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Study the effect of clomiphene on some physiological and histological changes associated with induced polycystic ovary syndrome in female albino rats

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***Abstract*—** Polycystic Ovary Syndrome (PCOS) is one of the most well-known and widespread disorder in women. The causes of PCOS are multiple and complex, resulting from interactions between diverse environmental and genetic factors. One of the treatments used for PCOS is clomiphene citrate. Clomiphene works by stimulating follicle development, follicle growth and multiplication. It is used to treat PCOS. Clomiphene treatment improves approximately 60-85% of ovulation cycles. This study aimed to determine the physiological and histological effects of clomiphene on female rats with induced polycystic ovary syndrome. This experiment was designed to study the histological and hormonal effects of clomiphene on induced PCOS in female rats. 40 female rats were randomly divided into four groups of 10 rats each and treated as follows: Group G1 (control group) was administered physiological saline for 58 days. Group G2: Administered 1% carboxymethyl cellulose for 28 days, followed by saline solution for 30 days. Group G3: Administered letrozole 1 mg/kg of body weight orally administered mixed with 1% carboxymethyl cellulose for 28 days to induce PCOS, followed by saline solution for 30 day. Group G4: Administered letrozole 1 mg/kg orally administered mixed with carboxymethyl cellulose for 28 days to induce PCOS, followed by clomiphene 100 mg/kg of body weight for 30 days. In the result showed in G3 a significant decrease ($P<0.05$) in activin, estrogen, FSH and Progesterone compared to G1and a significant increase ($P<0.05$) in LH, testosterone and inhibin compared to G1. The results showed in G4 non-significant difference ($P>0.05$) in activin, estrogen, testosterone, inhibin and FSH and also showed a significant decrease ($P<0.05$) in LH and Progesterone compared to G1. the histological examination results for the fourth group of ovaries showed an improvement in the ovary tissue, We conclude from this that the clomiphene drug worked to restore hormonal balance and repair ovarian tissue when given to rats with polycystic ovary syndrome.

***Keywords* —** clomiphene, rats, Polycystic ovary syndrome, letrozole.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a endocrine disorder collection of small, pearl-sized, regularly shaped cysts in the ovaries. These cysts surround immature, fluid-filled eggs and cause ovarian enlargement due to the significant dilation and enlargement of follicles (1). This leads to the destruction and reduction of granulosa cell layers, resulting in apoptosis (programmed cell death). Consequently, the theca cell layers increase, producing androstenedione, which is converted to testosterone. This results in symptoms of hyperandrogenism and anovulation (decreased ovulation), which causes the Graafian follicles to fail to mature, leading to a decrease in the number of corpus luteum cells (2).

First described by Stein and Leventhal in 1935, epidemiological data indicate that one in every 5-6 women suffers from various symptoms of polycystic ovary syndrome, affecting 5-10% of women with infertility, and it is believed that this disorder affects 6-21% of premenopausal women (3). The main cause of polycystic ovary syndrome is hormonal imbalance, which includes hyperandrogenism, increased secretion of luteinizing hormone, progesterone, and follicle-stimulating hormone, and the level of estrogen decreases. PCOS is usually associated with insulin resistance, inflammatory responses and stress (4).

Recent evidence suggests that oxidative stress and chronic inflammation contribute significantly to the development of PCOS and related metabolic conditions. Oxidative stress is associated with impaired ovarian steroid production, leading to increased estrogen production and infertility. Increased oxidative stress is observed in women with infertility and PCOS. Cytokines have been shown to play a direct role in regulating the complex balance of the hypothalamic-pituitary-ovarian axis and in maintaining a normal ovarian and menstrual

cycle. Cytokine imbalance, a key feature of chronic inflammation in PCOS, may contribute to this (5,6).

Clomiphene citrate (CC) is the preferred first-line drug for ovulation induction in women with PCOS who are experiencing anovulation and infertility. It has been used in women to treat ovulation disorders since the 1960s and is the recommended treatment for ovulation induction. It is a simple, affordable, and effective treatment for infertility, stimulating ovulation with minimal side effects and has radically changed infertility treatment since its commercial approval in 1967 (7).

Its use may increase the chances of twin pregnancies due to ovarian stimulation. Caution is necessary, as ovarian overstimulation may occur, requiring medical consultation. As a precaution, Clomiphene should not be used repeatedly or the dose increased without consulting a doctor to minimize the risk of these side effects (8).

Clomiphene citrate, which contains enclomiphene and zoclomiphene, is administered orally this synthetic drug is available as round, white tablets in a 50 mg Dose. However, response varies, and the likelihood of ovulation induction is increased in patients with obesity, insulin resistance, and hyperandrogenism (9). The drug is rapidly absorbed in the intestines and remains in the body for approximately 5 days (10).

Clomiphene citrate has a long half-life (5-7 days), which may have a negative effect on cervical mucus and the endometrium, leading to variability between ovulation and pregnancy (11). Clomiphene citrate inhibits estrogen receptors in the brain via a negative feedback mechanism and by blocking estrogen receptors in the hypothalamus. Alternatively, clomiphene citrate acts as an anti-estrogen, increasing the amplitude of gonadotropin-releasing hormone (GnRH) impulses (10). It also helps the anterior pituitary gland produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn helps follicles reach terminal maturity (12).

There are concerns regarding the use of clomiphene, including the potential risk of ovarian cancer and its anti-estrogenic effects on the endometrium. Prolonged use of clomiphene citrate for more than 12 treatment cycles increases the risk of ovarian cancer. Consequently, this drug should be used for a maximum of six cycles (13).

Women taking clomiphene citrate have experienced miscarriages associated with its use in clinical practice (14). Clomiphene citrate has been observed to reduce endometrial receptivity, leading to impaired endometrial growth and uterine blood flow. This results in endometrial thinning in 15–50% of patients, which can lead to implantation failure and early pregnancy loss (11).

This study aimed to determine the physiological and histological effects of clomiphene on female rats with Induced polycystic ovary syndrome.

MATERIALS AND MTHODS

Experimental induction of PCOS

In this study, 1 mg/kg of letrozole was used to induce polycystic ovaries in female albino rats. Letrozole was dissolved in 1% carboxymethylcellulose (CMC) powder by taking (1 mg) of CMC with 99 ml of distilled water, and then administered orally to the animals using a dosing device (15).

Experimental Animals:

This experiment was designed to study the histological and hormonal effects of clomiphene on induced PCOS in female rats. 40 female rats, age between (12-16 weeks)weighing between 150-250 g, were randomly divided into four groups of (10) rats each and treated as follows:

1- Group G1 (control group) was administered physiological saline for 58 days.

2- Group G2: Administered 1% carboxymethyl cellulose for 28 days gave it as stabilizer, followed by saline solution for 30 days.

3- Group G3: Administered letrozole 1 mg/kg of body weight orally administered with 1% carboxymethyl cellulose (16) (CMC) for 28 days to induce PCOS, followed by saline solution for 30 days.

4- Group G4: Administered letrozole 1 mg/kg orally administered with carboxymethyl cellulose for 28 days to induce PCOS, followed by clomiphene 100 mg/kg of body weight for 30 days.

Blood sample collection

The animals are anesthetized using a cotton ball soaked in an appropriate amount of chloroform, placed inside a sealed, transparent container. The animal is then quickly transferred into the container, which is resealed to ensure inhalation of the anesthetic. Once the animal is fully anesthetized, it is removed, and a blood sample is drawn directly from the heart using a sterile 5 ml syringe. The blood samples are immediately placed in sterile, non-anticoagulant gel tubes and centrifuged at 3000 rpm for 15 minutes to separate the serum. The serum is then collected in clean, dry and appropriately labeled Eppendorf tubes and stored in a refrigerator at -20°C until testing. The tests performed included analysis of the hormonal parameters LH, FSH, estrogen, testosterone, inhibin, Progesterone and activin .

Collection of tissue samples

After drawing blood from the female white rats, they were dissected directly by making an incision in the abdominal cavity from below towards the heart. The ovaries were removed, then washed with water to remove the blood on them. After that, the ovaries were preserved in 10% formalin in clean plastic containers after labeling them and sealed tightly for 48 hours until the histological preparations were carried out on them.

Statistical analysis

The results of the statistical analysis were subjected to the SPSS Program (V.25) to determine the differences between the means for the studied criteria for the different groups, based on finding the least significant difference (LSD). The results were then expressed as mean ± standard error and mean ± standard deviation. A probability value of ($P \leq 0.05$) was adopted as a significant difference between the means for all the studied criteria and statistical analysis was used in the experiment is analysis of variance (ANOVA) (17).

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RESULT AND DISCUSSION

The results in Table (1) showed a significant decrease (P<0.05) in FSH hormone levels in G3 compared to G1. Conversely, the results showed non-significant difference (P>0.05) in FSH hormone levels in G2 and G4 compared to G1. However, there was a significant increase (P<0.05) in FSH levels in G4 compared to G3, and non-significant differences were found between G1 and G2.

The results in Table (1) indicate a significant increase (P<0.05) in LH hormone levels in G3 compared to G1. Conversely, the results showed a significant decrease (P<0.05) in G4 compared to G1. The results also showed a significant decrease (P<0.05) in groups G2 and G4 compared to G3 , as well as significant differences between groups G1 and G2.

The results in Table (1) showed a significant decrease (P<0.05) in progesterone levels in G3 and G4 compared to G1. Conversely, the results showed non-significant difference (P>0.05) in progesterone levels in G2 compared to G1. They also indicated a significant increase (P<0.05) in progesterone levels in G4 compared to G3.

Table 1. Effect of clomiphene on Progesterone, FSH and LH on female albino rats induced with polycystic ovary syndrome

groups	PR (ng/mL)	LH (IU/L)	FSH (IU/L)
G1	12.07 ± 0.26 a	13.81 ± 2.52 c	10.30 ± 0.51 a
G2	11.42 ± 0.97 a	11.14 ± 1.03 b	9.88 ± 0.53 a
G3	6.87 ± 0.53 c	18.07 ± 1.35 d	6.78 ± 1.22 b
G4	9.08 ± 0.82 b	4.95 ± 1.39 a	9.60 ± 0.30 a

Values represent the mean ± standard error.
Same letters between any two mean values indicate no statistically significant difference at a probability level of (P > 0.05).
Unspecified letters indicate a statistically significant difference at a probability level of (P < 0.05).

The statistical analysis results shown in Table (2) revealed a significant decrease (P<0.05) in Estrogen hormone levels in the G3 of rats induced with polycystic ovaries compared to the G1. However, the results also showed non-significant difference (P>0.05) in estrogen levels in the G2 and G4 groups compared to G1. Furthermore, a significant increase (P<0.05) in hormone levels was found in G2 and G4 compared to G3. Finally, the results indicated no significant differences between the G1 and G2.

The results of the statistical analysis shown in Table (2) revealed non-significant difference (P < 0.05) in testosterone levels between groups G4 and G2 compared to G1. The results also showed a significant increase (P < 0.05) in testosterone levels in the G3 compared to G1. Conversely, the results showed a significant decrease (P < 0.05) in testosterone levels in groups G4, G2 and G1 compared to G3. The analysis also indicated non-significant differences between groups G1 and G2.

The results of the statistical analysis shown in Table (2) showed a significant decrease (P<0.05) in the level of activin hormone in groups G4 and G3 compared to G1. The results indicated non-significant difference (P<0.05) in the level of activin hormone in G2 compared to G1. In contrast, the statistical analysis showed a significant increase (P<0.05) in the level of activin hormone in G2 compared to G3. The results of the analysis also showed non-significant differences between the two groups G1 and G2.

The results of the statistical analysis shown in Table (2) showed a significant increase (P<0.05) in the level of inhibin hormone in G3 compared to G1. The results indicated no significant difference (P<0.05) in the level of inhibin hormone in G4 and G2 compared to G1. Conversely, the statistical analysis showed a significant decrease (P<0.05) in the level of inhibin in groups G4 and G2 compared to G3 rats in which polycystic ovaries were induced. The results of the analysis also showed non-significant differences between groups G1 and G2.

Table 2. Effect of clomiphene on some hormone parameters (activin, estrogen, testosterone, inhibin) in female albino rats induced with polycystic ovary syndrome

groups	Inhb. (pg/mL)	Act. (pg/mL)	Testo. (ng/dL)	E2 (pmol/L)
G1	146.58 ± 11.20 a	560.03 ± 76.04 b	0.20 ± 0.008 b	106.99 ± 5.57 b
G2	151.91 ± 11.99 a	558.26 ± 41.41 b	0.21 ± 0.011 b	119.38 ± 1.48 b
G3	378.59 ± 58.46 a	458.36 ± 34.12 a	0.33 ± 0.04 a	92.91 ± 13.25 a
G4	161.19 ± 6.77 a	515.81 ± 65.31 a	0.19 ± 0.005 b	109.28 ± 3.81 b
LSD	LSD= 29.46 P<0.001	LSD= 66.61 P=0.002	LSD= 0.02 P<0.001	LSD= 7.97 P<0.001

Values represent the mean ± standard error.
Same letters between any two mean values indicate no statistically significant difference at a probability level of (P > 0.05).
Unspecified letters indicate a statistically significant difference at a probability level of (P < 0.05).

Histological changes in the ovary

Histological sections of the ovaries in the control group, treated with physiological saline for 58 days, showed normal ovarian tissue with a cortex exhibiting follicular development, a primary follicle with a corpus luteum and a secondary follicle, as shown in Figure 1.

However, the group treated with CMC solution only for 28 days, followed by physiological saline for 30 days, showed normal ovarian tissue with normal Graafian follicles and brown cells compared to the control group (Figure 2).

The group in which polycystic ovaries were induced by letrozole over 28 days showed marked pathological changes in the ovarian tissue, including multiple subcapsular cystic follicles lined with thin granulosa cells, reduced corpus luteum development, severe vascular congestion in the medulla, and

significant eosinophilic inflammatory infiltration, as shown in Figures 3 and 4, compared to the control group (Figure 1). The showed that the histological examination results for the fourth group of ovaries showed an improvement in the tissue, represented by an increase in the number of Graafian follicles with the formation of corpora luteum, Figure (5), when compared to Figure (3) and (4) of the letrozole group. No pathological changes were observed in the tissue when examined microscopically when compared to the control group, Figure (1).

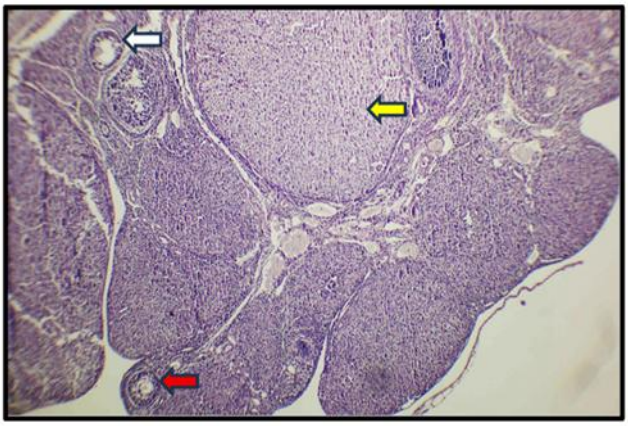

Figure 1. Histological section of a rat's ovary from the control group at 58 days showing the cortex with follicular development, the primary follicle (red arrow) with the corpus luteum (yellow arrow) and the secondary follicle (white arrow) (H&E X4).

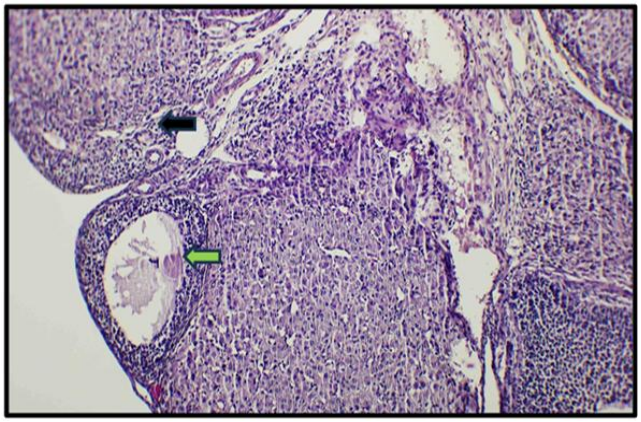

Figure 2. Histological section of a rat's ovary from the CMC group at 58 days showing Graafian follicle (green arrow) with interstitial cells (black arrow) (H&E) (X10

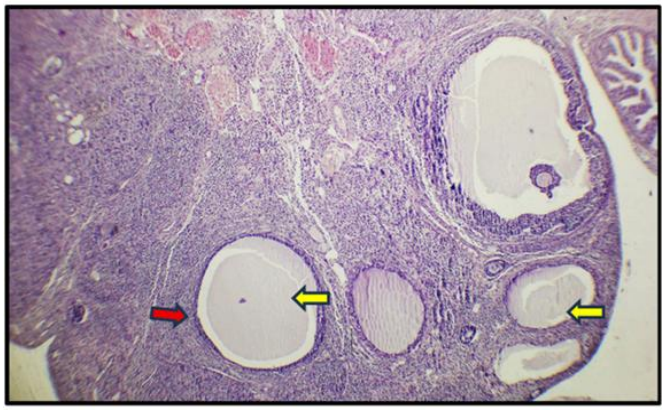

Figure 3. shows a Histological section of the ovary of rats in the letrozole group at 28 days old, revealing significant pathological changes in the ovarian tissue, represented by multiple cystic follicles under the capsule (yellow arrow) lined with thin granulosa cells (red arrow), with a lack of corpus luteum development (H&E X4)

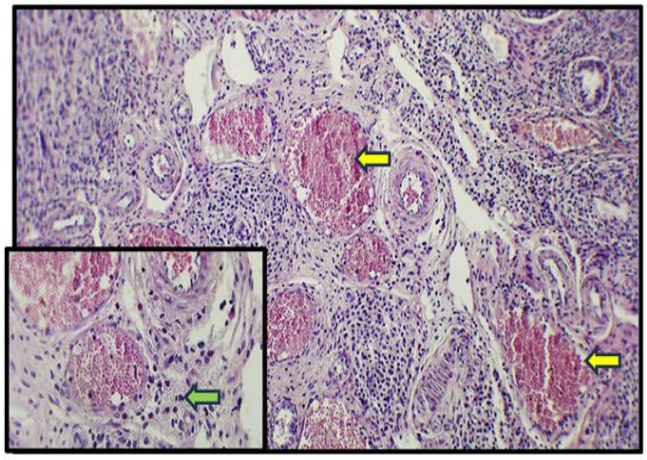

Figure 4. A pathological tissue section of the rat's ovary of the letrozole group at 28 days showing severe vascular congestion in the pulp area (yellow arrow) with severe infiltration of eosinophils (green arrow) H&E magnification (X40)

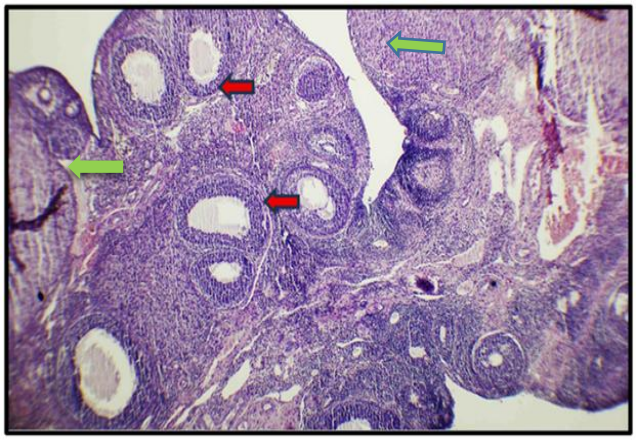

Figure 5. A histopathological section of the ovaries of rats that were given letrozole for 28 days and clomiphene for 30 days. An improvement in ovarian tissue is shown represented by an increase in the number of Graafian follicles (red arrow) with the formation of corpora luteum (green arrow) (H&E) X4

The results showed a significant decrease in FSH levels in the induced PCOS rats group compared to G1. This finding is consistent with (18). This decrease may be explained by letrozole promoting the increased androgen ratio by not stimulating FSH through aromatase inhibition (19). Letrozole induces anovulation, increases free radical production, and inhibits aromatase activity, leading to changes in sex hormone levels in an animal model of PCOS (20). Furthermore, the decrease in FSH levels impairs the ability of granulosa cells to convert androgens to estrogens, negatively impacting ovulation and follicular maturation (21).

A significant improvement in FSH levels was in G4, consistent with (22). This is attributed to the action of clomiphene, which blocks estrogen receptors, thereby disrupting the negative feedback loop of circulating endogenous estrogen. This leads to increased levels of FSH in the bloodstream (23). Clomiphene also inhibits negative feedback of endogenous estrogen on the hypothalamic-pituitary-adrenal axis, resulting in increased FSH secretion, follicular growth and ovulation (24). The results of the current study showed that clomiphene treatment achieved the best results compared to the other experimental groups, with a clear and significant improvement in FSH levels, bringing them closer to the normal values of the control group. This improvement is attributed to the effect of clomiphene's mechanism of action, which prevents negative estrogen feedback on the hypothalamic-pituitary axis, leading to increased FSH secretion (25)

The current results showed a significant increase ($P<0.05$) in LH levels in the induced polycystic ovary rat group compared to the G1. These results are consistent with (26, 27). This may be attributed to hyperandrogenism, which is the main feature of PCOS, with the ovaries being the primary source of increased androgen production, and to a lesser extent the adrenal glands. Increased adenomatous cell size and insulin resistance are also causes of increased androgen (28). This may also be explained by the enhancement of hyperinsulinemia in testosterone production in ovarian theca cells, leading to an imbalance in LH/FSH levels by increasing LH production and inhibiting FSH synthesis (29).

An improvement in LH levels was observed in the clomiphene-treated groups. These findings are consistent with (30). This may be attributed to clomiphene citrate acting as a selective estrogen receptor modulator, stimulating follicular growth through negative feedback mechanisms, thus ovarian stimulation. It also enhances the follicular response to FSH and LH, ultimately promoting ovulation (31).

Statistical analysis revealed a significant decrease in progesterone levels in the PCOS induced G3 compared to G1. These results are consistent with a study by Kandasamy et al. (26). This may be due to hyperandrogenism LH-induced hyperandrogenism inhibits progesterone synthesis, although this possibility requires further confirmation, according to Alkalby and Hamzah (32). This decrease in progesterone synthesis may be explained by the degeneration of granulocytes in this syndrome, which are unable to produce progesterone and estradiol in the serum, thus supporting these findings (20).

A significant improvement in progesterone levels was observed in the groups treated with clomiphene, and the results were consistent with (33). A previous study also indicated the positive effect of clomiphene citrate on reproductive hormones in women with PCOS (34). This is attributed to clomiphene promoting follicular growth by inhibiting the negative estrogen feedback mechanism and stimulating the release of gonadotropins from the pituitary gland, thereby enhancing fertility in women with PCOS (35).

The results showed a significant decrease in estrogen levels in the G3 Compare with G1. These results are consistent with the study by Sangar et al. (20). This is attributed to letrozole increasing the elevated androgen ratio by not stimulating FSH due to aromatase inhibition (19). Follicular growth is often arrested in polycystic ovary syndrome, leading to impaired follicular maturation. This impaired follicular growth results in decreased estradiol production, as the granulosa cells of the mature follicle are the main source of estradiol (36). Conversely, an improvement in progesterone levels was observed in the drug-treated groups. The current results are consistent with (34, 37). This may be attributed to the fact that clomiphene citrate acts as a selective estrogen receptor modulator, stimulating follicular growth through negative feedback mechanisms, which in turn stimulates the ovaries and ultimately promotes ovulation (31). Clomiphene is also a nonsteroidal anti-inflammatory drug that primarily affects estrogen production. It has notable effects on female hormonal balance, normal ovulation, and overcoming infertility problems associated with PCOS (38).

The results of the current study showed a significant increase in testosterone levels in the G3 compared to G1. These results are consistent with (39). This is because most women with PCOS experience hyperinsulinemia due to insulin resistance. Hyperinsulinemia promotes testosterone production in ovarian theca cells (29). Furthermore, a study by Frhan et al. (40) demonstrated that increased LH levels in PCOS patients lead to increased testosterone. The elevated testosterone levels in women with PCOS are also attributed to adrenal gland dysfunction, which causes the overproduction of androgenic hormones, particularly testosterone (41).

An improvement in testosterone levels was observed in the groups treated with clomiphene. These results are consistent with (22), whose study indicated a significant increase in serum LH and testosterone levels after daily treatment with clomiphene citrate. A study by Hinojosa-Amaia etal.,(42) also showed that clomiphene raises testosterone levels to normal levels in men. The reason for the low testosterone concentration in women is attributed to elevated LH and FSH levels. Testosterone concentration is directly affected by these two hormones. An increase in FSH levels leads to a decrease in testosterone levels and any decrease in their levels results in the stimulation of androgens, primarily free testosterone. This stimulates cells to reduce the role of estrogen in the blood by absorbing estrogen and releasing a protein that binds to androgens (43).

The results of the current study showed a significant decrease in the level of activin hormone in the PCOS group G3 compared to G1. The current results were similar to those of a study by (44,45). A previous study also indicated that serum activin A levels are lower in women with PCOS (46). This is attributed to elevated levels of the protein follistatin. Follistatin, a protein that binds to activin and prevents it from functioning, tends to have higher than normal levels in PCOS. This elevation means that the amount of active activin in the blood is lower because follistatin inhibits its activity and reduces its free concentration. Consequently, patients have a lower level of active activin (47). Alternatively, women with PCOS may have insulin resistance and excess weight, which can affect the balance between follistatin and activin and increase activin inhibition, leading to lower levels (48). An improvement in the

level of activin hormone was also observed in the groups treated with clomiphene. This may be attributed to the fact that clomiphene raises FSH via hypothalamic-pituitary signaling mechanisms, so it is possible that activin is spontaneously affected via the HPO axis without a direct study measuring this. The results of the current study showed a significant increase in inhibin-B levels in the induced PCOS G3 compared to G1. These results are consistent with (49). Elevated inhibin-B levels in PCOS patients are an important factor in assessing ovarian reserve. The reason for these elevated levels in PCOS patients is attributed to their increased fat mass, which leads to increased production of androgens and ovarian hormones, including inhibin-B, which inhibits FSH. Ovarian hyperactivity resulting from elevated androgen levels leads to increased inhibin-B production. Previous studies have indicated the potential importance of inhibins in normal follicular growth, and thus, inhibin deficiency may contribute to the follicular stagnation associated with PCOS (50).

A significant improvement in inhibin levels was also observed in the clomiphene-treated groups. These results are consistent with (51). This is attributed to the fact that clomiphene is a selective estrogen receptor modulator that prevents estrogen from providing negative feedback to the pituitary gland and higher brain regions (52). As a result, the pituitary gland produces greater amounts of FSH and LH. The elevated FSH stimulates the ovaries to develop follicles (which produce inhibin-B). Another possible explanation for the different responses to clomiphene in improving hormone levels is its preferential effect (53).

Histological sections of the ovaries in the group induced with letrozole over a 28-day course showed marked pathological changes in the ovarian tissue, including multiple subcapsular cystic follicles lined with thin granulosa cells, reduced corpus luteum development, severe vascular congestion in the medulla, and significant eosinophilic inflammatory cell infiltration. These results are consistent with Wang et al. (15) and with previous findings indicating that letrozole-induced polycystic ovary syndrome leads to typical polycystic ovaries, including an increased number of cystic follicles, reduced granulosa layers, and a marked decrease in corpus luteum (54). The results of the study conducted by Al-Yasari (55), in which polycystic ovary syndrome was induced, showed that cystic follicles appeared significantly. This feature is consistent with the pathological changes in the ovaries of women with polycystic ovary syndrome due to letrozole, which was used to induce this syndrome and is an effective aromatase inhibitor. This leads to the accumulation of excess androgens in the ovary, which then affects the growth and function of ovarian follicles, thus destroying them and causing the formation of cystic follicles on the surface of the ovary to a large extent.

The showed that the histological examination results for the fourth group of ovaries showed an improvement in the tissue While an improvement was observed in the clomiphene-treated group, with results showing an increase in the number of Graafian follicles and the formation of corpora luteum compared to the control group, no pathological changes in the tissue were observed compared to the control group. The results of the histological study are consistent with a previous study (56), which showed that histological sections of the ovaries of mice treated with clomiphene citrate showed a decrease in the number of cysts, an increase in the number of ovarian follicles, the presence of corpora luteum, and a decrease in the size of the nucleus in the region occupied by interstitial cells. Clomiphene citrate acts primarily on the hypothalamus, where it exhibits an anti-estrogenic effect and generates a feedback mechanism. Its effectiveness in inducing ovulation is attributed to the stimulation of follicle proliferation due to an increase in the secretion of FSH, which is accompanied by a proportional increase in LH (53).

CONCLUSION

We conclude from this that the clomiphene drug group worked to restore hormonal balance and repair ovarian tissue compared to rats with polycystic ovary syndrome.

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