The Histopathological Changes in Rat Ovary and Endometrium Following Long Term Clomiphene Treatment

Uzun Dönem Klomifen Tedavisinin Rat Ovaryum ve Endometriyumu Üzerinde Yaptığı Değişikliklerin Histopatolojik Yönden Araştırılması

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ABSTRACT Objective: Clomiphene citrate (CC) a nonsteroidal tissue selective estrogen receptor modulator is used for inducing ovulation. In this study, we aimed to investigate the histopathological changes and dose related effects of long term CC treatment on rat ovary and endometrium. **Material and Methods:** Four groups of female rats were included in the study. In group 1 (control) only saline was administered whereas in groups 2, 3 and 4 CC was (once a week) administered for 18 weeks at the doses of 1 mg/kg, 4 mg/kg and 8 mg/kg, respectively. Uterus and both ovaries were excised for histopathological evaluation. **Results:** Histopathological examination showed that papillary hyperplasia of the surface epithelium of the ovary was present in all CC treated groups, with the highest percentage and severity in group 4. The mean values of endometrial thickness and endometrial gland diameters in this group were lower than those in groups 1 and 2 ($p<0.05$). Diameters of ovaries and ovarian surface epithelium in group 4 were decreased with respect to control group ($p<0.05$). **Conclusion:** High doses and repetitive treatment with CC may have detrimental effects in terms of endometrial thinning and developing precancerous lesions in the ovaries.

Key Words: Clomiphene; pathology; endometrium; ovary; rats; time

ÖZET Amaç: Klomifen sitrat (CC) ovulasyon indüksiyonunda kullanılan, steroid yapıda olmayan, seçici özellikte östrojen reseptör modülatörüdür. Bu çalışmada, uzun süreli CC tedavisinin rat ovaryumu ve endometriyumundaki histopatolojik değişikliklerin uygulanan dozla ilişkisi araştırıldı. **Gereç ve Yöntemler:** Çalışma her grupta yedi dişi rat bulunan dört grupta yapıldı. Grup 1'e (kontrol) fizyolojik tuzlu su verilirken grup 2, 3 ve 4'e klomifen sitrat sırasıyla 1 mg/kg, 4 mg/kg ve 8 mg/kg dozlarda 18 hafta boyunca haftada bir kez oral yolla uygulandı. Çalışmanın sonunda uterus ve her iki ovaryum histopatolojik değerlendirmeler için eksize edildi. **Bulgular:** Histopatolojik çalışmalar, klomifen sitrat uygulanan bütün grupların ovaryum yüzey epitellerinde papiller hiperplazi meydana geldiğini gösterdi; grup 4'te papiller hiperplazinin şiddeti ve yüzdesi daha yüksek belirlendi. Bu grupta endometrial bezlerin çapları ve endometrial kalınlıklarının ortalama değerlerinin grup 1 ve grup 2'den daha düşük olduğu bulundu ($p<0,05$). Grup 4'teki ovaryum yüzey epiteli ve ovaryum çapları da kontrol grubu ile karşılaştırıldığında azaldığı saptandı ($p<0,05$). **Sonuç:** Yüksek dozda ve uzun süreli tekrarlanan klomifen sitrat uygulanması endometriyal kalınlıkta incelme açısından zararlı etkilere ve ovaryumda prekanseröz gelişmelere sebep olabilir.

Anahtar Kelimeler: Klomifen; patoloji; endometriyum; over; sıçanlar; zaman

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Clomiphene citrate (CC) a nonsteroidal tissue selective estrogen receptor modulator was approved for clinical use in 1967.[1-3] CC is the first line treatment for women with ovulatory disorders who are

normally estrogenized, i.e. predominantly those with polycystic ovary syndrome (PCOS), a syndrome which accounts for approximately 80% of anovulatory infertility.[4]

The exact mechanism of action of CC is not known, but CC may generate mainly antiestrogenic effects with some estrogenic sequence. It can therefore be considered as a selective estrogen receptor modulator. Acting as an antiestrogen, it competes with endogenous estrogen for hypothalamic binding sites, leading to an increased GnRH and hence of FSH and LH from the pituitary gland, which results in ovarian follicular maturation. This is followed by the preovulatory LH rise, ovulation and the subsequent development of the corpus luteum. It also competes with estradiol for binding sites at the hypophysis level.[5,6]

It is important to emphasize here that the administration of CC can restore ovulation ~73% of anovulatory patients, however the pregnancy rate related to CC treatment is significantly lower than expected (only around 36%). This discrepancy is believed to result from the negative effect of CC on the cervical mucus quality and its antiestrogenic activity on endometrium.[7-9]

There are several complications of ovulation induction. Functional ovarian cysts have often been observed after ovulation induction.[10] High order multiple pregnancy rates are also found to be increased due to CC treatment. Moreover, ovarian hyperstimulation syndrome is a potentially serious complication of ovulation induction.[11] It has also been reported that CC causes hepatic damage in rats, as shown by histopathological findings such as cytoplasmic vacuolations in hepatocytes, leucocytic infiltrations, congestion of blood vessels and hyperplasia of bile ducts.[12] Thin endometrium was reported as a common adverse effect of CC treatment. In this content, Randall and Templeton assessed follicular development and endometrial growth using transvaginal sonography in spontaneous and CC cycles and concluded that endometrial thickness was reduced in CC cycles.[13]

Lacoste et al. reported that CC caused significant histopathological changes on the epithelial surface of the tuba uterine in terms of ciliated cell loss, pseudostratification and nuclear abnormalities.[14] Ovarian and uterine abnormalities following CC treatment were reported [15,16] elsewhere, Ozdemir et al. investigated the effects of CC on ovarian, endometrial and cervical histologies in rat and showed an increase in granulosa, theca and luteal cells with high doses of CC. They also concluded that CC could be a risk factor for granulosa, theca and luteal cell tumors.[17]

In the last decades many investigators focused on the relationship between infertility treatment and cancer. The most commonly used ovulation inducing medications, CC and gonadotropins, were implicated in the etiology of ovarian cancer. Some investigators found a higher risk of developing cancer in women who received ovarian stimulation, whereas some researchers reported no correlation between the therapy and cancer.[18,19]

On the other hand, an increasing number of studies suggest that fertility drugs may have a special predisposition for the development of uterine cancers, which are recognized as hormonally responsive cancer type.[20] Breast cancer risk associated with CC has been studied in details.[21,22] However, investigations regarding breast cancer risk produced inconsistent results. Additional studies are needed to clarify the effects of fertility drugs on cancer risk, especially those used in conjunction with in vitro fertilization.

Thus, we aimed at assessing the effects of subchronic treatment of rats with CC in different doses, with the goal of determining whether CC causes histopathological changes in the uterus and ovaries.

MATERIAL AND METHODS

CHEMICALS AND REAGENTS

CC {2-[p-(2-chloro-1,2-diphenylvinyl) phenoxy] triethylamine citrate (1:1)}[12] was purchased from Kocak Ilac A.S., Istanbul, Turkey (Klomen®). This drug is categorized in the selective estrogen receptor modulators.[23] A stock solution of CC was prepared by dissolving CC in distilled water. CC was given to the rats orally (Po) by orogastric intubation via a tube.

ANIMALS AND TREATMENTS

All animal rights were observed. Then, the experimental procedures were approved by the Animal Ethics Committee of Afyon Kocatepe University, Afyonkarahisar, Turkey (AKUHEK; 5654, 30.10.03). Twenty eight virgin female Sprague Dawley rats showing a normal estrous cycle and weighing about 230 g and young age (7 months) were obtained from Selcuk University Laboratory Animal Breeding Research Center. All rats were allowed to acclimatize for a one week period before starting the experiment. All animals were kept under the same laboratory conditions in the Animal Services Centre Laboratory at Afyon Kocatepe University. The temperature was set at 25±2°C with relative humidity between 40-70% and the lighting was 12:12 h light:dark cycle. The rats were given free access to standard rat food and tap water ad libitum.

The rats were allocated into 4 groups, each containing 7 rats. Live body weight measurement of all rats in 4 groups were performed before the experiment. In group 1 (control), physiological saline was given by oral route. In groups 2, 3 and 4, CC was administered in the doses of 1 mg/kg, 4 mg/kg, and 8 mg/kg, respectively. Saline or CC administration was performed as a single dose a week for 18 weeks duration in each group. The doses of CC adopted in this study were based on the preliminary studies.[16]

LIVE BODY WEIGHT MEASUREMENT

At the beginning and at the end of the experiment, live body weights of all rats were measured and then mean values for each group were calculated.

TISSUE PREPARATION

Live body weight measurements of all rats were recorded at the final stage of the experiment. Following cervical dislocation uterus and both ovaries of rats were excised by means of laparotomy. The tissues were fixed in formaline solution for further histopathological studies. The paraffin blocks were placed in a microtome, and 4-5 μm thick sections were cut; these were stained with hematoxylin-eosin (H&E).

Histopathological examination of the sections stained with H&E was performed under a light microscope (Nikon E 600). Measurements were achieved with a digital camera (Nikon DXM1200) loaded with basic software. Sections were analyzed semiquantatively, taking into consideration that the changes would be particularly formed in the surface epithelium of the ovaries and in the endometrial tissue. Moreover, in the evaluation of the ovaries, diameter of the ovaries were measured and follicles in different stages were counted. Corpora lutea, an intermediary, endocrine active gland that alternately undergoes generation and degeneration in the course of the cycle were counted. The counting of the corpora lutea included both 'newly formed corpus luteum' and 'previously formed corpus luteum'. In the evaluation of uterine sections, thickness of endometrium and diameter of endometrial glands were also measured.

Diameter of the ovaries, endometrial thickness, and endometrial gland diameters were evaluated in 10 x objectives, whereas the thicknesses of the surface epithelium of endometrium and ovaries were measured using 40 x objectives of the microscope. The measurements of the surface epithelium were performed in regions where the epithelium is found to be the thickest in diameter. Moreover, the surface epithelium thickness of the ovary was measured in the thickest regions where papillary hyperplasia was absent.

While measuring endometrial gland diameters, large and narrow diameters in transverse sections were taken into consideration, and diameters of 5 glands were measured, then their mean values were calculated.

STATISTICAL ANALYSIS

SPSS for Windows Release 10.0.1 standart computer program was used for statistical analysis. Data were expressed as mean±SD. Differences between group were determined by both one way ANOVA and Kruskal Wallis H test. Student's-t paired test was used in order to compare the weight of rats of the beginning and the end of the experiment. For evaluation of the data for homogen variances Dunnett T test and for non homogen variances Dunnett

T3 test were performed. To investigate a relationship between variables, Pearson's chi-square test was performed. Results were considered statistically significant when $p<0.05$.

RESULTS

LIVE BODY WEIGHT

At the end of the experiment, an increase was observed in body weights of group 1 and group 2. On the other hand, the body weights of rats in groups 3 and 4 decreased (Table 1).

HISTOPATHOLOGY

The comparison among groups in terms of ovarian histology revealed that ovarian diameter, thickness of ovarian surface epithelium, primordial and preantral follicle and corpus luteum counts in the ovarian cross sections of group 4 were lower than those in the control group ($p<0.05$). It was also seen that ovarian diameter was lower in group 4 with respect to control and groups 2 and 3 ($p<0.05$), and thickness of ovarian surface epithelium and counts of antral follicle and corpus luteum in group 4 were lower than those in group 2 ($p<0.05$) (Table 2).

When the groups were investigated in terms of proliferation activity on the ovarian surface, the rats in the control group showed normal simple cuboidal epithelium characteristics with no pathological finding on the ovarian surface epithelium (Figure 1). However, 43% of the rats in group 2 (3 rats), 29% of those in group 3 (2 rats) and 72% of those in group 4 (5 rats) were found to have papillary hyperplasia on their ovarian surface epithelium (Figures 2, 3, 4, Table 3). The severity of papillary hyperplasia on the surface epithelium was found to be increased in direct proportion with the CC dose administered.

There was no significant proliferative change on the ovarian surface in control group rats (Table 3). However, 3 rats in the group 2 showed light proliferation in their ovarian surface (43%); one rat showed light (14.3%) and one rat showed mediate proliferation (14.3%) in group 3; one rat showed light (14.3%) and 4 rats showed severe (57.2%) proliferation in their ovaries in group 4.

The comparison of groups with respect to endometrial histology demonstrated that total endometrial thickness and endometrial gland diameters of the rats in group 4 were statistically lower than those in the control and group 2 ($p<0.05$) (Table 4).

TABLE 1: Mean values of live body weights of rats at the beginning and at the end of the experiment.

	Animal weight (g)		
Groups	**Beginning**	**End**	**p-value**
G1 (Control)	237.88±14.76	243.75±18.29	0.146
G2 (1 mg/kg CC)	223.25±14.56	226.13±16.10	0.472
G3 (4 mg/kg CC)	231.63±17.03	222.13±13.70	0.051
G4 (8 mg/kg CC)	234.63±15.62	217.38±20.84	0.069

Student's-t paired test was performed for dependent groups. G: Group; CC: Clomiphene citrate.

TABLE 2: Mean values of ovarian histological parameters in all groups.

	Groups				
Ovarian parameters	**G1 (Control)**	**G2 (1 mg/kg CC)**	**G3 (4 mg/kg CC)**	**G4 (8 mg/kg CC)**	**p-value**
Ovarian diameter (μm)	3681.50±550.92	3259.25±462.82	3191.75±640.26	2134.50±271.77	<0.05
Thickness of ovarian surface epithelium (μm)	15.23±1.658	19.09±4.93	13.16±3.33	10.57±3.34	<0.05
Primordial follicle count	8.25±3.05	6.75±2.12	4.63±1.50	1.63±1.06	<0.05
Preantral follicle count	25.63±4.98	33.75±16.03	18.75±6.75	14.63±6.78	<0.05
Antral follicle count	8.13±2.69	16.00±3.66	12.63±3.46	10.38±3.37	<0.05
Corpus luteum counts	13.88±6.81	10.63±3.70	7.50±4.81	1.88±1.88	<0.05

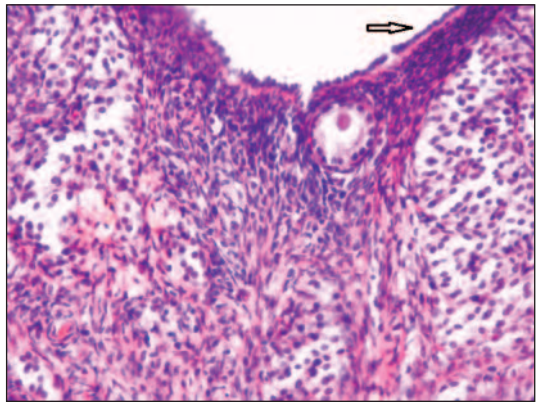

FIGURE 1: Photomicrograph of an ovarian section from group 1 (Control). Normal ovarian surface epithelium can be seen (H.E. x400).

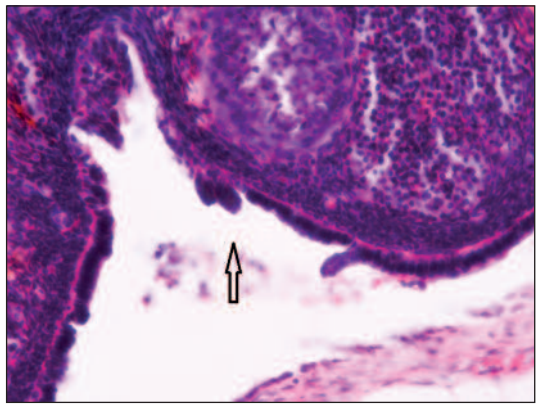

FIGURE 2: Photomicrograph of an ovarian section from group 2 (1 mg/kg CC). Epithelial tissue showing 1-2 papillary hyperplasia focus in a region of ovarian surface can be noticed (H.E. x400).

DISCUSSION

In the last four decades, fertility treatment is increasing and ovulation inducing drugs are widely used as independent therapies or during in vitro fertilization cycles. CC is one of the most widely used ovulation inducing drugs in the management of infertility due to its several advantages, such as its highly effectiveness in ovulation induction, relative safety, cheapness and facility of oral administration.[2] However, several complications of CC have been recognized. Functional ovarian cysts, ovarian hyperstimulation syndrome, and thinning of the endometrium are some of them.[10,11,24]

In recent years, fears of cancer possibility induced by ovulation inducing agents have been raised and addressed by various study reports.[25] Among ovulation inducing agents, CC has been implicated in the etiology of genital organ cancers and breast cancer.[22,26] It is therefore of great importance to clarify the relation between CC use and cancer.

The data in the literature regarding the relationship between ovulation induction and cancer is very conflicting. For instance, Rossing et al. reported that prolonged use of CC increased the risk of ovarian tumors, whereas Trabert et al. showed no association of ovarian cancer risk with ever use of CC among women evaluated for infertility.[19,27]

In our histopathological studies we observed that CC treated groups showed papillary hyperplasia on the ovarian surface epithelium, severity of which was increased proportional with the dose of CC administered.

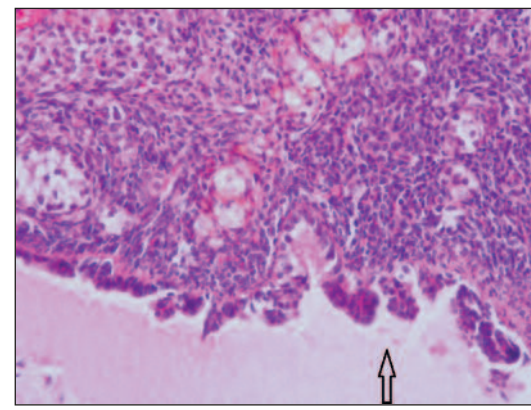

FIGURE 3: Photomicrograph of an ovarian section from group 3 (2 mg/kg CC). Epithelial tissue showing 3-4 papillary hyperplasia focus in a region of ovarian surface can be observed (H.E. x400).

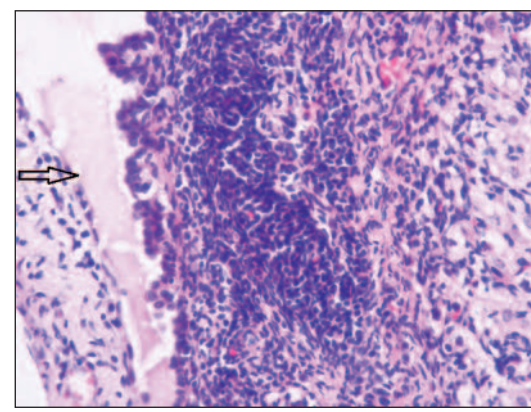

FIGURE 4: Photomicrograph of an ovarian section from group 4 (8 mg/kg CC). Epithelial tissue showing 6-7 papillary hyperplasia focus in a region of ovarian surface can be noticed (H.E. x400).

TABLE 3: Comparisons of the proliferative changes in the surfaces of ovaries for control and treatment groups.

	Proliferation of the ovarian surface epithelium			
Groups	**Minor* (n / %)**	**Moderate** (n / %)**	**Severe*** (n / %)**	**Total (n / %)**
G1 (Control)	-	-	-	-
G2 (1 mg/kg CC)	3/43	-	-	3/43
G3 (4 mg/kg CC)	1/14.3	1/14.3	-	2/28.6
G4 (8 mg/kg CC)	1/14.3	-	4/57.2	5/71.6

Significant differences are seen between the percentages of the three experimental groups in terms of proliferative changes (Pearson's chi-square test).

* Mild proliferation: Epithelial tissue showing 1-2 papillary hyperplasia focus in a region.

** Mediate proliferation: Epithelial tissue showing 3-4 papillary hyperplasia focus in a region.

*** Severe proliferation: Epithelial tissue showing more than 5 papillary hyperplasia focus in a region.

TABLE 4: Comparison of endometrial histology of control and experimental groups.

	Groups				
Endometrial parameters	**G1 (Control)**	**G2 (1 mg/kg CC)**	**G3 (4 mg/kg CC)**	**G4 (8 mg/kg CC)**	**p-value**
Endometrial thickness (μm)	588.09±82.16	590.21±123.60	390.49±112.72	349.10±49.14	<0.05
Thickness of endometrial surface epithelium (μm)	20.40±7.74	20.34±3.70	17.18±5.11	23.59±10.27	>0.05
Endometrial gland diameters (μm)	66.36±5.10	68.57±16.85	59.64±9.09	48.98±14.41	<0.05

Mean endometrial thickness is significantly lower in G4 when compared to control ($p<0.05$) and G2 ($p<0.05$).

Mean endometrial gland diameter is significantly lower in G4 when compared to control ($p<0.05$) and G2 ($p<0.05$).

In fact, unlike other malignancies involving the female genital tract such as cervical or endometrial carcinomas, precursor lesions of ovarian carcinomas have not yet been well defined, resulting in a failure to develop effective screening programs. But it has long been believed that most ovarian carcinomas arise from the ovarian surface epithelium or from invaginations of the surface epithelium into the ovarian stroma.[28,29] Furthermore, a well established hypothesis about the origins of ovarian malignancy is that a woman's risk of ovarian cancer increases with the number of ovulations she experiences.[30] After each ovulation, the ovulatory defect is repaired and remodeled by the ovarian surface mesothelium, and the repair process may result in the entrapment of surface mesothelial cells within the stroma as inclusion cysts.[31,32]

Thus, our observation of the presence of papillary hyperplasia on ovarian surface epithelium in CC treated rats may be interpreted as a predisposition to cancer or increased risk of cancer due to CC application. Supporting our findings, Ozcan et al. reported that dysplasia on the ovarian surface epithelium was noted in rats exposed to CC.[33] Additionally, a human study by Nieto et al. confirmed a possible association between ovulation induction therapy and ovarian epithelial dysplasia. [34]

Collectively, these results suggest a possible association between ovarian epithelial dysplasia/hyperplasia and CC therapy.

In our study endometrial gland diameters of the rats in group 4 were found to be lower than those in the control group ($p<0.05$). This means high doses of CC cause a decrease in endometrial gland diameters. Our findings are in agreement with a human study in which Sereepapong et al. reported that the number of glands per square millimeter and the mean diameter of the glands were lower in the CC treated cycles than in the control cycles.[9] The possible mechanism responsible here may be the apoptotic effects induced by CC in high dosages. Supporting the presence of an apoptotic effect of CC, Nutu et al. have reported that chronic CC treatment induced apoptosis in a fraction of uterine stromal cells.[35]

Our study showed that total endometrial thickness in group 4 (high dosage CC treated group) were lower than those in the control group

and group 2 ($p<0.05$). In group 2 (the lowest dosage CC treated group), these parameters were unaltered compared to control. Confirming our findings, many human studies reported that diminished thickness of endometrium was observed following CC treatment. For example, Takasaki et al. concluded that 41 out of 100 women were detected with a thin endometrium during a standard CC treatment cycle.[36] Haritha and Rajagopalan showed a significant reduction in endometrial thickness.[37]

Sereepapong et al. emphasized that endometrial thickness was similar in spontaneous and CC induced cycles.[9] But in their study CC was given at a low dosage. In fact, this may be an explanation for the differing results. Their results are also partially in agreement with ours in which endometrial thickness was not significantly altered in low dosage CC treated group (group 2).

Nevertheless, thinning of endometrium following CC treatment may be attributable to the antiestrogenic activity of CC. Although CC may cause an increase in serum estradiol levels, CC was proven to have an antagonistic activity on estrogen/estrogen receptor (ESR) signaling in female reproductive tissues, including endometrium, ovary and fallopian tube.[35] In this content, several studies have correlated endometrial thickness and pregnancy. Kovacs et al. examined a retrospective analysis of 1228 in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycles and concluded that the endometrium was thicker and the embryo quality was higher among women who became pregnant when compared with nonpregnant women after assisted reproduction.[38] Thus, endometrial thickness may be a factor contributing to the discrepancy between ovulation (60-85%) and pregnancy rates (around 36%) with the use of CC.[39,40] Since a thin endometrium is a critical factor for implantation failure, preventing CC induced thinning of the endometrium by adding exogenous estrogens during CC stimulation is important.[8]

A relationship between CC treatment and endometrial cancer investigated by many retrospective studies.[41] Ovulation stimulating agents increase serum estradiol levels causing unopposed supraphysiological levels of estrogen during the follicular phase of menstrual cycles of induced ovulation and therefore may also increase uterine cancer risk.[42] Confirming a possible relationship between CC and uterine cancer, Clark and McCormack reported that a single dose of CC in neonatal rats caused epithelial metaplasia, uterine cystic hyperplasia, and tumors of the uterus.[15] Jensen et al. proposed that CC may increase the risk of uterine cancer.[43]

However, we were unable to detect any supporting precancerous histological finding of endometrial carcinoma in this study similar to study of Benshushan et al. reported that they found no evidence of an association of CC treatment and higher risk of endometrial cancer in their case control study.[44]

In our study ovarian diameters were lower in group 4 with respect to control and groups 2 and 3 ($p<0.05$). Thickness of ovarian surface epithelium and counts of antral follicle and corpus luteum in group 4 were lower than those in group 2 ($p<0.05$). This may be explained that CC in high doses could be associated with apoptotic changes in the ovarian tissue. The mechanism responsible for these apoptotic changes may be the antiestrogenic effects of CC, though the possible mechanism by which CC exerts its antiestrogenic effect at the level of ovary is poorly understood .[40,45] In this content, as mentioned before, Chaube et al. reported that CC had an inductive effect on the apoptosis of ovarian granulosa cells and to reduce estradiol synthesis in vivo in rats.[46] They hypothesized that reduced estradiol level in ovary lead to poor development and maturation of oocytes and induced apoptosis after CC treatment.

Further findings in our study were; in low dose CC treated rats preantral and antral follicle counts increased compared to the control group. Apparent increase in antral follicles was noted. This means, follicle development was stimulated as expected in low dose CC treated group. A significant reduction in healthy follicles and corpus luteum was detected in group 4 and group 3, but the latter statistically was no significant. These results

suggest that CC in high dosages could have a toxic effect on growth and development of follicles and luteal cell populations. CC might induce a physiologic imbalance in the ovary-pituitary-hypothalamic axis, thus possibly cause a change in the follicular morphology and physiology, resulting in probable apoptosis.[47] Hence, CC induced apoptosis in ovarian follicular cells lead to reduced estradiol level in ovary and circulation which might result in poor development and maturation of oocytes leading to reduced ovulation.

The negative effects of CC can occur during follicle development, decreasing the number of healthy oocytes and hence embryos capable of leading to viable pregnancy. In the use of high dosage and long term CC treatment, ovulation induction can lead not only to higher incidences of ovulation failure but also poor pregnancy rates.

In addition, in our study the body weights of group 3 and 4 were found to be decreased at the end of the experiment when compared to the pre-treatment values, whereas control and group 2 values were found to be increased. These findings also confirm the toxic effects of high dosage, long term use of CC.

In conclusion, CC is the treatment of choice for women with infertility, however side effects and recent concerns of long term use, including an increased risk of cancer, thin endometrium, and apoptotic changes in genital organs should be taken into consideration. Hence, it might be advised to restrict the dosage and treatment duration of CC (i.e. 3-6 months) until the possible relationship between CC and genital organ malignancies become clarified by further studies.

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