Diabetes & Metabolic Syndrome: Clinical Research & Reviews 13 (2019) 1957–1961

Contents lists available at ScienceDirect

Diabetes & Metabolic Syndrome: Clinical Research & Reviews

journal homepage: www.elsevier.com/locate/dsx

Original Article

# Evaluation of antioxidant defense markers in relation to hormonal and insulin parameters in women with polycystic ovary syndrome (PCOS): A case-control study

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ARTICLE INFO

*Article history:*
Received 18 March 2019
Accepted 22 April 2019

*Keywords:*
Polycystic ovary syndrome
Insulin resistance
Oxidative stress
Cardiovascular
Glutathione

ABSTRACT

*Aim:* Polycystic ovary syndrome (PCOS) is a composite heterogeneous condition with multifactorial etiology like genetic, environmental factors and oxidative stress. The exact pro-oxidant and antioxidant status in PCOS patients has not yet been fully established. We designed prospective study aimed to explore the association of PCOS and oxidative stress and examine the relationship of oxidative stress biomarkers with insulin parameters.

*Methods:* Two groups were included: study group including 85 women with PCOS and control group of 85 healthy volunteers. Biochemical, Hormonal and insulin parameters were measured. Vitamin C, vitamin E, nitric oxide and activities of antioxidant enzymes were estimated using spectrophotometric methods.

*Results:* Subjects with PCOS had poor antioxidant status as reflected by significantly low levels of glutathione, vitamin C & E and considerably increased activities of antioxidant enzymes like glutathione peroxidase, glutathione reductase and glutathione transferase as compared to those without PCOS. At the same time insulin levels were found to be significantly high and a positive correlation between oxidative stress and insulin parameters was observed in PCOS.

*Conclusion:* Low levels of antioxidants and increased oxidative stress with insulin resistance along with the observed correlation between these parameters suggest that women with PCOS are under oxidative stress which supports the concept that oxidative stress is involved in PCOS pathophysiology. Thus oxidative stress could be a contributory factor to future cardiovascular disease risk in these women in addition to known features like dyslipidemia, central obesity, etc.



## 1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most common and multifaceted endocrinological disorder in adolescence and reproductive age women, with a stated prevalence of 4–12% [1]. According to ESHRE/ASRM consensus workshop at Rotterdam in 2003, the syndrome is diagnosed by the presence of two or more of the criteria, which are clinical and/or biochemical androgen excess, chronic oligo-ovulation or anovulation and polycystic ovaries [2]. This syndrome is associated with both reproductive and metabolic features. Reproductive complications include hyperandrogenism, ovarian dysfunction, pregnancy complications and infertility [3,4]. Moreover, metabolic complications include insulin resistance, dyslipidemia, impaired glucose tolerance, diabetes mellitus [5]. The mechanism underlying polycystic ovary syndrome (PCOS) has not been well defined. However latest studies have demonstrated that insulin resistance and associated hyperinsulinemia seem to play a role in the development of PCOS [6]. Insulin resistance appears to interfere with ovarian steriodogenetic defects as well as anovulatory mechanism [7]. Insulin resistance leads to metabolic syndrome and independently increases the risk for cardiovascular dysfunction [8]. Therefore, the

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https://doi.org/10.1016/j.dsx.2019.04.032
1871-4021/

Androgen Excess-PCOS Society has recommended to evaluate the cardiovascular risk in all PCOS women [9].

The incidence of PCOS has been closely related to oxidative stress and chronic low grade inflammation [10,11]. Oxidative stress is commonly referred as a state where oxidative forces surpass the antioxidant systems due to loss of the balance between them. Reactive oxygen species (ROS) like superoxide, hydrogen peroxide, and hydroxyl radical ions [12], are the agents of oxidative stress and are produced at low physiological levels mostly in the mitochondria and peroxisomes. Reactive oxygen species participate in the regulation of numerous essential physiological processes but when present in great concentrations they become toxic and damage body tissues. To deal with the oxidative stress, living systems have evolved protective physiological mechanisms in the form of antioxidants that counterbalance the effects of oxidants and have ability to dispose, scavenge, and suppress the free radical formation [13]. Polycystic ovary syndrome is associated with decreased antioxidant concentrations, and is thus regarded a state of increased oxidative stress [14,15]. The link between oxidative stress and insulin resistance has drawn attention. The recent studies in this area suggest that there could be a strong connection between insulin resistance, hyperandrogenism, inflammation and oxidative stress in the pathogenesis of PCOS. There is significant evidence that PCOS women have considerably increased protein oxidation, increased serum and urinary levels of lipid peroxidation products (in most studies measured as MDA), and raised total oxidative state [16,17]. But there are only limited data relating the degree of oxidative stress to insulin resistance in PCOS. Investigations have been impeded by limited availability of reliable biomarkers of oxidative stress for use in epidemiological studies.

In this study we intend to estimate blood concentrations of selected antioxidant markers in PCOS patients and to assess the association of oxidative stress markers with insulin parameters in patients with polycystic ovary syndrome.

## 2. Materials and methods

### 2.1. Study population

The study was approved by Institutional Board of Research Studies and was carried in the Department of Clinical Biochemistry, University of Kashmir. A total of 170 women were enrolled in the present study. Among them, 85 women had PCOS, while the other 85 were healthy control subjects. The PCOS women were diagnosed as using the Rotterdam criteria. The written consents were taken from the patients prior to study and all the subjects were fully informed about the study protocol.

Other related diseases, such as Cushing syndrome, adrenal congenital hyperplasia, and androgen-secreting tumors, hyperprolactinemia and patients with other medical diseases were excluded. The women in the control group were healthy volunteers, had normal menstrual cycles with duration of 4–5 days and a frequency of 25–30 days, with no signs of hyperandrogenism and normal sonographic appearance of the ovaries.

### 2.2. Anthropometric examination

All women underwent anthropometric assessment like measurement of weight, height, waist-hip circumference ratio and complete systemic examination. The details of menstrual history included menarche, duration, regularity, dysmenorrhea, flow and number of menstrual cycles per year. Oligo menorrhea was defined as an inter-menstrual interval of >35 days or a total of <8 menses per year and amenorrhea as absence of menstruation during last 6 months. Hirsutism assessment was done using modified Ferriman-Gallwey score. A score ≥8 was defined as Hirsutism [18]. Transvaginal ultrasound was used to identify polycystic ovaries, defined as the presence of at least one ovary>10 $cm^3$ or comprising at least 12 follicles 2–9 mm in diameter.

### 2.3. Biochemical assays

Blood samples collected after an overnight fast of ≥12 h on the second or third day of the menstrual cycle were used for the analysis. The levels of glucose, triglycerides, cholesterol, were measured using semi-automated analyzer.

### 2.4. Insulin assays

Fasting insulin levels were measured using ELISA kit and IR was estimated via the homeostasis model assessment insulin resistance index (HOMA-IR), as follows: HOMA-IR = fasting insulin (μIU/mL) × fasting glucose (mg/dl)/405, and quantitative insulin sensitivity check index (QUICKI) was defined as 1/[log fasting insulin (μIU/mL) + log fasting glucose (mg/dl)] [19]. High HOMA-IR, low QUICKI and low FGIR denote insulin resistance.

### 2.5. Hormonal assays

Luteinizing hormone (LH), Follicle-stimulating hormone (FSH) and testosterone analyses were carried out with a chemiluminiscence immunoassay.

### 2.6. Oxidant/antioxidant parameters assessment

Glutathione levels were determined by observation of absorbance of yellow colored product which forms as a result of reaction of Elman reagent with sulfhydryl groups in 412 nm, spectrophotometrically according to the method developed by Moren [20]. Glutathione peroxidase and glutathione reductase activity was assayed using the method of Sharma [21]. NADPH oxidation was recorded at 340 nm spectrophotometrically and the enzyme activity was evaluated as nmoles NADPH oxidized/min/ml of serum. GST activity was assayed using the method of Haque [22] using 1-Chloro-2, 4-Dinitro Benzene (CDNB). The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nmoles of CDNB conjugates formed/min/ml serum. Nitrite levels were determined by a colorimetric method based on the Griess reaction, in which nitrite is reacted with sulfanilamide and *N*-(1-naphthyl) ethylene diamine to produce an azo dye detected at 540 nm [23]. Ascorbic acid levels were assessed by the method of Tietz [24]. Vitamin E levels were calculated by the method of Baker [25]. Hydroxyl radical, produced from the $Fe^{3+}$-Ascorbate-$H_2O_2$ (Fenton reaction), was estimated by degradation of deoxyribose that produce thiobarbituric acid reactive species (TBARS) [26]. The absorbance was measured spectrophotometrically at 532 nm. The estimation of superoxide anion radical scavenging property was carried out as described by McCord and Fridovich [27] with some modifications.

### 2.7. Statistical analysis

The statistical analysis was achieved using SPSS for windows version 16.00 program. All data were reported as mean ± standard deviation (SD). Unpaired *t*-test was used for comparison of variables between the groups. Pearson Correlation was used for the correlation analysis. Regression analysis was done and statistical significance was defined as $p < 0.05$.

## 3. Results

The baseline clinical and hormonal parameters of study population are shown in (Table 1). There was no significant difference in terms of BMI and age and the parameters like weight, waist circumference and FG score were found to be significantly increased in PCOS women. Compared with controls PCOS women had elevated levels of LH and Testosterone. There was no significant difference between PCOS and control groups in terms of FSH.

The Insulin and biochemical parameters are summarized in (Table 2). Fasting insulin and HOMA-IR values were significantly greater in the study group whereas FGIR and QUICKI were lower in the study group which was statistically significant. The PCOS women had increased levels of glucose 1hr and cholesterol as compared to the controls. Although triglyceride levels were more in cases than controls but it did not reach statistical significance.

**Table 1**
Clinical and hormonal parameters of patients with Polycystic Ovary Syndrome and controls.

| Parameters | Cases N = 85 | Controls N = 85 | p-value |
|---|---|---|---|
| Mean Age (years) | 22.42 ± 4.20 | 21.32 ± 2.39 | 0.111 |
| Weight (Kg) | 59.04 ± 10.22 | 50.32 ± 6.95 | 0.001* |
| BMI (Kg/m$^2$) | 23.24 ± 3.72 | 22.39 ± 2.41 | 0.078 |
| FG-score | 10.10 ± 2.83 | 7.72 ± 1.51 | 0.001* |
| Waist | 87.92 ± 9.15 | 77.91 ± 7.72 | 0.001* |
| WHR | 0.94 ± 0.05 | 0.90 ± 0.08 | 0.001* |
| LH (IU/L) | 8.09 ± 4.72 | 5.63 ± 3.16 | 0.001* |
| FSH (IU/L) | 7.04 ± 2.74 | 6.31 ± 1.56 | 0.105 |
| Testosterone (mg/dl) | 43.69 ± 19.02 | 25.48 ± 9.81 | 0.001* |

Data was expressed as Mean ± SD, *P < 0.05 is considered significant, BMI, Body Mass Index, FG, Ferriman Galway score, LH, Leutinizing hormone, FSH, Follice stimulating hormone, WHR, Waist Hip Ratio.

**Table 2**
Insulin and biochemical parameters of patients with Polycystic Ovary Syndrome and controls.

| Parameters | Cases N = 85 | Controls N = 85 | p-value |
|---|---|---|---|
| Fasting Insulin | 14.96 ± 4.78 | 9.88 ± 2.49 | 0.001* |
| FGIR | 6.37 ± 1.59 | 9.57 ± 3.21 | 0.001* |
| HOMAIR | 3.28 ± 1.20 | 2.13 ± 0.60 | 0.001* |
| QUICKI | 0.32 ± 0.01 | 0.34 ± 0.01 | 0.001* |
| Blood glucose fasting (mg/dl) | 88.84 ± 9.05 | 89.33 ± 14.83 | 0.794 |
| Blood glucose-1 hour (mg/dl) | 120.29 ± 7.44 | 112.17 ± 8.32 | 0.001* |
| Blood glucose-2 hour (mg/dl) | 107.28 ± 12.71 | 105.27 ± 9.03 | 0.238 |
| Serum cholesterol (mg/dl) | 151.00 ± 18.63 | 139.91 ± 19.76 | 0.001* |
| Serum Triglycerides (mg/dl) | 115.02 ± 21.27 | 114.39 ± 27.17 | 0.867 |

Data was expressed as Mean ± SD, *P < 0.05 is considered significant, FGIR, Fasting glucose to insulin ratio, HOMAIR, Homeostasis Model Assessment, QUICKI, Quantitative insulin sensitivity check.

The activities of various antioxidant enzymes are shown in (Table 3). The activities of Glutathione peroxidase GPx, Glutathione reductase (GRs) and Glutathione-*S*-transferase (GST) were found to be considerably higher whereas the levels of glutathione, Vitamin C and Vitamin E were found to be significantly lower in the PCOS women compared to the control group. Although nitric oxide levels were slightly raised in cases than controls but it did not achieve statistical significance. Hydroxyl radical and superoxide radical scavenging activity were comparable in both groups with no statistical significance.

Correlation of oxidative stress parameters with insulin parameters and hormonal parameters are shown in Tables 4 and 5.GPx, GRs, NO were positively correlated with insulin and HOMA-IR and negatively correlated with QUICKI. Further, GRs are also positively corelated with FSH and Testosterone (Table 5). Regression analysis was carried out with weight, waist/hip, BMI, LH, FSH, testosterone, insulin, HOMA, FGIR, QUICKI, glucose, cholesterol, triglycerides as independent variables and GPx, GRs, GST, Glutathione as dependent variables. Using multiple regression analysis GPx activity showed significant association with FGIR (P = 0.013) and FG score (P = 0.014) in PCOS women with standardized coefficients respectively as (−0.653 and −0.295). GRs was found to be associated with testosterone (P = 0.057) and weight (P = 0.022) having standardized coefficients as (0.228, 0.288) respectively. GST was found to be significantly related with triglycerides (P = 0.017), insulin (P = 0.004) and FGIR (P = 0.007) with standardized coefficients respectively as (0.321, −0.774 and −0.719)

## 4. Discussion

PCOS is not only a gynecological disorder but also a comprehensive syndrome with a variety of associated metabolic disorders such as dyslipidemia, insulin resistance [7]. In the present study, higher mean Fasting Serum Insulin was recorded in PCOS subjects, higher mean HOMA-IR was recorded and QUICKI and fasting G/I ratio were lowered in PCOS subjects compared to controls. This was consistent with Shou-Kul et al. They confirmed in their study that the HOMA-IR of the PCOS women was significantly higher than that of the age-matched healthy women, which revealed that insulin resistance had a critical role in pathogenesis of PCOS [28].

PCOS is considered as a chronic systemic syndrome and is related with chronic inflammation and oxidative stress [29]. In this study, we found a significant decline in the levels of glutathione (GSH), vitamin C and vitamin E in women with PCOS when compared to controls. The decline in the levels of these non-enzymatic antioxidant parameters may be because of increased turnover, for inhibiting oxidative damage. Glutathione is an important antioxidant present in all the cells. It protects cells against free radicals, peroxides and other toxic compounds [30]. Indeed, depletion of glutathione increases the sensitivity of cells to

**Table 3**
Oxidative Stress parameters in patients with Polycystic Ovary Syndrome and controls.

| Parameters | Cases N = 85 | Controls N = 85 | p-value |
|---|---|---|---|
| Glutathione Peroxidase (nmoles NADPH oxidized/ml of serum) | 71.06 ± 8.53 | 67.00 ± 8.54 | 0.002* |
| Glutathione Reductase (nmoles NADPH oxidized/ml of serum) | 87.85 ± 16.77 | 73.74 ± 21.88 | 0.001* |
| Glutathione Transferase (nmoles of CDNB conjugates formed/μl serum) | 0.0039 ± 0.002 | 0.0029 ± 0.001 | 0.001* |
| Glutathione (nmol/ml) | 101.75 ± 27.45 | 112.80 ± 23.11 | 0.005* |
| Vitamin E (μmoles/L) | 3.24 ± 1.41 | 9.14 ± 4.44 | 0.001* |
| Vitamin C (mg/dl) | 5.17 ± 0.51 | 5.52 ± 1.14 | 0.011* |
| Nitric Oxide (μmol/L) | 7.96 ± 4.78 | 6.88 ± 2.49 | 0.066 |
| Hydroxyl radical scavenging activity (%) | 50.03 ± 13.02 | 53.10 ± 13.76 | 0.137 |
| Superoxide radical scavenging activity (%) | 25.05 ± 10.59 | 28.04 ± 9.17 | 0.051 |

Data was expressed as Mean ± SD,*P < 0.05 is considered significant.

**Table 4**
Correlation between different oxidant/antioxidant and insulin parameters.

| Variable | Insulin | | HOMA-IR | | QUICKI | | FGIR | |
|---|---|---|---|---|---|---|---|---|
| | r | P | r | P | r | P | r | P |
| GPx | 0.301 | 0.005* | 0.258 | 0.017* | −0.225 | 0.038* | −0.355 | 0.001* |
| GRs | 0.365 | 0.001* | 0.181 | 0.097 | −0.223 | 0.040* | −0.430 | 0.001* |
| GST | −0.111 | 0.313 | −0.034 | 0.759 | 0.017 | 0.877 | −0.062 | 0.573 |
| GSH | −0.009 | 0.936 | −0.074 | 0.501 | 0.067 | 0.541 | 0.000 | 1.000 |
| NO | 1.000 | 0.000* | 0.600 | 0.000* | −0.575 | 0.000* | −0.865 | 0.001* |
| Hydroxyl radical scavenging activity | 0.258 | 0.017* | 0.224 | 0.040* | −0.264 | 0.015* | −0.150 | 0.170 |

r = Pearson correlation coefficient.
*P < 0.05 is considered significant.

**Table 5**
Correlation of glutathione reductase with hormonal parameters.

| Variable | LH | | FSH | | Testosterone | |
|---|---|---|---|---|---|---|
| Glutathione Reductase (nmol/ml) | r | P | r | P | r | P |
| | 0.144 | 0.187 | 0.249 | 0.021* | 0.323 | 0.003* |

r = Pearson correlation coefficient.
*P < 0.05 is considered significant.

various aggressions and also has numerous metabolic effects. The low levels of glutathione may be associated with hyperglycemia due to insulin resistance associated with PCOS women. Vitamin E is the major *in vivo* chain breaking antioxidant and is particularly effective for inhibition of lipid peroxidation. Increased lipid peroxidation raises the need for lipid soluble antioxidant thus decreasing vitamin E levels in the PCOS. Victor et al. performed a study with PCOS and control group and they observed lower GSH levels in PCOS group [31]. They suggest that this is due to the association between insulin resistance and an impaired mitochondrial oxidative metabolism. On the other hand, Glutathione peroxidase (GPx), Glutathione reductase (GRs) and Glutathione-*S*-Transferase (GST) activities were significantly higher in women with PCOS than controls. In multi examinations, oxidative stress parameters are discovered to be positively correlated with androstenedione and testosterone levels in PCOS women [32]. The results of the present study showed that a significant positive correlation exists between glutathione reductase activity and serum testosterone and FSH. GPx, an oxidative stress inducible enzyme plays a crucial role in reducing peroxide formation and retains functional integration of the cell membranes [33]. GRs is concerned with the maintenance of reduced GSH levels by causing fast reduction of oxidized glutathione to reduced form. Any variation in their levels make cells susceptible to oxidative stress and hence cell damage. The GST is a group of multifunctional proteins, which acts as an enzyme for GSH conjugation reactions and plays an effective role in detoxification and the hepatic removal of potentially harmful hydrophobic compounds from blood [34]. Increased activities of these antioxidant enzymes may be a compensatory response to counter the effect against increased oxidative stress. Previous studies also showed increased levels of GPx, GRs and GST in PCOS women as compared to controls [35].

In the present study we found no significant difference in NO levels between control subjects and women with PCOS. Nitric oxide (NO) contributes to vessel homeostasis by preventing contraction of vascular smooth muscle and growth, platelet aggregation, and leukocyte adhesion to the endothelium. Humans with diabetes atherosclerosis or hypertension frequently show altered NO pathways [36]. Insulin has been demonstrated to increase NO release from cultured endothelial cells via a phosphatidylinositol-3 (PI-3) kinase pathway [37]. In the literature, there are similar studies to our results [38]. With multiple regression analysis decrease in antioxidants along with increase in antioxidant enzymes has certain effect on metabolic features related to PCOS.

Hydroxyl radical is an extremely reactive radical formed in biological systems and is capable of destroying almost every molecule found in living cells [39]. Superoxide is biologically essential as it can form hydroxyl radical and singlet oxygen [40]. Superoxide anion radical overproduction contributes to redox imbalance and is associated with detrimental physiological consequences [41]. Superoxide anions are precursor to active free radicals that have potential of reacting with biological macromolecules and thus inducing tissue injury [42]. In the present study we found no significant difference in superoxide radical scavenging activity and hydroxyl radical scavenging activity between control subjects and women with PCOS. In the literature, we could not find any study investigating superoxide radical and hydroxyl radical scavenging activity in women with PCOS.

The results of the present study showed that GPx, GRs, NO and hydroxyl scavenging activity were positively correlated with insulin and HOMA-IR and negatively correlated with QUICKI. Increased oxidative stress seems to be a detrimental factor leading to β-cell dysfunction, impaired glucose tolerance and promotes insulin resistance as seen in type 2 diabetes mellitus. This is evidenced by decreased insulin mediated signaling as a result of increased ROS mediated serine phosphorylation of insulin receptor substrate-1 (IRS-1), which results in suppression of insulin-stimulated tyrosine phosphorylation and activation of IRS-1 and consequently insulin resistance [43]. Seow et al. confirmed that insulin resistance in PCOS involves both receptor and post receptor defects, including defects in phosphatidylinositol 3-kinase and the GLUT-4 glucose transporter [44]. Obesity may play a role in the correlation between systemic oxidative stress and these conditions [45]. Chronic oxidative stress is mainly harmful for β-cells because pancreatic islets have the lowest levels of antioxidant enzyme expression, and β-cells have high oxidative energy requirements [46]. In addition, there is considerable evidence that increased oxidative stress also impairs insulin action, which might be due several factors like membrane fluidity alterations, reduced nitric oxide availability and increased intracellular calcium content [47,48].

## 5. Conclusions

In conclusion PCOS is associated with excessive oxidative stress.

The results of our study have shown increased antioxidant enzyme activities which may be a compensatory instruction in response to increased oxidative stress in PCOS. Decreased levels of glutathione, vitamin E and vitamin C specify that PCOS is linked with high oxidant status. Furthermore, our study revealed an association between oxidative stress markers and insulin parameters which are accepted risk factors for diabetes and future CVD.

## Conflicts of interest

The authors declare no conflict of interest related to this manuscript.

## Acknowledgement

Authors would like to acknowledge Department of Biochemistry/Clinical Biochemistry for providing all facilities to carry out this work. We also acknowledge all the participants who voluntarily participated in the study.

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