 3: A Photomicrograph in uterus of ovariectomized female albino rats represent: c,d: sections in phytoesterogens treated rats showing the intensity of ERα expression in the lining epithelium (LE) glandular epithelium (GE), tunica muscularis (M) and surface epithelium (SE) compared to control Figure a,b. Figures g,h: section in phytoesterogens treated rats showing the intensity of ERβ expression in the lining epithelium (LE), glandular epithelium (GE), connective tissue (CT), tunica muscularis (M) and surface epithelium (SE) compared to control Figures e,f. Figure i: section in phytoesterogens treated rats showing increase the intensity of VEGF expression around the blood vessels (BLV) and surface epithelium (SE) compared to control Figure j.

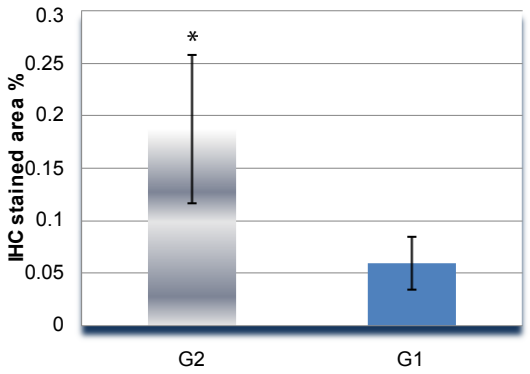

Figure 4: Effect of dietary soy phytoestrogens on IHC stained area % of ERβ expression in ovariectomized female albino rats.

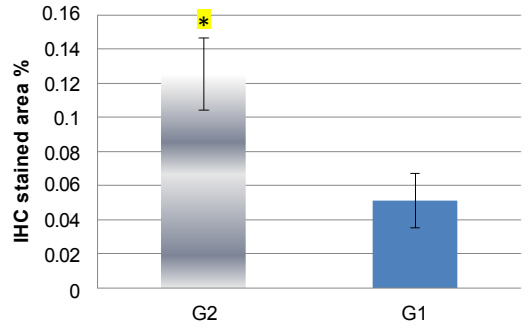

Figure 5: Effect of dietary soy phytoestrogens on IHC stained area % of VEGF expression in ovariectomized female albino rats.

The present study demonstrates that the expression of VEGF, an endothelial cell-specific mitogen and permeability factor, in myometrium, perimetrium and perivascular area was increased significantly ($P<0.05$) in soy phytoestrogens fed group (G2) than control (G1). This effect was associated with an increase in uterine vasculature with presence of newly formed blood vessels in G2. These findings agree with previous results of Ikeda et al. [70] and Mosquette et al. [71] who investigated the positive effect of phytoestrogens on VEGF and uterine vascularity in rat uterus. Previous study of Bausero et al. [25] demonstrated the role of VEGF *in vivo* angiogenesis and microvascular hyperpermeability within the uterus by a paracrine action may explain the presence of edema that observed in the current study. Also they added that expression of VEGF could be related to the changes in estrogen receptors concentration. So the proliferative and vascular effect of phytoestrogens on the uterus could be attributed to changes in expression of estrogen receptors and VEGF in ovariectomized female rats. VEGF is expressed in tissues with rapid vascular endothelial turnover such as ovary, uterus and placenta [72,73], and tumors [25]. The expression of VEGF was induced by estrogen, which demonstrated estrogen- responsive element sequences in the transcription regulatory domain in the VEGF gene [72]. It was known that some uterine endometrial cancers are estrogen-dependent in growth [74]. Therefore, VEGF might contribute to growth in some estrogen dependent uterine endometrial cancers [75].

The hyperexpression of VEGF could be involved in pathological situations, abnormal hyperpermeability and dilated capillaries and increase risk of uterine cancer [25]. This suggests that dietary phytoestrogens could predispose uterine neoplasia, so further studies should be carried out to clarify this point.

Conclusion

The current study demonstrates the effect of dietary phytoestrogens on ovariectomized rats uterus and their proliferative effect which is mediated by estradiol receptors expression. Also dietary phytoestrogens up regulate VEGF expression, that seems to follow the changes in estrogen receptors expression, and stimulate angiogenesis and hyper permeability in blood vessels that makes soy phytoestrogens may be used as a natural HRT in case of low levels of endogenous estrogens especially after ovariectomy or during ovarian hormonal dysfunction. But the use of these compounds should be concerned as they may predispose uterine neoplasia.

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