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Isoflavone increases the mRNA expression levels of IL-6 signal transducer glycoprotein 130 in human endometrial glandular cells

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Summary

Purpose of investigation: The authors examined whether isoflavone can effectively increase the secretion of cytokines associated with implantation from endometrial glandular cells. *Material and Methods:* Endometrial tissue was collected from 14 patients who underwent surgery for benign gynecological diseases in this hospital. The endometrial tissue was separated into stromal and glandular cells, and isoflavone was added to the glandular cells at a concentration of 0, 0.05, 0.5, and 5 μg/ml. In another group, an estrogen receptor antagonist was added together with isoflavone. After 24 hours, the expression levels of leukemia inhibitory factor, interleukin (IL)-6, IL-11, and IL-6 signal transducer glycoprotein 130 (IL-6/gp130) were measured by quantitative polymerase chain reaction. *Results:* A significant increase in the expression of IL-6/gp130 was found in glandular cells treated with isoflavone (0.5 μg/ml). The significant differences were not observed among the groups upon treatment with isoflavone and an estrogen receptor antagonist, indicating that isoflavone acted via the estrogen receptor. *Conclusion:* Isoflavone increases the mRNA expression levels of IL-6/gp130 in human endometrial glandular cells of early to mid secretary phase.

Key words: Isoflavone; Implantation; Endometrial glandular cells; Cytokines; Infertility.

Introduction

Infertility is a serious problem for many couples. One in six couples will encounter fertility problems, defined as the failure to achieve a clinical pregnancy after regular intercourse for 12 months [1]. There are various causes of infertility, such as tubal factors, ovulatory dysfunction, and male infertility. There is also unexplained infertility, which includes implantation failure [2].

Many cytokines are necessary for implantation of the fertilized egg into the endometrial glandular cell layer [3-5], such as leukemia inhibitory factor (LIF) [6], interleukin (IL)-6, and IL-11 [4, 7-9]. LIF, IL-6, and IL-11 belong to the IL-6 family and bind to IL-6 signal transducer glycoprotein 130 (IL-6ST/gp130). Importantly, the mRNA expression levels of these cytokines are known to be low during early to mid secretary phase in many patients with infertility [10].

Estrogen is a key hormone that is essential for the maintenance of pregnancy, and also promotes the secretion of IL-6 family members [4, 11, 12]. Interestingly, there is a substance in natural plants called isoflavone, which is a type of phytoestrogen and exerts similar effects to those of estrogen. There have been some reports that isoflavone contributes to the maintenance of pregnancy [13], or improves the pregnancy rate of patients with infertility [14].

There are two types of isoflavone: a glycosidic type that contains a carbohydrate chain, and an aglycone type that does not. The aglycone type is degraded by intestinal bacteria flora and is more readily absorbed by the intestine than the glycosidic type [15]. Therefore, the aglycone type exerts stronger effects in the body than the glycosidic type.

The aglycone-type isoflavone is further divided into three types: daidzein, genistein, and glycitein. They have estrogen-like effects, and act via the estrogen receptor [16]. However, it is not well understood as to how isoflavone acts to support the mechanism of pregnancy. Therefore, in the present study, the authors added isoflavone to human endometrial glandular cells of early to mid secretary phase, and quantified the expression levels of cytokines associated with implantation, to investigate the possible effects of isoflavone on implantation.

Materials and Methods

This study was approved by the ethics committee of Tokyo Medical University Hospital. After obtaining informed consent, endometrial tissue was collected from 14 patients who underwent surgery for benign gynecological diseases in this hospital. The patients were aged 27 to 41 years (33.8 ± 1.0 years) and had regular menstruation (25-32 days), no medical history or allergies, and had not taken any medication for two months before the surgery.

The authors confirmed that the patients did not have any space-occupying lesions in the endometrial cavity by transvaginal ultrasound and magnetic resonance imaging on preoperative

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examination.

The patients' basal body temperature and levels of estrogen, progesterone, and luteinizing hormone were measured on the day of surgery. All patients were in the early to mid secretary phase, and their endometrial tissue was collected by curettage during the surgery.

Uterine glandular cells were isolated and cultured as described previously, with some modifications [17, 18]. Briefly, the endometrial tissue was cut finely and incubated in Hank's balanced salt solution with 0.5% collagenase for three hours at 37°C in 5% CO_2 and stirred every hour for approximately one minute. The solution was then filtered with a 100 μm cell strainer liner membrane, and then with a 40-μm cell strainer liner membrane. Then, the 40-μm cell strainer liner membrane was reversed and washed with phenol-red free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% charcoal-treated fetal bovine serum (5% DCC-FBS), and the solution was collected. The solution was then centrifuged at 1,000 rpm for five minutes, and the endometrial glandular cells were collected.

A portion of the cells was immunostained with an antibody against cytokeratin (M3515) to confirm that they were endometrial glandular cells. The present cell samples were greater than 95% endometrial glandular cells by viewing. The isolated cells were cultured in 96-well plates in 100 μl of phenol-red-free DMEM supplemented with 5% DCC-FBS, 100 IU/ml penicillin, and 50 mg/ml streptomycin.

The authors used isoflavone aglycone AglyMax (daidzein: genistein: glycitein aglycones at a ratio of 7:1:2). Isoflavones were dissolved in dimethyl sulfoxide at a concentration of 5.0 mg/ml and then added to the medium of endometrial glandular cells at a final concentration of 0.05, 0.5, or 5 μg/ml. In another group, the estrogen receptor inhibitor ICI 182,780 (10 mM) was added to the cells together with isoflavone. Cells were collected after culturing with the various reagents for 24 hours. mRNA was extracted from the cells, and then purified and cDNA was synthesized using a gene expression cells-to-CT kit according to the manufacturer's protocol.

The mRNA expression levels of LIF, IL-6, IL-6ST/gp130, and IL-11 in glandular cells were measured by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR), according to the manufacturer's instructions. Quantitative RT-PCR was performed in a total volume of 20 μl of a mixture containing 6 μl DNA-free water, 10 μl universal PCR master mix, 2 μl gene expression assay, and a two μl cDNA sample in each 96-well plate. The gene expression assay sequences were LIF (Hs00171455_m1), IL-11 (Hs00174148_m1), IL-6 (Hs00985639_m1), and IL-6 signal transducer (gp130, oncostatin M receptor Hs00174360_m1). The following RT-PCR protocol was used: ten minutes at 95°C, followed by 40 cycles of amplification for 15 seconds at 95°C, and one minute at 60°C. Melting curve measurements were collected and analyzed. The mRNA level of each sample was normalized to that of glyceraldehyde phosphate dehydrogenase (GAPDH).

For the immunostaining of cell samples to confirm that they were glandular cells, cells were collected using liquid-based cytology, and thin-layer specimens were made, according to the manufacturer's protocol. Next, the specimens were heat treated with CC1 buffer for 60 minutes for antigen activation. Subsequently, cells were stained with an anti-pan-cytokeratin antibody (1:200), using the automatic immunostaining apparatus, according to the manufacturer's protocol (Figure 1).

Statistical analyses were performed using SPSS Statistics version 22 software. The mRNA expression levels of LIF, IL-11, IL-6, and IL-6ST/gp130 were compared between isoflavone-treated cells and control cells, using the Dunnett's test. A p-value of less than 0.05 was considered to demonstrate a statistically significant difference between two groups.

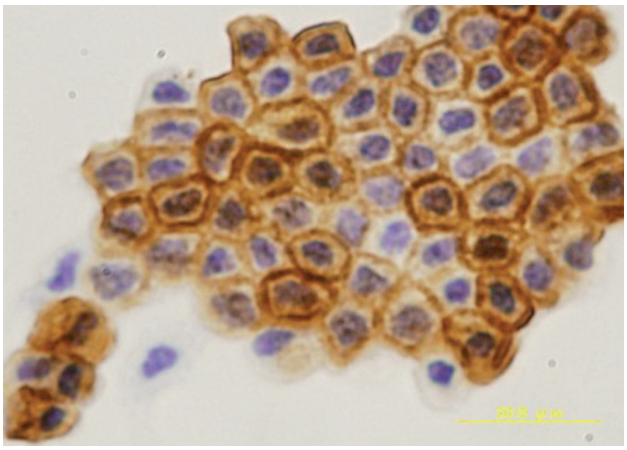

Figure. 1. — Confirmation of the cells used in this study as glandular cells. Anti-cytokeratin antibody staining of the endometrial glandular cells that were used in this study. Isolated cells were found to be 95% positive for cytokeratin, indicating that they were mostly endometrial glandular cells. Scale bar 100 μm.

Results

The authors first determined the optimal doses of isoflavone to use for their experiments, as well as the optimal time for collecting and analyzing the cells. The maximum recommended dose for the oral intake of isoflavone is 75 mg/day or less in Japan. However, considering that the maximum dietary intake of isoflavone is about 45 mg/day, the maximum recommended dose as a supplement is 30 mg/day. From the data of a previous study in humans [15] the authors calculated an intake of isoflavone 30 mg/day of isoflavone to result in a peak blood concentration, of approximately 0.5 μg/ml. Furthermore, as studies have shown that in humans the blood concentration of the aglycone-type isoflavone reaches a peak at four to eight hours after intake, and peaks out within 24 hours, the incubation time was set at 24 hours [15, 19, 20].

To investigate how isoflavone acts on endometrial glandular cells, the authors compared the effects of 0.05 μg/ml, 0.5, and 5 of isoflavone with the control group (0 μg/ml) (Figure 2). The mRNA levels of IL-6ST/gp130 were significantly increased upon treatment with 0.5 μg/ml isoflavone compared with the control group ($p < 0.05$). There was no significant difference, but expression of all genetic mRNA levels increased in the isoflavone addition group. However, there were little increases at the high concentration (five μg/ml), except IL-11 (Figure 2).

The authors next analyzed whether isoflavone acted via the estrogen receptor, using the estrogen receptor inhibitor ICI 182,780. They added ICI 182,780 (10 mM) to the cells

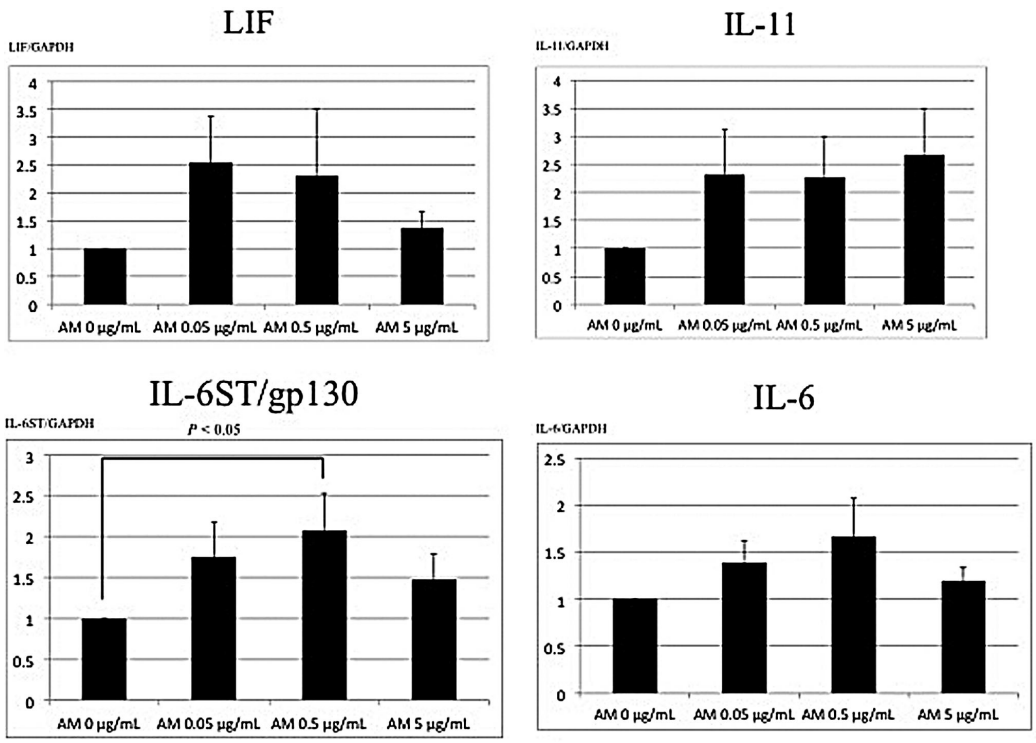

Figure. 2. — Cytokine mRNA expression levels in glandular cells upon treatment with isoflavone. mRNA expression levels of LIF, IL-11, IL-6, and IL-6ST/gp130 in glandular cells that were treated with various concentrations of isoflavone, as indicated, are compared to control. mRNA levels are expressed as ratios to GAPDH (n = 14). AM: isoflavone aglycone AglyMax.

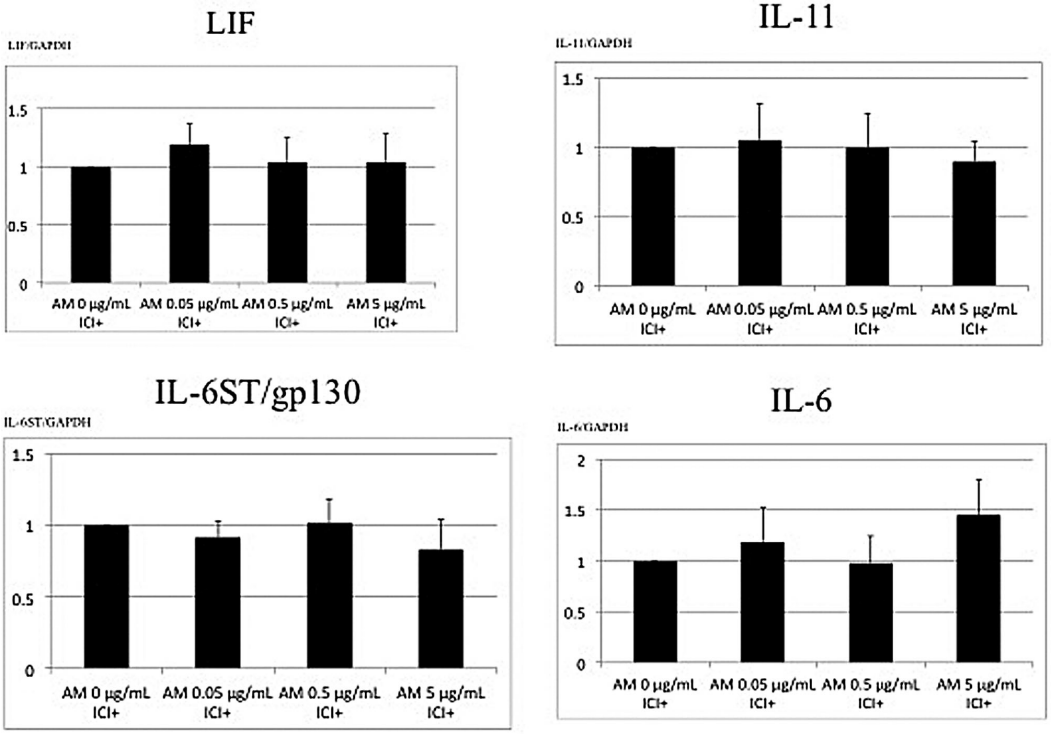

Figure. 3. — Cytokine mRNA expression levels in glandular cells treated with isoflavone and an estrogen receptor inhibitor. mRNA levels of LIF, IL-6, IL-6ST/gp130, and IL-11 mRNA in glandular cells treated with various concentrations of isoflavone and ICI 182,780 (10 mM), are compared to control. mRNA levels are expressed as ratios to GAPDH (n = 14). AM: isoflavone aglycone AglyMax.

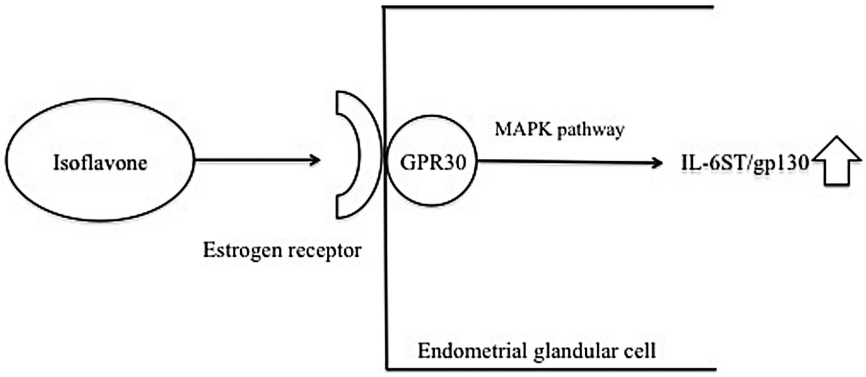

Figure. 4. — Proposed signal transduction mechanism in glandular cells upon treatment with isoflavone. Possible mechanism by which isoflavone acts to increase IL-6ST/gp130 mRNA levels in glandular cells. Isoflavone may act via the estrogen receptor, to activate the MAPK pathway and to subsequently increase the mRNA levels of IL-6ST/gp130 and mRNA.

together with isoflavone at 0, 0.05, 0.5, and 5 μg/ml. The significant different increases were not observed among all groups (Figure 3). Therefore, these results indicated that isoflavone exerts its effects via the estrogen receptor.

Discussion

In this study, the authors found that 0.5 μg/ml of isoflavone increased the mRNA expression level of IL-6ST/gp130 in endometrial glandular cells of early to mid secretary phase. There were also increases, not statistically significant, in the mRNA expression level of all genetic mRNA. Furthermore, when the authors added an estrogen receptor inhibitor together with different concentrations of isoflavone to the cells, there were no significant differences between the groups. Therefore, isoflavone is thought to act via the estrogen receptor.

In this study, the authors used the aglycone-type isoflavone, which comprises mostly daidzein, genistein, and glycitein. Daidzein is metabolized in the body into equol, O-desmethylangolensin, and dihydrodaidzein by intestine flora. The estrogen-like effects of equol were shown to be stronger than that of daidzein [21]. Glycitein was shown to have weaker estrogen-like effects in the body than the two other isoflavones [19]. Genistein showed the strongest estrogen-like effects in the body [16, 22]. The authors are unclear as to which of the three aglycone-type isoflavones exerted the estrogen-like effects in this study. However, each is known to have estrogen-like effects, and there have been no reports that the three types interfere with each other's effects. Therefore, the authors believe that the three isoflavones all exerted estrogen-like effects in their experiments.

The authors used endometrial glandular cells of early to mid secretary phase, because implantation is said to occur in this period, and isoflavone acts on the estrogen receptor, which was reported to be expressed in glandular cells [16]. The present results showed that isoflavone increases the expression levels of IL-6ST/gp130 mRNA via the estrogen receptor.

G protein-coupled receptor (GPCR) 30 is a member of the G protein-coupled receptor membrane proteins, and forms a complex with the estrogen receptor. It is expressed in the normal endometrium [23]. Furthermore, GPCR30 was shown to activate the mitogen-activated protein kinase (MAPK) pathway and increase IL-6 mRNA levels in a human endometrial cancer cell line [24, 25]. From these previous results, the mechanism of action of isoflavones acting via the estrogen receptor might be that isoflavone activates the MAPK pathway through the estrogen-receptor complex with GPCR30, and increases signal transducer and activator of transcription 3, which is located downstream of the MAPK pathway. Therefore, IL-6ST/gp130, the receptor of IL-6, may be increased upon treatment with isoflavone (Figure 4). Further experiments are required to confirm this hypothesis.

The present results showed that there was a significant increase in the mRNA levels of IL-6ST/gp130 upon the addition of isoflavone at 0.5 μg/ml. However, there was little increase at a higher concentration (five μg/ml) in most genetic mRNA expression. One possible reason for this is that the higher concentration of isoflavone inhibited endometrial cell proliferation [26]. Furthermore, in humans, it has been reported that side effects such as endometrial hyperplasia occurred upon the long-term administration of isoflavone (150 mg/day) [27]. The peak blood concentration upon the intake of isoflavone at 150 mg/day is expected to be greater than 0.5 μg/ml [15]. Therefore, a high concentration of isoflavone may cause adverse effects to the body, rather than increase the mRNA levels of cytokines associated with implantation. Further experiments are also required to clarify this point.

Conclusions

Isoflavone acted via the estrogen receptor in endometrial glandular cells to increase the secretion of cytokines such as IL-6ST/gp130, which are associated with implantation. Isoflavone might improve implantation failure by increas-

ing the levels of cytokines associated with implantation. The authors would like to give isoflavone to the patients with implantation failure to examine an effect in the future.

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References

[1] Farquhar C., Rishworth J.R., Brown J., Nelen W.L., Marjoribanks J.: "Assisted reproductive technology: an overview of Cochrane reviews". *Cochrane Database Syst. Rev.*, 2014, *12*, CD010537.
[2] Lindsay T.J., Vitrikas K.R.: "Evaluation and treatment of infertility". *Am. Fam. Physician*, 2015, *91*, 308.
[3] Singh M., Chaudhry P., Asselin E.: "Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors". *J. Endocrinol.*, 2011, *210*, 5.
[4] Saito S.: "Cytokine cross-talk between mother and the embryo/placenta". *J. Reprod. Immunol.*, 2001, *52*, 15.
[5] Saito S.: "Cytokine network at the feto-maternal interface". *J. Reprod. Immunol.*, 2000, *47*, 87.
[6] Stewart C.L., Kaspar P., Brunet L.J., Bhatt H., Gadi I., Köntgen F., Abbondanzo S.J.: "Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor". *Nature*, 1992, *359*, 76.
[7] Prins J.R., Gomez-Lopez N., Robertson S.A.: "Interleukin-6 in pregnancy and gestational disorders". *J Reprod Immunol.*, 2012, *95*, 1.
[8] Tawfeek M.A., Eid M.A., Hasan A.M., Mostafa M., El-Serogy H.A.: "Assessment of leukemia inhibitory factor and glycoprotein 130 expression in endometrium and uterine flushing: a possible diagnostic tool for impaired fertility". *BMC Womens Health*, 2012, *12*, 10.
[9] Marwood M., Visser K., Salamonsen L.A., Dimitriadis E.: "Interleukin-11 and leukemia inhibitory factor regulate the adhesion of endometrial epithelial cells: implications in fertility regulation". *Endocrinology*, 2009, *150*, 2915.
[10] Dimitriadis E., Sharkey A.M., Tan Y.L., Salamonsen L.A., Sherwin J.R.: "Immunolocalisation of phosphorylated STAT3, interleukin 11 and leukaemia inhibitory factor in endometrium of women with unexplained infertility during the implantation window". *Reprod. Biol. Endocrinol.*, 2007, *5*, 44.
[11] Sawai K., Matsuzaki N., Okada T., Shimoya K., Koyama M., Azuma C., et al.: "Human decidual cell biosynthesis of leukemia inhibitory factor: regulation by decidual cytokines and steroid hormones". *Biol Reprod.*, 1997, *56*, 1274.
[12] Sekine Y., Yamamoto T., Yumioka T., Imoto S., Kojima H., Matsuda T.: "Cross-talk between endocrine-disrupting chemicals and cytokine signaling through estrogen receptors". *Biochem. Biophys. Res. Commun.*, 2004, *315*, 692.
[13] Woclawek-Potocka I., Bah M.M., Korzekwa A., Piskula M.K., Wiczkowski W., Depta A., Skarzynski D.J.: "Soybean-derived phytoestrogens regulate prostaglandin secretion in endometrium during cattle estrous cycle and early pregnancy". *Exp. Biol. Med. (Maywood)*, 2005, *230*, 189.
[14] Unfer V., Casini M.L., Gerli S., Costabile L., Mignosa M., Di Renzo G.C.: "Phytoestrogens may improve the pregnancy rate in in vitro fertilization-embryo transfer cycles: a prospective, controlled, randomized trial". *Fertil. Steril.*, 2004, *82*, 1509.
[15] Izumi T., Piskula M.K., Osawa S., Obata A., Tobe K., Saito M., *et al.*: "Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans". *J. Nutr.,* 2000, *130*, 1695.
[16] Staar S., Richter D.U., Makovitzky J., Briese V., Bergemann C.: "Stimulation of endometrial glandular cells with genistein and daidzein and their effects on ERalpha- and ERbeta-mRNA and protein expression". *Anticancer Res.*, 2005, *25*, 1713.
[17] Zhang L., Rees M.C., Bicknell R.: "The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on oestrogen and progesterone challenges". *J. Cell. Sci.*, 1995, *108*, 323.
[18] Satyaswaroop PG, Bressler RS, de la Pena MM, Gurpide E.: "Isolation and culture of human endometrial glands". *J Clin Endocrinol Metab. 1979,48,*639.
[19] Setchell K.D., Faughnan M.S., Avades T., Zimmer-Nechemias L., Brown N.M., Wolfe B.E., *et al.*: "Comparing the pharmacokinetics of daidzein and genistein with the use of 13C-labeled tracers in premenopausal women". *Am. J. Clin. Nutr*., 2003, *77*, 411.
[20] Song T.T., Hendrich S., Murphy P.A.: "Estrogenic activity of glycitein, a soy isoflavone". *J. Agric. Food Chem.*, 1999, *47*, 1607.
[21] Atkinson C., Frankenfeld C.L., Lampe J.W.: "Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health". *Exp. Biol. Med. (Maywood)*, 2005, *230*, 155.
[22] Hopert A.C., Beyer A., Frank K., Strunck E., Wünsche W., Vollmer G.: "Characterization of estrogenicity of phytoestrogens in an endometrial-derived experimental model". *Environ. Health Perspect.*, 1998, *106*, 581.
[23] Kolkova Z., Noskova V., Ehinger A., Hansson S., Casslén B.: "G protein-coupled estrogen receptor 1 (GPER, GPR 30) in normal human endometrium and early pregnancy decidua". *Mol. Hum. Reprod.*, 2010, *16*, 743.
[24] Nishida M., Kasahara K., Kaneko M., Iwasaki H., Hayashi K.: "Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors". *Nihon Sanka Fujinka Gakkai Zasshi*, 1985, *37*, 1103.
[25] He Y.Y., Cai B., Yang Y.X., Liu X.L., Wan X.P.: "Estrogenic G protein-coupled receptor 30 signaling is involved in regulation of endometrial carcinoma by promoting proliferation, invasion potential, and interleukin-6 secretion via the MEK/ERK mitogen-activated protein kinase pathway". *Cancer Sci.*, 2009, *100*, 1051.
[26] Kayisli U.A., Aksu C.A., Berkkanoglu M., Arici A.: "Estrogenicity of isoflavones on human endometrial stromal and glandular cells". *J. Clin. Endocrinol. Metab.*, 2002, *87*, 5539.
[27] Unfer V., Casini M.L., Costabile L., Mignosa M., Gerli S., Di Renzo G.C.: "Endometrial effects of long-term treatment with phytoestrogens: a randomized, double-blind, placebo-controlled study". *Fertil. Steril.*, 2004, *82*, 145.

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