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The Effect of Metformin and Anastrozole on the Induced Polycystic Ovarian Syndrome in Rat. Morphological, Morphometric, and Histological Study

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Abstract
Background:
Polycystic ovarian syndrome is one of an important gynecologic disorder that attracts the attention of many scientists about its etiology, diagnosis and treatment.
It is characterized by enlargement of the ovary, many macroscopic and microscopic changes, with disturbance of menstruation and infertility.
Polycystic ovarian syndrome is a multifactorial disorder of unclear etiology. Its diagnosis depends on many criteria.
Many lines of treatment are used which give different responses.
Aim of study:
Follow up the treatment of polycystic ovarian syndrome with metformin and anastrozole through morphological, morphometric and histological studies.
Materials and methods:
Twenty-one day old female rats had been involved in this study. Forth of them received nothing and considered as a control group while the remaining received testosterone to induce polycystic ovarian syndrome. The animals with polycystic ovary had been divided into three subgroups; the first one received nothing, while the second one treated with metformin and the last subgroup received anastrozole as a therapy.
The morphological, morphometric and histological studies of each subgroups had been demonstrated and comparison between the results obtained of metformin and anastrozole treated subgroups had been analyzed.
Result:
The result collected from metformin treated subgroup revealed response to treatment, however, this response was inferior to the response of this syndrome to anastrozole. This was observed according to the morphological, morphometric and histological studies.
Discussion:
Metformin reduces insulin level in the blood which acts directly or systemically on the ovarian tissue to reduce androgen production, which in turn, improves the physiological cycle of sexual hormones. However, this improvement (reflected by macroscopic and microscopic results) did not approximate the normal value as anastrozole did. Anastrozole acts as aromatase enzyme inhibitor, therefore, inhibits aromatization of androgen into estrogen. This returned the hormonal cycle to almost normal status.

1. Introduction

Polycystic ovarian syndrome (PCOS) is one of the commonest endocrine and gynecological disorders affecting women during their reproductive life. It is characterized by enlargement of the ovary, increased ovarian follicular cyst number, failure of ovulation, infrequent or absence of menstrual cycle with infertility (Knochenhauer *et al*, 1998; Young, 1991).
The etiology of PCOS, although controversial, is still unclear till now (Ehrmann, 2005). However, there are many theories and concepts that may approximate the possibility of the explanation of the etiology of PCOS. These are:

1. There is significant cellular resistance to the circulating insulin, whether in obese or non-obese PCOS women (Venkatesan *et al*, 2001).
2. Hormonal imbalance: which is considered to be one of the most prominent features of PCOS, in the form of androgen excess, LH/FSH imbalance, and disturbance of serum estrogen level (Marx and Metha, 2003).
3. Genetic factor, since the syndrome could run in families (Fauser *et al*, 2011; Diamanti-Kandarakis *et al*, 2006).
4. Environmental factors, involving ethnic factor, race, and other environmental causes (Kauffman *et al*, 2002).
5. Inflammatory causes: patients with PCOS are more liable to have high levels of c-reactive protein, with disturbances in their immune response (Dehdashtihaghighat *et al*, 2013).

6. Developmental fetal abnormality (Tyndall *et al*, 2012; Walters *et al*, 2012).

The prevalence of PCOS is about 18% of women (although, 70% are undiagnosed previously) (March *et al*, 2010). Interestingly, PCOS affects obese women about 5-6 times more than that of non-obese ones (Alvarez-Blasco *et al*, 2006; Azziz *et al*, 2005).

The diagnosis of PCOS depends on many criteria, these are:

1- National Institute for Health (NIH) criteria (Zawadzki and Dunaif, 1992).
2- Rotterdam criteria (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004).
3- Androgen Excess PCOS Society criteria (Azziz *et al*, 2009).

The main macroscopical changes, that could be detected in the PCOS, are enlargement of the ovary (when the ovarian size is larger than the average normal one by 10 ml^3, it is considered as a PCOS criterion) (The Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). The ovarian cortex appears smooth, whitish-gray in *color*, with many subcortical cysts (0.5-1.5 cm in diameter) leading to coarse irregular outline surface (Ellenson & Pirog, 2010).

Microscopically, the ovary in PCOS reveals the following changes; hyperatrophy of theca interna, antral enlargement due to progressive follicular fluid accumulation which results, simultaneously, in degeneration of granulosa cells leading to thinning of the wall of the cyst. Besides , the theca cellular layer that surrounds the follicle is significantly thinner than that seen in normal ovary. Corpora lutea are scarcely present. Besides, hypercellular ovarian stroma and enlargement of central stroma are the characteristic findings in this disorder (Ellenson and Pirog, 2010; Chang, 2007).

The strategy for treatment of PCOS follows general line and specific one (Moran *et al*, 2009). The general line includes weight loss, avoiding weight gain, exercise, besides psychological therapy.

The specific treatment includes drugs therapy. One of these drugs is metformin (Tang *et al*, 2010), which is principally used in the treatment of type 2 diabetes mellitus. However, metformin is widely used in treatment of PCOS, mainly through its effect in reducing circulating insulin in hyperinsulinemia and even in patients with normal insulin level in their blood (Ehrmann, 2005). Clomiphene citrate, an estrogen receptor blocker, has been used to induce ovulation in PCOS for a long time. However, one of its drawback is the anti-estrogenic effects on the endometrial & cervical mucosae, which can lead to the failure of the pregnancy. This side effect makes clomiphene citrate is out of argument in the treatment of PCOS nowadays (Casper and Mitwally, 2012).

Other group of drugs, used in the treatment of PCOS, is aromatase enzyme inhibitors. Anastrozole is an example of aromatase inhibitors. It is a non-steroidal drug used mainly for the treatment of the breast cancer in postmenopausal women. This drug inhibits aromatase enzyme action, preventing the conversion of androgen into estrogen and hence, blocking estrogen production also blocks the action of estrogen receptors. However, it does not have the side effect of clomiphene citrate on the pregnancy (Sioufi *et al*, 1997).

2. Aim of the Study

Morphological, *morphometric*, and histological comparison between the effects of metformin and anastrozole on the ovary of the induced PCOS in rat as they are the two main therapeutic agents in the treatment of induced PCOS in rat.

3. Materials and Methods

Norway albino rats had been chosen in this study. They were kept under optimum conditions throughout the experiment, regarding temperature, light, and food.

Eight [8] mature male rats and eight [8] mature female rats had been collected. Each couple had been kept in one cage separately. After mating, pregnancy, and near delivery, the males were separated from the females.

The outcome of deliveries was forty [40] offspring. Twenty eight [28] of the offspring were females. The age of the new born rats had been calculated till reaching 21 days old. At this age, twenty one premature female rats had received 1 mg/100 g body weight/day of testosterone propionate (Sustanon “250” mg, 1 ml ampoule, N.V. Organon Oss Holland) daily for 28 days (Waltersw *et al*, 2012). The route of administration of testosterone was subcutaneous injection in the dorsum of the neck of the rat (Beloosesky *et al*, 2004). The remaining 7 premature females received subcutaneous injections of sesame oil for 28 days and considered as a control group.

After testosterone treatment, the 28 female rats were divided into groups and treated with different drugs as demonstrated in table-1:

Table-1: Grouping of female rats, involved in this experiment, with their different treatments

Group	Control	Experimental (testosterone-treated female rats)		
		Subgroup-I (TP-treated group)	Subgroup-II (MET-treated group)	Subgroup-III (ANA-treated group)
Number	7	7	7	7
Treatment received	Received nothing	Received nothing	Treated with metformin (Glucophage, 500 mg. tablet Merk Serono)	Treated with anastrozole (Arimidex 1 mg. tablet AstraZeneca)
Dose and rout of administration			20 mg/100 gm body weigh/day (Di Pietro *et al*, 2014; Kabiri *et al*, 2014). The dose had been given orally after dissolving the grinded tablet in the drinking water.	15 µg/kg body weight/day (Shirai *et al*, 2009). The dose had been given orally after dissolving the grinded tablet in the drinking water.
Duration of treatment			5 weeks	5 weeks

TP: testosterone propionate, MET: metformin, ANA: anastrozole.

During the last week of treatment, all the animals involved in this study had been exposed daily to vaginal smears to determine the phase of estrus cycle, since the animals had to be scarified during the pro-estrus phase.

Following anesthesia, both ovaries had been excised and removal of the surrounding adipose tissue had been performed.

The ovaries had been weighed, their size measured, then examined by naked eye (sometimes aided with a magnifying lens) to study the morphology of the ovary. The measurement of the ovarian size had been performed through putting the ovary inside a graduated cylinder (10 ml volume) filled with normal saline. The excess of fluid (before and after putting the ovary inside the graduated cylinder) had been aspirated and measured by using another 5 ml empty graduated cylinder. Then the ovaries had been examined using dissecting microscope, connected to 10 mega pixel digital camera to study the morphometry of the ovary.

After that, the ovary had been incubated in 10% formalin, then incubated in paraffin. Sectioning of the paraffinized ovary had been done, then the sections had been stained with hematoxyllin and eosin for histological examination.

During histological (microscopical) examination, the following histological parameters had been taken in consideration:

- Number of primary follicles
- Number of secondary and mature follicles
- Number of corpora lutea
- Number of large cystic follicles
- Number of small cystic follicles
- Number of cellular layers of theca interna
- Percentage of interstitial glands to the total stromal area.

Statistical analysis of the results obtained had been done using PASW statistics software (2009). The results were presented as measures of mean $\pm$ standard error of mean at 95% confidence. Independent t-test and one-way ANOVA were used to compare between the different groups of the experimental animals.

Note: p-value < 0.05 : statistically significant

p-value < 0.01 : highly statistically significant

4. Results

Regarding the ovaries of the control animals, they revealed morphological, morphometric, (fig. 1-a) and histological features of the normal ovaries.

The experimental animal subgroups revealed the following results:-

4.1. Morphological and morphometric result

- Subgroup-I (TP-treated subgroup only):
  The ovary of this group appeared smaller than that of the control one, with rounded, smooth, and whitish surface. Under dissecting microscope, the ovary revealed smooth rounded outline, with pale yellowish-white *color*ed cortex due to small, scanty numbers of blood vessels.

It could not possible to see corpus luteum. Multiple large cysts filled with fluid were detected scattered underneath thick ovarian cortex causing slight bulging of ovarian surface (fig. 1-b).

- Subgroup-II (MET-treated group):
  The ovary of this group was still smaller and lighter in weight than that of control one and ANA-treated subgroup, but slightly larger than TP-treated subgroup ovary.
  Morphological and *morphometric* examination demonstrated the same picture of TP-treated ovaries. However, the large cystic follicles appeared to be smaller and not bulging beyond the ovarian surface, giving the ovarian outline a contour smoother than that of TP-treated ovaries (fig. 1-c).
- Subgroup-III (ANA-treated group):

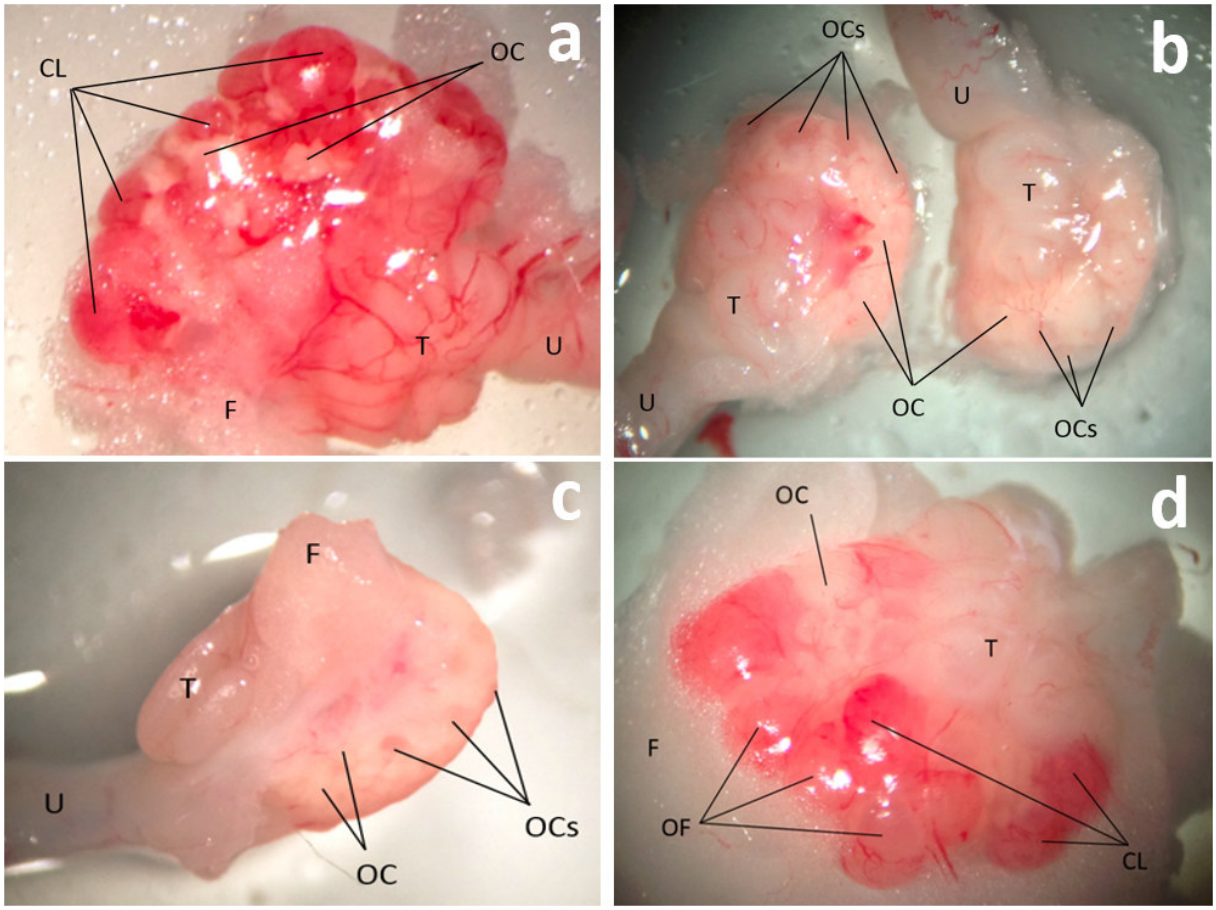

Figure 1: Gross ovarian appearance of control group (a), TP-treated group (b), MET-treated group (c), and ANA-treated group (d). Note the larger size, lobulated surface, red *color* and rich vasculature in the control and ANA-treated groups and the smaller size, smooth surface, pale *color* and poor vasculature in the TP-treated and MET-treated groups. U: uterus, T: uterine tube, F: fat, OF: ovarian follicles, OC: ovarian cortex, OCs: ovarian cysts, CL: corpus luteum. Dissecting microscope, 25X.

Generally, the features of the ovaries of this group were closer to those of the control one. The ovarian weight of this group was heavier significantly ($p < 0.05$) than that of TP-treated subgroup and that of MET-treated groups. Besides, the size of the ovaries returned approximately to the normal ovarian size, yet, it was larger than the ovarian size of the MET-treated group. The color of the ovary is dark red, while the ovarian outline was obviously irregular to a degree that the general oval-shaped ovary had been distorted.

Examination of the ovary under dissecting microscope elicited the following: ovarian surface was highly lobulated with large numbers of ovarian follicles of variable sizes, bulging out of the ovarian surface. The examination revealed also multiple dark red corpora lutea with prominent network of blood vessels. Both findings are responsible for the dark *color*ed ovarian surface (fig. 1-d).

4.2. Histological Features:

- Subgroup-I (TP-treated group):
  The main histological features of the ovaries in this group had been illustrated as follows: total absence of corpora lutea, large cortical cysts (5-8 in number) with their cavities containing remnants of antral fluid and scattered degenerated and necrotic granulosa cells could be seen. These numbers were statistically ($p<0.05$) higher than those of control group. The walls of these cysts were formed by layers of ovarian stroma that failed to differentiate into theca interna and theca externa layers. Theca interna cellular layers were highly significant ($p<0.01$) less in number (or even absent) than those in control group. The numbers of small cysts revealed highly significant ($p<0.01$) increment in comparison with the control group; however, their sizes were larger than those of the control one. Primary and secondary/mature follicles were highly significantly ($p<0.01$) less in number than the control one. The interstitial glands occupied the major part of the stroma (84%) which was significantly more than normal stroma (fig. 2-a).
- Subgroup-II (MET-treated group) and subgroup-III (ANA-treated group):
  The histological findings in these two subgroups will be demonstrated together in comparison and illustrated together as figures, since the aim of this study is to compare between the effect of metformin and anastrozole on PCOS.
  The following table (table-2) compares between the histological changes elicited in the ovaries of MET-treated animals and ANA-treated animals.

Table-2: Histological findings seen in MET-treated and ANA-treated subgroups

MET-treated subgroup	**ANA-treated subgroup**
1. Primary and secondary/mature follicles revealed significant increase in number in comparison with TP-treated group, but still significantly less than their numbers in normal ovaries ($p < 0.05$).	1. Primary and secondary/mature follicles were almost similar in number to those in the control group. However, they were significantly higher in number that those in MET-treated group ($p < 0.05$).
2. The corpora lutea developed in 3 animals out of the 7 animals involved in this subgroup.	2. The corpora lutea were obviously identified in the ovaries of all of the 7 animals involved in this subgroup.
3. Small cysts were more than control group, although this increment was statistically insignificant.	3. The small cysts were less in number than those in MET-treated group, yet this decrement was statistically insignificant.
4. The number of large cortical cysts was more than that in the normal ovary; however, this increase was statistically insignificant, but statistically significant if compared with the TP-treated subgroup.	4. The number of large cortical cysts was more or less comparable to that seen in MET-treated subgroup.
5. Theca interna surrounding large follicles, was made of up to (3-4) cellular layers, which were still significantly lower than that of control group. However, the number of theca layers revealed highly significant improvement compared with that of TP-treated group (fig. 2-b).	5. Theca interna cellular layers were almost identical to those seen in control group, and significantly more developed than those seen in MET-treated group. It was formed by 5-6 cellular layers (fig. 2-c).

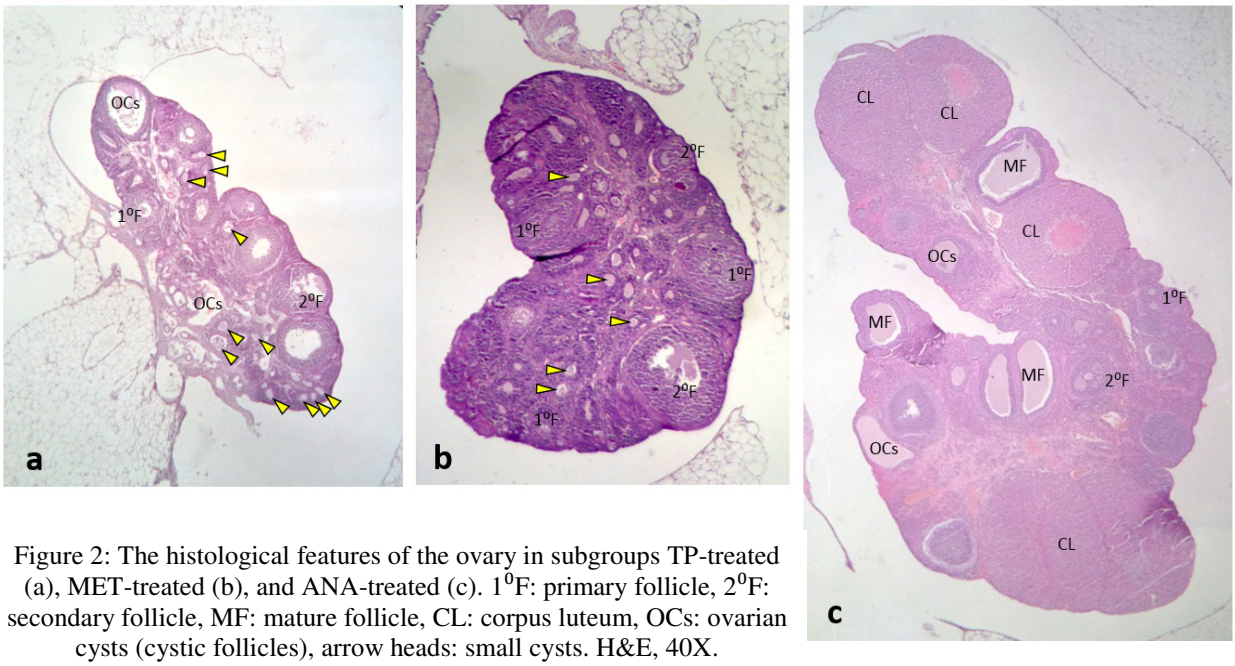

Figure 2: The histological features of the ovary in subgroups TP-treated (a), MET-treated (b), and ANA-treated (c). 1^0F: primary follicle, 2^0F: secondary follicle, MF: mature follicle, CL: corpus luteum, OCs: ovarian cysts (cystic follicles), arrow heads: small cysts. H&E, 40X.

5. Discussion

All the results (morphological, morphometic & histological) obtained in TP-treated subgroup I were in line with the picture of PCOS. The explanation for these changes is attributed to the excessive exogenous androgen administration that was injected to the animals of this subgroup. There is one exception that the ovary reduced in size in PCOS of this study, this due to the fact that in rodent the ovarian medullary size represents very small percentage in comparison with the cortex (Shirai *et al*, 2009). This leads to disturbance of normal physiological sex hormonal cycle leading to reduction of FSH and then estrogen levels which, in turn, leads to failure of maturation of primary follicles into secondary & the mature graafian follicles (Marx and Metha, 2003). This explains the presence of large number of small cysts & large cysts (Pradeep *et al*, 2002), since primary follicles will failed to be mature till reaching graafian follicle (Ingraham *et al*, 2000) & the process is limited to the increase in the size of primary follicles forming small cysts which in turn increase in size due to accumulation of a great amount of fluid forming large cysts. The enlargement, in turn, occurs on the expanse of the surrounding granulosa and theca interna cells leading to degeneration and necrosis of the cells of both layers & thinning or absence of theca layers.

The absence of corpora lutea reflected the failure of the ovulation due to disturbances of feedback mechanism between ovary & pituitary gland leading to hormonal imbalance (LH/FSH) (Serafini *et al*, 1986; Spinder *et al*, 1989). In another word, these histological changes occurred are the mirror to the disturbance of normal physiological sequence of sexual hormone from the ovary &pituitary gland due to increase in the level of androgen in the blood of the animal of this subgroups.

Regarding the morphological, *morphometric* and histological changes in the ovaries of the experimental subgroups II and III (which had been treated with metformin and anastrozole respectively), it was clear that the improvements in the polycystic ovaries had been observed on using these two therapeutic agents, although, the degree of these improvements varied according to which drug had been used.

The results elicited in ANA-treated subgroup of this study reflected restoration of almost the normal development of the ovary in the induced PCOS, which was much better than what occurred on using metformin treatment. This difference in response of the ovary to each administered drug, could be attributed to the mode of action of each drug on the polycystic ovary.

Before discussing the effect of metformin or anastrazole on the PCOS, it is better to understand the normal physiological sex hormonal cycle. The theca cell is the main source for androgen production in addition to the interstitial cells, which in turn under the cover and effect of FSH, will converted into estrogen by the action of aromatase enzyme. In the same time androgen production is under the effect of LH hormone. Estrogen hormone is the principle effector in maturation of the primary follicle till reaching mature graafian follicle (Attia *et al*, 2001).

Accordingly, disturbance of this normal physiological cycle will lead to failure of maturation of primary follicle (i.e. leading to increase in the number of primary follicle) and this lead to reduction in the number or even absence of mature follicle and replace by large cyst (Marx and Metha, 2003) which increase in

size on the expanse of the surrounding granulosa cellular layer. This accompanied with failure of ovulation i.e. absence of corpous luteum (Serafini *et al*, 1986). Besides, interstitial cellular bulk will increase in comparison to the total ovarian tissue since it considers as one of the principle source of androgen production inside the ovary.

From this brief review of hormonal physiology the question arises: whether the hyperandrogenemia and LH/FSH imbalance are the cause or the result of PCOS?

The treatment of PCOS via using metformin therapy, may be attributed to its systemic effect in lowering insulin level and so reducing androgen production (Ehrmann, 2005; Kabiri *et al*, 2014), or due to its direct effect on reducing androgen synthesis from ovarian tissue (Attia *et al*, 2001) and then inhibition of estradiol production (Mansfield *et al*, 2003). All this, in sequence, resumes almost normal FSH and LH secretion from the pituitary gland (Attia *et al*, 2001; Mansfield *et al*, 2003). Other possible explanation of the effect of metformin on PCOS is the reduction of ovarian stromal fibrosis seen in PCOS which acts as an obstacle against the rupture of mature follicles (Zhang *et al*, 2013).

However using metformin, as a therapy for PCOS, did not restor the hormonal physiological cycle in the body to the normal status but partially. Since histology of the ovary reflects the hormonal status and in this subgroup ovarian histology did not return to the normal one, so this means that the hormonal condition did not reach the reasonable level.

Regarding anastrozole, which is one of the aromatase enzyme inhibitors, its action results in the reduction or even cessation of estradiol synthesis. This action could occur directly through its effect on granulosa cells to reduce secretion of androgen, or indirectly through inhibition of conversion of the androgen into estrogen (Simpson, 2003). This will stimulate FSH secretion through negative feedback mechanism stimulating pituitary gland to secret this hormone, which in turn stimulate estrogen secretion from the ovary. This action can resume the normal hormonal feedback mechanism between the ovary and the pituitary gland, which results in increase secretion of level of FSH. In another words, anastrozole acts to resume the normal physiological cycle of pituitary gonadotropins which is disturbed in PCOS. The same results had been elicited by Shiari *et al* (2008) and Radwan (2010). Anastrozole can resume secretion of FSH and so LH/FSH ratio and restor the level of estrogen and progesterone to almost normal level and this is proved by reaching to almost normal ovarian histology as demonstrated in this study.

From the results obtained in this research with the discussion of these results, one can conclude that anastrozole is superior to metformin in the treatment of PCOS.

6. Recommendation

To solidify these results with their discussion, it is recommended to do hormonal assay including estrogen, progesterone, androgen, FSH and LH when each drug (used in this research) is used as a line of therapy.

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