Original Article

Insulin Ameliorates Folliculogenesis in An Experimental Model of PCOS Mice

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Abstract

Background: Insulin is an essential factor that controls female reproductive system. Insulin signaling via *Foxo1* and *Akt1* can improve steroidogenesis, cell proliferation, and protein synthesis. We aimed to determine the effect of insulin on possible changes in gene expression, hormonal status, and histological aspects of the ovary following the induction of the animal model of polycystic ovary syndrome (PCOS).

Materials and Methods: In this experimental study, 24 adult female NMRI mice weighing 25-30 g were randomly placed in three groups: control, PCOS (60 mg/kg dehydroepiandrosterone (DHEA) for 20 days, and PCOS+insulin (60 mg/kg DHEA for 20 days+100 µL insulin diluted in water twice a week for 30 consecutive days). Blood specimens were obtained from the heart and the serum levels of testosterone, progesterone, and estradiol were measured. Right, and left ovaries were removed for real-time polymerase chain reaction (PCR) and stereological study.

Results: DHEA injection significantly amplified the concentration of testosterone, progesterone, and estradiol. While insulin treatment amended the level of reproductive hormones. DHEA injection significantly reduced the expression levels of *Irs1-4, Pdk1, Pi3k,* and *Akt1-3* and raised the expression level of Caspase-3. However, insulin administration amplified expression levels of *Irs1-4, Pdk1, Pi3k,* and *Akt1-3*, and reduced Caspase-3. The total volume of ovarian tissue in mice receiving DHEA significantly declined compared to the control group. Besides, a substantial decrease was detected in the number of ovarian antral, Graafian, and primordial follicles and also in the total number of corpus luteum following DHEA administration. Comparison of structural alterations in ovarian tissue between the PCOS+insulin and the PCOS groups displayed that insulin administration improved the total number of Graafian, primordial, and antral follicles and also corpus luteum.

Conclusion: In general, short-term insulin treatment showed improvement in hormonal balance, folliculogenesis, and insulin resistance in the ovaries of the PCOS mice model.

Keywords: Folliculogenesis, Insulin, NMRI Mice, Ovarian Function, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is one of the prevailing endocrine conditions in women, disturbing metabolic and reproductive systems in its course as a cycle and showing different phenotypes individually (1). According to global standard documents, PCOS currently affects 5 to 15% of the population of women of reproductive age worldwide. In addition, statistics show that the incidence of PCOS has increased from 1.45% in 2007 to 1.91% in 2017 (2, 3). Until 2005, an average of 4.36 billion dollars was spent every year in the United States of America on the diagnosis and treatment of PCOS, of which more than 40% was spent on the management of developed diabetes mellitus (4). Despite its massive social and economic problem, not much attention has been paid to research on various aspects of this syndrome (5).

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PCOS is often diagnosed based on the presence of two out of the three main conditions (the Rotterdam criteria): ovulatory dysfunction, biochemical and/or clinical hyperandrogenism, and polycystic ovarian morphology. Although genetic plays a crucial role in the etiology of PCOS, the role of acquired risk factors is undeniable. Reasons such as obesity, low physical activity, and improper dietary increase the chance of developing the disease. A relatively wide range of complications of PCOS has been observed, which usually affect a person's normal life. Some disorders including metabolic syndrome, cardiovascular diseases and anovulatory infertility can all be associated with PCOS. Therefore, since there is no definitive cure for the treatment of PCOS, the aim of conventional therapies is mainly to reduce the suffering symptoms caused by the syndrome. Current remedies include oral contraceptive pills containing anti-androgenic progestin and insulin-sensitizers along with lifestyle modification and weight loss (1, 6).

It seems that in PCOS, insulin sensitivity reduces glucose uptake in oocytes by regulating the expression of glucose transporters of granulosa cells and thereby reducing energy metabolism (7).

The activity of insulin along with insulin-related absorption of glucose on ovarian cells and ovarian function is important in several areas of reproductive medicine. Although gonadotropins are the principal controllers in folliculogenesis, this process can also be modulated by insulin. In granulosa and theca cells, insulin signaling can increase steroid production via Akt serine/threonine kinase 1 (*Akt1*) and forkhead box protein O1 (*Foxo1*). It can also increase protein synthesis and cell proliferation in ovarian cells through *mTOR/ERK* as well as hinder apoptosis (8). Given that almost all the controlling mechanisms associated with the influences of insulin on folliculogenesis are not yet fully understood, further evaluations are needed to better identify the pathways of insulin function and the role of glucose in folliculogenesis.

It is notable that understanding the mechanisms related to the pathophysiology of PCOS will help to realize appropriate approaches to prevent and improve the symptoms of the syndrome. Hyperandrogenism triggered by insulin resistance and the particular impact of insulin on ovaries has been predicted to be determining factors in the development and progression of PCOS (1, 9). So, the goal of this study was to clarify the effect of insulin on possible changes in gene expression and hormonal and histological status of the ovary following the induction of PCOS in an animal model.

Materials and Methods

Animals

For this experimental research, 24 Prepubertal Naval Medical Research Institute (NMRI) female mice weighing 25-30 g were purchased from Pasteur Institute, Tehran, Iran, and cared for under standard conditions in the animal house of Shahid Beheshti University of Medical Sciences. The standard conditions for keeping animals were as follows: limitless access to water and food, 12-hour light/dark cycle, and normal room temperature (RT) (22 ± 2°C). After adapting to the conditions, mice (n=8 per group) were randomly placed in three main groups including control, the PCOS, and the PCOS+insulin. All experimental procedures were approved by the Committee of Ethics, Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1399.1120).

Induction of PCOS model and insulin therapy in mice

In the PCOS group, the previous method approved by Aragno et al. (10) was used to induce the PCOS model. For this purpose, 60 mg/kg of dehydroepiandrosterone (DHEA) dispersed in a 9:1 combination of sesame oil and 95% ethanol was injected subcutaneously (SC) in prepubertal female NMRI mice with a mean age of about 21 days for 20 days. For mice in the control group, a mixture similar to the PCOS group was administered without DHEA. Finally, in the PCOS+insulin group, DHEA-treated mice received intraperitoneal (IP) injection of 100 µL insulin (Sigma, St. Louis, MO, USA) diluted in water twice a week for 30 consecutive days.

Sample preparation for analysis

At the end of treatments, mice were deeply anesthetized and sacrificed by intraperitoneal (IP) injection of sodium pentobarbital. Next, transcardial perfusion was done using normal saline and fixative solution, comprising 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Afterward, the left ovaries were collected, stored in 10% formalin solution, and prepared for histological and stereological analyses. Right ovaries were also used for biochemical and molecular examinations. The whole ovarian samples [granulosa cells (GCs) and oocytes] were used in all groups.

Confirmation of PCOS induction using ovarian histological and biochemical analysis

To confirm the induction of PCOS, ovary samples were harvested, rinsed in normal saline, and fixed in 10% formalin solution for 48 hours at RT. Next, a set of ethanol and xylene solutions were used for tissue dehydration and clearing processes, respectively. Subsequently, the tissue samples were embedded in paraffin. Serial sections were prepared using a microtome, Leica RM2125 RTS, Germany) with 5 and 10 µm thickness. The hematoxylin and eosin (H&E) technique (Sigma, USA) was utilized to stain tissues. Observing a large number of cysts in ovarian tissue by light microscopy (Nikon; ECLIPSE E200, Tokyo, Japan), was considered as the major sign of PCOS.

In addition to histological confirmations, serum concentration of hormones including testosterone, progesterone, and 17 β-estradiol was analysed using the ELISA assay (CSB-E05104m for progesterone, CSB-E05101m for testosterone, and CSB-E05109m for 17 β-estradiol).

Table 1: Polymerase chain reaction (PCR) amplification primers

Genes	Primer sequences (5´-3´)	Temperature (ºC)	Product size (bp)
Irs1-4	F: ACGTTCCAAGTGGTGCATC R: ACGTTCCAAGTGGTGCATC	58	332
Caspase-3	F: AGTGGGACTGATGAGGAGATGG R: AGTGGAGTACAGGGAGAAGGA	57	151
Pdk1	F: CGGATCAGAAACCGACACA R: ACTGAACATTCTGGCTGGTGA	53	160
Akt1-3	F: TCTGACGGGTAGAGTGTGCGT R: CTACTTCCTCCTCAAGAATGA	59	160
Pi3k	F: TAGCTGCATTGGAGCTCCTT R: TACGAACTGTGGGAGCAGAT	58	155
β-actin	F: TCAGAGCAAGAGAGGCATCC R: GGTCATCTTCTCACGGTTGG	60	293

Quantitative-polymerase chain reaction analysis

The real-time polymerase chain reaction (PCR) technique was carried out to determine the expression level of genes involved in apoptosis and regulating insulin activity. The, total RNA was isolated from ovary samples using a specific kit (Life Technologies, Gent, Belgium) in accordance with the manufacturer's manual instructions. After that, cDNA was reverse-transcribed from 1 µg of the total RNA in a 20 µl reaction mixture (Takara Bio, Inc., Kusatsu-Shi, Japan). The absorbance ratio of all samples at 260 and 280 nm (A260/280) was approximately 1.808-2.014. Also, A260/230 ratios were about 0.3-0.7. Primer 3 Plus software and the exon-exon junction technique were used to design the reverse and forward primers (11). Before that, PCR primers were tested using the Primer-Blast tool available at the website, www.ncbi.nlm.nih.gov/tools/primer-blast (Table 1).

Tissue preparation and stereological assessment

Following ovarian tissue sampling, processing, and slicing, the systematic uniform random sampling (SURS) method was applied to take 10 slices of each specimen by collecting a random figure in the range of 1 to 10 and the selected samples were stained with H&E (12). Serial sections with thickness of 5 and 10 µm were organized to estimate the volume and the number of ovarian follicles, respectively. Thus, histological elements including the volume of the ovary and the total number of primordial, primary, secondary, antral, and Graafian follicles, and also the total number of corpus luteum were estimated. An unbiased counting frame with inclusion and exclusion limits was placed over the pictures and to estimate the z-axis movement, a microcator was fixed on the stage of the light microscope. Any ovarian follicles and corpus luteum coming into the highest focus within the next focal sampling plane were designated if they lay partially or totally inside the frame and did not contact with the exclusion edge. Moreover, the total number of ovarian follicles and corpus luteum was calculated using the optical dissector method and the following formulas (13, 14):

The numerical density (Nv) was deliberated as:

$$Nv=(\sum Q)/(\sum P\times h\times a/f)\times t/BA$$

(ΣQ) represents cell nuclei number, (ΣP) represents the total number of the unprejudiced calculating frame in entire fields of ovarian tissue, (h) represents the disector height, (a/f) represents the area of the frame, (t) represents the actual thickness of slice measured in all fields of tissue, and (BA) represents the block advance of the microtome which was fixed for 10 µm. Ultimately, the total number of ovarian follicles and corpus luteum (N_{total}) was measured by multiplying the numerical density (Nv) by the total volume:

$$N_{total}=Nv\times V$$

On the other hand, the total volume of the ovary was assessed via the Cavalieri method and the next principle (15, 16):

$$V_{total}=\sum p\times a/p\times t$$

In the corresponding formula, (Σp) means the total number of points superimposed on the microscopic pictures, (a/p) means the zone correlated with each point, and (t) means the thickness of the microscopic slices.

Statistical analysis

Data were evaluated by ANOVA and Tukey post hoc tests using the SPSS software, version 19.00 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered as significant difference among groups.

Results

Insulin treatment reduced the level of reproductive hormones following PCOS induction

Hormonal analysis indicated that DHEA injection significantly amplified the concentration of reproductive hormones in the PCOS group including testosterone ($P<0.001$, Fig.1A), progesterone ($P<0.05$, Fig.1B), and estradiol ($P<0.001$, Fig.1C) in comparison with the control group. Conversely, insulin treatment significantly reduced the concentration of testosterone ($P<0.05$, Fig.1A), progesterone ($P<0.05$, Fig.1B), and estradiol compared with the PCOS group ($P<0.001$, Fig.1C).

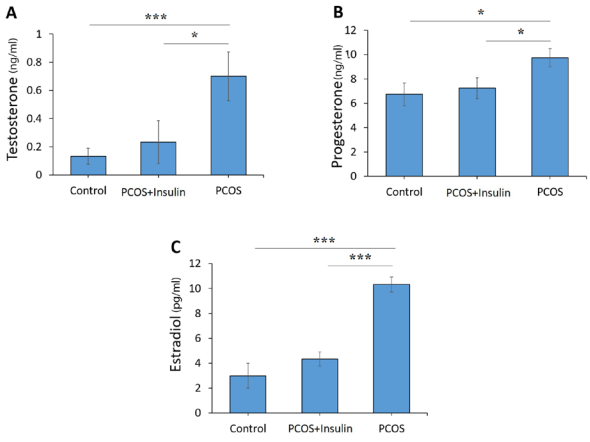

Fig.1: The effect of insulin administration on hormonal alterations following PCOS induction in NMRI mice. **A.** The effect of insulin administration on testosterone level. **B.** The effect of insulin administration on progesterone level. **C.** The effect of insulin administration on estradiol level. Results were reported as mean ± SD in study groups. PCOS; Polycystic ovary syndrome, *; P<0.05, and ***; P<0.001.

Insulin therapy caused alterations in histological parameters following PCOS induction

Histological analyses revealed significant changes in ovarian tissue following injection of DHEA. Numerous pre-antral and antral follicles, subcapsular cysts as well as atretic follicles were observed in the PCOS group. However, these changes were ameliorated following insulin treatment in the PCOS+insulin group (Fig.2). Hereafter, the total volume of ovarian tissue in mice receiving DHEA significantly declined compared to the control group (P<0.001, Fig.3A). While there was no considerable amendment in tissue volume in insulin-treated mice compared with the PCOS group (Fig.3A). The results attained from the stereological study exhibited a significant reduction in the total number of ovarian antral follicles (P<0.001, Fig.3B), Graafian follicles (P<0.001, Fig.3A), primordial follicles (P<0.001, Fig.3B), and the total number of corpus luteum (P<0.001, Fig.3A) following the use of DHEA compared to the control group. In contrast, the results revealed a significant increase in the total number of both ovarian primary and secondary follicles in the PCOS group in comparison with the control group (P<0.01 and P<0.001, respectively, Fig.3B). Evaluation of structural alterations in ovarian tissue among the PCOS+insulin and the PCOS groups showed that insulin administration improved the total number of some parameters including Graafian follicles (P<0.001, Fig.3A), corpus luteum (P<0.05, Fig.3A), primordial (P<0.001, Fig.3B), and antral follicles (P<0.001, Fig.3B).

Insulin administration amplified expression levels of *Irs1-4*, *Pdk1*, *Pi3k*, and *Akt1-3*, whereas reduced Caspase-3 following PCOS induction

Real time PCR analysis showed DHEA injection considerably declined the expression levels of *Pdk1* (P<0.001, Fig.4A), *Irs1-4* (P<0.05, Fig.4B), in comparison with the control group. In contrast, DHEA injection significantly raised the expression level of *Caspase-3* (P<0.001, Fig.4C) in comparison with the control group. Furthermore, the results showed that the DHEA injection considerably declined the expression levels of Pi3k (P<0.01, Fig.4D), Akt1-3 (P<0.05, Fig.4E) in comparison with the control group. On the other hand, insulin treatment significantly improved the expression levels of *Pdk1* (P<0.001, Fig.4A), *Irs1-4* (P<0.001, Fig.4B), *Pi3k* (P<0.01, Fig.4D), and *Akt1-3* (P<0.05, Fig.4E). In addition, insulin was able to significantly decrease the expression level of *Caspase-3* (P<0.05, Fig.4C) in comparison with the PCOS group.

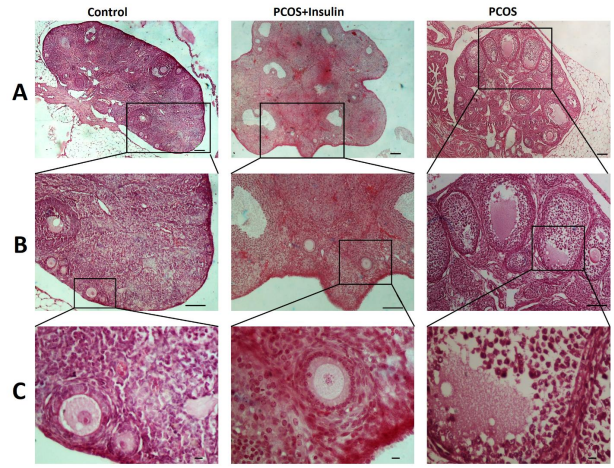

Fig.2: H&E-stained section of ovaries from all groups (control, the PCOS, and the PCOS+insulin). Follicles of various stages are present. In the PCOS group, substantial changes including atretic follicles, subcapsular cysts, and several antral follicles (marked area) were seen after injection of DHEA. In the PCOS+insulin group, a decline in the number of cystic and atretic follicles was observed (scale bar: **A.** 4x=100 µm, **B.** 10x=100 µm, and **C.** 40x=10 µm). PCOS; Polycystic ovary syndrome and DHEA; Dehydroepiandrosteron.

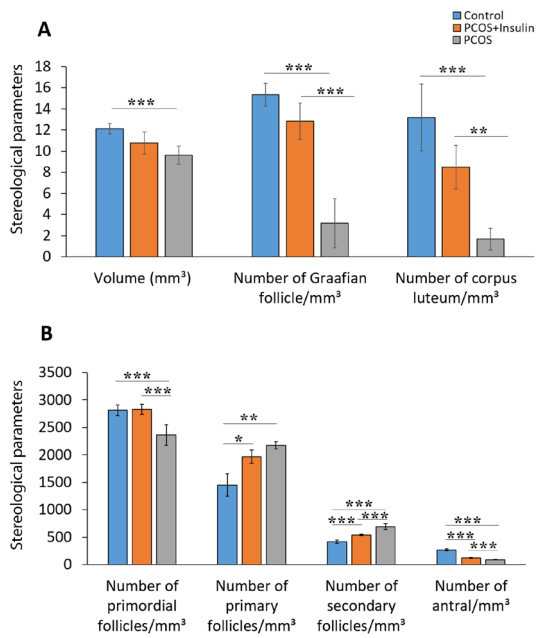

Fig.3: The effect of insulin administration on histological changes following PCOS induction in NMRI mice. **A.** Total number of Graffian follicles and corpus luteum as well as total volume of ovarian tissue. **B.** Total number of primordial, primary, secondary, and antral follicles in ovarian tissue. Results represented as mean ± SD in study groups. PCOS; Polycystic ovary syndrome, *; P<0.05, **; P<0.01, and ***; P<0.001.

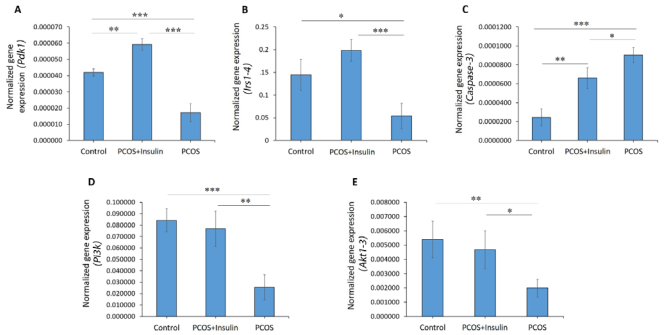

Fig.4: The effect of insulin administration on the expression of genes involved in insulin-related ovarian functions and apoptosis following PCOS induction in NMRI mice. **A.** *Pdk1*, **B.** *Irs1-4*, **C.** *Caspase-3*, **D.** *Pi3k*, and **E.** *Akt1-3*. Three biological replicates were analysed per gene. Results represented as mean ± SD in study groups. PCOS; Polycystic ovary syndrome, *; P<0.05, **; P<0.01, and ***; P<0.001.

Discussion

In the present investigation, the data indicated that the serum level of testosterone, estradiol, and progesterone as well as the mRNA expression level of the executioner *Caspase-3* significantly decreased in the PCOS+insulin group compared to the PCOS group. On the other hand, the data indicated a significant increase in the level of *Irs1-4*, *Pdk1*, *Pi3k*, and *Akt1-3* mRNA expression, as some of the key agents in the *Pi3k/Akt/Foxo3* pathway and also the number of Graafian follicles in the PCOS+insulin group compared to the PCOS group.

Most of PCOS patients show insulin resistance to some extent and it is widely accepted that insulin plays a great task in the pathophysiology of PCOS. It is also suggested that insulin sensitivity of body organs is decreased in most of PCOS patients, while insulin sensitivity of ovaries is preserved. On the other hand, there is another theory proposing the hypersensitivity of PCOS ovaries to insulin (17, 18). Additionally, it has been declared that insulin sensitivity of insulin targets in normal organs, as well as ovaries, is decreased in most PCOS patients, affecting only metabolic activities of insulin in cells and preserving its mitogenic effects (19). Based on previous studies, insulin promotes early stages of follicle development, decreases follicle apoptosis, and increases the release of reproductive hormones from ovaries, which leads to PCOS morphology (20, 21). But a complete and integrated representation of the pathophysiology of PCOS and the role of insulin is not depicted yet.

It is generally believed that a hormonal imbalance is an important indicator of PCOS. So, androgen excess is the crucial agent of the pathogenesis of this condition. Some studies introduced free testosterone level as the most sensitive factor to determine hyperandrogenemia (1). It is observed that estradiol level is increased in PCOS patients (22), but low levels of estrogens are also detected in other studies (6). We detected increased levels of testosterone, estradiol, and progesterone in PCOS mice compared to the control group, which confirms the induction of PCOS. Treatment with insulin decreased levels of reproductive hormones and approximated them to that of the control group. Although compensatory hyperinsulinemia in response to insulin resistance leads to hyperandrogenism, based on our results exogenous insulin reduced the serum level of testosterone. So, it seems that insulin might have a dose-dependent manner. Therefore, evaluation of the effects of insulin on PCOS, in different doses, can be a useful suggestion for finding more reliable data.

Another aspect of PCOS is impaired folliculogenesis and consequent oligo-anovulation. There are three main processes called initial recruitment, cyclic recruitment, and selection which lead to ovulation of a single dominant Graafian follicle in each cycle. During initial recruitment which repeats throughout the whole life, the primordial follicles turn into primary, secondary, and antral follicles respectively. Antral follicles undergo atresia except for a few of them which survive through cyclic recruitment due to cyclic FSH secretion after puberty. Selection happens when one of the recruited antral follicles grows faster than others and becomes a Graafian follicle (23). In PCOS, the initial and cyclic recruitment processes happen normally but the selection process is impaired, and no dominant follicle becomes Graafian. Resultant anovulation decreases the fertility rate and leads to the accumulation of many arrested antral follicles in the ovary. To investigate folliculogenesis in each group, we recorded the number of follicles in all phases of development.

The results of this study demonstrated that insulin treatment can normalize the state of PCOS ovaries somewhat similar to healthy tissue by ameliorating histopathologic effects of PCOS, especially with the prevention of the decline in the number of Graafian follicles as well as corpus luteum which can imply an increase in the ovulation process. It was reported that due to the accumulation of arrested follicles in the ovaries of PCOS patients, the volume of ovaries is increased. But our results showed PCOS ovaries are smaller than the PCOS+insulin group, and the PCOS+insulin group is smaller than the control group. It can be attributed to the density of primordial follicles in the PCOS group which is less than the PCOS+insulin and the control groups, while withthe density of primary follicles it is the opposite. It is consistent with the suggested over-activation of primordial follicles in the PCOS state, and it seems that insulin treatment modulated this activation. It is also noteworthy that although the density of secondary follicles in the PCOS group is greater than in the PCOS+insulin and the control groups, the density of antral follicles in the PCOS group is less than those in the two other groups. It can be compliant with the theory attributing the pathogenesis of PCOS to a disruption in the Hippo signaling pathway (24). It is believed that the Hippo signaling pathway adjusts the development of secondary follicles to antral follicles.

Insulin has two main signaling pathways, *Pi3k/Pkb* and *MAPK/ERK*. *Pi3k/Pkb* mainly regulates the metabolism and survival of cells and prevents apoptosis (18). There are cross-talks between insulin and LH or FSH pathways in ovaries. *Akt*, an agent in the lower stream of the *Pi3k/Pkb* pathway, is introduced as an important cross-talk in some studies (8), while others consider *Pi3k*, the molecule

in upper stream of the pathway. It was observed that over-activation of the pathway increases steroidogenesis in both granulosa and theca cells (25). It also increases protein synthesis and decreases apoptosis in granulosa cells (8). *Pi3k/Akt/Foxo3* pathway activation displays a vital function in the activation of primordial follicles (24). It was also detected that in PCOS patients, insulin resistance is the result of impairment in *Pi3k* signaling. In our study, we observed that in PCOS mice, expression of *Irs1-4*, *Pdk1*, *Pi3k*, and *Akt1-3* as some of the key factors in this pathway, is less than in control and the PCOS+insulin groups. Insulin treatment increased their expression and approximated it of the control group. *Caspase-3* is a factor that induces apoptosis in ovaries. Its level in the PCOS group was greater than in the control group. Furthermore, insulin treatment decreased *Caspase-3* expression and thus apoptosis in the ovary of PCOS mice (26).

Conclusion

Briefly, short-term insulin treatment shows improvement in hormonal balance, folliculogenesis, and insulin resistance in the ovaries of the PCOS mice model. For further evaluation, we recommend investigation of the effects of insulin treatment on fertility and survival of fetus as it has been reported that insulin in higher concentrations than physiological concentrations decreases the quality of the oocyte. Concomitant administration of insulin-sensitizing agents and insulin can also be a new possibility to enhance fertility rates among PCOS patients.

Acknowledegements

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Authors' Contributions

M.-A.A., V.E.; Designed this study and drafted the manuscript. M.K.G., S.R.; Conducted the stereological study and provided the clinical data and sample. M.A., S.M.; Helped to draft the manuscript and provided the molecular sample. A.Y., H.N.; Performed the statistical analysis and helped to draft the manuscript. A.G., A.K.G., A.A.; Carried out the animal model and molecular test. Sh.A., S.I., S.Z.; Carried out the hormonal test and helped with data analysis. All authors read and approved the final manuscript.

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