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Immunohistochemical Study on the PCNA–Immunoreactivity in the Uterus of Rats Ovariectomized or Treated With Antiestrogen Clomiphene Citrate

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Abstract: PCNA (proliferating cell nuclear antigen) immunoreactivity in the uterus of mature rats was investigated after long term administration of clomiphene citrate and ovariectomy.

In the luminal epithelium and glandular epithelium and endometrial stroma regions, few PCNA–immunoreactive cells were observed.

PCNA reactivity was estimated for the luminal and glandular epithelium and for the stromal cells.

Treatment of clomiphene citrate decreased the proliferation of the luminal epithelial cell and stromal cells to the same extent as ovariectomy. In both of these groups, the proportion of anti–PCNA positive cells in the glandular epithelium was significantly higher than in the luminal epihelium and stromal cells. Clomiphene citrate has a strong antiproliferative effect on the uterus of rats, and this antiestrogenic action is specific for cell types.

Key Words: Clomiphene citrate, Uterus, PCNA immunohistochemistry.

Introduction

Immunocytochemical descriptions of PCNA immunoreactive cells in the mature uterus have been researched by several researchers (1, 2, 3). Studies have been done on the effects on the uterus tissue of non–steroidal antiestrogens (species–specific antiestrogenic and estrogenic effects) (4, 5, 6, 7). Clomiphene citrate is known to be an ovulation inducer (8). Therefore, it is an antiestrogen because of its competitive inhibition of estrogen binding to estrogen receptors, and it is used as an estrogen antagonist in the treatment of human breast cancer (9). It is a triphenylene derivate which has both estrogenic and antagonist activity, and has the effect of regulating the hypothalamo–pituitary hormonal release. However, it is known to have estrogen agonist activity in the reproductive tracts. Of special interest is the fact that several clinical studies have found an increased incidence of endometrial carcinoma (11) and hyperplasia (12) in long term antiestrogen users. These effects, which have been observed in postmenopausal women, are thought to be due to estrogenic stimulation of the endometrium by antiestrogen.

Data derived from these experiments have shown not only various effects of the drug in different speices, but also multiple effects on endometrial receptivity, implantation capacity, egg transport, ovum maturation, blastocyt development and outcome of pregnanacy (11).

The effects of clomiphene citrate on the genital tract also depend upon the hormonal status of the subject under study and the duration of treatment (13).

The effects of estrogen antagonists on the uterus have been observed in the short–term treatment of immature or ovariectomized animals a hormonal situation comparable to that of the postmenopausal woman in laboratory investigations. In these animal models, they have been shown to display both weak agonistic and strong antagonistic actions in rodents. With regard to the use of Clomiphene citrate as a preventive agent in the uterine morphology of rats during preimplantation stages, pregnant animals should be suitable models for studying the effects of clomiphene citrate on the uterus. In intact mature rodents, long–term administration of clomiphene citrate causes a decrease in uterine weight as in ovariectomized rats (14), but which of the in the uterine epithelial morphology, the several uterine tissues

are involved in this antiuterotrophic effect has not precisely characterized.

The present study was designed to survey the effects of long–term administration of clomiphene citrate on proliferation of the uterus in mature rats. We used a monoclonal antibody against the proliferating cell nuclear antigen (PCNA) to estimate the proliferative activity in the luminal and glandular epithelium and in the endometrial stroma. Untreated mature and ovariectomized rats were used as controls.

PCNA is an auxilary protein for DNA polymerase delta (15), and is detectable by immunohistohemistry in cells which enter the cell cycle at late G1, with the strongest staining intensity during the S–phase (16).

Materials and Methods

Mature female Wistar rats were used for the present study. Animals were kept under controlled conditions of lighting and room temperature. The animals were fed ad libitum.

Clomiphene citrate (50 mg Clomen tablets, Yurtoğlu Company, Türkiye) 20 mg/kg/d was dissolved and administrated by the way of gastric lavage 6 times per week a period of 8 weeks (n:6). Eight mature female rats at different stages of the estrus cycle, and 6 rats ovariectomized in the 8–week period were used as controls. The animals were sacrificed under ether.

The uterine horns were removed, cut into several pieces and fixed overnight in 10% formaline solution. They were passed through an ethanol, xylene series and embedded in paraffin. The stage of the estrus cycle in the control animals was ascertained from the vaginal smear stained with Papanicola methods.

Immunohistochemical detection of PCNA was carried out with the monoclonal antibody PC10. All sera used were purchased from Dako. Sections were cut at 3 µm thickness and mounted on gelatin–coated glass slides, deparaffinized in three changes of xylol and dehydrated in graded ethanol. After washing in distilled water, the sections were placed in 10 mmol citrate buffer, pH 6.0, and cooked in a pressured cooker for 1 min. Care was taken to ensure that the sections were always covered with the buffer. The sections were cooled to room temperature for 20 min. All the following steps were carried out at room temparature.

Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide in methanol 30 min. rabit serum to reduce the unspecific binding.

They were then treated with PC10 for 60 min. The sections were rinsed in PBS and reacted with rabbit anti–mouse and administratired for 10 min. It was followed for 10 min by a PAP complex (rabbit: 1/100). The peroxidase reactions were developed in a solution of 3.3 diaminobenzidine tetrahydropchloride–H_2O_2 (0.003% w/v Tris–HCl buffer) for 15 min. Developed sections were lightly counterstained with Mayer's Hematoxylin, dehydrated in a graded series of ethanols, cleared in xylene and cover–slipped. For positive controls, sections of human palatine tonsil were used. All sections were examined with a light microscope.

To estimate the relative frequency of PCNA immunoreactive cell typs in the luminal and glandular epithelium, endometrial stromal cells were determined by counting a total number of 150 cells for each cell type, per section in a minimum of two sections from different uterine segments for each animal, and expressing the PCNA reactivity as the number of positive cells per 50 cells. The cells were counted at a magnification of 1000X.

For statistical analysis of the mean values of the proliferative reactivity, Student's t–test was used.

Results

Three immunoreactive cell types (luminal and glandular epithelium, stromal cells) for PCNA were identified in the uterus. Immunoreactivity was observed in positive control sections. Staining with the PCNA antibody was virtually confined to the nucleus, but did occasionally overlap into the cytoplasm. Cells with PCNA reactivity displayed dark homogeneous staining.

The percentage of immunoreactive cells in the uterus in the experimental group from was different from that of normal rats. The PCNA immunoreactive cells in the glandular and luminal epithelium and endometrial stromal cells were found to be positive in animals treated with Clomiphene citrate and ovariectomy. However, the proportion of PCNA immunoreactive cells also varied considerably between different glandular epithelia in all the rats examined. Epithelial and stromal cells with PCNA–positive nuclei were found in all the control group animals except those in diestrus phase.

Table 1 summarizes the regional distribution and relative frequency of the PCNA reactive cells in the uterus.

The proliferation was high during proestrus and metestrus, but intermediate during estrus (Figs 1a, b, c). In the glandular epthelium, a high proliferation reactivity was found during metestrus (Fig. 1b), whereas this was lower during proestrus (Fig. 1a), low and low during

Table 1. PCNA immunoreactivity in the uterus of rats after control, ovariectomy and Clomiphene citrate.

Experimental group		n epithelium	luminal epithelium	glandular	stromal cell
Control	metestrus	2	71.6	83.5	4.4
	diestrus	2	0	0	9.4+3.4
	proestrus	2	57.5±7.8	24.4+6.8	3.6+2.9
	estrus	2	24.2+6.7	5.5+1.6	7.2+3.1
Ovariectomy		7	1.5+1.1	6.2+4.1	1.3+0.9
Clomiphene citrate		6	1.0+1.1	9.2+4.9	0.3+0.3

estrus (Fig. 1c). Rats in diestrus did not display PCNA immunoreactivity in the luminal and glandular epithelia, but endometrial connective tissue displayed more anti–PCNA positive nuclei than in the other stages of the estrus cycle. The highest PCNA activities were found in the stromal cells in diestrus rats (Fig. 1d).

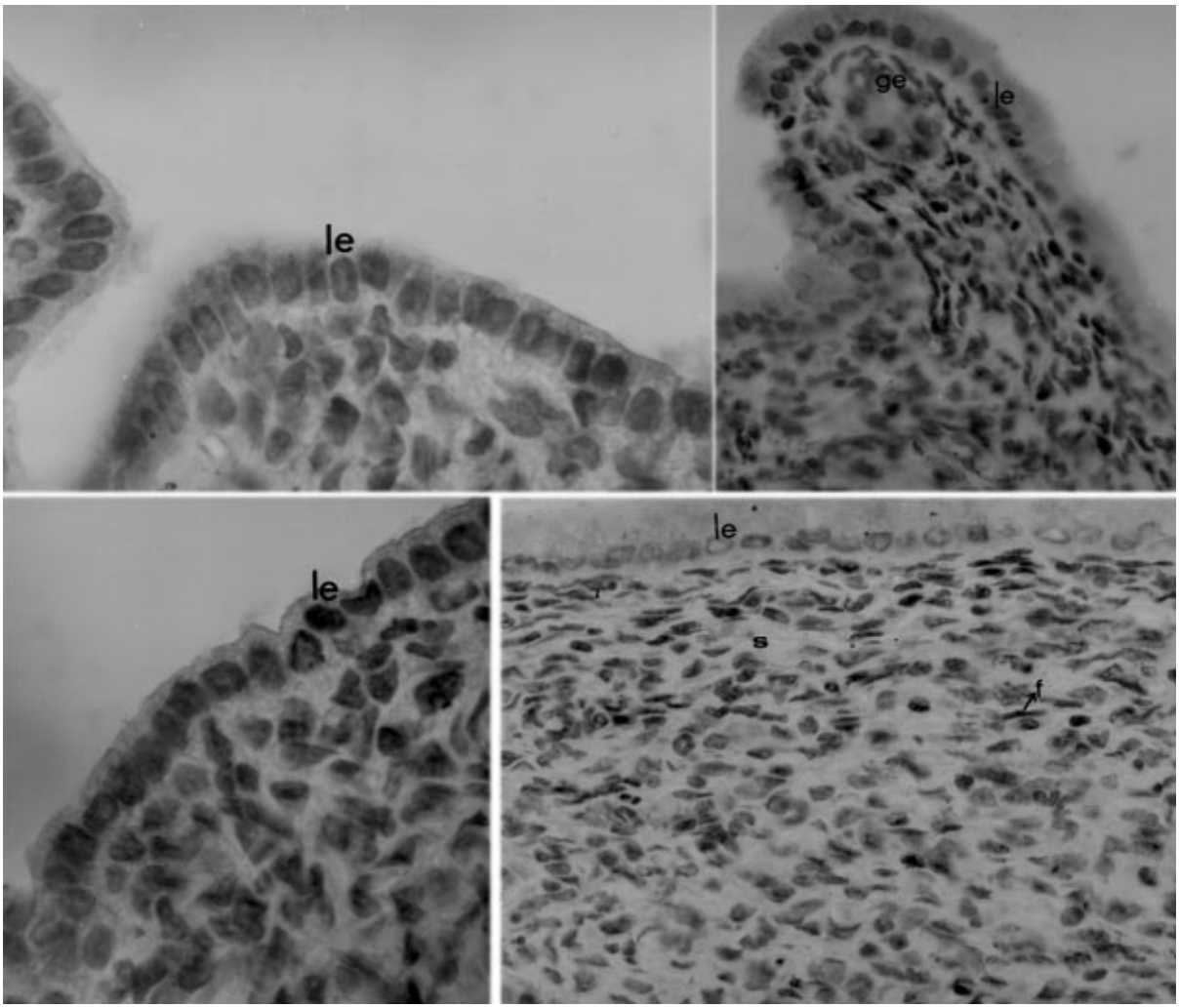

Figure 1. Immunohistochemical staining for PCNA in the uterus of rats. a) PCNA positive nuclei in the luminal epithelium from a rat in proestrus, X100. b) Positive cells in the luminal and glandular epithelium from a rat in metestrus, X40. c) A few positive luminal epithelium cells from a rat in estrus, X100. d) The highest PCNA positive activity in the stromal cells from a rat in diestrus. Luminal epithelial cells are PCNA negative, X40. le: luminal epithelium, ge: glandular epithelium, s: stroma, f: fibroblast.

In the ovariectomized rats, PCNA–positive cells were occasionally observed in the luminal and glandular epithelia (Fig. 2a). Whereas in some areas, the number of PCNA positive cells in the glandular epithelium was significantly lower than in the luminal epithelium (Fig. 2b).

Clomiphene citrate treatment suppressed anti PCNA immunoreactivity in the uterus. In the luminal epithelium and endometrial stroma, PCNA reactivities were very low (Fig. 3a), whereas in the glandular epithelium the proliferation activity was higher than in the luminal epithelium and stromal cells (Fig. 3b).

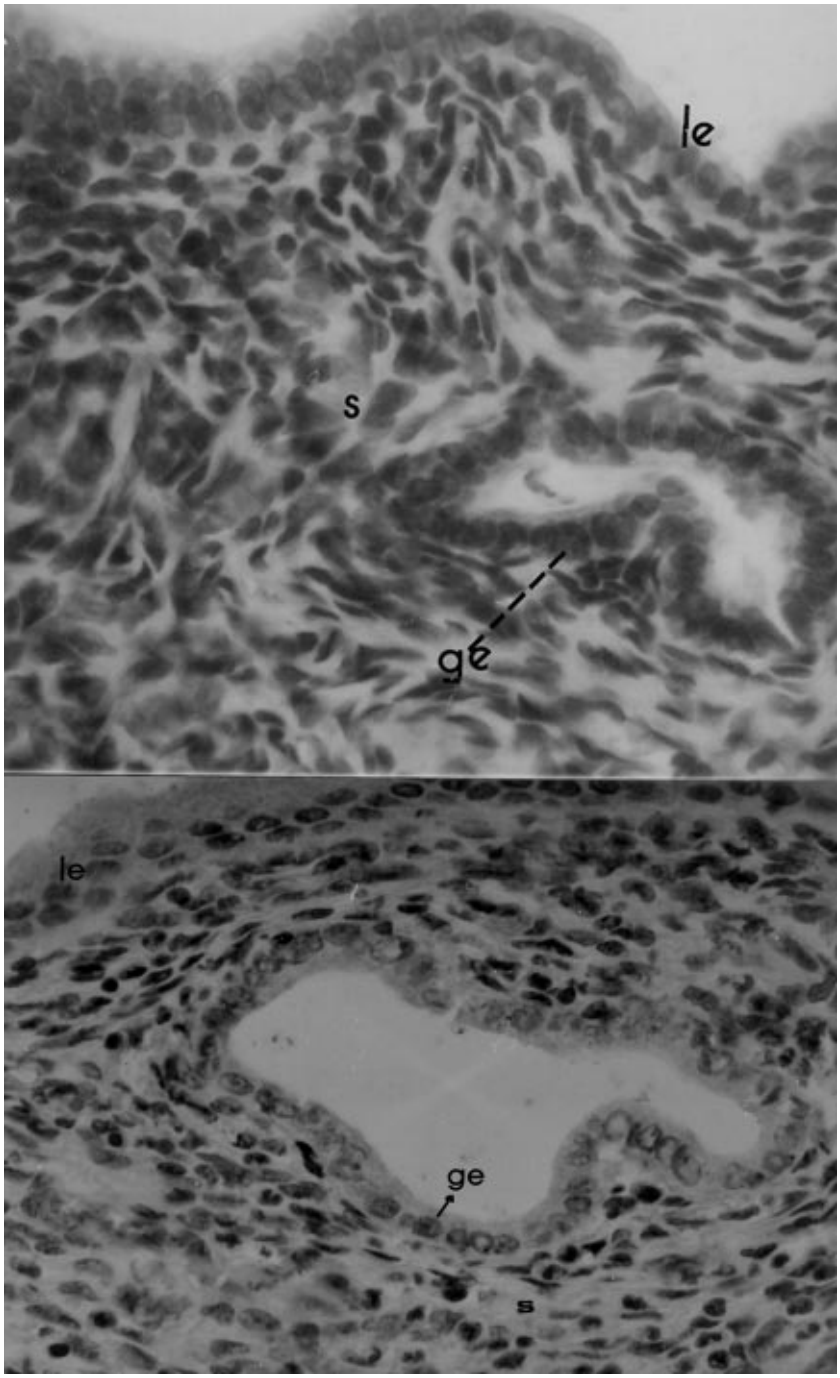

Figure 2. Immunochemical staining for PCNA in the uterus of rats after ovariectomy. a) PCNA positive cells are occasionally observed in the luminal and glandular epithelium cells, X40. b) PCNA positive cells are lower in the glandular epithelium than in the luminal epithelium, X40. le: luminal epithelium, ge: glandular epithelium, s: stroma.

The mean values of the proliferation activity of ovariectomized and Clomiphene citrate treated rats showed no statistical difference (P<0.01).

Discussion

The present immunocytochemistry investigated the effects of administration of Clomiphene citrate on the proliferative activity of epithelial and stromal cells in the uterus of mature rats. Untreated normal and ovariectomized rats were used as controls.

For the uterus tissue of the rat, PCNA may be suitable for detection of proliferation.

In the control group, PCNA reactivity varied with the phases of the estrus cycle. PCNA immunoreactivity in the epithelial cells in the two rats in diestrus was not present but the stages of the other estrus cycls in the control animals, PCNA reactivity was present in the epithelial cells.

The presence of PCNA–immunoreactive uterine tissues has been reported. Rumpel et al. in 1994 demonstrated a small number of PCNA immunoreactive cells in the uterus of mature rats after long–term administration of tamoxifen (3).

The results of our study demostrate the antiproliferative effect on the uterus of long term administration of Clomiphene citrate in mature rats. Antiproliferative response was strong in the luminal epithelium and in the endometrial stroma, as shown by the very low PCNA reactivity and weakness in the gland epithelium in the same areas. The antiestrogenic effects were comparable to those of ovariectomy. Rumpel et al. observed uterine atrophy following long–term treatment with tamoxifen in adult rats (17). Clomiphene citrate and its isomers show agonistic and antagonistic effects (18).

That estrogen antagonists inhibit the mitogenic effects (18) the uterine luminal epithelium to estrogens has been found during short, term experiments on immature rats with the antagonists nafoxidine and clomiphene citrate (19).

In our study, Clomiphene citrate inhibited the mitogenic responses such that mitotic figures were not found in the uterus epithelium or glands. There are differences in the biological properties of the antiestrogens (20), and the duration of treatment is more important. Martin (21) has shown that tamoxifen causes an initial stimulation of stromal mitosis in ovariectomized rats, but continuing the treatment decreases the number of proliferating stromal cells.

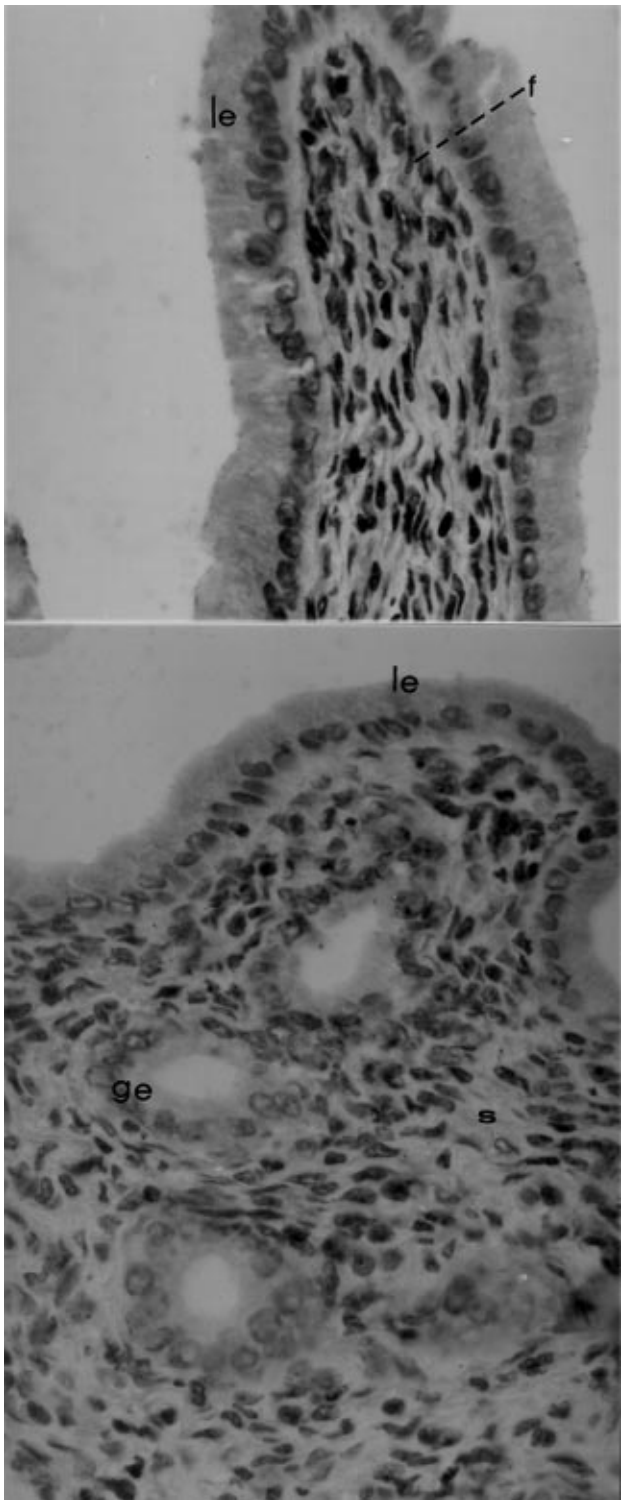

Figure 3. The uterus tissue after Clomiphene citrate treatment. a) Luminal epithelium and endometrial stroma are negative, X40. b) Many glandular epithelium display PCNA immunoreactivity whereas luminal epithelium and stromal cells are negative X40.

To assess the effects of steroidal antiestrogen on postnatal uterine development, rats given ICI 182.780 on postnatal days. Immunocytochemical analysis has revealed that antiestrogen reduces the uterine estrogen receptor, and immunoreactivity in all uterine cell types (22).

Poteat WL (1981) has shown that Clomiphene citrate does not induce glycogen deposition in the uterine luminal epithelium of pregnant rats as it did in ovariectomized rats. However, the drug did alter the epithelial morphology, which may be a factor in its postcoital contraceptive action (14).

Sato et al. (1996) demostrated that tamoxifen acts as an estrogen receptor inducer in the uterus and vagina of neonatal and ovariectomized adult mice (9).

In the uterus of immature or ovariectomized rats, tamoxifen causes several different estrogenic responses, such as increase in uterine weight and DNA content. On the other hand, the drug antagonizes the estradiol–induced uterine weight gain in ovariectomized rats (23).

We conclude that the present antiestrogens have essentially similar effects. Our conclusions are similar to those previously reported. Therefore, clomiphene citrate and its isomers have no known long–term effects on the uterus. Clomiphene citrate has an antiproliferative effect on the uterine epithelial and stromal cells. This effect is comparable to that of ovariectomy. Furthermore, the patterns of antiproliferative activity in luminal and glandular epithelial cells were observed to differ.

References

1. Yu CC–W, Wilkinson N, Brito MJ, Buckley CH, Fox H, Levison DA. Patterns of immunohistochemical staining for proliferating cell nuclear antigen and p53 in benign and neoplastic human endometrium. histopathology. 23: 367–371, 1993.
2. Li S, Nakayama K, Masuzawa H, Fujii S. The number of prolifeating cell nuclear antigen positive cell in endometriotic lesions differs from that in the endometrium. Analysis of PCNA positive cells during the menstrual cycle and in post–menopause. Virchows Archiv A Pathol Anat. 423: 257–263, 1993.
3. Rumpel E, Michna H, Kühnel W. PCNA–Immunoreactivity in the uterus of rats after treatment with the antiestrogen Tamoxifen. Ann Anat. 177: 133–138, 1995.
4. Furr B, Jordan VC. The pharmacology and clinical uses of tamoxifen. Pharmacol Ther. 25: 127–205, 1984.
5. Lu–YC, Chatterton RT Jr. Effects of the antiestrogen anordiol on ovulation and endometrial histology in the rat. 9(3): 257–68, 1993.
6. Lu–YC, Chatterton RT Jr. Effect of anordiol on ovarian hormone secretion, ovulation, and uterine and vaginal responses in the immature rat. 10 (2): 157–66, 1994.
7. Peters AJ, Wentz AC, Kazer RR, Jeyendran RS, Chatterton RT Jr. Estrogenic and antiestrogenic activities of anordiol: a comparison of uterine and vaginal responses with those of clomiphene citrate 52 (37: 195–202, 1995.
8. Karabinus DS, Gelety TJ the impact of sperm morphology evaluated by strict criteria on intrauterine insemination success. Fertil. Steril. 67 (3): 536–41, 1997.
9. Sato T, Ohta Y, Okamura H, Hayashi S, Iguchi T. Estrogen receptor (ER) and its messenger ribonucleic acid expression in the genital tract of female mice exposed neonatally to tamoxifen and riethylstilbestrol. Anat Rec. 244 (3): 374–85, 1996.
10. Correy JF. Marsden DE, Schokman FCM: The outcome of pregnancy resulting from clomiphene–induced ovulation. Aust NZ J Obstet Gynecol 22: 18–21, 1982.
11. Fornander T, Rutqvist LE, Cedermark B, Glas U, Mattsson A, Siljversward C, Skoog L, Somell A, Theve T, Wilking N, Askergen J, Hjalmer ML. Adjuvant tamoxifen in early breast cancer: occurence of new primary cancers. Lancet i: 117–120, 1989.
12. Lahti E, Blanco G, Kauppila A, Apaja–Sarkkinen M, Taskinen P, Laatikainen T. Endometrial changes in postmenopausal breast cancer patients receiving tamoxifen. Obstet Gynecol. 81: 660–664, 1993.
13. Clark JH, Guthrie SC. Agonistic and antagonistic effects of clomiphere citrate and its isomers. Biol Reprod. 25 (3): 667–72, 1981.
14. Poteat WL. Uterine morphology and glycogen deposition of pregnant rats after clomiphene citrate treatment during preimplantation stages. Experientia 37 (1): 84–6, 1981.
15. Bravo R, Frank R, Blundell pa, Mcdonald–Bravo H. Cyclin–PCNA is the auxilary protein of DNA polymerase–6. Nature. 326: 515–517, 1987.

16. Celis JE, Celis A. Cell cycle–dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: Subdivision of S phase. proc Natl Acad Si. 82: 3262–66, 1985.

17. Rumpel E, Schibert A, Duchesne MJ. Immunoshistochemischer Nachweis des Major Basic Protein (MBP) der eosinophilen Granulozyten im uterus der Ratte nach Gabe von Steroid hormonantagonisten. Ann Anat Suppl. 176: 118, 1994.

18. Clark JH, Markaverich BM. The agonistic–antagonistic properties of clomiphene citrate a riviev. Pharmacol Ther. 15: 467, 1982.

19. White Jo, Moore PA, Elder MG, Lim L. The relationship of the oestrogen and progestin receptors in the abnormal testosterone propionate or clomiphene citrate. Biochem J. 15: 196 (2): 557–65, 1981.

20. Terenius L. Structure–activity relationships of antiestrogens with regard to interaction with 17 β–oestradiol in the mouse uterus and vagina. Acta Endocrinol. 66: 431–447, 1971.

21. Martin L. Effects of antiestrogens on cell proliferation in the rodent reproductive tract. In: Sutherland RL, Jordan VC (eds) non–steroidal antiestrogens. Molecular Pharmacology and Antitumor activity Academic Press, Sidney New York London Toronto San Francisco. 1981.

22. Branham WS, Fishman R, Streck RD, Medlock KL, De–George JJ, Sheehan DM. ICI 182.780 inhibits endogenous estrogen–dependent rat uterine growth and tamoxifen–induced developmental toxicity. Biol Peprod. 54 (1): 160–7, 1996.

23. Jordan VC, Haldemann B, Allen KE. Geometric isomers of substituted triphenylethylenes and antiestrogen action. Endocrinology. 108: 1353–1360, 1981.