Research Article

Ameliorating effect of tocilizumab in letrozole induced polycystic ovarian syndrome in rats *via* improving insulin resistance and down-regulation of VEGF/ANGPT/PDGF

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Abstract

Introduction: Polycystic ovarian syndrome (PCOS) is the most common endocrinal pathology that causes anovulatory infertility. This study explored the possible therapeutic effect of tocilizumab (TCZ), an interleukin (IL)-6 receptor inhibitor in PCOS induced by letrozole in female Wistar rats, which settled both clinical and metabolic criteria as in PCOS patients. **Methods:** Letrozole (1 mg/kg) was administered per orally *(p.o.*) for a period of 21 days for the induction of PCOS. TCZ was then followed in a dose of either (4 mg/kg, *i.p.*) or (8 mg/kg, *i.p.*), once weekly for 3 doses. Metformin (2 mg/100 g/day, *p.o.*) was utilized as a standard group using 0.1% w/v CMC as vehicle. **Results:** The administration of TCZ significantly reduced the increase in body weight, ovarian diameter, and cysts, as well as abnormalities in serum sex steroid, insulin, glucose levels, and lipid profile levels demonstrated in PCOS group. Besides, TCZ significantly decreased the elevation in oxidative stress parameters, and normalized the depletion in antioxidant activity reported in the PCOS rats. Moreover, TCZ exhibited a significant reduction in the elevated serum levels of tumor necrosis factor (TNF)-α and IL-6. Finally, Letrozole induces a remarkable increase in ovarian angiogenic markers, vascular endothelial growth factor (VEGF), angiopoietin (ANGPT), and platelet-derived growth factor (PDGF), while TCZ was able to successfully reduce these markers to baseline levels. **Conclusion:** TCZ showed beneficial effects in Letrozole induced PCOS in rats. Its effect was parallel to that of metformin, the most widely used remedy in PCOS condition. Thus, its ability to block the IL-6 receptor, restore the ovarian function and exerts anti-androgenic, anti-metabolic, antioxidant, and anti-angiogenic effects may offer an advantageous therapeutics for PCOS.

Keywords: Tocilizumab, Metformin, Polycystic ovarian syndrome, IL-6, Angiogenesis, Insulin resistance.

Introduction

Polycystic ovarian syndrome (PCOS) is the most prevalent endocrinal pathology that causes anovulatory infertility (Broekmans et al., 2006), affecting 6–20% of reproductive age women (2004). In order to be diagnosed, the two hallmark criteria that must be met are oligoovulation/anovulation as well as clinical/ biochemical signs of polycystic ovaries and hyperandrogenism. (Azziz et al., 2009). PCOS is not restricted to being an ovarian disease, it is rather an exhausting multifactorial syndrome, presenting as frequent abnormalities of lipid and glucose metabolism (Karakas et al., 2010). Prolonged sequelae in PCOS include metabolic alternations, hyperinsulinemia, diabetes mellitus, obesity, amenorrhea/oligomenorrhea endometrial carcinoma, and cardiovascular disease (Franks, 1995, Kadowaki and Yamauchi, 2005, Escobar-Morreale et al., 2006).

To date, no single medication exists for PCOS condition. Currently available lines of treatment include insulin sensitizers like metformin and

derivatives of thiazolidinedione, are widely used in managing PCOS (Legro et al., 2013). Metformin, a biguanide derivative, improves insulin sensitivity, hinders hepatic glucose production, and suppresses androgen synthesis by ovarian theca cells (Cibula et al., 2005, Palomba et al., 2009). However, parallel to these benefits, metformin also has side issues including lactic acidosis, gastro-intestinal disturbances, mode changes, memory loss, and renal insufficiency (Palomba et al., 2009). Therefore, its long-term use as PCOS medication maybe not the perfect choice. Moreover, it tailors according to the symptoms, thus new strategy in treating and controlling the underlying etiopathologic condition of this syndrome still represents a pressing need in order to shift the treatment from an incomplete, symptomatic approach to a curative one.

Over the last few decades, research has emerged the low-grade inflammation (Duleba and Dokras, 2012) and the oxidative stress (Sabuncu et al., 2001) as the key contributors in the pathogenesis of this syndrome. Low grade inflammation is the focus of recent research, as it reinforces the development of ovarian dysfunction, metabolic aberration (Piotrowski et al., 2005, González et al., 2006), and is highly correlated with the androgen excess in this disorder (Gonzalez, 2012).

In PCOS, the proinflammatory state has been directly attributed to the pathogenesis, and the promotion of insulin resistance as well as the atherogenesis in this syndrome (Gonzalez, 2012). In obese patients, adipose tissue expansion results in tissue hypoxia and death. This in turn promotes a flow of MNC into the stromal-vascular compartment (Weisberg et al., 2003). These MNC change morphologically to become occupant macrophages and becomes the main source of tumor necrosis factor (TNF) -α and interleukin (IL) -6 production in adipose tissue. It also stimulates the paracrine secretion of cytokine in adipocytes (Fain et al., 2004), and sets up a vicious circle of inflammatory state and adipocyte cellular necrosis. The profuse release of the inflammatory mediators, in particular, IL-6 has been also reported in non-obese PCOS patients (Gonzalez et al., 1999, Vgontzas et al., 2006).

IL-6 is a crucial mediator in the inflammatory process and is included in cell proliferation, differentiation, and apoptosis. Recently, there is a growing body of evidence that places particular emphasis on the importance of IL-6 in the pathogenesis of PCOS. However, a final conclusion is still not definite about the association between IL-6 levels and PCOS (Escobar-Morreale et al., 2003, Vgontzas et al., 2006, Escobar-Morreale et al., 2011, Toulis et al., 2011). Other studies have shed the light on the presence of genetic polymorphisms in IL-6 promoter at positions −174G/C and one at −597G/A. There was a direct association between these polymorphisms with hyperandrogenism in PCOS, particularly with elevated serum concentrations of testosterone, 17-OH progesterone, and cortisol (Villuendas et al., 2002, Walch et al., 2004). Thus, there is a reason to believe that blocking IL-6 receptor signaling may hinder the consequences following the profuse release of such inflammatory mediators and might provide beneficial therapeutic effects to these patients.

Tocilizumab (TCZ), is an IL-6 receptor blocker which has been FDA approved for the treatment of osteoarthritis. TCZ has a wide range of biological effects includes anti-inflammatory, antioxidant (Ruiz-Limon et al., 2017), antihyperlipidemic (Hoffman et al., 2019) and antiangiogenic activities (Shinriki et al., 2009). TCZ exhibits anti-proliferative and apoptotic properties in many human cancer cell lines, like those derived from cancers of the oral cavity and lungs (Shinriki et al., 2009, Kim et al., 2015).

In the light of the role of IL-6 in the pathogenesis of PCOS, and the above-reported activities of TCZ, this study was done to examine the possible therapeutic effect of TCZ, and the role of inhibiting IL-6 activation in PCOS induced by letrozole, the aromatase inhibitor in adult female albino rats. This work focused on its effects on the histopathological changes of rats' ovaries, anthropometric changes, inflammatory markers, oxidative stress markers, hormonal changes, and the accompanying metabolic changes, with particular attention on its potential effects on insulin resistance, and certain angiogenesis key players of VEGF/ANGPT/PDGF pathway.

Materials and methods

Ethics

In the current study, animal handling, treatments, and scarification were operated following guidelines for the use and care of experimental animals. The protocol was approved by the Institutional Ethical Committee, Medicine Faculty, Minia University, Egypt. This approval is under the NIH Guide for taking care and use of laboratory animals (Council No. 154:2/2019).

Animals:

Eight to ten weeks old adult, virgin, female, Wistar albino rats weighing, 200 ± 7 g were conducted in this study. All animals were checked daily for 6-day ovarian cycle using vaginal cytology, and animals exhibiting at least three consecutive regular estrous cycles, were chosen from were purchased from Nahda University at BeniSuef (NUB) Animal House (Beni-Suef, Egypt). Animals were supplied with pellets of feed and drinking water ad libitum and kept for 1 week before the onset of the study to be adapted to the laboratory environment. They were maintained under controlled conditions of humidity, light, and temperature for a period of 35 days. All rats were randomly divided into following six groups consisting of 8 rats in each group:

(i) Control group (CMC; 2 mg/kg/day, *p.o.*),
(ii) Control group treated with TCZ (TCZ; 8 mg/ kg, *i.p.* once weekly for 3 doses).
(iii) PCOS group (letrozole; 1 mg/kg/day, *p.o.*), in which the rats received letrozole (aromatase inhibitor for 21 days (Kafali et al., 2004, Li et al., 2017).
(iv) Standard group (PCOS/metformin; 2 mg/100 g/day, *p.o.*),
(v) TCZ -I group (PCOS/TCZ; 4 mg/kg, *i.p.*.; low dose).
(vi) TCZ -II group (PCOS/TCZ; 8 mg/kg, *i.p.*.; high dose).

Letrozole was administered for 21 days, and vaginal smears were daily collected and examined to confirm the induction of PCOS, by a microscopic examination using an H&E stain. Letrozole administration was then continued till day 34, while metformin and the two TCZ doses were administered starting from day 21 till the day 34 of the experiment, Metformin as a daily oral dose and TCZ as a once weekly intraperitoneal injection on days 22, 28, and 34 of the experiment, then rats were sacrificed on the day 35 of the experiment.

Treatment protocol:

Letrozole (Sigma-Aldrich, Co. Egypt) was prepared by dissolving the drug in 0.1% aqueous solution of CMC as 1 mg letrozole dissolved in 2ml CMC (Li et al., 2017). Metformin (Sigma-Aldrich, Co. Egypt) was prepared by dissolving the drug in 0.1% aqueous solution of CMC, and was taken as 2mg/100gm body weight (Jahan et al., 2018), and TCZ, which was obtained from Actemra®; F. Hoffmann-La Roche, Basel, Switzerland, was taken as 4 and 8 mg/kg body weight (Hancerli et al., 2017), *i.p.* as a single *i.p.* injection per week and for 3 doses.

Blood and tissue sampling:

Rats were sacrificed on the 35th day after overnight fasting, rats were anesthetized with *i.p.* injection of urethane (25% in a dose of 1.6 gm/kg), anthropometric measurements were reported then blood was collected from the heart of the rats in heparinized syringes and allowed to clot at room temperature. Centrifugation was then followed at 3000 rpm for 15 min. The serum layer was then withdrawn and was kept at -20°C for hormonal and biochemical analysis and ovaries were cleaned in saline and made fat free and weighed. The right ovary was kept in 10% formalin for histopathology and the left ovary was kept at -80°C for the determination of antioxidant status.

Ovarian histology & histopathological examination

The Fixation of the ovary has been operated by 10% formalin. Then through a routine histological protocol, the ovaries were serially sectioned using a microtome (Thermo, Shandon finesse 325, UK) at 5μm thickness, where every 20th section was arranged on a glass slide. In order not to repeat follicles, we have selected 10 representative sections per rat ovary. Following the standard protocol, the slides were stained with hematoxylin and eosin. The slides were observed by a pathologist who was blind to the study groups, under a light microscope (Olympus, Japan) connected to a camera to capture the representative images.

The total number of follicles were counted by multiplying the number of follicles in the

examined sections by five as we used every 5th section in the analysis, then the percentage of each type of follicle was estimated (Meng et al., 2016).

Biochemical estimations

Measurement of fasting blood glucose (FBG)

Blood samples used for the determination of BGL were collected from the rats' tail veins. Blood glucose concentration was measured using the ACCU-CHEK Active blood glucose meter (Roche, Mannheim, Germany).

Lipid profile assay

Total lipid, total cholesterol (TC), triglycerides (TGs), and HDL (high-density lipoprotein) levels were estimated by using colorimetric kits procured from Biodiagnostic, Egypt.

Hormonal analysis

The concentration of plasma LH (luteinizing hormone) testosterone and serum insulin were measured by using Enzyme Linked Immuno Sorbent Assay (ELISA), via commercial kits provided by Elabscience, USA and the protocol was followed as given in the kit catalog. The concentration of LH, testosterone, and insulin were estimated from the standard curve.

Homeostasis model assessment of insulin resistance (HOMA/IR) was estimated by the following equation: serum glucose (mg/dl) × serum insulin (mIU/ml)/405 (Matthews et al., 1985).

Interleukins analysis

The concentration of plasma IL-6 and TNF-α was measured via ELISA, with the help of commercial kits provided by Elabscience Biotechnology, Houston, Texas, USA, and the procedure was followed as given in the kit catalog. The concentration of IL-6 and TNF-α were measured spectrophoto-meterically at 450 nm and were calculated by generating a standard curve.

Estimation of oxidative and anti-oxidant profiles

Ovarian tissue (20 mg) was homogenized in 400 µL of phosphate buffer (pH 7.4). Centrifugation of the homogenate was done at 12000 rpm for 30 min at 4 °C. The clear supernatant was aspirated to detect the antioxidant state of the following assays: Superoxide dismutase (SOD) activity was assessed by the method of Mc Cords and Fridovich (McCord and Fridovich, 1969). After 1.5 min of reaction, readings were noted at 420 nm and results were explained as units/mg of protein. Malondialdehyde, a measure of lipid peroxidation, was determined by following Mihara and Uchiyama's method (Mihara and Uchiyama, 1978). The readings were detected at wavelength 535 nm from spectrophotometer and results were expressed as nmol/gm tissue. Finally, by using Griess reagent nitric oxide, total nitrite was assessed colorimetrically by measuring its stable degradation products (Green et al., 1982), and results were detected at 540 nm and expressed as nmol/gm tissue.

Real-time Polymerase Chain Reaction (RT-PCR)

According to the manufacturer's instructions, RNA was extracted using the RNeasy kit (Qiagen). NanoDrop was used for quantification and checking the purity of RNA (260/280 ~1.8). cDNA was prepared using a High- Capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) in a VeritiTM 48-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Gene expression for vascular endothelial growth factor (VEGF), angiopoietin (ANGPT), and platelet-derived growth factor (PDGF)- A, was measured by Real-Time quantitative PCR (qPCR) which was performed using StepOne with specific primers and SYBR Green **(Table 5)**, mRNA levels were then normalized to GAPDH results **(Table 6)**. Real-time qPCR reaction conditions comprised initial denaturation at 95 °C for 10 min. Then, 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec were followed. A melt curve analysis was conducted at the end of each run of the SYBR Green protocol to prove the generation of specific PCR products. The fold change of each gene was calculated using the Delta-delta threshold (ΔCt) data analysis method. All tests were two-tailed and P values <0.05 were considered significant. Expression was measured using CT values normalized to that of GAPDH (ΔCT= CT (GAPDH)- CT (target) and then expressed as 2ΔCT. All samples were done in duplicate.

Ovarian immunohistochemistry for VEGF:

Immunohistochemical Analysis:

Multiple 5 µm sections were cut from all ovaries studied then, they were all deparaffinized, rehydrated, blocked endogenous peroxidases by 3% H_2O_2, and finally washed in phosphate buffer solution. By citrate buffer pH

6.0, antigen retrieval was conducted for all slides in the microwave for 30 min. An incubation of the slides with primary antibodies in a humidity chamber were then conducted at 4°C overnight. Biotinylated 2ndry antibody was applied followed by incubation with streptavidin-biotin (Lab Vision Laboratories) for 30 min each. The slides were then treated with DAB for 5 min in order to obtain brown staining for the positive cases. Washing with water was then done followed by counter-staining with Mayer's hematoxylin. Dehy-dration, clearing mounting, and covering the slides with slips, were then operated. Positive and negative controls were prepared with each run for both antibodies. The anti-VEGF antibody used was obtained as; Lab Vision Laboratories, ready to use.

Immunohistochemical scoring:

Under the light microscope, sections were examined for the positivity for VEGF. Sections stained for both primary antibodies were assessed by semiquantitative analysis of immun-ohistochemical staining. Representative images of VEGF are illustrated in figures 2. For the H-score assessment, 10 high power fields were randomly chosen. A score was then given to each slide based on the staining density as 0, 1, 2, or 3, in agreement to the presence of brown staining as negative, weak, intermediate, or strong, respectively. The total number of cells was counted in each field in addition; the number of cells stained with each intensity was counted and an H-score was given between 1 and 300, as 300 is considered 100% of strongly stained cells.

Statistical analysis

All values were expressed as mean ± SEM. The statistical analyses were operated using Graph Pad Prism (version 6.0; San Diego, CA, USA). Analysis of Variance (ANOVA) test was used for multiple comparisons followed by Tukey-Kramer as a post-ANOVA test. The results were considered statistically significant if the *p*-values were <0.05.

Results

Effect on body weight, body length, ovarian weight, and diameter changes

At the end of the experiment, PCOS induced rats showed a 27% increase in mean body weight when compared to the control group. In the contrast, both TCZ and metformin-treated groups did not show any significant difference in body weight when compared to the control group **(Table 1)**.

The weight and diameter of the right ovary were 72 mg ± 3.2 and 6.83 mm ± 0.17, and those of the left ovary were 70.5 mg ± 3.03 and 7.5 ± 0.22, respectively in cystic rats versus control group that were in the right ovary 48.13 ± 2.98 mg and 4.58 ± 0.20 mm, and were in the left ovary 49.50 ± 1.77 mg and 4.75 ± 0.31 mm. The metformin and TCZ-treated rats showed a significant decrease in ovarian weight and diameter as compared to untreated PCOS group **(Table 1)**.

Morphometric analysis

On Examination of the PCOS group of rats, we found a large number of cystic follicles instead of the normal growing follicles. However, the number of cystic follicles in TCZ-treated groups revealed a highly significant reduction ($P < 0.001$) and exhibited a rise in number of growing follicles at various stages of maturity. Comparing the metformin group to the PCOS, rats demonstrated a significant difference ($P < 0.001$) in the reduction of the number of cystic follicles **(Table 2).**

FBG, serum insulin, and HOMA/IR

In the PCOS group, there was a significant increase ($P < 0.001$) in FBG, serum insulin and HOMA/IR levels were observed compared to the control group. However, all the treatment groups showed a significant decrease ($P < 0.001$) in their levels different from PCOS induced group **(Table 3)**. Interestingly, these results show stronger ameliorating effects with TCZ treated group when compared to the metformin group, with a statistically significant reduction ($P < 0.05$), particularly as regards serum insulin levels.

Lipid profile: Total lipids, cholesterol, triglyceride, HDL

There was a profound increase ($P < 0.001$) in total lipids, cholesterol, triglyceride, and a decline in HDL in the PCOS induced group compared to the control group. Treatment with metformin and TCZ ameliorated all these parameters in comparison to the PCOS positive animals **(Table 3)**. TCZ treated group showed better ameliorating effects than metformin in improving these disturbed lipid profile values, with a statistically significant difference ($P < 0.001$) regarding the triglyceride results.

Hormonal assay of different treated groups:

Key ovarian steroidogenic enzymes testosterone

and LH showed a significant ($P < 0.001$) increase in PCOS induced rats when compared to control. A significant fall ($P < 0.001$) in testosterone and LH levels was observed in standard, low dose, and high dose groups of TCZ as displayed in **Table 3.**

Cytokine (IL-6 and TNF-α) levels

There was a remarkable increase in serum levels of IL-6 and TNF-α in the PCOS group in comparison to the control group **(Table 3).** However, treatment with TCZ and metformin prodigiously optimized the IL-6 values to baseline levels ($P < 0.001$). In TNF-α levels, TCZ significantly reduced it when compared to the PCOS group. Moreover, low and high dose of TCZ reported lower levels of TNF-α when compared to the metformin-treated group ($P < 0.01$ and $P < 0.05$, respectively).

Oxidative stress and anti-oxidant profiles

A remarkable decrease ($P < 0.001$) in SOD levels was realized in the ovarian tissue of letrozole-treated rats as compared to control. Metformin and TCZ treatment reversed these values near to baseline. A significant rise in ovarian lipid peroxides and total nitrite levels were noticed in the PCOS induced group that was remarkably decreased by the action of metformin and TCZ. Levels of MDA and total nitrite was statistically lower ($P < 0.05$) in Metformin treated group when compared to low and high dose TCZ treated groups **(Table 4).**

Angiogenesis markers

Ovarian angiogenesis has been shown to contribute to the emergence of PCOS pathogenesis. To further investigate whether TCZ effects on the expression of ovarian angiogenic factors, we measured the mRNA levels of VEGF, ANGPT, PDGFA in ovaries **(Table 5 & 6)**. Vascular endothelial growth factor (VEGF), mediates endothelial cell migration, and proliferation. Angiopoietin (ANGPT) family, critically contributes to the regulation of vessel stability and permeability (Carmeliet, 2003). Finally, the platelet-derived growth factor (PDGF) family plays a major role in pericytes recruitment to new vessels (Hoch and Soriano, 2003, Betsholtz, 2004). Besides, VEGF protein expression was detected in ovarian tissues from different treated groups. TCZ administration reversed the increased levels of VEGF, ANGPT, and PDGFA observed in PCOS rats to control values. Moreover, VEGF protein expression has shown remarkable increase in the ovarian tissues by immunohistochemistry. TCZ administration drives this increase back to normal levels, and significantly better than the effects of metformin **(Figure 2)**.

Number of ovarian follicles

In the PCOS group, the mean number of normal growing follicles and corpus luteum markedly decreased, on the other hand, a significant rise in the mean number of cystic and atrophic follicles has been detected in comparison to the normal control group ($P < 0.001$). In the groups treated with metformin and TCZ, the number of all types of normal follicles and corpus luteum has been increased significantly ($P < 0.001$) which is like normal ovaries indicating possible treatment for PCOS **(Figure 1, Table2).**

Table (1): The changes in body weight and length & ovarian weight and length in different groups

Parameter	CMC	TCZ.H	PCOS	PCOS/Met.	PCOS/TCZ.L	PCOS/TCZ.H
Initial body weight (gm)	202.3±5.90	203.5±7.02	201.5±8.24	202.3±7.40	200.8±5.540	201.8±7.38
Final body weight (gm)	220.7±4.11	222.0±6.22	254.5±5.28***	230.8±4.73 $^{\#}$	222.3±4.92 $^{\#\#}$	222.0±5.57$^{\#\#}$
Body length (cm)	17.98±0.57	18.15±0.48	19.53±0.92	18.90±0.63	18.77±0.58	19.74±0.57
Right ovary weight (mg)	48.71±3.37	54.50±3.87	72.00±3.20***	64.92±4.55**	56.33±2.58$^{\#}$	60.00±1.75$^{\#}$
Left ovary weight (mg)	49.50±1.77	48.80±2.40	70.50±3.03**	62.50±3.94	63.83±3.26 $^{\#}$	55.50±4.64$^{\#}$
Right ovary length (mm)	4.58 ±0.20	4.50 ±0.22	6.83±0.17***	5.75±0.31*$^{\#}$	5.67±0.25*$^{\#}$	4.83 ±0.31$^{\#\#\#}$
Left ovary length (mm)	4.75±0.31	4.80±0.37	7.50±0.22***	6.00±0.45$^{\#}$	6.00±0.26$^{\#}$	4.83±0.31$^{\#\#\#}$

Note. CMC: Carboxy-methyl cellulose; PCOS: poly ovarian cystic syndrome; Met.: metformin; TCZ.H: Tocilizumab (8mg/kg); TCZ.L: Tocilizumab (4mg/kg). Data represent mean ± SEM of 8 observations. Data were analyzed with one-way ANOVA followed by Tukey-Kramer test for multiple comparisons post-test.

*Significant difference from control group, (*, p<0.05; **, p<0.01; ***, p<0.001).

$^{\#}$Significant difference from PCOS untreated group, (#, p<0.05; ##, p<0.01; ###, p<0.001).

$^{\$}$Significant difference from PCOS treated by metformin group, ($, p<0.05; $$, p<0.01; $$$, p<0.001).

Table (2): Percentage of ovarian follicles in different groups

Pathology	CMC	TCZ.H	PCOS	PCOS/Met.	PCOS/TCZ.L	PCOS/TCZ.H
CF	Zero	Zero	68.97±3.33***	47.88±2.71$^{***\#\#\#}$	32.10±1.60** $^{*\#\#\#\$\$\$}$	19.53±0.70$^{***\#\#\#}$ $^{\$\$\$}$
CL	47.80±3.80	48.08± 2.60	11.86±1.02 ***	14.95±0.52***	18.46±1.02***	30.43±2.00$^{***\#\#\#}$ $^{\$\$\$}$
GF	41.25±2.12	42.33 ± 2.41	14.80± 1.94***	22.72±2.08$^{***\#\#\#}$	40.97±2.46$^{\#\#\#\$\$}$	37.24± 3.16$^{\#\#\#\$\$}$
PF	0.00± 0.00	0.00 ± 0.00	3.18 ± 0.44a***	12.70 ±1.28***	8.95 ± 0.43$^{***\#}$	10.00 ± 0.63$^{***\#\#}$
O	9.35± 0.23	9.68 ± 0.44	1.50± 0.14***	2.13±0.27***	2.77± 0.25$^{***\#}$	2.90 ± 0.14$^{***\#\#}$

Note. CL: corpus luteum; CF: cystic follicle; GF: growing follicle; PF: primordial follicle; O: oocyte; CMC: Carboxy-methyl cellulose; PCOS: poly ovarian cystic syndrome; Met.: metformin; TCZ.H: Tocilizumab (8mg/kg); TCZ.L: Tocilizumab (4mg/kg). Data represent mean ± SEM of 8 observations. Data were analyzed with one-way ANOVA followed by Tukey-Kramer test for multiple comparisons post-test.

*Significant difference from control group, (*, p<0.05; **, p<0.01; ***, p<0.001).

$^{\#}$Significant difference from PCOS untreated group, (#, p<0.05; ##, p<0.01; ###, p<0.001).

$^{\$}$Significant difference from PCOS treated by metformin group, ($, p<0.05; $$, p<0.01; $$$, p<0.001).

Table (3): Serum parameters in different groups

	CMC	TCZ.H	PCOS	PCOS/Met.	PCOS/TCZ.L	PCOS/TCZ.H
TNF-α (Pg/ml)	19.10±2.46	21.41 ± 2.76	91.47 ± 3.98***	59.15±3.44***###	38.93±2.92**## #$$	42.80±3.56***# ##$
IL-6 (Pg/ml)	28.25±1.52	28.20 ± 1.39	46.60 ± 1.37***	34.18 ± 2.24#	31.32 ± 1.83##	31.82 ± 2.12#
Total lipid (mg/dL)	317.4±48.45	326.5± 12.77	1074± 30.24***	620.5±31.51***###	651.1±53.12**###	548.9±33.90*** ###
Cholesterol (mg/dL)	214.1±1.05	215.5 ± 0.82	231.9 ± 1.09 ***	218.0± 1.07**##	220.7±1.11***###	221.8±1.12***# ##
Triglycerides (mg/dL)	50.87±3.27	57.09± 2.794	155.0 ± 8.5***	129.1±3.48***###	94.25±5.69***# ##$$$	81.76±3.46**### $$$
HDL(mg/dL)	49.47±1.02	47.22 ± 1.41	28.55 ± 1.70***	39.30 ± 2.88*#	42.68 ± 2.26##	45.43 ± 3.44###
Insulin level (ng/ml)	1.92 ± 0.11	2.20 ± 0.18	5.03 ± 0.28***	3.07± 0.14**###	2.15 ± 0.20###$	2.55 ± 0.16###
BGL (mg/dL)	79.83±3.43	78.33 ± 3.92	134.8 ± 3.11***	99.00±2.10**###	99.80±2.65**###	88.33 ± 5.07###
HOMA-IR	0.97 ± 0.14	0.85 ± 0.11	4.40 ± 0.26***	2.30±0.14***###	1.78 ± 0.14*###	1.77 ± 0.11*###
Testosterone (ng/ml)	2.68 ± 0.19	3.15 ± 0.19	6.12 ± 0.30***	3.32 ± 0.40###	3.48 ± 0.30###	3.96 ± 0.30*###
LH (mIU/ml)	2.43 ± 0.26	2.34 ± 0.28	6.38 ± 0.31***	3.13 ± 0.46###	4.25± 0.14**###	4.16± 0.17**###

Note. CMC: Carboxy-methyl cellulose; PCOS: poly ovarian cystic syndrome; Met.: metformin; TCZ.H: Tocilizumab (8mg/kg); TCZ.L: Tocilizumab (4mg/kg); TNF-α: tumor necrosis factor –α; IL-6: interleukin -6; HDL: high-density lipoprotein; BGL: Blood glucose level; LH: luteinizing hormone. Data represent mean ± SEM of 8 observations. Data were analyzed with one-way ANOVA followed by Tukey-Kramer test for multiple comparisons post-test.
Significant difference from control group, (, p<0.05; **, p<0.01; ***, p<0.001).
#Significant difference from PCOS untreated group, (#, p<0.05; ##, p<0.01; ###, p<0.001).
$Significant difference from PCOS treated by metformin group, ($, p<0.05; $$, p<0.01; $$$, p<0.001).

Table (4): Ovarian oxidative state in different groups

	CMC	TCZ.H	PCOS	PCOS/Met.	PCOS/TCZ.L	PCOS/TCZ.H
MDA (nmol/gm)	9.11 ± 0.18	10.16 ± 0.50	24.34±0.95***	13.12 ± 0.56**###	14.61 ± 0.55***##	16.59 ± 1.07***###
Total nitrite (nmol/gm)	35.54 ± 2.45	35.91 ± 1.92	84.85 ±4.32***	51.87 ± 1.75**###	64.44 ± 3.18***##	53.98 ±2.70**###
SOD(U/gm)	1070 ± 42.17	954.9± 68.76	558.0±40.24***	805.9 ± 45.75**##	923.6 ± 28.20###	852.7±35.87*##

Note. CMC: Carboxy-methyl cellulose; PCOS: poly ovarian cystic syndrome; Met.: metformin; TCZ.H: Tocilizumab (8mg/kg); TCZ.L: Tocilizumab (4mg/kg); MDA, malondialdehyde; SOD, superoxide dismutase. Data represent mean ± SEM of 8 observations. Data were analyzed with one-way ANOVA followed by Tukey-Kramer test for multiple comparisons post-test.
Significant difference from control group, (, p<0.05; **, p<0.01; ***, p<0.001).
#Significant difference from PCOS untreated group, (#, p<0.05; ##, p<0.01; ###, p<0.001).
$Significant difference from PCOS treated by metformin group, ($, p<0.05; $$, p<0.01; $$$, p<0.001).

Table (5): The primers specific for RT-PCR of VEGF, ANGPT, PDGFA and GAPDH.

Gene name	Forward primer	Reverse primer
Rat -GAPDH	5'CACCATCTTCCAGGAGCGAG 3'	5'GGCGGAGATGATGACCCTTT 3'
Rat- VEGF	5'AAAAACGAAAGCGCAAGAAA 3'	5'TTTCTCCGCTCTGAACAAGG 3'
Rat- ANGPT	5'ATGCGCCCTTATGCTAACAG 3'	5'TTTAGATTGGAAGGGCCACA 3'
Rat- PDGFA	5'CAAGTGCCAGCCCTCAAG 3'	5'TTCCTGACATACTCCACTTTGG 3'

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; VEGF: Vascular endothelial growth factor; ANGPT: angiopoietin; PDGFA: platelet –derived growth factor-A.

Table (6): mRNA expression of VEGF, ANGPT, and PDGFA (relative to the control)

	CMC	TCZ.H	PCOS	PCOS/Met.	PCOS/TCZ.L	PCOS/TCZ.H
VEGF	1.00±0.00	1.03±0.06	15.29±0.69***	4.50±0.39$^{***\#\#\#}$	3.17 ±0.28$^{**\#\#\#}$	1.68±0.16$^{\#\#\#\$\$\$}$
ANGPT	1.00 ± 0.00	1.02±0.46	37.65 ± 0.42***	4.37±0.42$^{***\#\#\#}$	3.2 ±0.42$^{**\#\#\#}$	2.40± 0.20$^{\#\#\#\$}$
PDGFA	1.00± 0.00	0.97±00.0	1.34 ± 00.69***	3.30±00.28$^{***\#\#\#}$	1.18±00.07$^{\#\#\#\$\$\$}$	2.43±00.16$^{*\#\#\#}$

Note. CMC: Carboxy-methyl cellulose; PCOS: poly ovarian cystic syndrome; Met.: metformin; TCZ.H: Tocilizumab (8mg/kg); TCZ.L: Tocilizumab (4mg/kg); VEGF: Vascular endothelial growth factor; ANGPT: angiopoietin; PDGFA: platelet –derived growth factor-A. Data represent mean ± SEM of 8 observations. Data were analyzed with one-way ANOVA followed by Tukey-Kramer test for multiple comparisons post-test.
Significant difference from control group, (, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).
#Significant difference from PCOS untreated group, (#, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$).
$Significant difference from PCOS treated by metformin group, ($, $p<0.05$; $$, $p<0.01$; $$$, $p<0.001$).

Figure 1

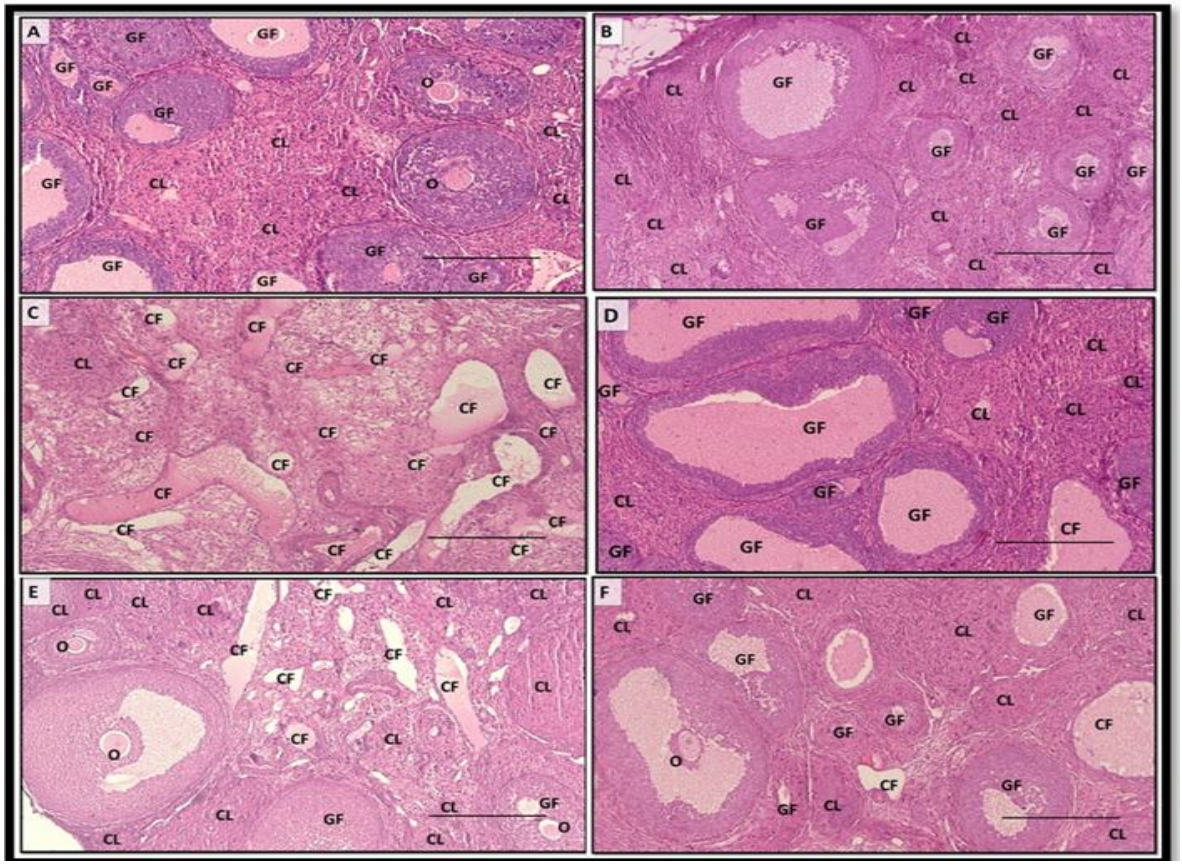

Figure 1: Sections of studied ovaries stained with H&E in 200x magnification. In normal ovaries of control rats, receiving either CMC or TCZ **(Figures 1A and 1B,** respectively**)**, there are ovarian growing follicles at variable developmental stages and several fresh corpora lutea **(Figures 1A and 1B)**. The PCOS group shows predominance of cystic follicles with limited number of growing follicles and corpora lutea **(Figures 1C)**. Metformin treated group of the studied rats shows decreased number of cystic follicles and marked increase in the number of both growing follicles and corpora lutea **(Figure 1D)**. TCZ low dose treated group show increased number of follicles of different stages of the Graafian follicle maturation pathway similar to the normal control group **(Figure1E)**. TCZ high dose treated group show decreased number of cystic follicles and marked increase in the number of follicles of different stages of the Graafian follicle maturation pathway similar to the normal control group **(Figure 1F)**. Data represent mean ± SEM of 8 observations. Data were analyzed with one-way ANOVA followed by Tukey-Kramer test for multiple comparisons post-test. CL: corpus luteum; CF: cystic follicle; GF: growing follicle; PF: primordial follicle; O: oocyte.

Figure 2

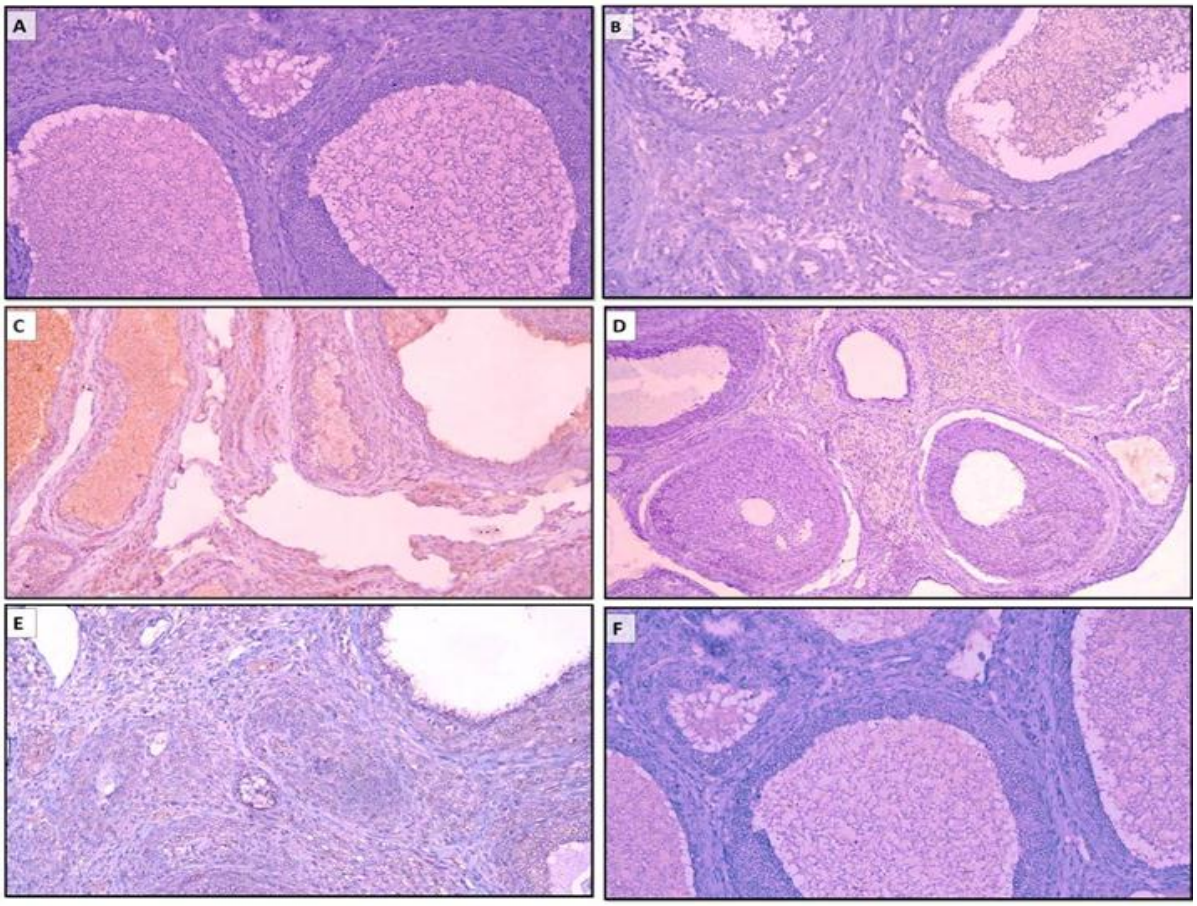

Figure 2: Immunohistochemical stained sections for VEGF examined from ovaries of different groups. Original magnification was 200x. Staining is considered positive if the cytoplasm stained brown. Sections examined from the control groups, receiving either CMC or TCZ **(Figures 2A and 2B,** respectively**)**, showed negative expression for VEGF **(Figure 2A and 2B)**. Strong VEGF expression appeared in PCOS group **(Figure 2C)**. Sections examined from metformin treated group showed weak positive expression for VEGF **(Figure 2D)**. Sections examined from low dose of TCZ treated group showed weak positive expression for VEGF **(Figure 2E)**. Sections examined from high dose of TCZ treated group showed weak positive expression for VEGF **(Figure 2F)**. Data represent mean ± SEM of 8 observations. Data were analyzed with one-way ANOVA followed by Tukey-Kramer test for multiple comparisons post-test.

Discussion

The current study succeeded in displaying the development of PCOS model in female Wistar rats following the method of Kafali et al., 2004. Letrozole, a non-steroidal aromatase inhibitor, represses total body aromatization, folliculogenesis, and ovarian function (Homburg, 2009, Maliqueo et al., 2013). Administration of letrozole for 3 weeks to adult rats develop PCOS condition that depicts features that are very much similar to human PCOS (Kafali et al., 2004). Vaginal smear histology is a confirmation for working of this model and a major indication for ovarian physiology. Regular examination of vaginal smears indicated the absence of cycling and the presence of persistent vaginal cornification, indicating the development of the ovarian cysts (Kafali et al., 2004).

In addition, this model exhibits a remarkable increase in body weight in PCOS model rats (Gambineri et al., 2002), while TCZ treated rats showed a significant drop in these abnormal values up to normal levels. This finding may be explained by the fact that 15-30% of the circulating IL-6 are indeed secreted by adipose tissues under basal conditions (Mohamed-Ali et al., 1997). Also, several studies demonstrated a parallel relationship between obesity and serum levels of IL-6 and CRP (Menezes et al., 2018), as well as a decrease in IL-6 levels upon weight loss (Bastard et al., 2000). This may indicate a possible role for IL-6 in obesity/adiposity. Besides the weight gain, the ovaries demonstrated a significant increase in both ovarian weight and length in PCOS rats and looked hyperemic and bulgy as a result of the effects of the inflammatory cytokines, e.g., TNF and IL-6. Using TCZ, as an IL-6 inhibitor, normalized these abnormalities and improved the inflammatory microenviroment in comparison to the standard therapy by metformin.

The hormonal alternations of letrozole induced rats exhibit a hyper-androgenized state responsible for disrupted ovarian physiology (Jahan et al., 2016). The disturbance in the usual hypothalamic-pituitary gonadal axis increases both LH and testosterone progressing into a disease status (Mendonca et al., 2004, Doi et al., 2005). As evidenced, LH triggers testosterone secretion in a thecal layer of ovarian follicles initiating such abnormalities (Fukuda et al., 2009). The present study shows marked elevation in LH and testosterone levels in comparison to control animals reflecting the hyperandrogenism state in PCOS condition. TCZ was able to successfully restore LH and testosterone levels back to control level. The present data is in accordance with the work of Taskin et al., 2015 which shows a regulatory effect for TCZ and a depressing effect over the progesterone level in a rat model of ovarian hyperstimulation syndrome.

Besides, PCOS is a metabolic disorder connected to type 2 diabetes mellitus and exhibits a close cross-talk between insulin and inflammatory signaling pathway. It manifests by hyperglycemia and ending gradually by insulin resistance (Boudreaux et al., 2006). In our study, PCOS induced rats showed a remarkable increase in fasting blood glucose levels and insulin resistance declared by a significant increase in HOMA/IR. This is in harmony with the earlier reports about the induction of hyperglycemia in Letrozole induced PCOS rats (Maharjan et al., 2010).

The administration of TCZ significantly prevented the elevation in fasting blood glucose, insulin resistance, and HOMA/IR, indicating the beneficial effect of TCZ in preventing insulin resistance and diabetic complication. Interestingly, Metformin showed similar results with a moderately lower efficacy in comparison to TCZ. Our data may be explained by the previous findings that chronic exposure to IL-6 induces insulin resistance by inhibiting insulin receptor autophosphorylation of IRS-1 and IRS-2 in liver (Klover et al., 2003), and by diminishing transcription of IRS-1, GLUT-4, and peroxisome proliferator-activated receptor γ in adipose tissue (Lagathu et al., 2003, Rotter et al., 2003). Moreover, IL-6 acutely induces glucagon synthesis, α-cell proliferation and prevents α-cell apoptosis leading to a status of α-cell dysfunction and increased proportion of α cells relative to β-cell (Ellingsgaard et al., 2008). This indeed may represent key events in T2DM physiology and pathology. In addition, TCZ has shown efficacy to decrease insulin levels and HOMA/IR in patients with rheumatoid arthritis and type 2 diabetes in some recent clinical studies (Schultz et al., 2010). Improving insulin resistance in PCOS by the use of TCZ represents an important mechanistic bonus in alleviating this complex syndrome.

One of the sequelae of PCOS is dyslipidemia. i.e. decreased HDL-C and higher levels of plasma total lipids, total cholesterol, and triglycerides (Wild et al., 2011). Imbalances in the lipid profile are assigned to hyperandrogenemia (Croston et al., 1997, von Eckardstein, 1998). The present work demonstrated comparable data in the lipid profile. PCOS control group exhibited a remarkable increase in total lipids, TC, TG's, and a decrease in HDL levels. TCZ displayed its antihyperlipidemic action by significantly decreasing serum total lipids, TC, TG's, while increasing HDL levels. Metformin showed parallel results with a moderately lower efficacy in comparison to TCZ. Our results were evidenced by a previous study showing a potential cardiovascular protective role of TCZ treatment against the cardiovascular load in RA patients, by reducing the elevated HDL level, and a tendency to normalize adipokine profile changes (Hoffman et al., 2019).

Aside from hyperglycemia and inflammation, oxidative stress also participates in PCOS (Yeon Lee et al., 2010). Too much production of ROS happens as a result in Super Oxide (SO) anion reaction in cytochrome P450 and oxygenase reactions in mitochondria (Burton and Jauniaux, 2011). Alternations in the mitochondrial function lead to changes in the production of adenine triphosphate (ATP), which is essential for gametogenesis results in cellular injury and DNA damage (Liu et al., 2000). Also, several reports pointed to the role of increased oxidant levels in the ovaries and its contribution to increased androgen production and polycystic ovaries (Sabuncu et al., 2001). Superoxide dismutase assists in detoxifying SO anion by its conversion to H2O2 (Agarwal et al., 2012). MDA is the breakdown output of polyunsaturated fatty acids peroxidation, where lipid peroxidation is one of the important markers for oxidative tissue damage, as it stimulates free radical damage to the fatty acid cell membrane which leads to cell damage and necrosis (Gaweł et al., 2004). NO is an inflammatory mediator produced by iNOS consequent to the stimulation of variable cytokines in pathological states (Gaston et al., 1994, Bloomfield et al., 1997). In the present study, SOD was significantly diminished in the PCOS group while MDA and total nitrite were significantly increased, and concomitant treatment with TCZ counterbalanced these activities. These results were in parallel to metformin results to rebalance the ovarian oxidative load, but with higher effects to metformin compared to TCZ. Thus, by the ability of TCZ to block the IL-6 signaling may be a direct and an additional mechanism by which TCZ ameliorates the PCOS disease state in these rats. This following the earlier reported antioxidant capacity of TCZ in RA patients (Ruiz-Limon et al., 2017).

IL-6 levels were previously reported to be increased in PCOS, and contribute to the inflammatory cascade and the progression of the pathogenesis of PCOS (Vgontzas et al., 2006, Escobar-Morreale et al., 2011, Toulis et al., 2011). Our results showed a significant increase in IL-6 as well as in its upstream cytokine inducer, TNF-alpha, protein levels in serum of PCOS rats. A remarkable decrease in their expressions was shown with the administration of TCZ and with higher efficiency compared to metformin. This finding is in unison with previous studies in patients with RA, which demonstrated a reduction in IL-6 serum levels with TCZ administration (Nishimoto et al., 2014). Previous studies showed that TCZ provides a rebalance in the production of various immune cells in RA patients, thereby providing a protective response regarding inflammatory conditions (Samson et al., 2012, Pesce et al., 2013). The suppressing effect of TCZ on intrinsic IL-6 production has been attributed to its inhibitory effect on Th17 cells (Mills, 2008). This may explain our results for the reducing effects of TCZ on IL-6 production, through its suppression to Th17. This can be considered as an important biologic finding with major beneficial consequences in the inflammatory cascade in PCOS cases.

Angiogenesis is an important recently studied contributing factor in the pathogenesis and the development of the PCOS conditions. Variable angiogenic factors contribute to the formation of a perfect thecal vasculature to secure appropriate nutrition and hormonal supply to the developing follicle. VEGF, ANGPT, and PDGF are some of the main angiogenic factors, the alternations in their levels have been previously reported in PCOS. In our study, data showed a dramatic increase in both the mRNA and the protein expression of VEGF, ANGPT, and PDGFA, while TCZ was able to restore

their expression back to normal levels. Previous reports have shown a remarkable efficacy for TCZ to suppress the angiogenesis proliferation and mRNA expression of VEGF, which was attributed as the main mechanism for its suppressing efficacy for the *invivo* tumor growth of oral squamous cell carcinoma (Shinriki et al., 2009). This direct mechanism adds another beneficial increment for the protective effects of TCZ against letrozole-induced PCOS in rodents.

The PCOS ovaries showed multiple ovarian cysts and lack of the growing follicles, corpus luteum, oocytes, granulosa, and theca cell layers, which is similar to other studies. The anovulatory state of the PCOS, which is responsible for the failure of conception, results in the decreased number of the corpus luteum. Corpus luteum controls the menstrual cycles through progesterone secretion that prepares the uterus for conception. On the contrast, treatment with TCZ and metformin showed significant restoration of the normal ovarian structure which is evident by the increased number of the growing follicles and oocyte production in addition to the decreased number of the cystic follicles. These results were more evident and highly significant in the TCZ rats treated with the high dose. A lower level of improvement was detected in TCZ treated rats with low dose and metformin-treated rats but still significant.

Conclusion

Our results demonstrated that TCZ is a powerful remedy with the capability to battle metabolic and endocrine comorbidities in PCOS. TCZ, through its high as well as its low doses, exhibited beneficial effects on hormonal indices, lipid profile, and insulin resistance. Also, it provided strong anti-oxidant properties and recovered ovarian cysts into normal follicles. TCZ aborts IL-6 effects, suppresses its expression, and remarkably rebalances the angiogenesis imbalance in PCOS. While using high doses of TCZ provided the maximal effects, the use of its low doses still achieved dramatic improvement. Thus, its anti-androgenic potentials and the restoration of ovarian function may offer an advantageous therapeutics for PCOS. Future studies are in demand to investigate TCZ efficacy with various dose profiles in humans either alone or as adjuvant therapy in order to cure PCOS conditions.

Author Contributions

YFI, AH and HAB were responsible for suggestion of the hypothesis and for experimental design. YFI contributed to the performance of experiments, collection of the samples, data statistics. YFI was responsible for manuscript writing and composition. ND performed the histopathology and the immunohistochemistry work and KT performed the polymerase chain reaction experiments and analysis. YFI supervises the project. All authors have participated in and approved the final manuscript. All data were generated in-house and that no paper mill was used.

Conflict of Interests

The authors stated no potential conflict of interests considering the authorship, research, and publication of this manuscript.

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