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Original paper

Effects of single injection of phytoestrogens or estrogen receptor α or β agonists on uteri in ovariectomized rats

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Abstract

The effects of phytoestrogens and agonists for estrogen receptor α and β (ERα and ERβ, respectively) on uteri were examined morphologically in ovariectomized rats. First, ovariectomized rats received an injection with 1mg of coumestrol (CM), genistein (GS), daidzein (DZ),β-estradiol (E_2) or oil, and 24 hours later, uteri were fixed after weighing and histological examinations were performed. Next, 0.5 or 1.0 mg propyl pylazole triol (PPT, ERα agonist), 1.0 or 1.5 mg diarylpropionitrile (DPN, ERβ agonist) or solvent was injected and the uteri were examined in the same manner. The mean uterine weight and height of epithelial cell in the E2 group were higher than those in those of oil-treated rats. In the CM group, Uteri were larger but height of epithelial cell was not higher than those in the oil group. In the GS and DZ groups, histological features of uteri and epithelial cells were comparable to those in the oil group. Uterine weights and height of epithelial cell in all PPT groups were higher, compared to those in solvent-treated group. In all DPN-treated rats uteri were larger than those in the solvent group but lower than those in the PPT groups. Epithelial cells in the DPN group were similar to those in the solvent group. These results indicate that estrogenic effects on the uterus of CM are highest among phytoestrogens but lower than E2. Estrogenic substances increase uterus through mainly ERα but ERβ is also involved in the mechanism.

Key Words： Phytoestrogen, estrogen receptor α, estrogen receptor β, uterus, female rats

Introduction

Estrogen is an important factor in the maintenance of uterine activity in all female mammals, because ovariectomy induces severe reduction of the uterine tissues and supplementation with estrogen recovers them. In female rats, the blood level of estrogen changes with a 4-day cycle and preovulatory increases of estrogen in the morning of the day before ovulation act on the forebrain and induce the estrous condition and ovulation (see review, Freeman,1994).

Activities of estrogen are mediated mainly by two subtypes of nuclear receptor of estrogen (ER): ERα and ERβ (Katzenellenbogen and Korach 1997; Kuiper et al.1998b; Enmark and Gustafsson 1999). Mowa and Iwanaga (2000) reported that ERα-mRNA was abundant in the luminal and glandular epithelial cells of rat uteri and moderate signals were also detected in the

subepithelium stromal cells and muscle. On the other hand, ERβ-mRNA was found only in the subepithelial stromal cells of the uterus.

Phytoestrogens, which are chemical compounds such as isoflavone, coumestan and lignan groups in plants, have been verified to bind ERs (Jordan et al., 1985; Dixon, 2004). The isoflavons, genistein and daidzein, and the coumestan, coumestrol, have a higher binding potency to ERβ, compared to ERα (Kuiper et al., 1997). Among the phytoestrogens, coumestrol shows the highest binding potency to ERβ and the binding affinity to ERβ is 1.4 times that of estradiol (Kuiper et al., 1998a).

Many phytoestrogens have been shown to affect the reproductive functions in animals including humans (Cornwell et al., 2004; Whitten and Naftolin, 1998). Phytoestrogens have the potency to influence not only the female reproductive tract in adult female rats, but also the neonatal rat brain in sexual differentiation of the brain functions (Kouki et al., 2003; 2005).

A frontier report of Bennetts et al. (1946) showed that severe reproductive problems, such as infertility, dystocia and prolapse of the uterus, were observed in Australian sheep grazing on clover pastures. Constant infertility syndrome was observed in sheep and cattle exposed to high levels of isoflavones and coumestans, a condition that became known as 'clover disease' (See review Adams,1996). Genistein and daidzein are abundant in soy beans and their products (Wang and Murphy,1994; Franke et al., 1994) .

Oral or subcutaneous administration of phytoestrogens has been found to have numerous effects on the reproductive organs of rats, mice and livestock (see review Whitten and Naftolin, 1998). Increase in uterine weight has been reported after long-term feeding of clover silage in ovariectomized ewes (Nwannenna et al., 1995). The uterine weights of animals eating diets that include more than 2.4% soy extract were found to increase and edematous stroma, endothelial hyperplasia, glandular leukocyte infiltration and hemorrhagic extravasations were observed (Gallo et al., 1999). In female rats, long-term oral administration of genistein or daidzein induced uterine growth (Santell, 1997). Oral administration of coumestrol (Whitten and Naftoline 1992) for a few days also increased the uterine weight. Rapid increase in the uterine weight has been reported to result from the injection of genistein to rats (Diel,2004). Thus, coumestan and isoflavone are effective in increasing uterine tissue in rats. However, the specific roles of the subtypes of ER in increasing uterine tissue by estrogen or phytoestrogens have remained obscure.

In this experiment, to compare the effects of phytoestrogens on the uterus, coumestrol, genistein or daidzein was injected to ovariectomized rats and the uterine tissue examined histologically 24 hr after the injection. Next, to elucidate the role of ERα and ERβ, the ERα agonist, propyl pylazole triol (PPT) or the ERβ agonist, diarylpropionitrile (DPN), was injected and the uterus was observed histologically in the same manner as described above

Materials and Method

Female Wistar rats at 8 weeks of age (250-300g) were purchased from Takasugi Animal Farm (Saitama, Japan) and kept in a light- (14L:10D, lights off at 19:00) and temperature- (22-24°C) controlled room. Food and water were accessed ad libitum. All rats were ovariectomized under ether anesthesia. One week after the ovariectomy, female rats were injected with phytoestrogens or estrogen receptor agonists. Their uteri were examined histologically one day after injection. All experiments were conducted in accordance with the Regulations for Animal Experimentation at Waseda University (Approval No.10J005)

Histological changes in the uteri in the estrous cycle.

As a preliminary experiment, changes in the condition of the uterus in accordance with the 4-day estrous cycle: proestrus (P), estrous (E), diestrus 1 (D1) and diestrus 2 (D2), were observed. The estrous cycle was determined by a change in the cell types in vaginal smears. Vaginal smears were taken for about 10 days and rats showing 2 or more regular estrous cycles of 4 days were used. In each stage of the estrous cycle, the animals were sacrificed by an over-dose of ether and their uteri were analyzed histologically after weighing.

Effects of phytoestrogens on uteri

All rats were ovariectomized under ether anesthesia and one week after ovariectomy, several kinds of phytoestrogens, estradiol (E_2) or 0.1 ml sesame oil (control) were injected subcutaneously. The injection dose of all phytoestrogens and E_2 was 1 mg/rat. Coumestrol (CM, LKT Laboratories, USA, 7 rats), genistein (TS, Nakahara Science Lot No. 0006, Japan, 6 rats), daidzein (DZ, Nakahara Science, Japan, 6 rats), and 1,3,5(10)-Estratriene-3,17β-diol (E_2, SIGMA Lot No.70K1206, USA, 6 rats) were suspended in 0.1 ml sesame oil (KANTO chemical, Japan). Twenty-four hours after the injection, all rats were autopsied by an overdose of ether after vaginal smears were taken. Their uteri were analyzed histologically after weighing.

Effects of ERα and ERβ antagonists on uteri

All of the methods except for the preparation of drugs were the same as described above. One week after ovariectomy, an ERα agonist, 4,4',4"- (4-Propyl-(1H)- pyrazole- 1,3,5-triyl), trisphenol (PPT, TOCRIS, BatchNo:6A/92906, USA) or the ER β agonist, 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN, TOCRIS, BatchNo:4A/94463,USA), was injected subcutaneously. The injected doses of PPT were 0.5 (9 rats) and 1 mg (10 rats) and of DPN were 1 (9 rats) and 1.5 mg (8 rats). Both ER agonists were dissolved in 0.1 ml of sesame oil, including 10% ethanol. A 0.1-ml aliquot of solvent was injected as the control. Since ERβ distribute restricted areas in the uteri, compared to ER α, injecting dose of DPN was higher than PPT.

Histological analysis

Adipose tissue was trimmed off quickly from the resected uteri. Next, the uteri were weighed and then fixed in bouin's fluid. Ten-μm-thick paraffin sections of the uteri were prepared and stained with haematoxylin and eosine. Uterine tissues were observed under light microscopy.

Statistical analysis

Student'st test or one-way analysis of variance (ANOVA) followed by a post hoc test were carried out to compare the ovarlan (ovarian) weights among the groups. Results were considered to be significant at the level of $p<0.05$.

Results

Histological changes of uteri in the estrous cycle

Mean uterine weights at stage P, E, D1 and D2 were 699 ± 22, 651 ± 42, 469 ± 37, and 580 ± 57 mg, respectively (Fig 1). Uterine weights at P and E were higher than those at D1 ($P<0.03$). Epithelial cells of the uteri at all stages were columnar with a clear nucleus in the center of the cells (Fig 2). Although epithelial cells at P and E seemed to be slightly taller than those at D1 and D2, there was no remarkable difference. Stroma of the endometrium, and the muscle layer were developed at all stages. There were no significant morphological differences in the uterine glands in the estrous stages except for some in P and E.

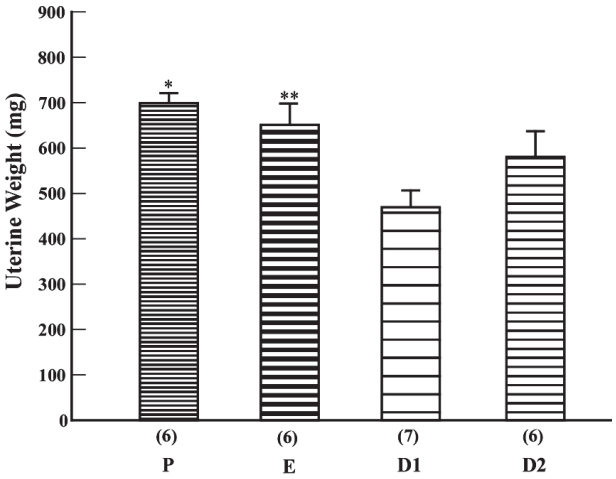

Fig. 1. Mean uterine weights in the proestrus (P), estrus (E) or diestrus 1 and 2 (D1 and D2, respectively) of female rats.
* $P<0.005$ vs D1. ** $P<0.03$ vs D1

Effects of phytoestrogens on the uteri

The mean uterine weight in the oil group was 180 ± 17 mg (Fig 4) and was lower than the mean weights in all of the estrous cycle stages (Fig.3). Histological examination showed that the endometrium and muscle layer were scanty, compared to those of the intact groups (Fig 3) and the height of the epithelium was low, consisting of cuboidal shaped cells with a little cytosol (Fig 4). The uterine glands were reduced. In the E_2 group, the uterine

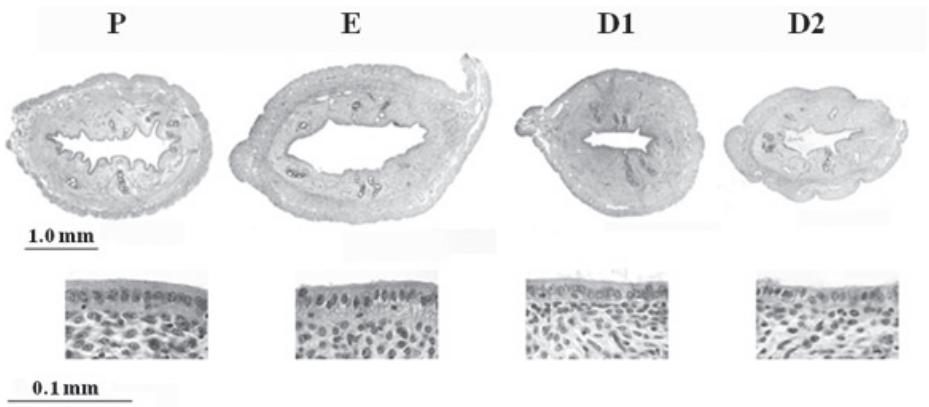

Fig. 2 Representative photomicrographs of a uterine frontal section and uterine tissue with epithelium (H-E stain) in the proestrus (P), estrus (E) or diestrus 1 and 2 (D1 and D2, respectively) of female rats.

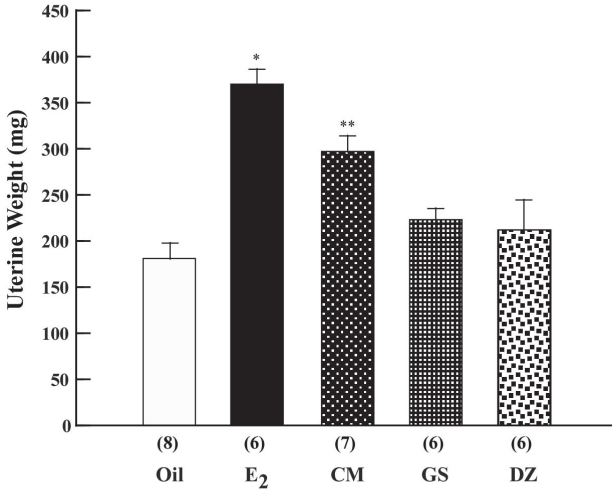

Fig. 3. Mean uterine weights in ovariectomized rats 24 hrs after injection with 1 mg estradiol (E2), phytoestrogens or oil. Phytoestrogens: CM:coumestrol; GS:genistein; DZ; daidzein.

* P<0.001 vs Oil, GS and DZ, P< 0.05 vs CM. ** P< 0.03 vs oil.

weight was 370 ± 16 mg, which was higher than that in the oil group (P < 0.001). Both the endometrium and myometrium of the E2 group uteri were larger than those in the oil group. In this group, the epithelium consisted of tall columnar cells with a clear nucleus in the basal portion of the cells. Large uterine glands were seen in the endometrium.

The mean uterine weight in the CM group was 297 ± 17 mg, which was higher than that in the oil group (P<0.03). In the GS and DZ groups, the mean uterine weights were 223±12 and 212±33 mg, respectively, but there were no statistically significant differences among these groups and the oil group. In the CM group, the endometrium and myometrium were developed and comparable to those in the E_2 group (Fig 4). However, the epithelium did not consist of tall columnar cells and there were no differences when compared to the oil group. Uterine glands were not developed well. In the GS and the

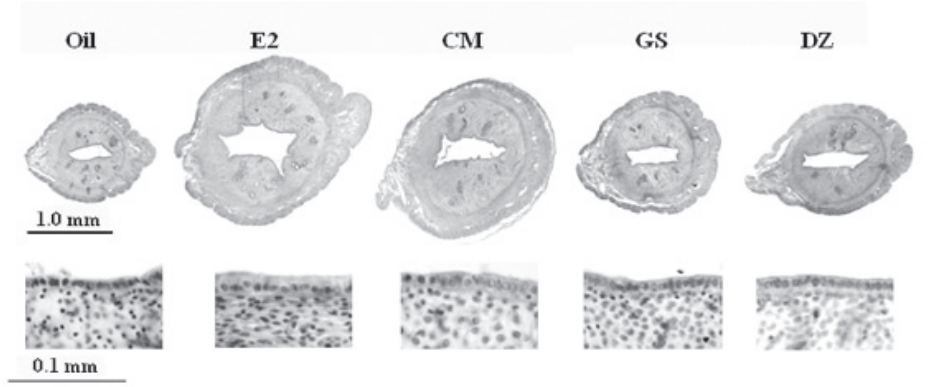

Fig. 4. Representative photomicrographs of a uterine frontal section and uterine tissue with epithelium (H-E stain) in ovariectomized rats 24 hrs after injection with 1 mg estradiol (E2), phytoestrogens or oil. Phytoestrogens: CM:coumestrol; GS:genistein; DZ; daidzein.

DZ groups, the endometrium and myometrium were not developed as well as those in the oil group and the epithelial cells and uterine glands were also similar to those in the oil group.

Effects of ERα and ERβ antagonists on the uteri

Mean uterine weights in the control group were 233 ± 8 mg (Fig.5). Morphological features in all parts of the uteri in the control group were similar to those in the oil-treated group. In the 0.5-mg and1.0-mg PPT groups, the mean uterine weights were 404 ± 20 mg and 393 ± 26 mg, respectively (both P<0.001 vs. control). Histological examination showed development of the endometrium and muscle layer of the uteri in the PPT group and both were larger than their counterparts in the oil group (Fig. 6). In this group the epithelium consisted of tall columnar cells, in comparison with the control group. The nuclei of the epithelial cells were clear in the PPT group. The uterine glands were not as large as in the intact groups.

In the 1.0-mg and 1.5-mg DPN groups, the uterine weights were 310 ± 21 mg and 327 ± 9 mg, respectively, which were higher values than that of the control group (both P<0.005). In the DPN groups, the endometrium and muscle layer were developed when compared to the control group, but seemed to be small in comparison with the PPT groups. The epithelium was lower than in the PPT groups. The uterine glands did not differ from those in the PPT groups.

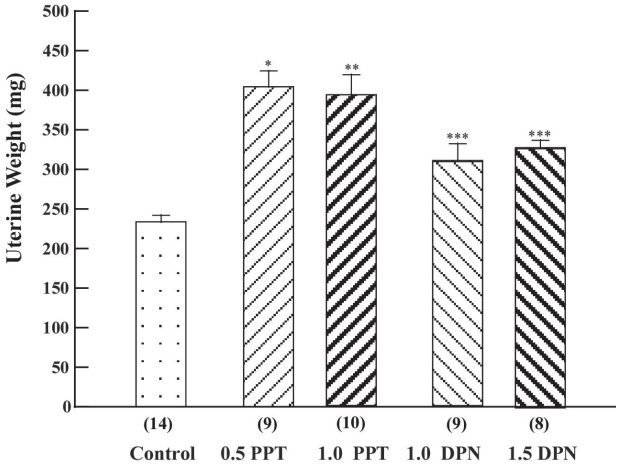

Fig. 5. Mean uterine weights in ovariectomized rats 24 hrs after injection with estrogen receptor α or β agonist (PPT or DPN) or solvent (Control). Injected doses of PPT were 0.5 mg or 1.0 mg. Injected doses of DPN were 1.0 or 1.5 mg.
* P< 0.001 vs Control, P<0.05 vs 1.0 and 1.5 DPN. ** P< 0.004 vs Control. *** P<0.005 vs Control.

Discussion

In accordance with the phases of the estrous cycle, small changes in the uterine weights were observed in this experiment, although the uterine weights in the proestrous and estrous phases were a little higher than in the diestrous phase in female rats. It is well known that the level of estrogen increases in the morning of the proestrous phase in rats (see review, Freeman 1994). Increase of uterine weights in proestrus and estrus phases are thought to be caused by an increase of estrogen secreted from matured follicle in the morning of proestrous phase, because 7 hrs is sufficient for the increase of uterine tissue by estrogen (Diel,2004). In

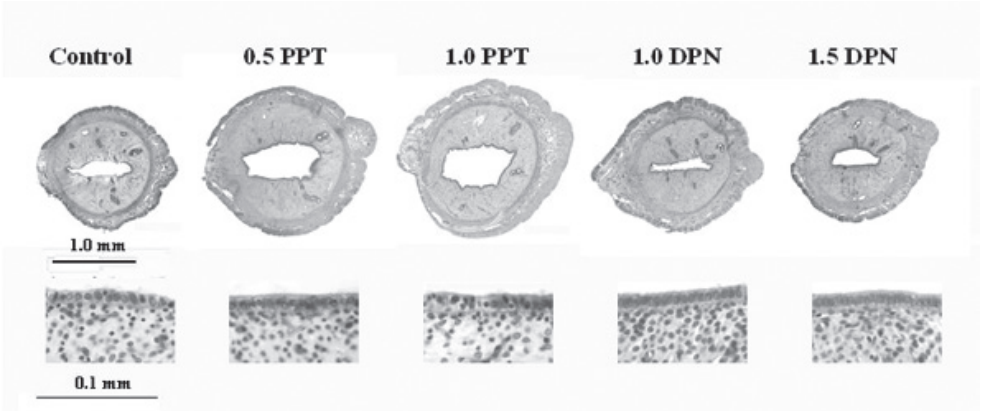

Fig. 6. Representative photomicrographs of a uterine frontal section and uterine tissue with epithelium (H-E stain) in ovariectomized rats 24 hrs after injection with estrogen receptor α or β agonist (PPT or DPN) or solvent (Control). Injected doses of PPT were 0.5 mg or 1.0 mg. Injected doses of DPN were 1.0 or 1.5 mg.

addition, basal secretion of estrogen throughout all estrous cycle phases reduces the range of change in uterine weights during the 4-day estrous cycle.

In this experiment, ovariectomy decreased the uterine weights to one-third to half of those in intact female rats in all phases of the estrous cycle and reduced the mucosa and muscle. Single injection with 1 mg of E_2 recovered the uterine weights and increased the mucosa and muscle layer with change in the luminal epithelial layer from cuboidal to columnar cells one day after injection in ovariectomized rats. It has been shown that mitotic activity in the luminal epithelium increases within 24 hrs of an injection with estrogen in rat uteri (Clark ,1971). However, the uterine weights in estradiol-treated ovariectomized rats were almost half the levels in the proestrous phase. This suggests that a single injection of estrogen is insufficient to recover the mucosa and muscle of the uterus to intact levels in ovariectomized rats.

In the phytoestrogen-treated ovariectomized rats, only 1mg CM was effective in increasing uterine weights and stroma, but not in epithelial cells. One mg GS and DZ were not enhanced uterine tissue. Injections of synthetic isoflavone had been reported to have no effects on the uterine weights of ovariectomized rats (Yamazaki, 1986). However, Diel et al. (Diel,2004) showed that an increase in uterine weights appeared 7 hrs and not 24 hrs after a single injection of 2.5 mg/kg bw GS in rats, suggesting that different molecular events lead to an increase in uterine tissue due to GS. Long-term oral administration of GS (Santell et al., 1997) as well as coumestrol (Whitten and Naftoline,1992) was also found to increase the uterine weight.

Accordingly, coumestan, such as CM, has a strong potency to increase the level of cellular activity in the rat uterus. In contrast, isoflavones such as GS or DZ have only a weak effect in ovariectomized rats. The higher potency of CM in increasing uterine weight than GS and DZ can be thought to be due to high binding affinity of CM to ER (Kuiper et al.,1997). However, in the experiment using an ER- ligand binding assay in the rat uterus, the relative binding affinity of CM to ER was found to be only 0.9 % that of E_2 and that of Genistein and Daidzein was half that of coumestrol (Branham et al., 2002). Furthermore, it has been reported in immature female rats that there is a difference in effects on the uterus between CM and E_2, because estrogen depleted cytosol ER resulting in the accumulation of nuclear ER, whereas CM increased cytosol ER (Marverich et al., 1995). Thus, effects of phytoestrogen on rat uteri is not strong.

CM and GS were reported to bind strongly to ERβ rather than ERα (Kuiper et al.,1997; 1998a: Harris et al., 2002a). In the PPT group, the uterine weights were higher than in the solvent control groups and the epithelial cells were similar to those in the E_2 group in this experiment. DPN also induced a slight increase in uterine weight, but the epithelial cells were not developed. Long-term treatment with PPT was found to be more effective for increasing the uterine weight in rats, because daily treatment with PPT for 6 weeks increased the uterine weight to the same level as treatment with E_2 in ovariectomized rats (Harris et al., 2002b). These results indicate the dominant role of ERα in changes in uterine tissue caused by estrogen.

In the uteri, both ERα and ERβ mRNA are present, but the level of ERα mRNA is much higher (see review Kuiper et al., 1998 b). In the report of a study using immunohistochemistry and in situ hybridization, ERα was observed in uterine epithelial, stromal and muscle cells, but ERβ was not detected in rats at the D1 stage of the vaginal estrous cycle. (Pelletier et al.,2000). The distribution of ER subtypes in the uteri is thought to be changed through the estrous cycle, because the levels of ERα mRNA in stage P was twice that in stage D1, but ERβ mRNA did not change throughout the estrous cycle (Wang et al.,2000). Furthermore it was shown that ERα immunoreactivity in the estrous stages of E and P were higher than in stage D in uterine epithelial, stroma and muscle cells (Wang et al.,2000).

In ovariectomized rats, both the mRNA levels of ERα and ERβ increased 24 hrs after treatment with 2.5 μg of E2; moreover, a high level of ERα mRNA and a low level of ERβ mRNA were expressed in the epithelial and stromal cells (Wang et al., 1999). In this paper, ERα was detected immunohistochemically in 65% of stromal cells, 90% of epithelial cells and all gland epithelium

cells. Furthermore, although the level of mRNA for ERα was higher than in the control, expression of ERα in the epithelial cells was decreased 3 hrs after a single injection with 4 μg/kg of E_2 in ovariectmized rats (Nephew et al., 2000). Thus, up-regulation and down-regulation for ERα by estrogen occurs at an early stage of weight increase of uterine tissues.

In ERα knockout mice, even if ovariectomized and treated with E_2, severe hypoplasia of the uterus were seen and the mean uterine weight was half that of wild-type females (Couse et al., 2000). Luminal and glandular epithelial cells of the uterus in ERα knockout mice are cuboidal (Couse et al., 2000). In contrast to ERα knockout mice, in ovariectomized ER β knockout mice, after treated with estrogen, uterine weight increased to the same level as that of wild mice (Lindberg et al., 2002). From these immunohistochemical and knockout experiments and the present results of phytoestrogen and ER subtype agonists, uterine weight gain by phytoestrogen is caused by stimulation mainly to ERα in many tissues in the uterus.

On the other hand, there was a slight increase in uterine weight caused by DPN in the present study. ERβ knockout female mice have been reported to become subfertile in terms of the frequency and size of litters (Couse et al., 2000; Harris, 2007; Krege et al,. 1998). In ERα knockout mice, ERβ has been shown to exert some functions of the uterus (Dupont et al., 2000). It has been revealed that ERβ modulates ERα activity in the uterus of immature rats. (Weihua et al., 2000; Frasor et al., 2003) . Coexistence of ERα and ERβ mRNA has been found in neuronal cells of rats (Chen et al, 1999). ERα and ERβ form a heterodimer and the binding affinity of the heterodimer to genes is comparable to that of ERα (Cowley et al., 1997). Thus the role of ERβ can not be excluded from the regulation of uterine tissue by estrogen. In addition, it should be noted that ER β is an important factor in the down-regulation of the EGF receptor, which is associated with decidualization of stromal cells by estrogen on the cell membrane in the mouse uterus (Wada-Hiraike et al., 2006). Further experiments are needed to clarify the relationships between ERα and ERβ in regulating the uterus.

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卵巣除去ラットの子宮に対する植物エストロゲンおよびエストロゲン受容体αまたはβ作動剤一回投与効果

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要　旨

卵巣除去ラットにおける子宮に対する植物エストロゲンとエストロゲン受容体α（ERα）およびβ（ERβ）作動剤投与による形態的な変化を調べた。実験１では卵巣除去ラットに1mgのcoumestrol（CM），genistein（GS），daidzein（DZ），β-estradiol（E_2）またはoilを皮下投与し、24 時間後に子宮を固定した。子宮は重量測定後、組織学的検索を行った。実験２ではERα作動剤であるpropyl pylazole triol（PPT、0.5または1.0 mg），ERβ作動剤であるdiarylpropionitrile（DPN, 1.0 または 1.5 mg)、またはその溶媒を投与し、最初の実験と同様に24時間後子宮の検索を行った。その結果、E2投与群の平均子宮重量と上皮細胞の高さはoil群より高かった。CM投与群では子宮がoil群より重くなっていたが、上皮細胞の高さは変わらなかった。GSとDZ群では子宮重量も上皮細胞もoil群と変わりはなかった。PPTを投与されたラットではどちらの量でも溶媒投与群に比較して子宮重量が重く上皮細胞の高さも高かった。一方、すべてのDPN群で子宮重量は溶媒投与群より重かったが、PPT投与群より軽かった。DPN群の上皮細胞の高さは溶媒群と同程度であった。これらの結果は植物エストロゲンの中ではCMが子宮に対して最も強い増加効果を持つが、E_2と比較するとかなり弱いこと、エストロゲンは子宮に対して主としてERαを介して作用するがERβも関与している可能性を示すものである。

Key Words：　植物エストロゲン, エストロゲン受容体α、エストロゲン受容体β、子宮、雌ラット

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