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The Mode of Therapeutic Action of Metformin and Aromatase Enzyme Inhibitors in the Treatment of Induced Polycystic Ovary Syndrome in Rat through using Immunohistochemical Technique

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Abstract:
Background Polycystic ovary syndrome is one of well-known disorder that affects females during their reproductive period. The etiology and the pathogenesis of this disorder are still unclear since many scientists consider some factors are the causes of this disorder while the others proved them as a result of this disorder.
In general, this disorder chaptalized mainly by hormonal imbalance which reflected itself as pathological changes which mainly summarized by multiple large cysts associated with clinical features mainly disturbance of menstruation and infertility.
Two parameters play a principal role in pathogenesis and a guide line in the progression of the treatment. These are aromatase enzyme activity and estrogen receptor B distribution.
Now a day many lines of treatment are involved in the treatment of polycystic ovary syndrome, but the two important one are the use of antihyperinsulinemic drugs (like metformin) and aromatase inhibitors (like anastrozole).
The aim of this research is to study the mode of action of these two drugs through using immunohistochemical techniques and illustrate their effect on aromatase enzyme activity and estrogen receptor B distribution in different ovarian tissues.
Materials and methods: Twenty-six premature female Norway albino rats aged 21 day had been involved in this study. Five of them considered as controls while the others received testosterone for 4 weeks to induce polycystic ovary and considered as experimental group. The experimental group divided into 3 subgroups, the first one received nothing while the second one was treated with metformin and the third one was treated with anastrozole. In all the groups, the aromatase enzyme activity and estrogen receptor B distribution had been examined through using immunohistochemical technique.
Result: The results revealed improvement in the syndrome reflected through increase in the activity of aromatase enzyme besides increase in the estrogen receptor B distribution in certain ovarian tissues. However the intensity of aromatase activity and the intensity of estrogen receptor expression varied according to the treatment used.
Discussion: Both drugs, used in the treatment of polycystic ovary syndrome, were act through normalization of the sexual hormonal situation in order to preserve the normal histology and the physiological function of the ovary. However, anastrozole proved to be much better as a therapy than metformin in this line.

1. Introduction

Polycystic ovary syndrome (PCOS) is considered to be one of the common endocrine disorder that the females suffer from during their reproductive period of life (Aziz *et al*, 2004). The syndrome called so since the ovaries have multiple cortical cysts distorting their smooth outline (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). It affects about 28% of obese women and about 5% of non-obese ones (March *et al*, 2010).

The etiology of this disorder is still not clear since so many factors integrat in its development (Ehrmann, 2005). Although, many factors could be shared in PCOS etiology, like hyperinsulinemia (Venkatesan *et al*, 2001), hormonal imbalance (Marx and Metha, 2003), chronic inflammatory process (Repaci *et al*, 2011) and other causes, yet, some of these etiological factors could be considered as consequences of PCOS rather than its causes.

The diagnosis of PCOS is controversial as its etiology, since many criteria were considered by the endocrinologists and the gynecologists for making a diagnosis. However, whatever the character of these diagnostic criteria, the principle factors shared among them are the disturbance of sex hormones that leads to infertility, menstrual disturbances besides other systemic symptoms (Azziz *et al*, 2004). This hormonal disturbance results in the distortion of the normal physiological cycle between the pituitary gland and the ovaries through feedback mechanism (Guyton and Hall, 2010). One of the crucial steps in this cycle is mediated by aromatase enzyme (Diamanti-Kandarakis, 2008).

Aromatase is the microsomal cytochrome P450 enzyme that function is the conversion of androgen to estrogen (Cole & Robinson, 1990). The activity of aromatase enzyme could be detected in many normal tissues,

mainly the ovaries, brain, adipose tissue, muscles, liver, besides abnormal tissues like breast cancer (Nelson and Bulun, 2001). The main source of estrogen in premenopausal period is the ovary, while during the postmenopausal period the fatty tissue is the first line in estrogen production (Santen *et al*, 1990). Regarding the distribution of aromatase enzyme in the ovary, it is varied between its different tissues and changing during the different developmental stages of the ovary during menstrual cycle (Stocco, 2008).

Another line that one has to search for to understand the pathogenesis and to follow up the course of the treatment of PCOS, is estrogen receptors. These receptors are groups of proteins spread intracellularly and activated by estrogen (Hosokawa *et al*, 2001). They are present in different tissue and organs in the body, yet, the ovaries being the first site among other organs in expressing estrogen receptors (Taylor and Al-Azzawi, 2000).

Estrogen receptors are of two types: estrogen receptors alpha (ERα) and estrogen receptors beta (ERβ). The most important type, regarding its role in ovarian function, is ERβ which is present in the granulosa cells, theca interna cells, and corpus luteum (Drummond and Fuller, 2012). On the other hand, ERα is present mainly in the ovarian stroma (Sánchez-Criado *et al*, 2005).

The treatment of PCOS includes many lines, one of them is the use of antihyperinsulinemic agents (like metformin). Another line of PCOS treatment is the recent use of aromatase inhibitors (like anastrozole) to induce ovulation, instead of the old approach of using clomiphene citrate (which is estrogen receptor blocker) due to the unwanted side effects of the latter (Kamat *et al*, 2002). The mode of action of these two agents (metformin and anastrozole) in PCOS treatment is quite different.

2. Aim of the study

A trial to study the mode of action of metformin and aromatase inhibitors (in this study, anastrozole) in PCOS, using immunohistochemical technique to study the activity of aromatase enzyme and the intensity of distribution of estrogen receptors B within various ovarian tissues and cells in the induced PCOS female rat model when these two therapeutic agents had been used.

1. Materials and Methods

The experimental animals used in this study were Norway albino rats that were kept under optimal conditions throughout the study. Six mature males and six mature females were kept as pairs in isolated cages. After mating and pregnancy period, the males were separated from the pregnant females before delivery. The pregnancy outcome of the six dams was 36 pups; including 28 females and 8 males. Two female pups died during the first postnatal week, and the remaining 26 female pups were left with their mothers till the age of 21 days, they were divided into 4 groups and started to have specific treatment as shown in table 1.

Table-1: Grouping of the animals, with their treatment

Grouping	Control animals	Experimental animals (testosterone treated females)		
		Subgroup-I: testosterone treated subgroup (only)	Subgroup-II: metformin treated subgroup	Subgroup-III: anastrozole treated subgroup
Number	5	7	7	7
Treatment received	Equivalent volume of vehicle (sesame oil) injected subcutaneously.	Testosterone propionate 1mg/100g of body weight/day (dissolved in sesame oil) gave as subcutaneous injections for 28 days.	Testosterone propionate 1mg/100g of body weight/day gave as subcutaneous injections for 28 days, followed by oral metformin (Glucophage 500mg tablet, Merck-Serono) 20 mg/100 g of body weight, prepared by dissolving the tablet in drinking water, for 5 weeks (Heibashy *et al* 2013; Elia *et al*, 2006).	Testosterone propionate 1mg/100g of body weight/day gave as subcutaneous injections for 28 days, followed by oral anastrozole (Arimidex 1 mg, AstraZeneca tablet) 15 µg/kg of body weight, prepared by dissolving the tablet in drinking water, for 5 weeks (Shirai *et al*, 2009).

Regarding the experimental groups, the rats received 1 mg/100 g of body weight/day of testosterone propionate (TP) (Sustanon 250 mg/1 ml ampoule, N.V. Organon OSS Holland) daily for 4 weeks. Testosterone propionate had been given by subcutaneous injection at the back of the neck of the premature female rats (Walters *et al*, 2012).

All the animals (control and experimental groups) had been exposed in the last week of treatment to daily vaginal smears, and accordingly, animal sacrifice had been done during proestrous phase of estrous cycle (Goldman *et al*, 2007).

After anaesthesia, perfusion fixation had been performed, then the ovaries had been excised. Then the fatty tissue surrounding the ovary was removed and then the ovaries were paraffinized, sectioned at 5-6 µm thick sections and prepared for immunohistochemical study.

The immunohistochemical study of the ovaries had been performed to investigate two important parameters:

1. Aromatase enzyme (P450arom) activity.
2. Estrogen receptor beta (ERβ) distribution intensity.

To achieve these studies, streptavidin-biotin (LSAB) complex method had been used to utilize the extraordinary affinity of streptavidin for biotin (Chilkoti *et al*, 1995), using anti-aromatase antibody and anti-estrogen receptor beta antibody, respectively. The following ovarian tissues and cells that had been studied for the immunoreactivity of aromatase enzyme activity and the distribution of ERβ were:

- Granulosa cells of primary, secondary and mature follicles,
- Corpora lutea,
- Theca interna cells of secondary and mature follicles ,
- Granulosa cells in the wall of large cystic follicles,
- Interstitial glands.

Positive immunoreactivity for both parameters was detected by brownish discolouration of the investigated tissues and cells. The intensity of immunohistochemical reaction was scored using semiquantitative immunohistochemical scoring method (Tyndall *et al*, 2012) for both parameters, as demonstrated in table-2.

Table-2: Scoring of immunohistochemical reaction

Intensity of reaction	Intensity of staining	Score
• Negative reaction	No brown staining	Zero
• Weak reaction	Faint brown staining	1
• Moderate reaction	Intermediate brown staining	2
• Strong reaction	Dark brown staining	3

4. Results

The results obtained in this study after performing the immunohistochemical techniques can be divided into two sets: the activity of aromatase enzyme within the ovarian tissues, and the intensity of estrogen receptor B ditribution within the ovarian tissues.

a. The activity of aromatase enzyme:

- Control group: aromatase activity was strongly evident in the granulosa cells of the large antral and mature follicles, and was more clear in the mural granulosa cells. Granulosa cells of the primary follicles revealed weak reactivity. Moderate reaction was demonstrated by corpora lutea cells, Theca cells and interstitial glands revealed negative enzyme reaction (Fig.1-A).
- TP-treated subgroup: primary and secondary/mature follicular granulosa cells revealed weak immunoreactivity. However, enzymatic activity in primary follicular theca cells was relatively stronger than those of the control group. About half of the cells in the wall of large cysts revealed moderate immunoreactivity. No reaction could be illustrated in corpora lutea since no corpora lutea had been developed in this group. Theca cells and interstitial glands had weak immunoreactivity (Fig 1-B).
- MET-treated subgroup: about 40% of granulosa cells of secondary/mature follicles elicited moderate enzymatic reaction, while cells of the primary follicles showed weak reaction. Corpora lutea, although detected in almost half of the animals in this group, revealed weak activity. Theca cells and interstitial glands revealed no reactivity (Fig.1-C)
- ANA-treated subgroup: 80% of granulosa cells in the primary and secondary/mature follicles revealed moderate enzymatic immunoreactivity, while cells in the wall of large cysts stained weakly (i.e. weak enzymatic activity). Regarding corpora lutea, the same intensity of enzymatic reaction and percentage of cells involved, could be detected as that of the granulosa cells of antral/mature follicles. Theca cells and interstitial glands showed no enzymatic activity (Fig.1-D).

b. The intensity of ERβ distribution:

- Control group: the estrogen receptors beta in the ovaries of this group revealed their strongest distribution (the strongest immunoreactivity) in the granulosa cells of the antral/mature follicles. Strong ERβ reactivity could be detected in the theca cells, while the primary follicles revealed weak reactivity. Corpora lutea illustrated moderate uneven distribution of immunoreactivity within their cells. Interstitial

glands showed strong immunoreactivity to ERβ.

- TP-treated subgroup: moderate receptor distribution could be demonstrated in the granulosa cells of primary and secondary follicles, while 30% of theca cells revealed strong immunoreactivity. No reaction could be demonstrated within corpora lutea since no corpus luteum was developed in the ovaries of this group. The interstitial cells had the strongest immunoreactivity among all ovarian cells and tissues of this group.
- MET-treated subgroup: granulosa cells of antral/mature follicles showed moderate- strong immunoreactivity to ERβ, while the cells of the primary follicles had a weak receptor distribution. Less than half of theca cells revealed weak reaction. Moderate immunoreactivity could be illustrated in the

5. Discussion

The philosophy behind the treatment of PCOS, whatever the line of treatment used, is to restore the normal physiological sex hormones cycle between the ovary and pituitary gland. This is the aim of the different PCOS treatment approaches whatever their different modes of action.

One of the main PCOS treatment lines is the antihyperinsulinemic therapy (metformin). Metformin could act locally on the ovary (Attia *et al*, 2001; Mansfield *et al*, 2003), and systematically through its antihyperinsulinemic effect (Ehrmann, 2005). Both of these mechanisms can lead to a reduction in the ovarian production of androgens & improve the state of hyperandrogenemia that characterizes PCOS. Reduction in androgen levels can correct its feedback inhibitory effect on the pituitary gland, resuming –to a certain degree- the production of sufficient amounts of FSH and LH necessary to restore the normal ovarian function(John E Hall 2011). This increase in the level of FSH& LH will stimulate aromatase enzyme to convert the endogenous androgen into estrogen(Merlotti *et al*2011). Although this metformin-mediated improvement in gonadotropin (FSH) secretion is still subnormal, it appears to be able to induce the growth of some primary and secondary follicles to mature follicles with some increase in aromatase activity in their theca cells. On the other hand, LH secretion improvement after metformin treatment, is still below the normal level to restore normal ovulation, as evident by the small (less than half) number of animals which could ovulate & subsequently developed corpora lutea with weak aromatase immunoreactivity in this subgroup.

Regarding anastrozole treatment, the results obtained were almost similar to those of the normal (control) ovary. Anastrozole is an inhibitor of aromatase enzyme, it prevents the aromatization of androgens into estrogen in the ovary, reducing the amount of estrogen produced by the ovary. This reduction is believed to stimulate the anterior pituitary gland to secrete higher amounts of FSH and LH via negative feedback mechanism. This will stimulate aromatase enzyme to convert androgen into estrogen hormone (Barett *et al* 2010) which, in turn, acts to stimulate the maturation of ovarian follicles and induce ovulation, respectively. This explains its high expression of aromatase enzyme in the mature follicles of this subgroup. Besides, resumption of LH surge is evident by the resumption of ovulatory function of the ovary (Mescher 2013), as revealed by the large number of corpora leutea seen in all of the ovaries of this group of animals, with the increment in aromatase activity in the cells of those corpora leutea.

Regarding estrogen receptor beta distribution, it is well known that the site where estrogen could express its action is estrogen receptor. Therefore, wherever estrogen is needed, estrogen receptors are present and the intensity of distribution goes parallel with the estrogen need of the different ovarian tissues (Kurl and Morris 1978). According to this concept, the map of ERβ distribution within ovarian tissues, which was demonstrated through using immunohistochemical technique in this study, could be understood.

The control group revealed weak activity in the primary follicles, while the granulosa cells of the mature follicles elicited the strongest of ERβ immunoreactivity among the ovarian tissues. This agrees with the previous hypothesis since the follicle needs the highest concentration of estrogen for maturation and formation of the graafian follicle (Hall 2011), this has illustrated itself as a strong intensity of immunoreactivity (reflecting high intensity of ERβ) in the granulosa cells of the mature follicles. On the other hand the primary follicles, which have started the process of maturation, revealed weak activity. Corpus luteum revealed uneven moderate activity, which could be explained by the fact that the granulosa cells of the mature follicle that remain after ovulation, are responsible for this result.

Regarding TP-treated subgroup, the aromatase enzyme converts androgen into estrogen under the umbel of FSH action (Diamanti-Kandarakis, 2008). However, this conversion is more than need of ovarian tissues, which leads to negative feedback mechanism preventing FSH secretion by the pituitary gland, which in turn reduces the endogenous estrogen production (Hall 2011), reflected as a minimal ERβ distribution. Accordingly, the results of ERβ distribution in TP-treated subgroup had been illustrated as mentioned in this study. Since the negative feedback mechanism would reduce FSH secretion from the pituitary gland, no mature graafian follicle could be found, and failure of ovulation will occur, evidenced by the absence of corpora lutea in the ovaries of this group (Sadler 2012). Although negative feedback mechanism of hormonal secretion from the pituitary gland will be established, yet small amounts of estrogen was still present in the circulation, and

limited number of ERβ were present, which illustrated themselves as a moderate immunoreactivity in the primary and secondary follicles.

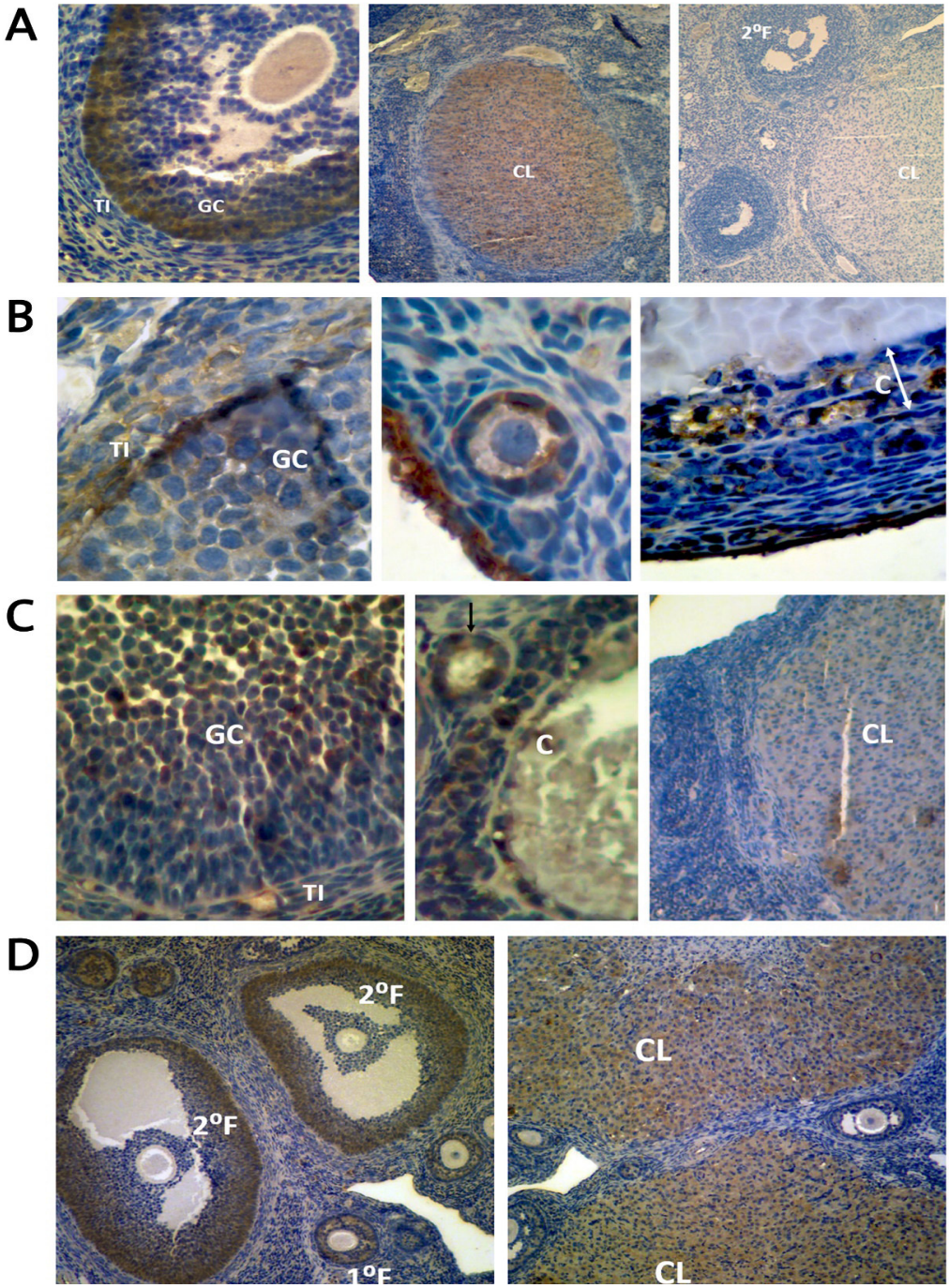

Figure 1: Aromatase enzyme activities in the different ovarian tissue in the studied groups; (A) control group, (B) TP-treated subgroup, (C) MET-treated subgroup, & (D) ANA-treated subgroup. TI: theca interna, GC: granulosa cells, CL: corpus luteum, C: cyst wall, 1ºF: primary follicle, 2ºF: secondary follicle, arrow: primary follicular cells (in MET-treated subgroup).

Corpora lutea, which were seen in almost half of the animals in this subgroup. The cells of interstitial glands showed a strong immunoreactivity(Fig.2- A & B).

- ANA-treated subgroup: granulosa cells of the primary and secondary/mature follicles in this subgroup showed moderate immunoreactivity, while theca cells revealed strong receptor distribution. All of the animals in this subgroup showed well developed corpora lutea, which demonstrated strong immune-histochemical reactivity in their cells. The interstitial glands demonstrated an intense distribution of ERβ (Fig.2- C & D).

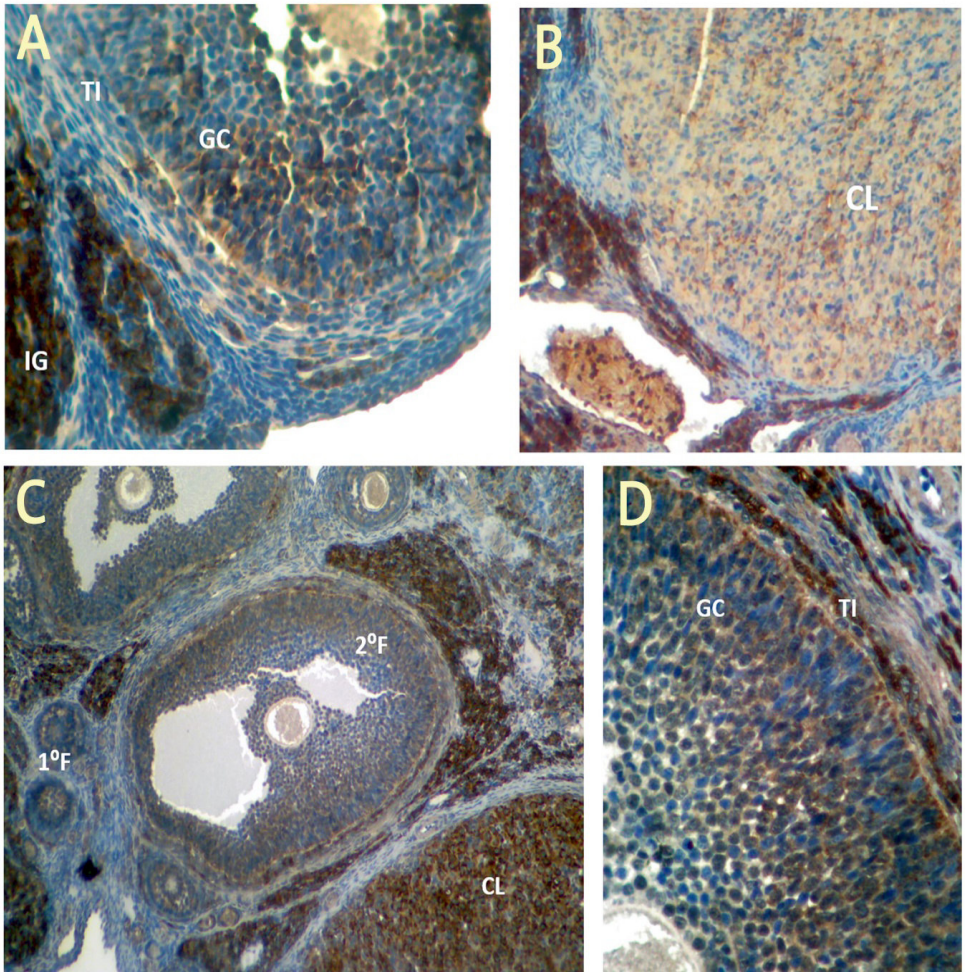

Figure 2: Estrogen receptor beta distribution in MET-treated group (A & B) and ANA-treated group (C & D). TI: theca interna, GC: granulosa cells, IG: interstitial glands, CL: corpus luteum, 1ºF: primary follicle, 2ºF: secondary follicle.

The distribution of ERβ in MET-treated subgroup and ANA-treated subgroup will be discussed together to match the aim of this study. The results revealed an obvious difference in ERβ distribution between these two subgroups, which could be attributed to the different modes of action of the treatment lines used in each subgroup.

Starting with MET-treated subgroup, as mentioned above, metformin is known to act locally (Attia *et al*, 2001; Mansfield *et al*, 2003), and systematically (Ehrmann, 2005) to reduce androgen synthesis by the theca cells, and hence, reduces estrogen production temporarily since androgen conversion into estrogen will be reduced (Cole and Robinson, 1990). This mechanism will stimulate the pituitary gland (through feedback mechanism) to resume its normal physiological secretion of gonadotropins, mainly FSH. FSH, which restore follicular maturation and estrogen production by the granulosa cells, will resume the normal physiological function of the ovary (Hall 2011). Besides, FSH induces a cofactor that coactivates ERβ in granulosa cells

(Cheng *et al*, 2002). Accordingly, the intensity of ERβ distribution that has been demonstrated using immunohistochemical methods, will be decided. However, this process of metformin-mediated hormonal normalization appears to be still suboptimal, i.e: metformin could not restore the physiological levels of sex hormones to the normal level, since prevention of hyperandrogenemia could be still subnormal. This was reflected clearly by deficiency in immunohistochemical reactivity regarding the distribution of ERβ and its intensity in different ovarian tissues, obviously seen in corpus luteum which was only detected in less than half of the animals, reflecting ovulatory failure in more than half of female animals in this subgroup.

Regarding ANA-treated subgroup, anastrozole is an aromatase enzyme inhibitor that acts by preventing the aromatization of androgens to estrogens. This leads to a reduction in estrogen level produced by conversion of androgen into estrogen in the ovarian follicles. The reduction in estrogen levels stimulates the pituitary gland to secrete more FSH and LH. The increased FSH level will stimulate the aromatization of the androgen in theca cells into estrogen (Barett *et al*, 2010), which appears to be enough to restore the follicular growth and maturation. The higher amount of LH secreted from the pituitary gland then induces ovulation in many mature follicles and resume normal maturation of corpora leutea, resuming the ovarian physiology to almost normal state. This is reflected by the results obtained in this subgroup via the immunohistochemical technique. According to the concept mentioned in the beginning of this discussion, since estrogen hormone is present, and the ovarian tissues that need estrogen could be detected, therefore ERβ distribution and intensity could be illustrated as had been shown in ANA-treated subgroup in this study. Besides, the sign indicating that anastrozole restored ovarian function near normal level is the resumption of ovulation: this study has demonstrated that all of the experimental animals treated with anastrozole have developed large corpora lutea, a definitive sign of ovulation (Sadler 2012).

To summarized the conclusion, PCOS therapy, specifically the medications used in this study (metformin and anastrozole), acts to standardize the ovary to restor its normal physiological function, whatever the mode of their action. In sequence this will preserve the normal physiological sexual hormonal cycle between the ovary and the pituitary gland leading to normal physiological function of the ovary, regarding maturation of the follicle and ovulation.

In this study, anastrozole proved to be superior in returning the ovary to its normal physiology than metformin did.

Recommendation:

To make the results and discussion solid enough, it is recommended to do hormonal assay including estrogen, FSH, LH and androgen in each subgroups received the different therapies.

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