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Original Article

Effect of soy isoflavones on implantation losses in Wistar rat: implication of progesterone receptors, vascular endothelial growth factor and estradiol receptors alpha

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Abstract

Background: Implantation is a crucial period determining the success of a full pregnancy. Endocrine disruptors such as phytoestrogens (PEs) were thought to adversely influence embryonic implantations. However, the mechanism by which they upset implantation was not fully elucidated. **Aims:** The effect of administering soy isoflavones on the implantation of Wistar rats was studied through the detection of progesterone receptors (PR), vascular endothelial growth factor (VEGF), and estradiol receptor alpha (ER-α) protein expression at gestation day 6 (GD6). **Methods:** Eighteen cyclic female Wistar rats were distributed into two groups, group A: control (n=9) were fed with a casein based diet, and group B (n=9) were fed with a casein diet and gavaged 50 mg/kg/day soy isoflavones' extract 40% starting from gestation day zero (GD0) to GD6. Feed intake, body weight (BW), body gain, and uterine weights were recorded. At the end of GD6 the number of corpora lutae (CLs) and implantation rates were recorded. Histopathology and immunohistochemistry (IHC) for PR, VEGF, and ER-α protein expression in implanted uteri were performed. **Results:** Soy isoflavones significantly reduced feed intake, weight gain, uterine weights CL numbers, and implantation rates of the treated pregnant dams. The endometrium of the soy treated dams showed less proliferation than that of the control. Immunostaining percentage of PR and VEGF proteins significantly reduced in soy treated dams compared to the control. However, the mean expression percentage of ER-α exhibited significant elevation in the soy treated dams in comparison to the control group. **Conclusion:** Implantation losses caused by soy isoflavones seemed to be due to the down regulation of PR that failed to down regulate ER-α action and decreased VEGF production.

Key words: ER-α, Implantation, Isoflavones, Progesterone, VEGF

Introduction

Implantation is a pivotal stage in the stabilization of a successful full term pregnancy (Oludare and Iranloye, 2016). It assimilates the most decisive step of the reproduction process in numerous species (Achache and Revel, 2006). High pregnancy rates are the main target of farmers looking to maximize the reproduction of their herds (Norwitz *et al*., 2001; Borini *et al*., 2006).

In rats, implantation occurs within approximately 24 h between 4th and 5th day of pregnancy (Spósito *et al*., 2011). Many predisposing factors influence the effectiveness of implantation including sex steroids, prostaglandins, cytokines, nitric oxide, and definite anti-inflammatory drugs (Kodaman *et al*., 2004; Oludare and Iranloye, 2016). Progesterone and estradiol are the main sex steroidal hormones that interact for the success of implantation in animals and humans alike (Kodaman *et al*., 2004). Moreover, vascular endothelial growth factor (VEGF) expression plays a dramatic role in increasing vascular endometrial permeability and cell proliferation at the implantaion site (Rabbani and Rogers, 2001).

Phytoestrogens (PEs) are non-steroidal molecules of plant origin (Amal *et al*., 2014). They are categorized into isoflavones, coumestans, flavonoids, and lignans and are all considered food supplements for humans and animals (Meena and Sreenivasula Reddy, 2014). They are also considered as endocrine disruptors with estrogen like activity. Therefore, they alter animals' reproductive physiology by binding to alpha and beta estrogenic receptors (Ebaid *et al*., 2016). The consumption of phytoestrogenic plants in humans has been found to exert a variety of adverse effects in woman including infertility and increased incidence of polycystic ovary syndrome (Amini *et al*., 2015).

Isoflavones also interfere with rodent sexual development (Casanova *et al*., 1999) causing infertility (Jefferson *et al*., 2005, 2006), behavioral disorders, and increases in the volume of the sexually dimorphic nuclei in female rats (Branham *et al*., 2002). Isoflavones have also been found to antagonize the progress of pregnancy in sheep (Morley *et al*., 1966), cows (Kallela *et al*.,

1984), and women (Chandrareddy *et al*., 2008), due to their adverse effects on the hormones as well as the receptors responsible for successful implantation (Amal *et al*., 2014).

Previous studies have demonstrated controversial results concerning the effect of PEs on preimplantation physiological events. Some of them have shown that PEs have favorable effects on implantation regulatory genes such as transforming growth factor beta 1, Homeobox A10, glycodelin-A, integrin αV, leukemia inhibitory factor and milk fat globule epidermal growth factor 8 expression (Suzuki *et al*., 2017). However, other studies suggested that PEs were associated with perimplantation embryonic losses through the alteration of transcription factors such as octamer-binding transcription factor 4 (Oct-4) and caudal-related homeobox 2 (Cdx2) (Amal *et al*., 2014). Mechanisms implicated in preimplantation embryonic losses and the failure of embryo implantations require further investigation, thus our aim is to validate the effect of soy isoflavone dietary supplements on fetomaternal dialogues at the time of implantation through assessing progesterone receptors (PR), VEGF, and estradiol receptor alpha (ER-α) protein expressions.

Materials and Methods

Animals

A total of 23 female Wistar rats (200-205 g) and 5 males weighing (210-220 g) were used in this study, all purchased from Lab. Animal House, Animal Vaccination Centre, Helwan. They were housed in plastic cages; the females kept separately three per each cage and males were all kept in a large cage. Food and water were offered *ad libitum*. Animals were acclimatized 2 weeks before beginning the experiment and fed with a casein based diet according to NRC (1995). Males were fed with the control (casein based) diet. Animal Ethics and Experimentation Committee at Suez Canal University ratified all the experimental proceedings (protocol No. 2018-57).

Reproductive procedure

Cyclicity of females was monitored by daily vaginal smears. Eighteen females out of 23 showed regular estrous cycle. One mature male was left in a cage with two proestrous virgin females in overnight. Mating was assured by the presence of sperms in the vaginal smears or the detection of the vaginal plug and gestation day zero (GD0) was designated accordingly (Piesta *et al*., 2009; Gaffer *et al*., 2018). The pregnant females were isolated from the mating cages and replaced in separate cages (3 females/cage). Feed intake was registered from GD0 of pregnancy to gestation day 6 (GD6). Female rats were kept under daily observation. At GD6, the females were weighed then sacrificed to study their early implanted uteri.

Design of experiment

Mated females were divided into two equal groups: group A (n=9), served as the control and was fed with a casein based diet, and group B (n=9) was fed with a casein based diet and gavaged 50 mg/kg/day soy isoflavone extract 40% (JMS Vitamins, USA) in ultrapure water (Sepehr *et al*., 2007). Each g contained 436 mg isoflavones. The total content of soy isoflavones extract were genistein (132 mg/gm) and daidzein (304 mg/gm), as tested by high-performance liquid chromatography (HPLC).

Feed intake, body weight (BW), and weight gain

Cumulative feed intake was determined from GD0 to GD6. Body weights were monitored daily and BW gain was obtained by subtracting final weight from initial weight for each experimental animal.

Uterine weights and implantation rate at GD6

Mated females were sacrificed under the effect of diethylether inhalation anesthesia. Implanted uteri were dissected from 9 females/group (both control and isoflavones groups) at GD6. Uteri were weighed for each dam. Before immersion in 10% neutral buffer formalin saline the number of corpora lutae (CLs) was counted under stereomicroscope (BOECO, Germany). Implantation sites were also counted to calculate the implantation rate percentage according to Hiremath *et al.* (2000) where:

Implantation rate (%) = number of implantation sites/total number of functional CLs × 100

Histopathology

Implanted uteri were immersed in 10% neutral buffer formalin saline. They were then processed and stained with haematoxylin and eosin (H&E) according to Bancroft and Gamble (2008). Image J software (version 1.51n software, NIH, USA) was used to determine the area percentage of uterine stroma in 10 random microscopic fields for each animal per group. The area percentage of uterine stroma was calculated (Cline *et al*., 2002) as follows:

The area percentage of uterine stroma (%) = area of uterine stroma/total area of uterine wall × 100

Immunohistochemistry (IHC) of PR, VEGF, and ER-α

The paraffin embedded implanted uteri were subjected to 5 µm sections used for PR, VEGF, and ER-α IHC according to Helmy *et al*. (2014). The primary antibodies used were (Cat No. MA1-410, Thermo Fisher Scientific Co., UK), (Cat No. MS-750-R7, Thermo Fisher Scientific Co., UK), and (Cat No. MA1-16629, Thermo Scientific Co., UK) at concentrations 1:200, 1:100 and 1:50, respectively. Image analysis for IHC stained area percentage was performed using Image J program according to Elgawish *et al*. (2015).

Statistical analysis

All data in the present experiment were expressed as mean ± SEM. Graphpad prism software (version 7, San Diego, USA) was applied to analyze the differencees

between soy isoflavones and control groups using Student's t-test. Significance was assigned at a probability value of <0.05.

Results

Feed intake, body weight, weight gain, and uterine weight

Soy isoflavones treated group showed significant (P<0.05) reduction in the cumulative food intake than control group starting from GD0 till GD6. Body weight was non-significantly altered while a significant decrease (P<0.05) was noticed in BW gains of soy treated dams as compared to the control (Table 1).

Uterine weights were significantly (P<0.01) diminished in the isoflavones treated dams compared to the control (Table 1).

Table 1: Feed intake, body weight, body weight gain, uterine weight, No. of CLs, implantation rate percentage and area percentage of uterine stroma in control and soy isoflavones treated dams

Parameters	Group	
	Control	Soy isoflavones
Cumulative feed intake (g)	130.9 ± 2.1	122.3 ± 3.1*
Body weight (g)	233.5 ± 10.8	228.3 ± 2.9
Body weight gain (g)	14.5 ± 2.1	8.3 ± 1.8*
Uterine weight (g)	1.2 ± 0.0	0.9 ± 0.1**
Number of CLs dam at GD6	10.4 ± 0.4	11.6 ± 0.2
Implantation rate at GD6 (%)	97.1 ± 1.5	81.5 ± 4.1*
Area percentage of uterine stroma (%)	46.9 ± 3.4	33.7 ± 2.2**

Data were expressed as mean ± SE. * Significant difference along rows at (P<0.05), and ** Significant difference along rows at (P<0.01). CLs: Corpora lutae, and GD6: Gestation day 6

Number of CLs and implantation rate percentage

The number of CLs showed non-significant variation in both groups while implantation rate percentages revealed significant (P<0.05) reduction in the soy isoflavones group compared to the control (Table 1).

Uterine histopathology

Uterine histopathological examinations revealed that the control group (Fig. 1A) had an average uterine stroma. The administration of soy isoflavones reduced the size of uterine stroma (Fig. 1B) and decreased its proliferation compared with the control at GD6. Moreover, both number and size of uterine blood vessels (BV) were normal in control (Fig. 1A); while, dramatically reduced in the soy isoflavones treated dams (Fig. 1B). The control group showed higher number of uterine glands (Fig. 1C) compared to their low number in soy isoflavones treated dams (Fig. 1D). The endometrial epithelium in control group (Fig. 1E) revealed normal architecture; however, it showed hyperproliferation in soy isoflavones treated dams (Fig. 1F). Furthermore, the area percentage of uterine stroma reduced significantly (P<0.01) in soy isoflavones dams as compared to the control group (33.7 ± 2.2 vs 46.9 ± 3.4%), respectively (Table 1).

Immunohistochemistry

The immunostaining of PR was demonstrated as brownish intranuclear as well as intracytoplasmic reaction in lining epithelium (LE), glandular epithelium (GE), connective tissue (CT), and tunica muscularis (M) (Fig. 2). The control group showed more intense immunoreactive staining of PR (Figs. 2A and 2C) than isoflavones treated dams (Figs. 2B and 2D).

Immunoreactivity of VEGF was clearly shown as brownish intracytoplamic reaction in GE, CT, M, and endothelial BV (Figs. 3). This reaction was more intense in the control group (Figs. 3A, 3C and 3E) than the isoflavones treated dams (Figs. 3B, 3D and 3F).

On the other hand, ER-α immunoreactivity was clearly demonstrated as weak intracytoplasmic signals in LE, CT, M and around BV in the control dams (Figs. 4A, 4C and 4E). The reaction was more intense in LE, CT, and M in the isoflavones treated dams (Figs. 4B, 4D and 4E).

The immunostained area percentage of PR and VEGF showed marked reduction (P<0.01) in the implanted uteri of soy treated dams compared to the control group (Fig. 5). However, immunostained area percentage of ER-α

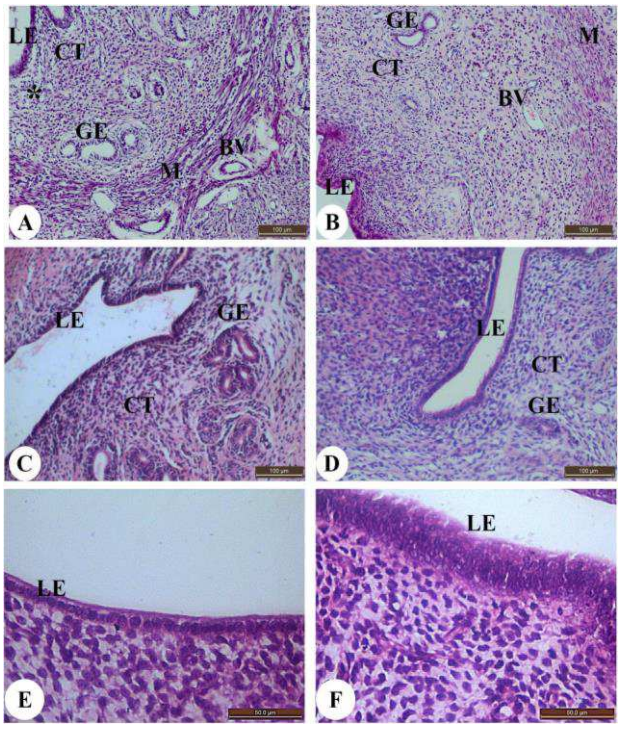

Fig. 1: Photomicrographs of uterus sections of pregnant female Wistar rats at GD6. Figs. **A**, **C**, **E**: Sections in control rats showed lining epithelium (LE), glandular epithelium (GE), connective tissue (CT), endothelial blood vessels (BV), and tunica muscularis (M). The sections showed normal LE and CT edema (*) with ample angiogenic changes (**A**, **C**). Figs. **B**, **D**, **F**: Sections in 50 mg/kg isoflavones treated rats showed hyperplasia in luminal epithelium (**F**) compared to the control group (**E**). Reduced number of uterine glands and indistinct CT edema and decreased angiogenic changes (**B**, **D**). All figures were H&E stained

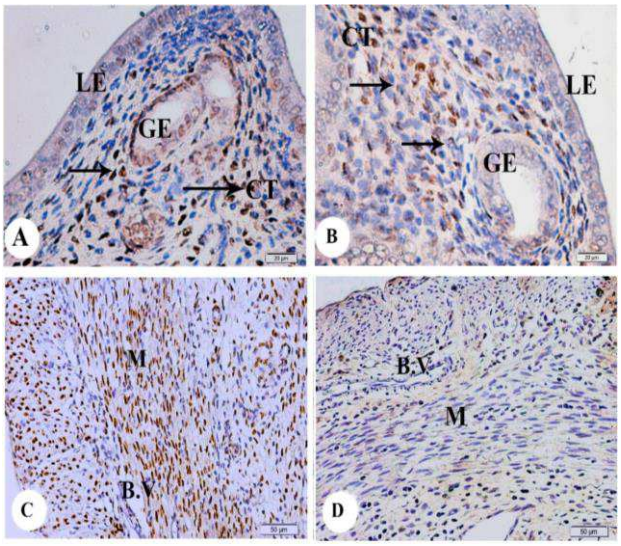

Fig. 2: Photomicrographs of uterus sections of progesterone receptors (PR) immunostaining in pregnant female Wistar rats at GD6. Figs. **A**, **C**: Sections in control rats showed intense positive intranuclear as well as intracytoplasmic immunostaining reactions in lining epithelium (LE), glandular epithelium (GE), endothelial blood vessels (BV), tunica muscularis (M), and connective tissue (CT). Figs. **B**, **D**: Sections in 50 mg/kg isoflavones treated dams showed less immunoreactivity than the control as well as a lower number of immunoreactive cells

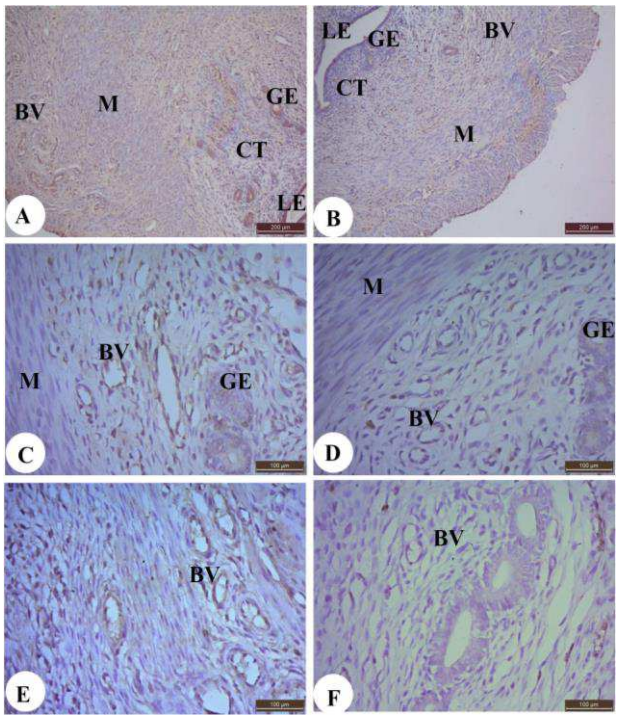

Fig. 3: Photomicrographs of uterus sections of vascular endothelial growth factor (VEGF) immunostaining in pregnant female Wistar rats at GD6. Control dams (**A**, **C**, **E**) showed intense positive intracytoplasmic immunostaining reaction in connective tissue (CT), glandular epithelium (GE), tunica muscularis (M), lining epithelium (LE), and endothelial blood vessels (BV) Figs. (**A**, **C**, **E**). Soy isoflavones treated dams (50 mg/kg) demonstrated less immunoreactivity than the control as well as a lower number of immunoreactive cells (Figs. **B**, **D**, **F**)

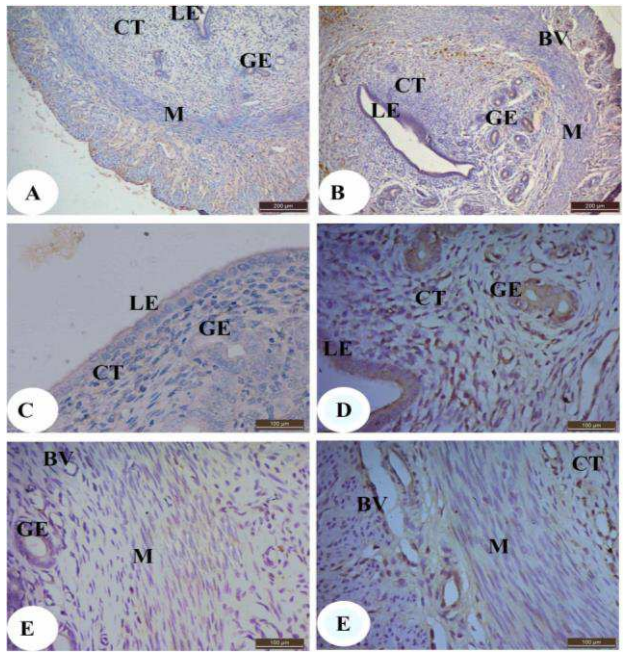

Fig. 4: Photomicrographs of uterus sections of estradiol receptors alpha (ER-α) immunostaining in pregnant female Wistar rats at GD6. Control dams (**A**, **C**, **E**) showed weak positive intracytoplasmic immunostaining reactions in lining epithelium (LE), connective tissue (CT), tunica muscularis (M), glandular epithelium (GE), and around blood vessels (BV). Soy isoflavones treated dams (50 mg/kg) demonstrated intense immunoreactivity in lining epithelium (LE), CT, and M than the controls (Figs. **B**, **D**, **F**). GD6: Gestation day 6

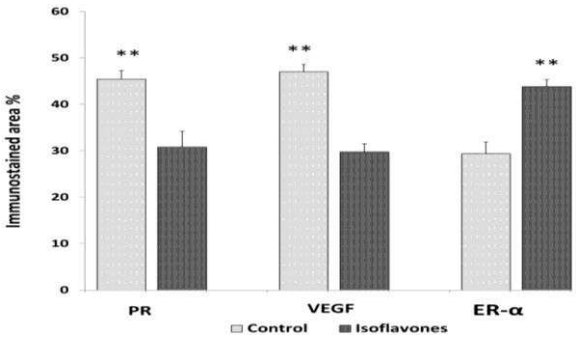

Fig. 5: Immunostained area % (mean±SEM) of progesterone receptors (PR), vascular endothelial growth factor (VEGF), and estradiol receptor alpha (ER-α) in implanted uteri of control and soy isoflavones treated dams at gestation day 6 (GD6). ** indicates significant difference at P<0.01

revealed a highly significant (P<0.01) elevation in soy isoflavones dams compared to the controls (Fig. 5).

Discussion

Around implantation time, the uterus is unique in its vascular progression as it establishes fetomaternal anastomosis and decidua formations that provide the uterus and embryo with nutrition and oxygen (Cha *et al*., 2012). Implantation events seem to be complicated but are well organized. The expressions of PR, VEGF, and ER-α in the uterine tissue during this period are fundamental for implantation (Matsumoto and Sato,

2006). The current study manipulated the anti-implantation effect of soy isoflavones on these three interrelated factors.

The present study demonstrated a significant reduction in feed intake of pregnant dams administered with soy isoflavones compared to the controls. These results were in harmony with those of Soucy *et al*. (2005) and Amal *et al*. (2014). The reduced feed intake in the isoflavones treated group could be attributed to the estrogen mimicking effect of isoflavones (Messina, 2010) that cause appetite repression (Wade, 1975). Moreover, the estrogenic actions of soy isoflavones play a role in body fat regulation, decreasing leptin production from fat cells. Leptin has a direct influence on neuropeptide Y levels that regulate feeding behavior, consequently reducing feed intake (Szkudelska *et al*., 2000). The reduced feed intake in this study influenced the BW gain of pregnant dams. Furthermore, the reduced BW gain may be due to the reduced implantation rates in the soy isoflavones treated group. Successful implantation is accompanied with uterine edema and increments in uterine weights (Rockwell *et al*., 2002) as observed in the control dams.

The reduction of implantation rates in the soy isoflavones group in the present study was similar to those of Amal *et al*. (2014). The physiology of implantation is multifactorial; estrogen and progesterone are two steroid hormones that regulate the transcription of several genes by binding to their cognate receptors PR and ER-α. Moreover, the VEGF plays a crucial role in implantation events and is fundamental for sustaining pregnancy (Rockwell *et al*., 2002). Our results suggest that soy isoflavones significantly decreased PR and VEGF while increasing ER-α during early implantation thus causing a predisposed reduction in implantation percentages. Both steroid hormone receptors PR and ER-α play roles in murine implantation biology. After the ovulatory estrogen surge, ER-α was expressed at luminal epithelium, causing their proliferation. Once pregnancy proceeded, the combination of stromal PR with its specific ligand, progesterone produced from CL, led to reduced ER-α epithelial proliferation (Wetendorf and DeMayo, 2012). In the current study, soy isoflavones produced lower PR proteins in LE, CT, and GE compared to the control that failed to down-regulate the observed ER-α dependent epithelial proliferation in this group. Moreover, PR could induce the proliferation of stromal cells and GE where they are expressed to help decidualization. The over regulation of ER-α could be attributed to soy isoflavones; genestein and daidzein that are considered selective estrogen receptors ligands (Setchell, 2001).

The reduction of VEFG protein in the implanted uteri of the soy isoflavones treated group was accompanied with reduced vascular permeability and edema. This was confirmed by the declined number of uterine BW in the histopathological sections. This abnormal decrease could be attributed to the sever reduction in PR in the isoflavones treated group where PR is necessary for VEGF expression and uterine endothelial angiogenesis (Matsumoto and Sato, 2006; Goddard *et al*., 2014; Tan *et al*., 2014). Moreover, VEGF is a chief mediator for the promotion of vascular permeability that takes place in uterine tissue as well as decidualization (Douglas *et al*., 2014). An unexpected finding of the present study was that soy isoflavones led to the over regulation of ER-α and the down regulation of uterine VEGF and angiogenesis. However, estrogen and its receptors are considered inducers for uterine microvasculature and VEGF (Mueller *et al*., 2000). This could be attributed to the selective estrogen receptors' modulator nature of genestein and daidzein that occupies the receptors exerting an antagonist effect (Thongon *et al*., 2017) rather than an agonist effect.

In conclusion, soy isoflavones caused embryonic losses at the time of implantation. These embryonic losses were related to the reduced PR that failed to promote uterine edema and microvasculature as well as a reduced VEGF expression. Moreover, the reduction of PR aborted to down regulate ER-α which is necessary for implantation.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

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