Original Article

A Histomorphological Comparative Effects of Metformin, Pioglitazone, Repaglinide and Acarbose on Polycystic Ovary in Mice

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Received: 02 Nov 2019 Accepted: 27 Apr 2020

Abstract

Background & Objective: Polycystic ovary syndrome (PCO) is one of the most common causes of infertility due to anovulatory in women. The use of insulin-sensitizing agents was one of the treatments for this syndrome. The main purpose of this study was to evaluate the effects of Metformin, Pioglitazone, Ripaglinide and Acarbose on histomorphological changes of polycystic ovaries.

Materials & Methods: In this study, Polycystic ovaries were induced by injection of testosterone enanthate (TE) in immature female mice, for four weeks, then they had been divided into five groups; the control, Metformin, Pioglitazone, Repaglinide and Acarbose groups. Body weight, the ratio of ovary-to-animal weight, ovarian diameter, histomorphological changes of ovaries and characteristics of ovarian tissues were studied. Tukey test was used to calculate these parameters ($P \leq 0.05$).

Results:TE significantly increased the percentage of cystic Follicular and decreased follicular growth compared to treatment groups. The body weight, the ratio of ovary-to-animal weight and ovarian diameter, in all groups showed a significant decrease compared to the control group ($p<0.05$). In Metformin and Pioglitazone treated group, the number of degenerated oocytes, pyknotic-granlosa cells and vascularization were decreased and luteinization can be seen only in these groups. There is a significant reduction in mean growth of primordial, primary, pre-antral, cystic and atretic follicles in the treatment group. However, the mean number of pre-antral follicle showed a remarkable increase. The average number of antral follicles increased in metformin and pioglitazone groups ($p<0.05$). The results showed that Metformin and Pioglitazone groups cause a meaningful decrease in the ratio of ovary-to-animal weight ($p<0.05$).

Conclusion: According to these results, Metformin and Pioglitazone have the same effects and can compensate for the damages due to PCOs. These drugs can develop follicular growth. Repaglinide can compensate for the damages in some cases, and, Acarbose has a negative effect on follicular growth.

Keywords: Polycystic ovary syndrome, Metformin, Pioglitazone, Acarbose, Ripaglinide, Mouse

Introduction

Polycystic ovary syndrome (PCOs) is the most common endocrine , metabolic and genetic disorder in women (1). The cause of this syndrome is not known yet. PCOs occurs with heterogeneous clinical symptom including menstrual cycle disorders, hyperandrogenism, hirsutism, severe acne, alopecia, increased body mass, metabolic disorders such as high levels of blood insulin and insulin resistance, the risk of

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diabetes type II in older ages, cardiovascular disease, bilateral ovarian enlargement, anovulation and infertility (2). It is estimated that 35%-50% of women with PCOs have impaired glucose tolerance test. Type II diabetes has been determined with three pathophysiological abnormalities including impaired insulin secretion, peripheral insulin resistance and overproduction of glucose in the liver. The abdominal, obesity can be seen in all patients (3). The basic treatments of PCOs are the utilization of ovulation-inducing factors such as Clomiphene Citrate, insulin-sensitizing drugs, ovarian laparoscopy and losing weight (1). Identifying the relationship between PCOs and hyperinsulinemia and its role in the manifestation of hyperandrogenism and ovarian follicle developmental disorder in people with PCOs cause several studies on the effects of blood glucose-lowering drugs; in recent year. About 85 percent of regular menstrual cycles and ovulation have been improved using these drugs along with exercise and a decrease in carbohydrate diet (4).

Metformin is the primary member of Biguanids and one of the most common drugs in diabetes control. Metformin is known as insulin-sensitizers with dominant action on the liver causing a decrease in gluconeogenesis, with hepatic insulin resistance reduction. Blood glucose-lowering action of Biguanids is independent of pancreatic beta-cell function (5). Metformin activates a protein kinase called AMPK (5 AMP-activated protein kinas) which can be effective in most tissues (6). Metformin is the most common insulin reducing drug for PCOs treatment and gestational diabetes, causing significant improvement in the regulation of the menstrual cycle in order to restore ovulation and increases fertility (7). Weight loss caused by Metformin is usually accompanied by a loss of adipose tissue (8). Jyoti et al reported that Metformin has been effective in returning menstrual cycle and pregnancy in patients with PCOs and insulin resistance and is able to correct follicular development by increasing insulin sensitivity in PCOs patients (9). Safwat et al demonstrated that treatment with Metformin in patients with PCOs resistant to Clomiphene Citrate can induce ovulation and increases pregnancy, and therefore improves infertility (10).

Pioglitazone is another drug in Thiozolidindiones (TZDs) class, which is used to treat type II diabetes. Pioglitazone activates PPAR (Peroxisome Proliferator Activated Receptors) receptor which works as steroid and thyroid nuclear receptors. PPAR is activated by pioglitazone and increases the sensitivity of insulin and stimulates fat cell differentiation. Pioglitazone is able to induce ovulation, increasing the rates of ovulation and pregnancy as well as reduction of hyperandrogenism and hirsutism in PCOs patients (11). Paschou et al (2019) reported that Pioglitazone significantly improved hyperandrogenism and hirsutism in women with PCOs (12). Aroda et al (2009) reported that Pioglitazone improves ovarian androgen production and insulin metabolism in polycystic women (13).

Repaglinide is a new antidiabetic drug, in Meglitinide drugs by Carbamoylbenzoic acid structure. This drug with new contents of oral anti-diabetic drugs is similar to Sulfonylureas compounds but differs in molecular structure, mode of action and excretion (14). Repaglinide is binding to sulfonylurea receptor, which has specific sites on the B-cells of the pancreas. The insulinotropic function of Repaglinide has stimulated by inhibition of the potassium ion and ATP-sensitive potassium channel located in the membrane of pancreatic B-cells. This action causes a cell membrane depolarization and calcium ions influx entry into the cell through voltage-dependent valves, followed by increasing the concentration of intracellular calcium and stimulates the release of insulin from beta cells (15). The modification of insulin release, development and maturation of oocytes could have been done by Calcium (16). Repaglinide can be a good alternative for patients with gastrointestinal side effects caused by Metformin, with better control of blood glucose levels (14). Repaglinide, in comparison with Sulfonylureas, has fewer hypoglycemia side effects while preventing an increase in blood glucose levels after eating (17).

Acarbose is an intestinal alpha-glucosidase enzyme inhibitor which is widely used to treat the patient with diabetes type II as well as to prevent type II diabetes in people with impaired glucose tolerance (18). Acarbose causes a delay in absorption of complex carbohydrates in the intestinal mucosal layer, by competitive inhibition of alpha-glucosidase enzyme in the brush border of the small intestine. As a result of this delay, entering of glucose into the bloodstream can cause blood glucose levels to become minimized after feeding. It also can lead

to reducing the body weight and hypoglycemia occurring after feeding (19). Acarbose is more effective in the reduction of insulin resistance and hyperandrogenemia and also improves ovulation and regulates the menstrual cycle. Due to the intestinal absorption delay of carbohydrates, it seems that the use of Acarbose is an appropriate selection during pregnancy (18). Penna et al (2005) reported that Acarbose can cause weight loss and abdominal obesity in women with PCOs (20). Derosa and Maffioli (2012) proposed that the addition of Acarbose to Metformin has positive effects on glucose and lipid metabolisms, including decreases in the concentration of TG, LDL and total cholesterol, in comparison with Metformin alone (21).

Considering the importance of antidiabetic drugs on PCOs and prevalence of Metformin in the treatment of this syndrome (22), this study compares the effects of Metformin, Pioglitazone, Repaglinide and Acarbose on growth improvement of polycystic ovarian follicles and the treatment effects on damages caused by polycystic ovarian induction under the same condition.

Materials & Methods

Laboratory animals

In this research, 40 immature (5-7 weeks old) female NMRI mice with a body weight of 20-25 g were selected. In order to adapt to the new environment, they were housed under controlled conditions of light (12/12 h light/dark), at a temperature of 25 ° C and were allowed free access to sufficient food and water for 10 days.

In order to induce polycystic ovary, the daily injections of testosterone enanthate dissolved in sesame oil, 1 mg/100g of body weight, subcutaneously in the back of the neck, were performed for 4 weeks (23). Then, they have been divided into five groups with eight members.

1. The control group: receiving no drug treatment for two weeks.
2. The Metformin group: receiving 250 mg/kg of Metformin (24), (Sami Saz Company, Iran) Intrapentoneal injection (IP) for two weeks.
3. The Pioglitazone group: receiving 15 mg/kg of Pioglitazone (25), (Sami Saz Company, Iran) orally daily for 2 weeks.
4. The Repaglinide group: receiving Repaglinide (Sami Saz Company, Iran) 30 µgr/ mouse for 2 weeks Intraperitoneally (26).
5. Acarbose group: receiving 50 mg/kg of Acarbos (27), (Sami Saz Company, Iran) by orally daily for 4 weeks.

Ovarian histology:

In this study, the mice were killed by cervical dislocation and with the creation of a longitudinal T form slot in the abdomen, and the ovaries were extracted. The ovaries -after removing surrounding adipose and connective tissues- were weighted by digital scale and fixed in a 10% formaldehyde solution. After routine histological preparation, paraffin blocks were provided, the sections less than 5µm in diameter were prepared and stained by Hematoxylin-Eosin (H & E) protocol.

Histological studies:

All measurements of ovarian diameter were done by Dino Capture program. Histological changes in the control and treatment groups were evaluated by light microscopy (the lack of intended character marked with "- ", and having the character according to low, moderate and severe, respectively shown by "+", "++" and "+++"). The ovarian follicle counts were performed by an optical microscope with 40x and 100x magnification. Then, the follicles were divided into six groups as follow;

Primordial follicle (with a unilaminar of squamous granulosa cells around the oocyte), Primary follicle (with a layer of cuboidal granulosa cells around the oocyte), Preantral follicle (with multi-laminar of granulosa cells), the antral follicle (with antrum), the cystic follicle (thin-walled, lined by one or more layers of granulosa cell, filled with clear fluid) and atretic follicle (with degeneration follicular wall).

Statistical analysis:

IBM SPSS (version-19) was used for statistical analysis. Mean ± standard deviation of the number of follicles at each stage of follicle development was calculated and compared among studied groups. To compare the percentage of the

follicles in each stage of follicular growth, we used one-way ANOVA (one-way analysis of variance) and Tukey post hoc test. We also determined the significant coefficient of data at 95% significance level.

Results

For comparing different methods of treatments in PCOs, we used testosterone enanthate and the one-way analysis of variance (ANOVA) revealed that testosterone enanthate causes increasing cystic follicles and reduction of follicular development ($p \leq 0.05$). This observation showed that the minimum ovary/animal, weight was found in Metformin and Pioglitazone treatment groups. But in Repaglinide and Acarbose treatment groups, there is no significant difference in this ratio. This observation showed the similarity in mean weight of the mouse in treatment groups, but the control group showed the greatest mean weight compared to other groups ($p \leq 0.05$). The mean diameters of the ovary in the treatment groups were similar and showed a significant decrease compared to the control group ($p \leq 0.05$) (Table 1).

Our microscopic investigation showed that the control group had irregular, wavy appearance germinal epithelium, thin tunica albuginea, a large degree of hyperkeratosis in antral follicles, large amounts of degenerated oocytes and moderate to high disrupted amounts of granulosa cells. The nuclei of granulosa cells became pyknotic to some extent, the tissue seemed highly vascularized and no luteinization was seen in its layers (Figure 1). Ovaries of treatment groups with Metformin and Pioglitazone showed reduction in hyperkeratosis, the number of degenerated oocytes and pyknotization of granulosa cells. Also, the tissue was highly vascularized and luteinization between layers was observed (Figure 1). In the Repaglinide and Acarbose treatment group the ovary showed less hyperkeratosis, fewer number of degenerated oocytes, less pyknotization of granulosa cells; and highly vascularized tissue compared with control group but these changes are increased when they compared with Metformin and Pioglitazone groups and also luteinization between layers was not seen (Figure 1 and Table 2).

Data comparison by the ANOVA for the quantitative count of follicles in each phase of

Table 1: Body and ovary weight and diameter of ovary in the control and treatment groups (ANOVA, $P < 0.05$).

Groups	Body weight (gr)	the ratio of ovary weight to body weight (mgr.)	Ovary diameter (µm)
Control	28.8±0.62^{a}	0.166±0.58^{a}	845.28±11.22^{a}
Metformin	23.8±0.74^{b}	0.105±0.80^{b}	523.16±19.51^{b}
Pioglitazone	23.7±0.34^{b}	0.109±0.37^{b}	548.11±18.59^{b}
Ripaglinide	23. 2±0.54^{b}	0.154±1.01^{a}	587.18±25.92^{b}
Acarbose	22.2±0.42^{b}	0.192±1.01^{a}	713.18±74.19^{ab}

Table 2: Ovarian histology change in control and treatment groups

Groups	Germinal epithelium	Tunica Albuginea	Hyperkeratosis	Oocyte degeneration	Granulosa cells disrupted	Pyknotic granulosa	Blood vessels	Latinization
Control	+	+ +	+ + +	+ + +	+ +	+ +	+ + +	-
Metformin	+	+ +	+	+	–	–	+	+
Pioglitazone	+	+ +	+	+	–	–	+	+
Ripaglinide	+	+ +	+	+ +	+	+	+ +	-
Acarbose	+	+ +	+ +	+ +	+	+	+ +	-

-: no character observed, +: moderate character, ++: moderately sever character, +++: very sever character

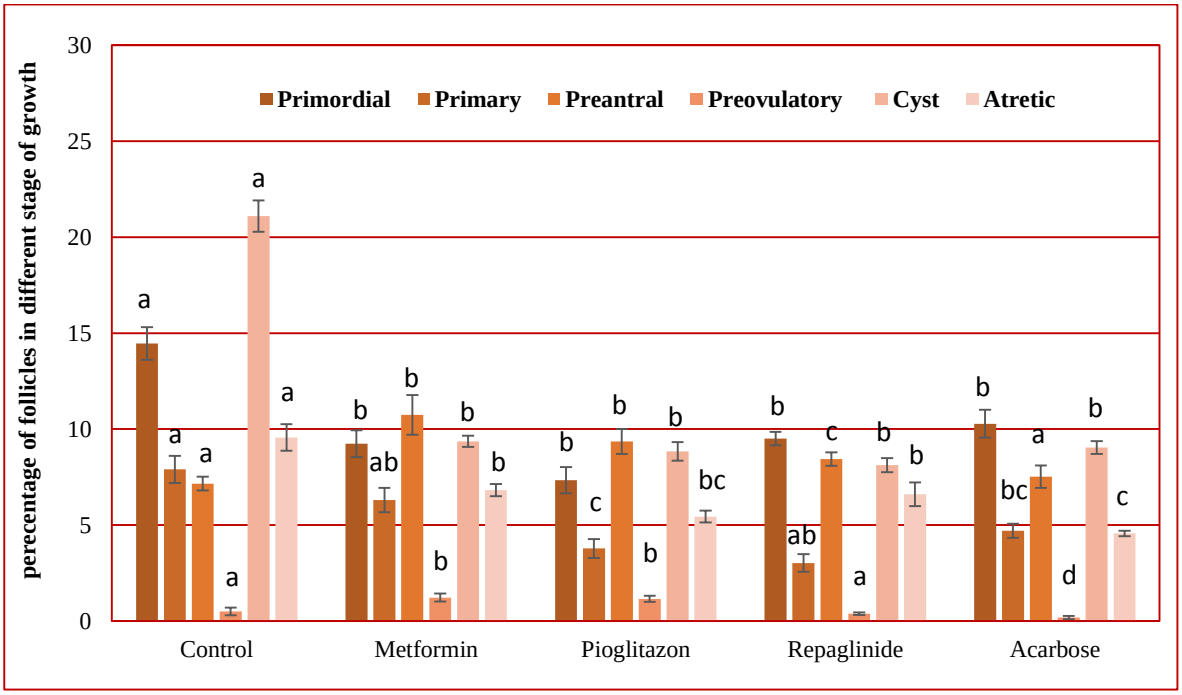

Chart 1: Percentage of follicles in different stages of growth in control and treatment groups. (ANOVA, *P* <0.05). a & b are the symbols to show exiting significant differences between the control and treatment groups.

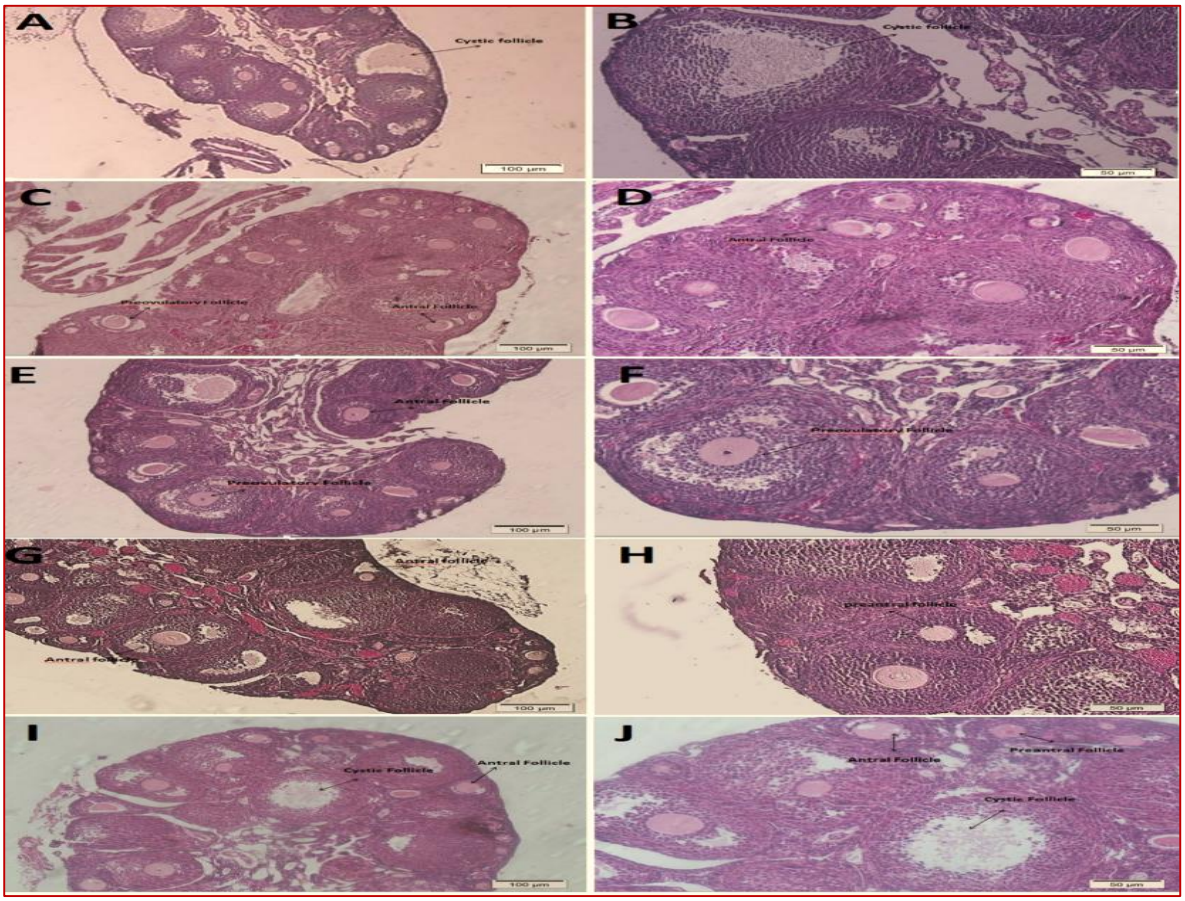

Fig 1: Photomicrographs of mice ovaries in control group and treatment groups. (A, B): Control The number of cystic follicles increased and follicles growth showed decrease. (C, D): Metformin, (E, F): Pioglitazone, the numbers of preantral follicles, antral follicles and ovarian follicular growth increased. (G, H): Ripaglinide,. (I, J): Acarbose the numbers of cystic follicles decreased. Ripaglinide can compensate the damages in some cases but Acarbose didn't have any effective role on improvement the ovarian follicular growth. (magnification×100 ×50) (H&E staining).

follicular growth demonstrated that although the average number primordial, primary, preantral and atretic follicles in treatment groups were similar, there was a significant decrease compared to the control group. The average number of preantral follicles in treatment groups is increased in comparison with the control group. The average number of antral follicles in the Metformin and Pioglitazone groups were similar but more than the other groups and control group ($p \leq 0.05$) (chart 1 & figure 1).

Discussion

In the present study, the histomorphological effects of Metformin, Pioglitazone, Repaglinide and Acarbose in polycystic ovaries of mice were investigated. Body weight, the ovary/animal weight, diameter of ovaries, follicle count and histological changes were evaluated. One of the most common histological changes in PCOs is large ovaries that resulted from discontinuous ovulation (28). PCOs are associated with dysfunction of adipose tissue metabolism and women with PCOs have hypertrophic fat cells. Due to insulin resistance, which can be seen in diabetes mellitus, type II. Although obesity is not a stimulating factor, it can be an accelerator of PCOs (29). Several methods, such as losing weight, were effectively used in the induction of ovulation and infertility treatments in PCOS women (1). Researchers - reported that Metformin decreases BMI in women with PCOs (30, 31). Glueck et al used Pioglitazone for 13 women with PCOs who used Metformin before- and suggested that adding Pioglitazone to Metformin were caused improvement in insulin resistance and reduction of blood lipids (32). De Souza et al reported that treatment with Pioglitazone causes the reduction of the glucose tolerance levels, triglyceride and free fatty acid levels; and improved insulin-resistant in diabetic patients (33). Other studies showed that Repaglinide causes a greater improvement in insulin-resistant diabetic patients compared to Glibenclamide, but was equally effective in glycemic control (34). Also, Ripaglindine can be effective in losing weight of diabetic patients (35). Researchers reported that Acarbose causes a significant decrease in BMI (36), reducing the risk of cardiovascular disease in patients with glucose intolerance and T2DM, as well as decreasing body weight and serum triglycerides (29). Our results agree with the previous studies and showed that Metformin, Pioglitazone, Repaglinide and Acarbose treatment groups showed a significant decrease in animal weight and decrease in ovarian weight and diameter, compared to the control group. The greatest decrease in ovarian weight and diameter were seen in Metformin and Pioglitazone treated groups. The most important diagnostic criteria for PCOs compare to normal ovary, was the wider tunica albuginea and increase the dispersion of hyperkeratosis, which is similar to order studies (28,37). Findings in all treatment groups are reduction of histological characteristics of polycystic ovaries, such as fewer degenerated oocytes, pyknotization of granulosa cells and tissue vascularization. The histological characteristics of ovaries in Metformin and Pioglitazone treatment groups were determined similar to each other; whereas luteinization can only be seen in the Metformin and Pioglitazone treatment groups. Therefore, all treatment groups act in order to compensate for the tissue damage caused by polycystic ovaries and this compensation was more significant in Metformin and Pioglitazone groups. Over recent decades, several animal models have presented for the induction of PCOs and they have been useful in understanding the pathogenesis. The best models are those that have been exposed to excess androgens before puberty in this period, testosterone injections do not affect the morphology of ovarian follicles; but after that, it leads to polycystic, anovulation, increases the atretic follicles and presence of unhealthy oocytes. Moreover, the treatment of PCOs causes smaller ovaries compared with healthy ones (23). Bloosesky et al (2004) showed that daily subcutaneous (SQ) injections of 1mg/100g of body weight of testosterone propionate dissolved in sesame oil induced PCOs in mice. Histological analysis of these mice clearly showed the presence of cystic follicles, anovulation and lack of corpus luteum compared with control group. Confirming of these reports, the obtained data from the average of follicles showed that polycystic ovary in mice can be induced by a dose of 1 mg/100 g body weight of testosterone enanthate. The most important diagnostic criteria in PCOs are the increased number of growing follicles and cystic follicles (23), arrested growth of early antral follicles and increase the number of atretic follicles in comparison with normal ovaries (37). Histological analyses of rats, which were exposed to uniform light for thirteen weeks

showed that uniform light causes the induction of PCOs in these animals. In this study no difference was observed in the reproductive parameters in PCOs animals compared with the control group (38). Anovulation in PCOs is characterized by the arrest of growth of antral follicles that indicated the development arrest of follicles until the preovulatory phase (39). Kocak et al (2006) reported that Metformin causes ovulation and increase the rate of pregnancy in PCOs women and so infertility is decreased (40). Also Metformin increases the ovulation induction and the live-birth and pregnancy rate in PCOs patients (10). Nagao et.al (2019) reported that Pioglitazone causes insulin-resistant, hyperandrogenism and ovulation rates in PCOs women to improve, also Pioglitazone was effective on lipid profile and able to induce ovulation, increase the rate of ovulation and pregnancy in PCOs women. Generally, increasing insulin therapy for PCOs patients leads to a significant improvement in their fertility and this treatment takes place in different positions such as; steroid secretion by ovarian cells, insulin-sensitivity in muscle, adipose tissue and adipose redistribution. However, before using it in clinical practices, several long tests should be processed to estimate the potential risks of these ligands and use it, especially during the first week of pregnancy, the monitoring should be carefully performed (11).

Ziaee et al (2012) reported that Pioglitazone in insulin-sensitivity and some cardiovascular diseases was more effective than Metformin, but it will not reduce BMI and body weight in PCOs patients (41). Ma et.al (2014) reported that both Metformin and Repaglinide were caused reduction in Triglycerides and improved glycemic patients with type II diabetes. However, Repaglinide has no effect on insulin-sensitivity but leads to improvement of the β-cells function (14).

Different studies showed that Repaglinide has a greater effect on reducing blood glucose after eating in comparison with Glibenclamide, while Glibenclamide was more effective for the reduction of fasting blood glucose (17). Other studies showed that Acarbose is similar to Metformin and it is effective in the ovulation rate of PCOs patients resistant to Clomiphene citrate (42) and Acarbose with Clomiphene Citrate, in compare with Metformin; Acarbose causes an increase in ovulation induction and reduction of BMI in infertile women with PCOs (43). The results of some studies showed there were no responses to Acarbose and this can cause by the short follow-up period in the patients (44) and our results agree with these studies. Histological analysis of ovary sections in control group clearly showed the existence of cystic follicles and accumulation of preantral, anovulation and lack of corpora luteum; also the number of primordial follicles significantly increased in compare with treatment groups; the number of preantral, cystic and atretic follicles in treated groups significantly decreased in comparison with control group. Histological observation of ovaries in Metformin and Pioglitazone groups were similar and showed a reduction in preantral follicles at the subcortical areas of ovaries. Also, more reduction of polycystic ovary characteristic (having numerous cysts and increase in primordial and atretic follicles) was revealed in these mice, in comparison with Repaglinide and Acarbose treatment groups; whereas preovulatory follicles only in both groups of Metformin and Pioglitazone significantly increased in comparison with other groups. Preovulatory follicles in the Repaglinide group showed no difference with control group, while the lowest preovulatory follicles were observed in the Acarbose group.

Conclusions

Although Metformin and Pioglitazone treatment groups cause reduction of ovary/animal weight ratio, animal weight loss, reduction of diameter in ovary and compensation for damage caused by polycystic ovarian tissue, these treatments lead to increase in ovarian follicular growth. Repaglinide and Acarbose also cause reduction in animal and ovarian weight and reduction in diameter of ovaries and could partly compensate for tissue damage caused by polycystic ovary, while Acarbose didn't have any effective role on improvement the ovarian follicular growth.

Acknowledgments

This paper presents data as part of a master's thesis of the first author with code 862082. This research was conducted based on international laws of society for the prevention of cruelty to animals (SPCA) that was founded in 2006 in the United States and adopted in the Ethics Committee of Razi University, Kermanshah, Iran.

This study was supported by Kermanshah Razi University. We should warmly appreciate the valuable contribution of Sami Saz Company, Tehran, Iran.

Conflict of Interests

The authors report no declarations of interest.

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مقاله پژوهشی

مقایسه اثرات داروهای متفورمین، پیوگلیتازون، ریپاگلینید و آکاربوز بر هیستومورفولوژی تخمدان پلی کیستیک موش

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تاریخ دریافت مقاله: ۱۳۹۸/۰۸/۱۱ تاریخ پذیرش مقاله: ۱۳۹۹/۰۲/۰۸

چکیده

زمینه و هدف: سندروم تخمدان پلی کیستیک شایع ترین علت ناباروری ناشی از عدم تخمک گذاری در زنان می باشد. یکی از راههای درمان این سندروم استفاده از داروهای حساس کننده به انسولین است. هدف اصلی این پژوهش ارزیابی تاثیر داروهای متفورمین، پیوگلیتازون، ریپاگلینید و آکاربوز بر هیستومورفولوژی تخمدان پلی کیستیک می باشد.

مواد و روش ها: در این مطالعه القای تخمدان پلی کیستیک با تزریق تستوسترون انانتات به موش های نابالغ برای مدت ۴ هفته انجام گرفته است. سپس موش ها در پنج گروه مساوی تقسیم شدند. کنترل ، متفورمین ، پیوگلیتازون ، ریپاگلینید و آکاربوز. وزن بدن، نسبت وزن تخمدان به وزن حیوان ، قطر تخمدان، تغییرات هیستولوژی تخمدان ها و ویژگی های بافتی تخمدان ها بررسی گردیده است. برای این مطالعه از تست آماری توکی استفاده شد ($P\leq0.05$).

نتایج: تستوسترون انانتات بطور قابل ملاحظه ای باعث افزایش میانگین فولیکول های کیستیک و باعث کاهش رشد فولیکولی در مقایسه با گروه درمان شده است. وزن بدن، نسبت وزن تخمدان به وزن حیوان ، قطر تخمدان در همه گروه ها بطور قابل ملاحظه ای کاهش یافته است در مقایسه با گروه کنترل($p<0.05$). در گروه متفورمین و پیوگلیتازون تعداد اووسیت های دژنره شده، سلول های گرانولوزای پیکنوزه شده و رگزایی کاهش یافته و لوتئینی شدن فقط در این گروهها مشاهده شد. کاهش معنی داری در فولیکول های پریموردیال، پرایمری، پره آنترال، کیستیک و آترتیک در گروه های درمانی دیده شد. هر چند که میانگین فولیکول های پره آنترال بطور قابل ملاحظه ای افزایش را نشان داد. میانگین فولیکول های آنترال در گروه های متفورمین و پیوگلیتازون افزایش معنی داری را نشان داد($p<0.05$). نتایج نشان داد که نسبت وزن تخمدان به وزن حیوان درگروه های متفورمین و پیوگلیتازون بطور معنی داری کاهش یافته بود($p<0.05$).

نتیجه گیری: بر طبق این نتایج متفورمین و پیوگلیتازون اثرات مشابه دارند و میتوانند آسیب های ناشی ازتخمدان پلی کیستیک را جبران کنند. این داروها میتوانند رشد فولیکولی را توسعه دهند. ریپاگلینید نیز میتواند تا حدودی آسیب های ناشی از تخمدان پلی کیستیک را جبران کند و آکاربوز اثر منفی بر رشد فولیکولی دارد.

کلمات کلیدی: سندروم تخمدان پلی کیستیک، متفورمین، پیوگلیتازون، آکاربوز، ریپاگلینید، موش

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