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Follicular fluid redox balance and DNA damage according to PCOS phenotype

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Abstract. – OBJECTIVE: Classification of polycystic ovary syndrome (PCOS) according to their phenotypes is important in terms of understanding which parameter has clinical and laboratory implications. This study was designed to measure the follicular fluid total oxidant capacity (TOC) and total antioxidant capacity (TAC) and DNA degradation products of 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in patients with different PCOS phenotypes undergoing In-Vitro Fertilization/Intra-Cytoplasmic Sperm Injection (IVF/ICSI).

PATIENTS AND METHODS: Thirty women who were diagnosed with PCOS and twenty infertile patients who did not have the clinical and laboratory findings of PCOS were included. Women carrying at least two of the three parameters below were considered to have PCOS. (1) Biochemical or clinical manifestations of hyperandrogenism (HA); (2) Ovulatory dysfunction (OD); (3) Polycystic ovarian morphology (PCOM). Patients were then classified into four different PCOS phenotypes: (1) Phenotype A is also known as classical PCOS and meets all three criteria (HA/OD/PCOM). (2) Phenotype B has two criteria, HA and OD. (3) Phenotype C consists of HA and PCOM criteria. (4) Phenotype D is the non-hyperandrogenic form and consists of OD and PCOM criteria. Antagonist protocol was used in both PCOS and control groups. During oocyte pick-up, follicular fluid of the dominant follicle was collected. TAC and TOC levels, which are redox balance markers, and 8-OHdG levels, which are DNA degradation products, were measured in follicular fluid samples (FF).

RESULTS: Follicular fluid 8-OHdG levels of all four types of phenotypes were significantly higher than the control group. When the phenotype groups were evaluated among themselves, FF-8-OHdG levels of each group were found to be similar. Serum TOC levels of each phenotype group were found to be significantly higher than the control group. TAC levels of the patients in the control group were significantly higher than the other four phenotype groups. Oxidative stress index (OSI) values were significantly higher in all four phenotype groups compared to the control group. OSI values of phenotype B and D groups were significantly higher than phenotypes A and C.

CONCLUSIONS: In each phenotype of PCOS, TOC and OSI increased while TAC decreased. Increased OSI leads to DNA degradation and an increase in the level of 8-OHdG. The cumulative effect of oxidative stress and DNA degradation may be the main mechanism of PCOS-related subfertility.

Key Words:
PCOS, Redox balance, TAC, TOC, 8-OH-2dG, Follicular fluid.

Introduction

Folliculogenesis is a physiological process accompanied by oxidative stress from the primordial follicular stage to the dominant follicular stage. In the ovulatory process, in addition to the changes made by the luteinizing hormone (LH) peak in the target follicle, a significant increase in the production of reactive oxygen species (ROS) occurs and contributes to follicle rupture[1-3]. Free radicals consist of three different compounds, such as superoxide, hydrogen peroxide, and hydroxyl, which are unstable and reactive compounds and become stable by gaining electrons from outside[3]. As long as it is balanced by antioxidant enzymes and ROS scavengers under physiological conditions, ROS does not cause an adverse effect. If the ovarian cells have enough antioxidants enzymes, the development of ROS-related follicular cell damage is prevented. Since redox pathologies due to excessive ROS production during follicular development will be reflected in the follicular fluid, we can have information about the functions of the cumulus-oocyte complex by measuring the total oxidant capacity (TOC) and total antioxidant capacity (TAC). In addition to superoxide

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dismutase, glutathione peroxidase, and lipid peroxides, the levels of oxidative DNA degradation products can be measured in follicular fluid[4,5]. Follicles that do not have a healthy oxidant/antioxidant balance are adversely affected by both fertilization, formation of good quality embryos and implantation rates[6,7].

Oxidative stress is accepted as the etiology of infertility in many infertile couples. Increased ROS production may adversely affect both oocyte developmental capacity and implantation rates[5,8]. Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies with subfertility in women of reproductive age. Although the main cause of subfertility due to PCOS is not known clearly, ovulatory dysfunction and hyperandrogenemia are blamed[9,10]. There are not enough studies on how the follicular fluid redox dynamics of PCOS patients change in patients undergoing assisted reproductive technology[11]. However, low cumulative pregnancy rates due to ovulation dysfunction and hyperandrogenemia suggest a problem in follicle development capacity and fertilization. Exogenous gonadotropins administered in addition to the syndromic effects of PCOS may increase ROS production by causing intra-follicular iron accumulation[12]. Increased ROS levels during *In-Vitro* Fertilization/Intra-Cytoplasmic Sperm Injection (IVF/ICSI) may cause cell damage by disrupting the oxidative balance (TOC>TAC) in the developing follicle. The indicator of DNA damage due to oxidative stress is 8-OHdG. The increase of this marker is associated with low fertilization rates[13,14].

Classifying PCOS patients according to their phenotypes is important in terms of understanding which parameter has clinical and laboratory reflections. Using the criteria of hyperandrogenemia (HA), ovulatory dysfunction (OD), and polycystic ovarian morphology, PCOS patients can be divided into four different phenotypes[15,16]. This study was designed to measure the levels of TAC, TOC, and 8-OHdG, which are redox balance and DNA degradation products, in the follicular fluid of patients undergoing IVF/ICSI from four different PCOS phenotypes. It will be evaluated how each parameter changes according to the PCOS phenotype and how they affect the follicular redox balance.

Patients and Methods

Thirty patients who were diagnosed with PCOS according to revised Rotterdam criteria[15,16] and decided on IVF/ICSI were included in the study. Twenty infertile patients who did not have the clinical and laboratory findings of PCOS and who decided to undergo IVF/ICSI were accepted as the control group. Patients in the control group were selected from non-hyperandrogenic infertile patients with regular menstrual cycles. Those carrying at least two of the three parameters below were considered to have PCOS. (1) Biochemical or clinical manifestations of hyperandrogenism (HA); (2) Ovulatory dysfunction (OD); (3) Polycystic ovarian morphology (PCOM) characterized by 12 or more follicles with a diameter of 2-9 mm in ultrasonographic evaluation. Patients were then classified into four different PCOS phenotypes according to the National Institutes of Health (NIH)'s 2012 phenotypic extension of the Rotterdam definition[15,16]: (1) Phenotype A is also known as classical PCOS and meets all three criteria (HA/OD/PCOM). (2) Phenotype B has two criteria, HA and OD. (3) Phenotype C consists of HA and PCOM criteria. (4) Phenotype D is the non-hyperandrogenic form and consists of OD and PCOM criteria. Exclusion criteria were determined as non-PCOS conditions that cause OD and HA. Women with type 2 diabetes, thyroid and pituitary diseases, androgen-secreting ovarian or adrenal tumors, Cushing's syndrome, adrenal hyperplasia, and cardiovascular diseases were excluded from the study. Those who used hormonal or lipid-lowering drugs and those who used antiandrogen and oral contraceptives in the last six months were also excluded from the study. Venous blood samples were taken from the patients in the ovulatory PCOS and control groups on the third day of their spontaneous menstrual cycle. Blood was collected on the third day of the progesterone withdrawal bleeding from the anovulatory PCOS patients to evaluate basal hormone and biochemical parameters. Age and body mass index (BMI) measurements were recorded. Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and fasting insulin levels were measured. The homeostatic model assessment (HOMA-IR) formula was used for calculating insulin resistance.

Antagonist protocol was used in both PCOS and control groups of patients. On the third day of spontaneous or induced cycles, all patients were started on recombinant follicle-stimulating hormone, taking into account age, ovarian reserve, and previous doses. Initial rFSH doses differed in PCOS and control groups. Follicular development was followed by both ultrasonog-

raphy and serum estrogen level measurements. Gonadotropin releasing hormone (GnRH) antagonist treatment was started when the leading follicle was 14 mm or on the 7th day of rFSH treatment. When the diameter of at least two leading follicles reached 17 mm, or more, triptoreline acetate (Gonapeptyl 0.1 mg/ml, Ferring, Istanbul, Turkey) was administered in the PCOS group, and a single dose of recombinant human chorionic gonadotropin (hCG) injection was administered in the control group to trigger ovulation. Oocyte pick-up was performed in patients in both groups 36 hours after ovulation triggering. The follicular fluids of the dominant follicle of both groups were frozen in RNA-later until analysis. Total embryo freezing was performed in some patients due to the risk of ovarian hyperstimulation syndrome. For this reason, the reproductive outcomes of the groups were not included in the study.

Follicular Fluid Sampling and 8-OHdG, TAC and TOC Analysis

Samples collected from a dominant follicle were analyzed to assess follicular fluid 8-OHdG levels. Follicles with a diameter of 17 mm or more in the right or left ovary and containing metaphase II (MII) oocytes were considered dominant. If the first aspirated follicle contained a large amount of blood or contained MI/ germinal vesicle (GV) oocyte, follicular fluid of other follicles was collected. No follicular flushing was performed before aspiration. Debris, blood clot, and cumulus cells in the collected fluid were removed with a hyaluronidase solution. The follicular contents were centrifuged at 3,500 g for 5 min. Clean samples remaining on the centrifuge tube were frozen and stored. After the frozen follicular fluid samples were thawed, 8-OHdG, TAC, and TOC levels were measured.

TAC and TOC levels were measured by using TAC or TOC kits (Rel Assay, Mega Medicine Industry, Gaziantep, Turkey). Follicular fluid (FF)-TAC and FF-TOC levels were measured as previously described. TAC results were given as mmol/L and TOC results as μmol/L. TOC/TAC value was taken as oxidative stress index (OSI) and the result was given as arbitrary unit[17]. Follicular fluid 8-OHdG levels were measured using the human ELISA kit and according to the manufacturer's recommendations (Sunred Bioscience, Shanghai, China). The measuring range of the kit was 1-100 ng/mL. Intra- and inter-assay coefficients of variability (CV) values of the 8-OHdG kit were 10% and 12%, respectively. The minimum measurable limit was 0.55 ng/mL.

Statistical Analysis

Analysis of DNA degradation, TAC, and TOC results obtained from FF measurements was performed with SPSS 21 (IBM Corp., Armonk, NY, USA) program. The Shapiro-Wilk test was used to determine data distribution patterns. One-way ANOVA test was used to compare ELISA, TAC, TOC, laboratory, hormonal and metabolic parameters of four different phenotypes and control groups. The correlation between parameters was evaluated by Pearson's correlation analysis. Results are presented as mean±standard deviation. $p<0.05$ was considered statistically significant in all comparisons.

Results

The distribution of demographic, metabolic, and hormonal parameters of both groups is presented in Table I. Although there was an increasing trend in the PCOS group in terms of age and BMI, the difference was not significant. When PCOS patients were classified according to phenotypes,

Table I. Demographic and hormonal parameters of PCOS and control groups.

	PCOS	Non-PCOS control	*p*-value
N	30	20	
Age (y)	27.5 ± 3.22	26.8 ± 2.81	> 0.33
BMI (Kg/m^2)	26.2 ± 2.30	25.7 ± 3.65	> 0.07
LH (mIU/mL)	11.2 ± 2.54	5.87 ± 1.33	< 0.02
FSH (mIU/mL)	4.99 ± 1.02	5.11 ± 2.09	> 0.40
Testosterone (ng/dL)	55.1 ± 6.43	37.5 ± 4.07	< 0.01
HOMA-IR	9.54 ± 3.20	3.83 ± 1.07	< 0.03

Polycystic ovary syndrome (PCOS), body mass index (BMI), luteinizing hormone (LH), follicle-stimulating hormone (FSH), homeostatic model assessment (HOMA-IR).

the number of patients in the phenotype A group was significantly higher than the other groups (36.6%). The total number of patients for phenotypes A (36.6%) and B (23.3%) was higher than the sum of phenotypes C (23.3%) and D (16.6%). The total number of patients in phenotypes A and B comprised more than half of all patients (n=18 *vs.* n=12). Serum LH and testosterone levels of the PCOS group were significantly higher than the control group. Similarly, the HOMA-IR values of the patients in the PCOS group were significantly higher than the control group. Serum FSH values were recorded as similar between the two groups. When subgroup analysis of PCOS patients was performed according to phenotypes, no significant change was found in metabolic, hormonal and demographic findings. The increase in androgen levels was more pronounced in phenotype A than in other phenotypes. However, this upward trend in androgens did not reach statistical significance.

FF-8-OHdG levels of all four phenotypes were significantly higher than the control group. When the phenotype groups were evaluated among themselves, FF-8-OHdG levels of each group were found to be similar. Serum TOC levels of each phenotype group were found to be significantly higher than the control group. TAC levels of the patients in the control group were significantly higher than the other four phenotype groups. OSI values were significantly higher in all four phenotype groups compared to the control group. OSI values of phenotype B and D groups were significantly higher than phenotypes A and C (Table II).

Discussion

Polycystic ovarian syndrome is the most common cause of subfertility and endocrine anomaly in women of reproductive age. The term polycystic ovarian syndrome is not sufficient to reflect the complex nature of the disease. PCO has a wide laboratory and clinical spectrum with endocrine and metabolic problems on the one hand and menstrual irregularity and subfertility on the other[18,19]. The fact that different organizations are not in a clear consensus on the PCOS diagnostic criteria causes difficulties in revealing the main causes of the disease. Clinical or biochemical hyperandrogenism and chronic oligo-anovulation are essential components of the disease pattern. Polycystic ovarian morphology is another parameter used in the classification of the disease. In the 2012 NIH consensus panel, PCOS was divided into the following four phenotypes using three different criteria. Phenotype A (HA+OD+PCOM), phenotype B (HA+OD), phenotype C (HA+PCOM) and phenotype D (OD+PCOM). The phenotypic classification made the disease groups clearer and available for clinical research[15,16].

When we separated PCOS patients according to phenotypes, the groups with the highest OSI were phenotypes D and B. Oxidative damage was significantly higher in both phenotypes compared to phenotypes A and C. While TOC levels increased, TAC levels decreased, resulting in an increase in the OSI values of these two phenotypes. Phenotype D consisted of OD and PCOM criteria. The absence of HA in the phenotype may explain the high OSI values. In phenotypes A and C, the common criterion is the presence of HA. Therefore, high oxidative damage in phenotype D may be associated with the absence of HA. However, this needs to be clarified by detailed experimental and clinical studies.

Ovulatory dysfunction has long been recognized as the main cause of PCOS-related subfertility. However, the fact that pregnancy rates did not increase significantly despite medical treatment of OD brought up the idea that the endome-

Table II. Follicular fluid 8-OHdG, TAC, TOC and OSI values of each phenotype and control groups.

	Phenotype A	Phenotype B	Phenotype C	Phenotype D	Control
N (%)	11 (36.6)	7 (23.3)	7 (23.3)	5 (16.6)	20
8-OHdG (ng/mL)	33.4 ± 5.09	35.9 ± 4.87	33.6 ± 3.22	39.7 ± 11.8	13.9 ± 2.09*
TOC (μmol/L)	56.7 ± 5.12	61.2 ± 6.88	59.5 ± 2.90	63.6 ± 6.30	28.7 ± 2.01*
TAC (mmol /L)	1.20 ± 0.01	0.97 ± 0.23	1.49 ± 0.20	0.87 ± 0.58	3.22 ± 1.50*
OSI (TOC/TAC) (arbitrary unit)	4.72 ± 3.55	6.30 ± 5.60	3.99 ± 3.90	7.31 ± 5.77	0.89 ± 1.20*

*Shows statistical signicance between each phenotype group *vs*. control group. Data are presented Mean ± SD. Total oxidant capacity (TOC), total antioxidant capacity (TAC), 8-hydroxy-2'-deoxyguanosine (8-OHdG), oxidative stress index (OSI).

trium of PCOS patients is also defective. Indeed, the PCOS endometrium has both a progesterone receptor defect and resistance to the physiological effects of progesterone. Since adequate progesterone cannot be produced, estrogen dominance is present in the endometrium[20,21]. Estrogen receptor alpha (ERα) and androgen receptor (AR) expression are increased in PCOS endometriums[21]. There is no significant increase in circulating estrogen levels.

Although the term ovulatory dysfunction often describes chronic oligo or anovulation, the idea that PCOS patients have a disorder in the dynamics of follicular development has gained weight[11]. Oxidative stress and increased ROS production are important factors that impair the developmental capacity of the follicle. If the developing follicle has sufficient antioxidant and ROS-scavenging enzymes, it can be protected from oxidative stress. Follicular fluid is an important biological fluid because it reflects the total metabolic activity and redox balance of the cumulus-oocyte complex[22]. Our study, which investigated intrafollicular redox balance in PCOS patients for the first time, is of critical importance in terms of opening up the idea that ovulatory dysfunction may be related to redox balance disorder and DNA damage. Follicular fluid levels of TOC, which is an oxidative stress indicator of four different PCOS phenotypes, were found to be significantly higher than the control group. However, when the phenotypes were classified among themselves, TOC levels were found to be similar. This finding is evidence of impaired redox balance in the follicle, independent of the PCOS phenotype.

The presence or absence of hyperandrogenemia or ovulatory dysfunction did not cause a significant change in TOC levels. Similarly, the presence of PCOM did not significantly affect oxidative stress[23,24]. The oxidant capacity indicator TAC levels, located at the other end of the redox balance, were found to be significantly lower in all four phenotype groups compared to the control group. When subgroup analysis was performed according to phenotypes, it was determined that TAC levels did not change. In summary, while TOC levels increased in all four PCOS phenotypes, TAC levels decreased significantly. For the redox balance to be restored, ROS and scavenging enzymes must remain in balance[25,26]. The shift of the redox balance towards TOC in each phenotype suggests that decreased antioxidant capacity is responsible for the decreased fertility in PCOS. The fact that TAC and TOC ratios are similar in all phenotypes supports the fact that PCOS is a syndrome that stimulates oxidative stress regardless of the underlying cause. In the presence of oxidative stress, decreased intrafollicular oxygenation may impair oocyte development and lead to cleavage and chromosomal segregation defects[27,28]. In addition, oxidative stress impairs follicular vascularization. For all these reasons, increased oxidative stress index (OSI=TOC/TAC) in PCOS patients may cause a decrease in embryo quality and implantation rates by impairing oocyte developmental capacity. The presence of increased OSI in each phenotype group suggests that phenotypic variables do not have a significant effect on the formation of oxidative stress.

We do not know the mechanism cause of impaired redox balance in PCOS patients. However, as the increased number of follicles occupies a large part of the ovarian cortex, it may lead to oxidative stress by causing a decrease in food and oxygenation. Minimal oxidative stress is necessary for follicular development and fertilization. Overproduction of ROS negatively affects both follicular development and embryo formation[29]. Increased ROS can cause DNA damage in granulosa cells and oocytes. The biomarker of DNA damage in biological fluids is 8-OH-2dG. An increase in this marker may lead to poor fertilization and poor embryo quality[30]. The fact that 8-OH-2dG levels are higher in all four PCOS phenotypes compared to the control group is important evidence that oxidative stress due to PCOS causes DNA damage. DNA damage in granulosa cells adversely affects the developmental potential of oocytes. Contrary to the increase in TOC, the increase in fertilization rates in proportion to the increasing TAC levels is evidence of the importance of antioxidant pathways in embryo development. Decreased TAC, increased TOC, and increased 8-OH-2dG levels in PCOS patients, regardless of phenotype, may explain the cumulatively reduced pregnancy rates. Since increased oxidative stress will impair DNA integrity, the subsequent development of oocytes may be disrupted even if they are at the MII stage.

Conclusions

Most of the drugs administered during antiretroviral therapy (ART) cause changes in the redox balance of the follicular fluid. Increased

iron ratios due to exogenous gonadotropins may cause an increase in ROS production in the follicle[12]. Since the antagonist protocol was applied to all PCOS and control group patients included in the study, it would not be correct to attribute the redox balance disorder to ART drugs. Despite the small number of cases, it is the first study showing that intrafollicular redox balance is impaired in PCOS classified according to phenotypes. We found that while TOC and OSI increased, TAC decreased in each phenotype of PCOS patients. Increased OSI leads to DNA degradation and an increase in the level of 8-OH-2dG. The cumulative effect of oxidative stress and DNA degradation may be the main mechanism of PCOS-related subfertility. A clearer conclusion can be reached with comprehensive studies evaluating the relationship between intrafollicular redox markers and embryological parameters and reproductive outcomes.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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Ethics Approval
The study was initiated after obtaining Gynolife Hospital Ethical approval (Document No.: 2022/40, Approved date: 08/03/2022).

Informed Consent
All patients included in the study gave written informed consent.

Availability of Data and Materials
Data is available after the approval of all authors.

Authors' Contribution
All authors contributed to the study conception and design. The study was designed by, MO. Patient selection and material collection were performed by MO, MA. Demographic and clinical data were collected by MO, MA. The collection of literature data and the processing of the obtained data were done by MO. Statistical analyzes were performed by MO, MA. The first draft was written by MO. All authors approved the final version of the manuscript. All authors gave permission for the article to be published.

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