

## Expression of Estrogen Receptor $\alpha$ and $\beta$ in the Uterus and Vagina of Immature Rats Treated with 17-Ethinyl Estradiol

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**ABSTRACT.** The action of estrogen on target organs has been actively studied with the discovery of estrogen receptor (ER)  $\beta$ . This study was carried out to examine the expression of ER $\alpha$  and ER $\beta$  in the uterus and the vagina of immature Sprague-Dawley rats treated with 17-ethinyl estradiol (EE). Twenty days old rats were subcutaneously treated with EE at the doses of 0 (vehicle control), 0.03, 0.3, 1.0, 3.0, and 10.0  $\mu\text{g/kg/day}$  for three consecutive days. The treatment of EE at the doses of 0.3, 1.0, 3.0 and 10.0  $\mu\text{g/kg/day}$  significantly increased the weights of the uterus and vagina of rats ( $p < 0.01$ ) and retained fluid in the uterus of rats. At the high doses of 3.0 and 10.0  $\mu\text{g/kg/day}$ , the treatment of EE caused an increase in the uterine height, hypertrophy, and a decrease in the expression of ER $\alpha$  and ER $\beta$  in the uterine luminal and glandular epithelium. The treatment of EE at the doses of 3.0 and 10.0  $\mu\text{g/kg/day}$  also caused cornification and a decrease in the expression of ER $\alpha$  and ER $\beta$  in the vaginal epithelium. These results suggest that the EE treatment decrease the expression of ER $\alpha$  and ER $\beta$  in the uterus and vagina of immature rats and that may be associated with the morphological changes such as increase in the uterine height, hypertrophy of the uterine epithelium, and cornification of the vagina.

**KEY WORDS:** estrogen receptor  $\alpha$  and  $\beta$ , 17-ethinyl estradiol, uterus, vagina.

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Xenoestrogens (dietary or environmental estrogens) might affect the endocrine and reproductive systems and they are associated with the development of hormone-dependent cancers [5, 9, 21]. Estrogens influence the growth, differentiation and function of female reproductive organs such as uterus and vagina via estrogen receptor (ER). A specific estrogen binding was observed in some tissues of ER $\alpha$  knock-out (ERKO) mice, indicating a presence of different type of estrogen receptor [13]. ER $\beta$  was cloned from the rat [11] and sequenced from the human and the mouse [8, 19]. The presence of ER $\alpha$  predominates in uterus and vagina, whereas ER $\beta$  is present in a wide range of tissues including uterus, ovary, heart, lung and bladder [16]. The recent discovery of a new isoform of estrogen receptor (ER)  $\beta$  has stimulated the study for estrogen action on target organs. There are a few data about the expression of ER $\alpha$  and ER $\beta$  in rats treated with estrogenic compounds. The expression patterns of ER $\alpha$  and ER $\beta$  concurrently after administration of an exogenous estrogenic compound need to be studied. Among the several xenoestrogens including chlorinated organic compounds, plastics, pharmaceuticals, and fuel constituents, 17-ethinyl estradiol (EE) was a representative contraceptive, which had been usually used in combination with progesterone agents and tested in many clinical trials [2, 7, 10]. In this study, we investigated the morphological changes and the expression patterns of ER $\alpha$  and ER $\beta$  in the uterus and the vagina of immature rats treated with EE.

## MATERIALS AND METHODS

**Animals and chemicals:** Immature Sprague-Dawley female rats were obtained by the Department of Laboratory Animal Resources, the National Institute of Toxicology Research, Food and Drug Administration (Seoul, Korea). The animals were housed in polycarbonated cages with a 12 hr : 12 hr light-dark cycle and controlled humidity (55 ( 10% RH) and temperature ( $23 \pm 2^\circ\text{C}$ ). They were fed autoclaved PMI Feeds, Inc., Certified Rodent Diet #5014 and provided with chlorinated tap water *ad libitum*. EE was purchased by Shering Pharmaceutical Co. (Berlin, Germany).

**Treatments:** EE was injected subcutaneously to immature rats with the age of 20 days for three consecutive days. EE were dissolved in 95% ethanol and diluted to a final concentration with corn oil (Sigma Chemical Co., U.S.A.). All test solutions were prepared daily prior to the injection. The injection volume per rat did not exceed 4.0 ml/kg.

**Measurement of the weight of body, uterus, and vagina:** Twenty-four hours after the last treatment, rats were killed by cervical dislocation. The body of uterus was cut to open the uterine wall just above its junction with the cervix and at the junction of the uterine horns with ovaries, and carefully blotted to remove the excess fluid with filter papers. The vagina was removed from the uterus at the level of the uterine cervix.

**Histopathological examination:** Sections of the uterus and the vagina were examined after a serial process including fixation in 10% neutral phosphate buffered formalin, routine processing with alcohol and xylene, embedding in paraffin, sectioning at 4  $\mu\text{m}$ , and staining with hematoxylin

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and eosin (H&E).

**ER expression:** The sections (4  $\mu\text{m}$ ) of uterus and vagina were mounted on a silanized slide (S3003, Dako, Denmark) and subjected to de-waxing, rehydration, and endogenous quenching peroxidase activity using hydrogen peroxide (3% v/v). After washing with double-distilled water for 5 min, the sections were subjected to microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 10 min at 750 watts power. The sections were allowed to cool and were blocked with serum for 1 hr. ER $\alpha$  antibody (SC-7207, Santa Cruz Biotechnology, U.S.A.) and ER $\beta$  antibody (06-629, Upstate Biotechnology, U.S.A.) were diluted 1/400 and 1/600, respectively. The sections were incubated with the ER $\alpha$  antibody or the ER $\beta$  antibody for overnight at 4°C. Immuno-reaction complexes were detected by a avidin-biotin affinity system (SC-2018, Santa Cruz Biotechnology, U.S.A.) and visualized with 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. The sections were counterstained with Mayer's hematoxylin and examined under a light microscope. Additionally, several control sections were produced by omission of ER $\alpha$  antibody. For specific control for ER $\beta$ , the omission of antibody or pre-incubation of ER $\beta$  peptide (#12-368, Upstate Biotechnology, U.S.A.) was performed.

**Statistical analysis:** Data for the weights of body, uterine, and vagina of rats were statistically evaluated by one-way ANOVA. For a significant difference between treatment groups and the respective control, the Bonferroni *t*-test was performed at the level of  $p < 0.05$ .

## RESULTS

**Weight of body, uterus, and vagina:** There was no significant difference in body weight between the EE-treated groups and the vehicle control group. The treatment of EE increased the weights of the uterus and the vagina in a dose-dependent manner (Table 1). There were significant differences in the weights of the uterus and the vagina between the control and the EE-treated rats at the doses of 0.3, 1.0, 3.0, and 10.0  $\mu\text{g/kg/day}$  ( $p < 0.01$ ). The retention of fluid in the uterus was also found in the EE-treated rats.

**Histopathological examination in the uterus and vagina:** The treatment of EE at the high doses of 3.0 and 10.0  $\mu\text{g/kg}$

increased evidently the height of uterine luminal epithelium and caused hypertrophy of luminal and glandular epithelium in the uterus. Cornification of the vaginal epithelium was also observed in rats treated with EE at the doses of 1.0, 3.0 and 10.0  $\mu\text{g/kg}$ .

**ER expression in the uterus:** ER $\alpha$  expression in many luminal and glandular epithelial cells of uterus in control rats was characterized by a strong nuclear and weak cytoplasmic staining pattern. ER $\alpha$  was also localized to some nuclei of stromal and muscular cells (Fig. 1A). The treatment of EE at the doses of 3.0 and 10.0  $\mu\text{g/kg}$  decreased the expression of ER $\alpha$  in luminal and glandular epithelial cells and stroma and muscle cells in the uterus (Fig. 1B). Meanwhile, the expression of ER $\beta$  in control rats was characterized by a nuclear and cytoplasmic staining pattern similar to that of ER $\alpha$  (Fig. 1C). The ER $\beta$  was expressed in most of the luminal and glandular epithelial cells. Although ER $\beta$  expression was present in the endometrial stroma and myometrium of the uterus, the number of ER $\beta$ -positive staining cells was fewer than that of ER $\alpha$ . The treatment of EE decreased the ER $\beta$  expression in the luminal and glandular epithelial cells, but caused no change in the ER $\beta$  expression in the stroma and muscle cells in the uterus (Fig. 1D). Some cells showed a loss of nuclear staining.

**ER expression in the vagina:** In control rats, ER $\alpha$  and ER $\beta$  in the vaginal epithelial cells and stromal cells were stained intensively. The ER $\alpha$  was present in most of epithelial cells and in some stromal cells (Fig. 2A). The degree of the ER $\alpha$  staining intensity in the vagina was weaker than that in the uterus. The expression of ER $\beta$  was also weaker in the vaginal epithelium and stromal cells than that in the uterus. Meanwhile, EE treatment at the doses of 3.0 and 10.0  $\mu\text{g/kg}$  decreased the expression of ER $\alpha$  in the vagina epithelium (Fig. 2B). There was a moderate decrease in staining intensity of ER $\alpha$  in the lower portion of the vaginal epithelium. There was also a decrease in ER $\alpha$  staining cells in the upper portion of the vaginal epithelium. The positive staining of ER $\beta$  was observed in some epithelial and stromal cells in the vagina of the control rats (Fig. 2C). EE-treatment at the doses of 3.0 and 10.0  $\mu\text{g/kg}$  caused a decrease in the expression of ER $\beta$  in the vagina epithelium, but no alteration of the expression of ER $\beta$  in the stroma (Fig. 2D).

Table 1. Effects of 17-ethinyl estradiol on the weight of body, uterine, and vagina of rats

Group	Treatments	No. of rats	Body Weight	Uterine Weight	Vaginal weight
			(g)	(mg)	(mg)
1	Vehicle control	6	38.3 $\pm$ 4.4	16.1 $\pm$ 4.0	19.8 $\pm$ 4.7
2	EE (0.03 $\mu\text{g/kg}$ )	6	40.8 $\pm$ 2.9	17.2 $\pm$ 2.4	22.5 $\pm$ 4.7
3	EE (0.3 $\mu\text{g/kg}$ )	6	36.9 $\pm$ 2.7	31.0 $\pm$ 6.7**	29.1 $\pm$ 3.1**
4	EE (1.0 $\mu\text{g/kg}$ )	6	40.4 $\pm$ 2.2	94.7 $\pm$ 7.8**	63.4 $\pm$ 8.1**
5	EE (3.0 $\mu\text{g/kg}$ )	6	38.1 $\pm$ 3.6	102.3 $\pm$ 9.0**	55.6 $\pm$ 12.7**
6	EE (10.0 $\mu\text{g/kg}$ )	6	41.5 $\pm$ 3.5	110.7 $\pm$ 11.2**	56.3 $\pm$ 7.1**

EE : 17-ethinyl estradiol.

Data represent mean  $\pm$  S.D.

\*\* Significantly different from the vehicle control ( $p < 0.01$ ).

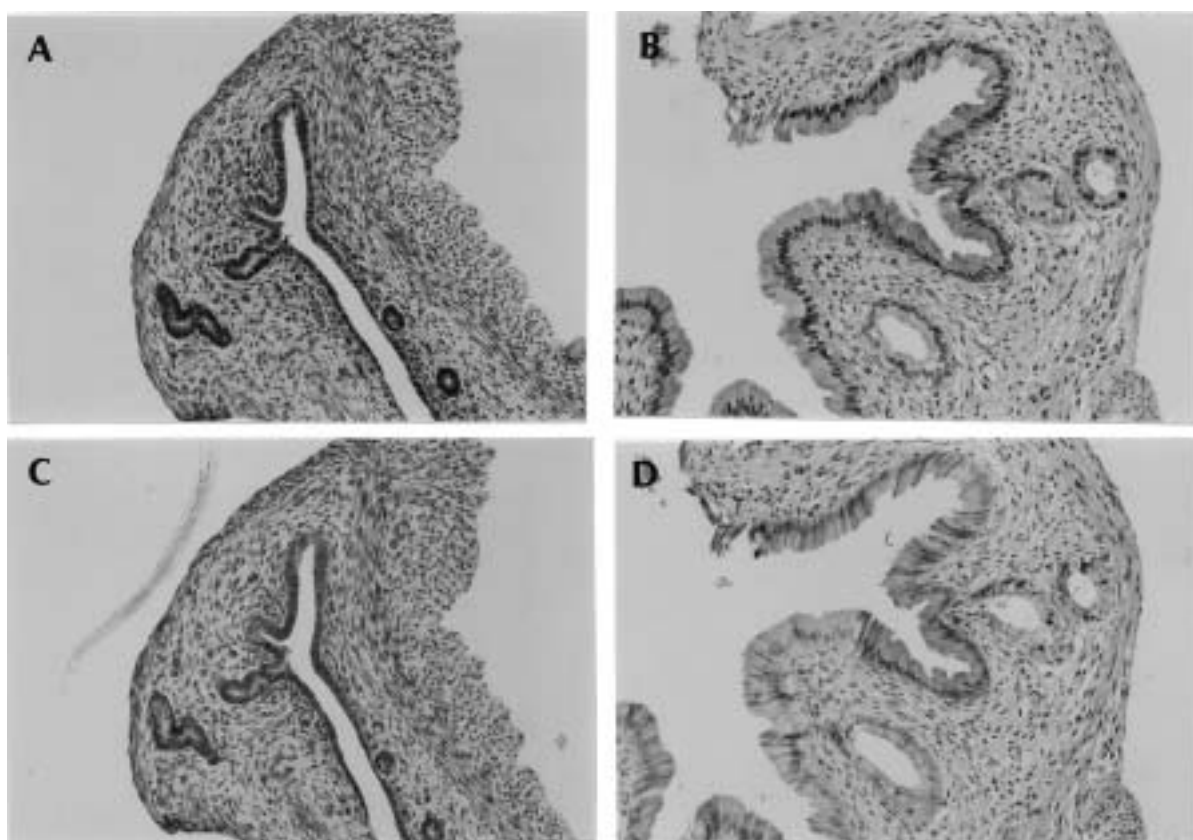


Fig. 1. Immunohistochemical staining for estrogen receptor (ER) expression in the uterus of rats. (A) ER $\alpha$  expression of the vehicle control rat, (B) ER $\alpha$  expression of the rat treated with 17-ethinyl estradiol 10  $\mu$ g/kg body weight. (C) ER $\beta$  expression of the vehicle control rat, (D) ER $\beta$  expression of the rat treated with 17-ethinyl estradiol 10  $\mu$ g/kg body weight. Magnification:  $\times 250$ .

## DISCUSSION

The uterus and vagina regulated by female sex steroids has been used as the target organs to evaluate the estrogenic action of a compound [12]. The uterus is composed of heterogeneous cell types (stroma, luminal epithelial, glandular epithelial, and smooth muscle), that undergo continuous synchronized changes of proliferation and differentiation in response to changes in levels of circulating estrogen and progesterone. In this study, EE-treatment increased wet and blotted weight of the uterus and vagina in a dose-dependent manner. The treatment of EE at the high doses including 3.0 and 10.0  $\mu$ g/kg/day caused uterine hypertrophy and cornification of vagina. The decrease in the expression of ER $\alpha$  and ER $\beta$  was also observed at the high doses above 1.0  $\mu$ g/kg. These findings suggest that the morphological changes may be associated with the alteration of ER expression. Although the treatment of EE at the low doses below 1.0  $\mu$ g/kg had no significant changes in the uterus and vagina, as measured by above parameters, the effects were numerically dose-dependent. The additional measurements such as uterine epithelial cell height [3], quantitative uterine ER content [4, 17], and gene expression [6] might need to evaluate fur-

ther effect of EE on female reproductive organs.

The transcriptional effects of EE in the body are mediated by two distinct ERs, ER $\alpha$  and ER $\beta$ . A definitive role for ER $\alpha$  in the uterotrophic effect of EE was confirmed in adult female ER $\alpha$  knockout mice, where there is loss of estrogen responsiveness [13]. ER $\beta$  is present in both endometrium and myometrium in rats, and its function in the uterus is reported to play an important role in the modulation of the expression of ER $\alpha$  [16]. Staining for ER $\alpha$  or ER $\beta$  antibody was localized to the nuclei of epithelial cells and stromal and muscle cell types in the uterus of rats. The pattern of the expression in rats was similar to that in human tissue [18]. Some cells had both nuclear and cytoplasmic staining patterns. The treatment of EE caused a decrease in the expression of ER $\alpha$  or ER $\beta$  in the uterine luminal and glandular epithelial cells, and stroma and muscle cells as well as a loss of staining uniformity. Uterine cell types might have different response to estrogen with respect to the degree of proliferation [14] and steroid hormone receptor expression [15]. A recent study showed that ER $\alpha$  protein was reduced in response to estrogen through rapid proteolysis without alteration to mRNA [1], representing that the direct proteolysis of receptors might be an early event in the estrogenic action.

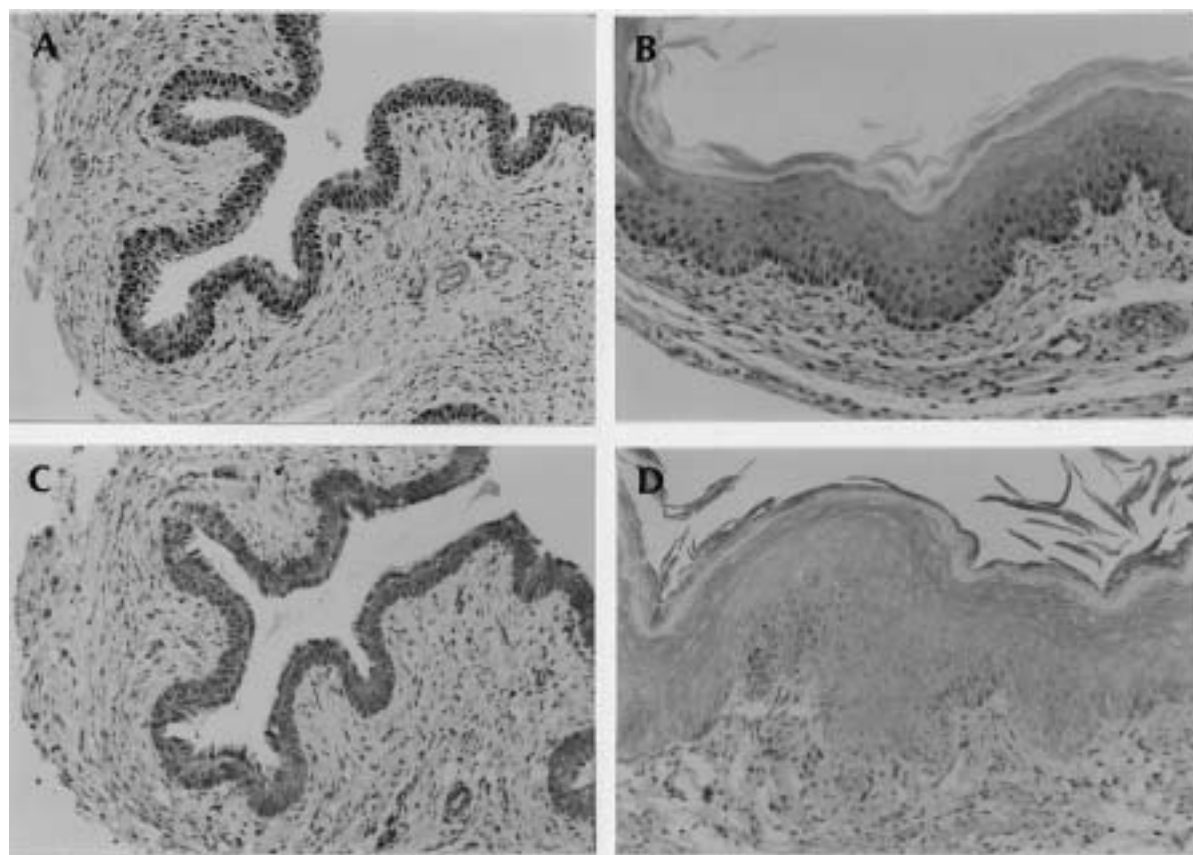


Fig. 2. Immunohistochemical staining for estrogen receptor (ER) expression in the vagina of rats. (A) ER $\alpha$  expression of the vehicle control rat, (B) ER $\alpha$  expression of the rat treated with 17-ethinyl estradiol 10  $\mu$ g/kg body weight. (C) ER $\beta$  expression of the vehicle control rat, (D) ER $\beta$  expression of the rat treated with 17-ethinyl estradiol 10  $\mu$ g/kg body weight. Magnification:  $\times 250$ .

Time course studies with estradiol represented that ER mRNA in the uterus of rats decreased initially, but returned to a basal level at 16–20 hr after injection [22]. Therefore, it seemed to be important to set the relevant time points. Recently, ER $\beta$  was reported to have an action of antiproliferative function in the immature uterus and a regulatory action on ER $\alpha$  [20]. Thus, the decreased number of two ER subtypes might contribute to loss of control in cell proliferation.

The treatment of EE at the high doses of 3.0 and 10.0  $\mu$ g/kg caused a decrease in the expression of ERs as well as a remarkable cornification in the vaginal epithelium. The treatment of EE caused a loss of uniform staining intensity of ER $\alpha$  and ER $\beta$  in the vaginal epithelial and stromal cells. It was thought that EE treatment might induce the entrance of estrous cycle in immature rats, thereby resulting in the cornification and the alteration of staining intensity of ERs in the vaginal epithelium.

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