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The Effect of Berberine on Over Function in Female Rats with Letrozole-Induced Polycystic Ovary Syndrome

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Abstract: Background: The term premature ovarian failure is defined as a persistent decrease in ovarian function in women. Platelet-rich plasma contains growth factors. In our study, the aim was to test the effectiveness of platelet-rich plasma that was tried in patients who did not benefit from routine infertility treatments on experimental animals. **Material and Methods:** In the study, 40 female rats were used, and to obtain platelet-rich plasma eight male rats were used. The animals used in the study were randomly divided into four groups, with 10 animals in each group, prospectively. At the end of the 21st day, rats in all groups were euthanized by taking intracardiac blood. Following anti-mullerian hormone measurement, histological analysis, follicle count and real time-polymerize chain reaction analyzes were made **Results**: In our study, the highest anti-mullerian hormone concentration was measured only in the group given platelet-rich plasma and the lowest value was measured in the group with ovarian damage. The results of Bcl-2 m-RNA expression analysis by real time-polymerize chain reaction also showed that the lowest expression occurred in the group with ovarian damage. The results of the correlation analysis showed that there was a statistically significant, negative moderate correlation between the ovarian damage group and the ovarian damage + Platelet-rich plasma group. **Conclusions**: As a result, it is evaluated that platelet-rich plasma can be used as a preventative in women who are treated with cyclophosphamide and similar agents and subsequently develop ovarian side effects that are very difficult to treat.

Keywords: Platelet-Rich Plasma, Cyclophosphamide, Anti-Mullerian Hormone, Bcl-2, Real Time-Polymerize Chain Reaction.

INTRODUCTION

Polycystic ovary syndrome (PCOS) represents the most frequently seen endocrine-metabolic syndrome in women of reproductive age [Azziz, R, 2018]. Prevalence of the syndrome differs between 6.8% and 18% depending on the diagnosis criteria [Deswal, R. *et al*., 2020]. It is characterized by hyperandrogenism and chronic anovulation. It is an inherited, complex, polygenic, and multifactorial disease. Interactions between multiple proteins and genes affected by epigenetics and environmental factors are considered triggers of the disease. Women with PCOS are at high risk for glucose intolerance and type 2 diabetes mellitus. Clinical manifestations such as hepatic steatosis, hypertension, obstetric complications, and psychosexual disorders are common in PCOS cases [Witchel, S.F. *et al*., 2019]. Although significant progress has been made in the pathophysiology and treatment of PCOS in recent years, problems regarding disease management continue [Witchel, S.F. *et al*., 2019; Mohamed-Hussein, Z.A. *et al*., 2009].

Various treatments have been suggested for infertile women with PCOS [Wang, J. *et al*., 2017; Zeng, X.L. *et al*., 2016]. Even though treatments like clomiphene citrate, metformin, and gonadotropins have been reported, there is insufficient evidence to back up the effects. The subject of how to manage PCOS has not been studied substantially [Rocha, A.L. *et al*., 2019; Jin, P. *et al*., 2018].

Different methods exist to construct an experimental PCOS model [Abbott, D.H. *et al*., 2005; Kafali, H. *et al*., 2004]. The use of letrozole, an aromatase inhibitor, is one of these methods. In studies performed on rats, ovaries with multiple large follicle cysts, irregular menstrual cycle, and anovulation were observed after letrozole administration [Kafali, H. *et al*., 2004]. It has been observed that all letrozole-treated rats were anovulatory and developed polycystic ovaries with structural changes very similar to those seen in human PCOS [Wang, M.X. *et al*., 2020].

Berberine (BBR) is prescribed for various metabolic disorders and infertility treatment [Li, M.F. *et al*., 2018]. BBR, whose antioxidant activity is well known, is an alkaloid widely used in traditional medicine applications against type 2 diabetes mellitus and cancer [Rondanelli, M. *et al*., 2020]. Recently, some evidence has been proposed showing that BBR can be used against PCOS and insulin resistance [Li, M.F. *et al*., 2018-Mirzaee, F. *et al*., 2021).

Apoptosis proceeds in two ways, namely extrinsic and intrinsic (mitochondrial) pathways [Chipuk, J.E. *et al*., 2004]. The Bcl-2 family of proteins controls the intrinsic pathway. One of the pro-

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apoptotic proteins in this family is the Bax protein. The Bax gene is a pro-apoptotic member of the Bcl-2 gene family [Martinou, J.C. *et al*., 2011].

Infertility can be prevented during PCOS treatment by eliminating the disorders in the hormonal axis and oxidative damage [Basheer, M. *et al*., 2018]. It has been determined that antioxidant compounds reduce reactive oxygen radicals and show beneficial effects in reversing the damage in PCOS patients. Our study aims to examine the effects of Berberine, whose antioxidant activity is well known, on experimental animals that have PCOS formed with letrozole.

MATERIALS AND METHODS

Experimental Animals

In the study, 50 regular-cycled, 10-12 weeks old adult female Wistar rats weighing 200±40 grams were used. The subjects were fed with standard pellet feed and city water in a 12-hour light, 12-hour dark period, and a constant temperature of 21-23 °C. Rats were randomly divided into five groups with ten animals in each group.

The relevant Ethics Committee approved the protocol regarding the procedures performed on animals. All work and procedures were carried out following the Guide for the Care and Use of Laboratory Animals [Clark, J.D. *et al*., 1997].

Study Design and Collection of Samples

The animals used in the study were randomly divided into five groups, with ten animals in each group prospectively.

Group 1 (Control Group): Rats were given 1% carboxymethyl cellulose (CMC) for 21 days.

Group 2 (PCOS) (Letrozole): Rats were given 1 mg/kg letrozole in 1% CMC orally using a gavage for 21 days.

Group 3 (Letrozole + Metformin): Rats were given 1 mg/kg letrozole in 1% CMC orally using a gavage for 21 days. Afterward, 2 mg/kg of Metformin were given for the next 15 days.

Group 4 (Letrozole + Berberine): Rats were given 1 mg/kg letrozole in 1% CMC orally using a gavage for 21 days. Afterward, 150 mg/kg of Berberine were given for the next 15 days.

Group 5 (Letrozole + Metformin+ Berberine): Rats were given 1 mg/kg letrozole in 1% CMC orally using a gavage for 21 days. Afterward, 150 mg/kg Berberine and 2 mg/kg Metformin were given for the next 15 days.

After 36 days, administration of Berberine and Metformin to rats in Groups 3, 4, and 5 was ceased. Rats were sacrificed after their ovarian tissues were extracted with a 50 mg/kg ketamine/15 mg/kg xylazine combination with anesthesia.

At the end of the experiment, the ovarian tissues of the rats in all of the groups were removed under anesthesia, stained with hematoxylin & eosin (HE), and follicle assessment was performed to determine the ovarian reserve histopathological analysis. RT-PCR from ovarian tissue performed M-RNA expression analyzes of Bcl-2 and Bax genes. Total Oxidant Status (TOS) and Total Antioxidant Status (TAS) levels, which are oxidative stress parameters, were determined in ovarian tissues.

Histopathological Analysis

The researchers carried out a histopathological examination. The researcher was unaware of the groups during the study. The removed ovaries were kept in 10% formalin for 72 hours, then cleaned with alcohol and xylene, increasing from 70% to 100%. The samples were coated with paraffin at 60°C after cleaning. Sections of 5 µm thickness were taken from the paraffin blocks for analysis. Sections were stained with HE and examined under a photomicroscope (Nikon Eclipse i5, Tokyo, Japan).

Follicle Count

Multiple sections were taken from each ovary to determine the number of follicles. Those with oocyte nuclei from the follicles were included in the analysis and classified into five groups. Primordial, primary, secondary, antral, and atretic follicles form five groups.

Real Time-PCR

Primarily, ovarian tissues were homogenized for analysis. For this purpose, a tissue homogenizer (Next Advance, USA) was used. According to the manufacturer's instructions, RNA isolation was performed using the PureLink RNA Mini Kit (Invitrogen, USA).

Accordingly:
The appropriate volume of lysis buffer containing 2-mercaptoethanol was added to the sample. The lysate below was placed in the collection tube and centrifuged for two minutes at 12,000 g.

1.5 volumes of 100% ethanol and tissue lysate were added to an appropriately sized RNase-free tube and vortexed.

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700 µL of the sample was transferred to the cartridge (Spin Cartridge) and transferred to the collection tube, centrifuged at 12,000 g for 15 seconds at room temperature, and the liquid part was discarded. 350 µL wash buffer one was added to the cartridge (Spin Cartridge) and centrifuged for 15 seconds at 12,000 g at room temperature.

80 µL of DNase mixture was added to the surface of the cartridge membrane and incubated for 15 minutes at room temperature.

Once more, wash buffer one was added and centrifuged. 500 µL of washing buffer two was added to the cartridge with ethanol, centrifuged at room temperature, and discarded the liquid part.

The cartridge was placed in a recovery tube (Recovery Tube), added RNase-free water, and incubated for one minute at room temperature. Centrifuged for 2 minutes at 12,000 x g, the liquid portion was discarded.

The quantity and quality of purified total RNA were determined with the Quant-iT™ RiboGreen™ RNA Test Kit using a fluorescent microplate reader (UV absorbance 260 nm).

The obtained RNA was stored at -80oC.

cDNA synthesis was done in a palm cycler device.

While interpreting our results, the concentration value of our target genes was proportioned to the concentration value of the reference (housekeeping) gene, and the variation of the results obtained compared to the control group were examined. In our study, the beta-actin gene was used for this purpose.

Primers that were used:
BAX Forward AGACACCTGAGCTGACCTTG
BAX Reverse GTTGTTGTCCAGTTCATCGCC
BCL-2 Forward GGTGAACTGGGGGAGGATTG
BCL-2 Reverse AGAGCGATGTTGTCCACCAG

2-ΔΔCt Calculation
Accuracy and reliability in PCR analyses are related to the efficiency of PCR. For this purpose, the mRNA expression level of the target genes (Bcl-2 and Bax) is encountered with the reference gene (Beta Actin), the 2-ΔΔCt method is widely used. In our study, the Bcl-2 expression values of the samples were transformed by the 2-ΔΔCt method. Statistical analyzes were made on the values obtained.

Measurements of TAS and TOS Levels
According to the manufacturer's instructions, the TAS measurement of the tissue was done with the Total Antioxidant Status Assay Kit (Rel Assay Diagnostics brand). (Mega Tıp San ve Tic Ltd Sti, Sahinbey/Gaziantep/TURKEY). Absorbance reading was done with the ChemWell 2910 brand Elisa Reading Device (Awareness Technology, Inc. Martin Hwy. Palm City, USA). The results were given as mmol Trolox Equiv./L prt.

According to the manufacturer's instructions, the TOS measurement of the tissue was done with the Total Oxidant Status Assay Kit (Rel Assay Diagnostics brand). (Mega Tıp San ve Tic Ltd Sti, Sahinbey/Gaziantep/TURKEY). Absorbance reading was done with the ChemWell 2910 brand Elisa Reading Device (Awareness Technology, Inc. Martin Hwy. Palm City, USA). The results were given as µmol H2O2 Equiv./L prt.

Statistical Analysis
The Kruskal Wallis test examined the significant difference between the groups. When a statistically significant difference was detected between the groups, the Mann-Whitney U test determined which groups caused the difference. Spearman Correlation analysis was performed to determine the correlation between the groups in the research.

RESULTS

Primordial, primary, secondary (antral), and atretic follicle counts were performed for all groups. Primordial, primary, and secondary (antral) follicle numbers were higher in the Letrozole-induced PCOS group than in others. The number of atresia follicles was lower in the PCOS group. There was no significant difference between the groups regarding the number of atresia follicles. However, the difference between the groups in terms of primordial, primary, and secondary (antral) follicle numbers was statistically significant ($p<0.01$). It was determined that the difference was because the number of secondary (antral) follicles was higher in the PCOS group than in the other groups. The findings are consistent with PCOS. The images obtained from the histopathological examination are shown in Figure 1.

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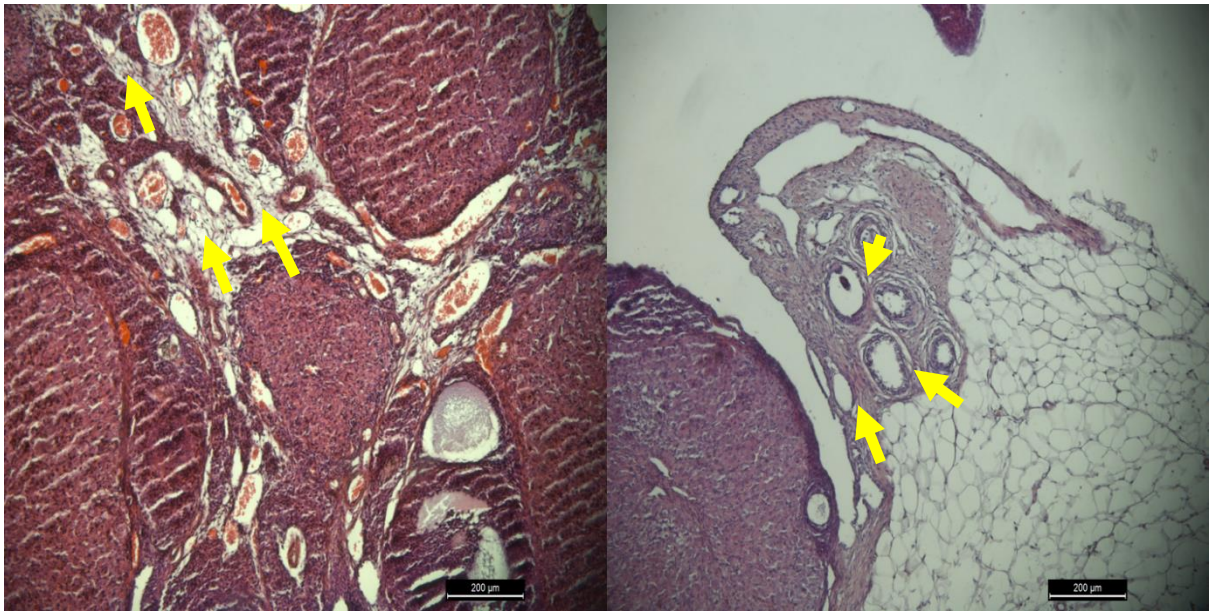

Figure 1: Images obtained as a result of the histopathological examination. Different follicles painted in different forms. Yellow arrows show the different follicles painted in different forms

The average serum TOS and TAS concentrations obtained in our study are shown in Table 1. According to the Table, the highest TOS value was measured in the group in which PCOS was formed with Letrozole. The lowest TOS value is in the control group. Among groups 3, 4, and 5, in which therapeutic agents were applied, the lowest value was determined in the Letrozole + Metformin + Berberine group. It was determined that difference between the groups was statistically significant. According to further analysis, this difference is due to the control group. When TAS values were examined, it was seen that the highest value was determined in the Letrozole + Metformin + Berberine group. The lowest TAS value was measured in the group where PCOS was formed with Letrozole. According to statistical analysis, there is a significant difference between the groups. Analysis results showed that the difference was due to the difference between the Letrozole-induced PCOS group, the Letrozole + Metformin, and Letrozole + Metformin + Berberine groups (Table 1).

Table 1: TOS and TAS Analysis by Groups*

Groups		n	Mean Rank	SD	x^2*	p	Significant Difference**
TOS (mmol Trolox Equiv./L)	1. Control	10	1.702	3	15.44	p<0.01	**1<2, 3, 4, 5**
	2. PCOS (Letrozole)	10	6.524				
	3. Letrozole + metformin	10	6.608				
	4. Letrozole + Berberine	10	5.476				
	5. Letrozole + Metformin + Berberine	10	4.385				
TAS (µmol H2O2 Equiv./L)	1. Control	10	0.374	3	16.08	p<0.05	**2<3** **2<5**
	2. PCOS (Letrozole)	10	0.229				
	3. Letrozole + metformin	10	0.471				
	4. Letrozole + Berberine	10	0.362				
	5. Letrozole + Metformin + Berberine	10	0.495				

*Kruskal Wallis Test, **Mann Whitney U Test

In our study, mRNA expression analysis of Bcl-2 and Bax proteins was performed by RT-PCR. Analyses were made on values transformed with the 2-ΔΔCt method. According to the results, there is a significant difference between the groups in Bcl-2 and Bax. In the Bcl-2 analysis, it was determined that the lowest gene expression was in the Letrozole-induced PCOS group, while the highest expression was in the Letrozole + Metformin + Berberine group. Further analysis

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determined that said difference was caused by the difference between the Letrozole-induced PCOS group and the other groups. In the analysis performed on Bax, the significant difference detected between the groups was more pronounced than the significant difference found in the Bcl-2 analysis (Bcl-2 $p<0.05$ and Bax $p<0.01$). The significant difference determined in the Bax analysis was examined with further statistical analysis, and it was determined that the difference resulted from the difference between the Letrozole-induced PCOS group and the Letrozole + Metformin + Berberine group (Table 2).

Table 2: Anti-Mullerian and 2 Base Delta Ct Analysis by Groups*

Groups		n	Mean Rank	SD	x^2*	p	Significant Difference**
BAX ($2^{-\Delta\Delta Ct}$)	1. Control	10	28.322	3	24.44	p<0.01	**5<2**
	2. PCOS (Letrozole)	10	29.036				
	3. Letrozole + metformin	10	28.605				
	4. Letrozole + Berberine	10	27.331				
	5. Letrozole + Metformin + Berberine	10	25.479				
Bcl-2 ($2^{-\Delta\Delta Ct}$)	1. Control	10	26.933	3	19.08	p<0.05	**2<1, 3, 4, 5**
	2. PCOS (Letrozole)	10	24.225				
	3. Letrozole + metformin	10	26.518				
	4. Letrozole + Berberine	10	26.720				
	5. Letrozole + Metformin + Berberine	10	27.945				

*Kruskal Wallis Test, **Mann Whitney U Test

The correlation between the TOS/TAS values and Bcl-2 and Bax expression results was studied. TOS values decreased in the treatment group. The lowest values are in the Letrozole + Metformin + Berberine group. TAS values are higher in the treatment group. The highest values are in the Letrozole + Metformin + Berberine group. Bax and Bcl-2 expression levels showed a change towards recovery in the treatment groups compared to the PCOS group. Bax expressions levels are the lowest among the treatment group, and Bcl-2 expression levels are the highest in the Letrozole + Metformin + Berberine group. Combined use of BBR and Metformin appears to benefit more than that standalone use. In standalone use, BBR is more effective than Metformin. TOS, TAS values, and Bcl-2 and Bax expression results were studied with correlation analysis, and no significant difference between groups was found ($p>0.05$).

DISCUSSION

Primordial, primary, secondary (antral), and atretic follicle numbers were counted in all groups with the histopathological examination performed in our study. Primordial, primary, and secondary (antral) follicle numbers were higher in the Letrozole-induced PCOS group. However, the number of atresia follicles is lower. The results obtained in our study support the findings obtained in some studies on PCOS. According to the results of our study, the total oxidant level was the highest, and the total antioxidant level was the lowest in the Letrozole-induced PCOS group.

Antral follicle count is considered an excellent parameter to determine the severity of reproductive dysfunction in PCOS. High antral follicle number is associated with significantly increasing androgens and LH/FSH ratio [Christ, J.P. *et al*., 2015]. Previous studies have indicated that ten or more follicles arranged peripherally around the dense core stoma can be used to diagnose PCOS [Alsamarai, S. *et al*., 2009], while 12 or more follicles are more reliable [Jonard, S. *et al*., 2003]. Some authors suggested that the number of follicles should be 15 [Fox, R. *et al*., 1991]. According to the Rotterdam criteria, at least 12 follicles ranging from 2 mm to 9 mm in the entire ovary indicate PCOS [PCOS, 2004]. In some updated guidelines published, studies support increasing the number of follicles to 25 [Coelho Neto, M.A. *et al*., 2018].

Oxidative stress is defined as the imbalance between oxidants and antioxidants and the formation of excessive amounts of reactive oxygen products. Many studies have revealed that oxidative markers are significantly increased in patients with PCOS compared to normal, which is considered a potential inducer of PCOS

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pathogenesis. Various oxidative stress biomarkers, including TAS and TOS, were analyzed to study the role of oxidative stress in the pathogenesis of PCOS. A meta-analysis study reported that according to age and body mass index, circulating biomarker concentrations increased by approximately 47% in women with PCOS compared to controls [Murri, M. *et al*., 2013]. In another study, biomarker levels in PCOS patients were compared with healthy controls. Biomarker levels were significantly higher in the PCOS group [Kuşçu, N.K. *et al*., 2009]. Zhang et al. also found that serum biomarker levels in PCOS patients were significantly higher than in the control group [Zhang, D. *et al*., 2008]. Palacio et al. compared PCOS patients with body mass index and age-matched controls and showed that PCOS patients had higher erythrocyte biomarker levels than controls [Palacio, J.R. *et al*., 2006].

On the contrary, studies show that serum biomarker levels in PCOS patients are similar to those of controls [Dursun, P. *et al*., 2006]. TAS can be defined as serum's ability to scavenge oxygen free radicals. Some studies determined that TAS levels in PCOS did not show a significant difference compared to controls [Murri, M. *et al*., 2013]. Fenkci et al. compared TAS levels in PCOS patients with matched controls. TAS levels were significantly lower in PCOS patients [Fenkci, V. *et al*., 2003]. Verit et al. found that TAS levels were significantly high [Verit, F.F. *et al*., 2008].

Different results have been obtained from studies on antioxidant concentration in PCOS. Further studies are needed to clarify the relationship of PCOS with antioxidants. This indicates the importance of studies on PCOS in rats. It is a generally accepted approach to examine the mechanisms of the pathophysiology of diseases with animal studies before humans.

Many studies show that positive and negative regulation play a role in the morphological changes of apoptotic cells and that they change depending on the signal transduction system. Members of the Bcl-2 family, which are among the signal transduction systems, are considered the primary regulators of apoptosis. Bcl-2 and Bax are the best-characterized members of this family. Bcl-2 is expressed in human ovarian tissues, but the mechanism by which Bcl-2 inhibits apoptosis has not been determined. In our study, administration of therapeutic agents to rats with Letrozole-induced PCOS increased Bcl-2 mRNA expression. It is thought that this increase leads to a decrease in the oxygen-free radicals involved in the pathogenesis of PCOS and, therefore, to the improvement of PCOS. These data are consistent with previous studies' results [Bas, D. *et al*., 2011; Raei Sadigh, A. *et al*., 2020].

Bcl-2 functions as an antagonist of the Bax protein. Bax is the protein homolog of Bcl-2. Studies have shown that Bax expression is more common than Bcl-2 and is expressed in male testis and female ovarian cells. The proapoptotic effect of Bax protein is inhibited with the anti-apoptotic protein Bcl-2. Increased expression of Bax protein triggers apoptosis. According to the results of our study, the application of therapeutic agents after forming PCOS via Letrozole in rats decreases Bax expression. This is thought to be effective in PCOS recovery by reducing oxygen-free radicals. According to a study examining in vitro and in vivo the apoptosis/proliferation signals of granulosa cells in PCOS patients, Bax expression has increased in patients [Raei Sadigh, A. *et al*., 2020]. This has been supported by other studies that have determined that Bax is involved in the pathogenesis of PCOS [Chen, Y. *et al*., 2021].

BBR is an agent whose efficacy in PCOS disease has been studied in various human and animal studies.

In a study in which PCOS was created with Letrozole in rats, it was found that BBR could improve serum hormone levels and improve insulin resistance [Shen, H.R. *et al*., 2021]. In the same study, morphological lesions of the ovary and apoptosis were also improved with BBR treatment [Shen, H.R. *et al*., 2021]. In another study, insulin resistance decreased, and testosterone levels decreased due to BBR administration in animals with PCOS by injecting dehydroepiandrosterone [Yu, J. *et al*., 2021]. Apoptosis significantly decreased. Expression levels of NF-kB, TNF-α, IL-1, IL-6, and caspase-3, which are high in PCOS, decreased with BBR administration [Yu, J. *et al*., 2021]. In another animal study in which PCOS was created with Letrozole, it was stated that BBR up-regulated GLUT4 by PI3K/AKT activation and suppressing the MAPK pathway, thereby improving PCOS pathologies [Zhang, N. *et al*., 2020].

The results of a study in which studies examining the effect of BBR on PCOS were analyzed highlight that there is not enough data to conclude the effects of BBR in PCOS, especially on insulin resistance (Li, M.F. *et al*., 2018). In the study,

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BBR and Metformin were examined as therapeutic agents. There was no significant difference between the effects of BBR and Metformin. Metformin combined with BBR did not provide superiority to Metformin alone (Li, M.F. *et al*., 2018). Some studies have the opposite of these results. The results of a study report that BBR is safe and promising for PCOS and that its low side-effect profile supports this claim (Rondanelli, M. *et al*., 2020).

CONCLUSION

Information on the pathophysiology and treatment of PCOS is quite detailed and complex. There are different opinions among researchers on this issue. Similarly, a consensus has not been reached on the treatment of PCOS. There is no clear data on the effects of the promising agents such as BBR or of the combined use of these agents. This situation has revealed the importance of animal studies on PCOS. It is essential to examine the arguments about pathophysiology and treatment in appropriate animal models.

In our study, a PCOS model was created in rats, and data on the pathophysiology and treatment of PCOS were obtained with different analyzes. The effects of oxygen radicals and antioxidants in the pathophysiology were examined by both TAS/TOS and gene expression, and reliable data were obtained.

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