Association of Prooxidant–Antioxidant Balance with Clinical and Laboratory Parameters and Its Relation to Different Drug Regimens in Polycystic Ovary Syndrome Women with Normal BMI

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Abstract Polycystic ovarian syndrome is one of the most common hormonally leading cause infertility disorders. The effect of oxidant-antioxidant imbalance on disease progression has been studied in many disorders. The present study was aimed to evaluate prooxidant–antioxidant balance (PAB) in patients with polycystic ovarian syndrome compared to healthy subjects. We also studied the possible effect of treatment with available drugs on serum PAB. In this case–control study 100 polycystic ovary syndrome (PCOS) patients and 100 healthy individuals were enrolled in the study. The laboratory features of patients and controls like as serum LH and FSH concentration and hematological examinations were collected. PAB was evaluated by a colorimetric method. Serum PAB value was significantly higher before treatment compared to after treatment and healthy subjects. PAB values were also higher in subjects with irregular menstrual cycle compared to normal subjects. Our results represented that serum PAB values has an indirect significant correlation with serum LH concentration. We also found that drugs

regimen containing spironolactone effectively reduced the serum PAB values. Our results showed that PCOS patients had increased level of PAB and treatment with spironolactone mainly decreases the level of serum PAB. Our results indicate that the measurements of PAB may be used as a potential laboratory marker for assessment of PCOS patients.

Keywords Follicle stimulating hormone · Luteinizing hormone · Prooxidant–antioxidant balance · Polycystic ovarian syndrome · Spironolactone · Treatment

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrinological disorders in women during their reproductive years exhibiting a wide spectrum of clinical manifestations. PCOS women commonly have features of hyperandrogenism and the primary cause of PCOS is probably multifactorial in origin [1, 2]. PCOS determined by metabolic dysfunction features including insulin resistance, obesity and diabetes and these abnormalities can change the body mass index (BMI) [3]. Excessive body fat and central adiposity in PCOS increases the risk of developing cardiovascular disease and type II diabetes, and its prevalence increases with age. Hirsutisms, acne, anovulatory infertility and also cardiovascular and endothelial disorders due to hormonal imbalance are the most common complications in PCOS patients [4].

Previous studies revealed that alteration in ovarian or adrenal androgens result in abdominal adiposity in PCOS women [5, 6]. High deposition of lipid in abdominal adipose tissue can cause adipose tissue dysfunction [7, 8], local and systemic cytokine excess [9, 10] and oxidative

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stress [11, 12]. The individual variability in the androgen excess and abdominal adiposity is the possible reason for the clinical heterogeneity of PCOS. Anovulation therapeutics, antidiabetic agents, gonadotropins, aromatase inhibitors, antiandrogens, oral contraceptives and other therapies like Medroxyprogesterone acetate and statins are the most common pharmacological approaches for treatment of PCOS. Among them, more attention has been paid to clomiphene citrate (Clomid, Sanofi), metformin (Glucophage, Bristol-Myers Squibb), spironolactone (Aldactone, Pfizer), oral contraceptive pills (OCPs) and medroxyprogesterone acetate (MPA) [13].

Several lines of evidence support a role for oxidative stress in PCOS [14]. Oxidative stress is determined by an imbalance in the prooxidant and antioxidant systems as a result of overproduction of reactive oxygen and nitrogen species or decreased clearance of these species by antioxidant agents [15]. Both the oxidant and antioxidant status should be determined for evaluation of prooxidant–antioxidant balance (PAB). Growing evidence indicates that chronic and acute overproduction of reactive oxygen species (ROS) under pathophysiologic conditions is essential for the development of PCOS [16]. The reactive oxygen species leads to many pathogenic events such as chronic inflammation, elevated levels of oxidized proteins (protein MDA) and antiendometrial antibodies, insulin resistance and hyperandrogenism. Furthermore, the increase in ROS was seen both in obese and lean PCOS when compared to matched controls and was independent of obesity [16–18]. Oxidative stress may also induce proliferation of ovarian mesenchymal cells in patients with polycystic ovarian syndrome [19]. Although several studies show the importance of oxidative stress caused by oxidants–antioxidants imbalance in the pathogenesis of PCOS, we were unable to find data in the literature regarding the effects of pharmacological agents on PAB in PCOS patients.

Therefore, in the present study, we measured the prooxidant–antioxidant balance in patients with PCOS by a previously modified PAB assay. Besides, we determined the possible correlation between PAB value with laboratory and clinical parameters. Furthermore, because the measurement of PAB is a simple, rapid, and inexpensive diagnostic tool, we also investigated the probable use of prooxidant–antioxidant balance as a risk factor that could be estimated along with other risk factors, for diagnosis or monitoring of treatment in PCOS patients.

Materials and Methods

Subjects: A total of 200 individuals (100 newly diagnosed PCOS patients, mean age: 25.29 ± 7.32 years and 100 weight-matched healthy controls, mean age: 26.32 ± 8.02 years) were consecutively enrolled in the

study. If a patients had two of the following criteria, it was diagnosed as PCOS according to the Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group 2003 [20]: menstrual cycle disorders such as oligomenorrhea (cycles lasting longer than 35 days) or amenorrhea (cycles lasting longer than 3 months), clinical or biochemical signs of hyperandrogenism (hirsutism) (Ferriman–Gallwey score of more than seven [21], obvious acne or alopecia [22], an elevated total testosterone (normal range 0.5–2.6 nmol/l) and/or DHEAS (normal range 6–123 µg/dl) and/or androstenedione (normal range 0.3–3.3 ng/ml)) and sonographically diagnosed polycystic ovaries (at least one ovary with at least 12 follicles with a diameter of 2–9 mm each or a volume > 10 ml). Control group subjects were healthy volunteers with normal menstrual cycles and who had no clinical or biochemical features of PCOS. The majority of the control group consisted of students and hospital staff. Depending on the purpose of treatment-regulating the menstrual cycle or pregnancy-a pharmacological treatment regimen included clomiphene citrate, metformin, spironolactone, OCPs and medroxyprogesterone acetate, were considered. All patients and controls were of Kurdish descent. All participants were informed about the survey and freely signed and dated the consent form. The protocol was approved by the ethics committee of the Kurdistan University of Medical Sciences. The study was conducted according to the Declaration of Helsinki for Medical Research Involving Human Patients and approved by the local research ethics committee. Subjects with a known history of thyroid disorder, hyperprolactinemia, infectious diseases, diabetes mellitus, hypertension, congenital adrenal hyperplasia, androgen-secreting tumors, signs or symptoms of other androgen-secreting tumors, or other endocrinopathies were excluded from the study.

The BMI was calculated as weight (kilograms) divided by height (meters) squared (kg/m^2). The BMI values of $\geq 30 \text{ kg/m}^2$ were considered as obese.

Biochemical and hematological analysis: Venous blood samples were obtained in the follicular phase of a spontaneous cycle before treatment and after a 6 months period of pharmaceuticals consumption. Aliquots of the EDTA-whole blood were used for white blood cell count by cell counter (Sysmex hematology analyzer kx-21, Sysmex Canada, Inc.). Samples were immediately centrifuged, and serum was separated and frozen at -20°C until assayed. Serum levels of FSH and LH were measured with specific enzyme-linked immunosorbent (ELISA) assay from Monobind Inc. (Lake Forest, CA 92630, United States) and for hypersecretion of LH, LH:FSH ratio ≥ 2 was considered to be a cut-off point.

Measurement of serum PAB value: PAB was determined according to a previously described method [23, 24].

Table 1 Demographic and anthropometric variables of studied groups

Studied individuals	Age (years) mean $\pm$ SD	Marital status (married/single) number (%)	Menstrual cycle status (normal/oligomenorrhea/ amenorrhea) number (%)
Healthy controls (n = 100)	26.32 $\pm$ 8.02	56(56)/44(44)	100(100)/0(0)/0(0)
PCOS (n = 100)			
Before treatment	24.87 $\pm$ 7.14	24(48)/26(52)	13(26)/37(74)/0(0)
After treatment	25.7 $\pm$ 7.5	15(30)/35(70)	5(10)/43(86)/2(4)

PCOS polycystic ovarian syndrome

Briefly, standard curve were plotted using varying proportions (0–100 %) of 250 μ M hydrogen peroxide with 3 mM uric acid (in 10 mM NaOH). Based on the hydrogen peroxide concentration in the reaction, the peroxidase enzyme oxidase 3,3',5,5'-tetramethylbenzidine (TMB) substrate and produced a visible blue dye. After stopping the reaction by Hydrochloric acid in an endpoint assay a yellowish product produces that may be read at 450 nm. The values of the PAB are expressed in arbitrary HK units, which correspond to the percentage of H₂O₂ in the standard solution. The values of the unknown samples were then calculated based on the values obtained from the above standard curve.

Sonography: Pelvic sonography (Nermio 30, Toshiba, Japan) was carried out on day 2 of menstrual cycle in both cases and controls.

Statistical Analysis: Data analysis was performed by using SPSS for Windows, version 16 (SPSS Inc., Chicago, IL). The respective distributions were compared using the Wilcoxon test. Results were presented as mean $\pm$ SD if normality assumption met; otherwise median $\pm$ IQR (intermediate quartile range) was used, and $p < 0.05$ was considered statistically significant. Wherever applicable, the independent samples *T* test or Mann–Whitney statistical tests were used to compare mean/median differences between two experimental groups. One-way ANOVA followed by Post Hoc, Tukey, and Dunnett tests were used to analyze mean differences between groups. For the evaluation of PAB values, Spearman's correlation coefficients were calculated with each of the parameters of interest in cases with PCOS and controls separately. In all performed hypothesis tests, a p value < 0.05 was considered as statistically significant.

Results

The demographic and anthropometric characteristics of the groups are shown in Table 1. Of 100 PCOS patients, 80 cases were recognized as oligomenorrhea and 2 patients as amenorrhea. Besides, patients received first line therapy with OCPs (10 cases), spironolactone (33 cases),

clomiphene (3 cases), metformin (29 cases) and medroxyprogesterone (44 cases). In addition, eleven patients were treated with a combination medication regimen consist of metformin, medroxyprogesterone and spironolactone, nine patients with metformin and medroxyprogesterone and nine cases with medroxyprogesterone and spironolactone. Furthermore, all patients had positive sonography reports. In contrast, there was not any evidence of PCOS in medical ultrasound results of control individuals. In addition, of patients group, 95 had normal BMI and 5 persons were obese.

The serum PAB values in different groups are shown in Fig. 1. PAB values in patients with PCOS were significantly higher than those in the control group (50.5 ± 24.9

Fig. 1 Serum PAB values in different studied groups. According to the plot, the difference of serum PAB values was statistically significant: *between under treatment PCOS patients and healthy controls; **between newly diagnosed PCOS patients and healthy controls. PAB prooxidant–antioxidant balance, PCOS polycystic ovarian syndrome

Table 2 Concentrations of different laboratory parameters in studied subjects

	LH (mIU/ml)	FSH (mIU/ml)	Hct (% of blood)	Hb (g/dl)	PAB (HK)
Healthy controls (n = 100)	10.53 ± 6.15	9.83 ± 19.95	39.73 ± 4.6	13.29 ± 1.58	28.6 ± 23.5
PCOS (n = 100)					
Before treatment	14.68 ± 13.91	5.53 ± 12.46	41.45 ± 2.1	13.9 ± 0.84	53.25 ± 31.41
After treatment	11.08 ± 8.81	8.32 ± 18.92	41.02 ± 2.65	13.78 ± 1.1	43.57 ± 38.57

Data is presented as mean ± SD

LH luteinizing hormone, FSH follicle stimulating hormone, Hct hematocrit, Hb hemoglobin, PAB prooxidant–antioxidant balance

Table 3 Corresponding value of clinical indices according to menstrual cycle status in studied subjects

	LH (mIU/ml)	FSH (mIU/ml)	Plt ($\times 10^5/\mu\text{l}$)	PAB (HK)
Normal menstrual cycle (n = 118)	10.85 ± 8.74	8.46 ± 19.13	2.3 ± 0.48 ^b	36.22 ± 30.76 ^a
Irregular menstrual cycle (n = 80)	13.35 ± 11.9	8.16 ± 16.04	2.8 ± 0.54 ^b	53.49 ± 31.69 ^a

Data is presented as mean ± SD

LH luteinizing hormone, FSH follicle stimulating hormone, Plt platelet, PAB prooxidant–antioxidant balance

^a *p* value <0.0001; ^b *p* value <0.05

Table 4 Serum PAB value in relation to type of drugs

	Under treatment patients					New cases
	Clo	OCPs	Met	Mp	Spi	
Serum PAB value (HK)	41.87 ± 23.02	41.13 ± 35.25	40.3 ± 36.76	39.6 ± 34.95	31.34 ± 28.26 ^a	53.25 ± 31.41 ^a

Data is presented as mean ± SD

Clo clomiphene citrate, OCPs oral contraceptive pills, Met metformin, Mp medroxyprogesterone acetate, Spi spironolactone, PAB prooxidant–antioxidant balance

^a *p* value <0.006

vs. 28.6 ± 23.5) (*p* < 0.01). In addition, before treatment, patients had significantly (*p* < 0.05) increased level of PAB than after treatment (53.25 ± 31.41 vs. 40.57 ± 38.57).

In the second part of our study, we assessed the laboratory outcomes in studied subjects (Table 2). Our results showed that the concentration of LH is higher in patients than controls. Furthermore, we found higher level of FSH in controls than patients. With regards to hematological evaluations, our results showed that there are a trend to increase in hematocrit and hemoglobin in patients compared to controls.

We also assessed the association between laboratory features of studied subjects and menstrual cycle status (Table 3). Based on our results, platelet count was significantly higher in patients with oligomenorrhea than healthy subjects. The results also showed that the serum LH has higher concentration in patients with irregular menstrual cycle compared to healthy subjects. Similarly, the concentration of serum FSH was lower in patients with menstrual cycle disorder compared to healthy.

We also evaluated the PAB values in three studied groups according to menstrual cycle status (Table 3). Our results showed that patients with menstrual cycle disorder had significantly higher PAB values than normal subjects (*p* < 0.0001).

In the next part of our study, we evaluated the PAB values in the under treatment group with regards to type of medication (Table 4). According to Table 4, regardless to the type of drugs, all under treatment patients had lower PAB values compared to new cases. Our results clearly showed that patients who were treated with spironolactone had greater reduction in PAB values and this was statistically significant (*p* < 0.006). Although the PAB values were decreased in other patients, we could not find such significant differences for this reduction.

Table 5 shows the PAB values in different types combined regimen of therapeutic drugs. According to this table, patients who were treated with medroxyprogesterone and spironolactone had the lowest values of serum PAB followed by treatment with metformin, medroxyprogesterone and spironolactone and metformin and

Table 5 Serum PAB value in relation to different type of combined drugs

	LH (mIU/ml)	FSH (mIU/ml)	PAB (HK)
1	9.8 ± 6.96	15.07 ± 30.89	25.13 ± 25.11 ^a
2	19.35 ± 16.54	14.88 ± 24.51	57.96 ± 46.98 ^a
3	14.32 ± 9.04	4.43 ± 2.48	22.51 ± 17.12 ^a

Data is presented as mean ± SD

LH luteinizing hormone, FSH follicle stimulating hormone, PAB prooxidant–antioxidant balance

1 Metformin, medroxyprogesterone and spironolactone; 2 Metformin and medroxyprogesterone; 3 Medroxyprogesterone and spironolactone

^a p value <0.05

medroxyprogesterone ($p < 0.05$). We also analyzed the laboratory outcomes of studied subjects in different types combined regimen of therapeutic drugs (Table 5). Based on our results metformin, medroxyprogesterone and spironolactone decreased the serum LH concentration and increased FSH concentration compared to the other regimen, although these changes were not statically significant ($p > 0.05$).

Finally, we analyzed the possible correlation between PAB values and laboratory outcomes of studied subjects. As shown in Table 6 serum PAB value had statically significant positive correlation with LH concentration. However, we could not find any association between PAB value and other laboratory outcomes (data not shown).

Discussions

In the present study we evaluated the possible effect of the most common medications which are used for treatment of PCOS on serum pro-oxidant anti-oxidant balance. Overall, our results showed that these drugs significantly decreased the serum PAB in under treatment patients compared to new patients.

There are several line of studies which has been evaluated the oxidative stress markers in PCOS patients. It has been shown that patients with PCOS have higher oxidative stress. Kuşçu et al. [4] showed that blood malondialdehyde (MDA) is significantly higher in the PCOS group compared to healthy controls (0.12 ± 0.03 vs. 0.10 ± 0.03 , $p = 0.01$) and this elevation in MDA concentration was not related to obesity.

Furthermore, they also showed that MDA level is significantly increased in young, non-obese PCOS patients even in the absence of insulin resistance when compared with controls (0.125 ± 0.03 vs. 0.101 ± 0.03 , $p = 0.03$) [4]. Significantly higher levels of MDA in PCOS patients compared with controls were also found by Palacio et al. [25], Sabuncu et al. [26]. Although BMI and age were not recorded in the study of Zhang et al. [27], however, they also demonstrated that serum MDA levels in PCOS patients were significantly higher than those of controls. On the other hands, Karadeniz et al. [28] found MDA levels in PCOS patients had no significant difference with those of controls (5.38 ± 2.47 vs. 4.475 ± 2.06 , $p > 0.05$). They also suggested that the presence of insulin resistance in PCOS patients has no effect on MDA levels. Similar to Karadeniz et al. study, Dursun et al. [29] found that serum MDA levels in PCOS patients were similar to those of BMI- and smoking status matched controls.

Protein carbonyl as a marker of protein oxidation was also evaluated in some studies. Increased levels of protein carbonyl in PCOS patients compared to controls have been shown in Fenkci et al. [30] study. They suggested that free radicals damage proteins in PCOS patients [30]. Furthermore, they showed that there was a strong association between insulin resistance and protein oxidation in PCOS.

Besides, Năcul et al. [31] studied nitric oxide (NO) levels in PCOS patients and healthy controls and reported NO were similar in both groups. Moreover, they showed that NO was related to the presence of insulin resistance in PCOS patients.

On the other hands, changes in serum and peritoneal fluid antioxidant concentrations have been studied in different types of infertility such as idiopathic infertility, tubal infertility, and endometriosis patients [16]. It has been reported that antioxidants have important roles in the female reproductive system and in the pathogenesis of female infertility [4].

Since the effect of the prooxidant and antioxidant molecules in serum is additive, various methods have been proposed for the separate measurement of the total oxidant or antioxidant status [23]. For the evaluation of the PAB, the determination of both the oxidant and the antioxidant status is often necessary. Therefore, the estimation of PAB is indirect and consequently not precise. To our knowledge, the present study is the first and only study that evaluates the concomitant measurement of the prooxidant burden and

Table 6 Correlation between PAB with LH and FSH in Studied individuals

	LH (mIU/ml)	FSH (mIU/ml)	Statistical parameter
Serum PAB value (HK)	0.048	0.371	p value
	0.207	−0.094	r_s

r_s Spearman correlation coefficient

the antioxidant capacity in PCOS patients. Our results clearly showed that, in PCOS patients, serum PAB value is increased and this elevation is significantly higher than healthy controls. In addition, we showed that serum PAB value has a direct correlation with LH and indirect correlation with FSH concentration. We found that patients with higher serum LH concentrations had higher PAB values, and patients with menstrual cycle disorders also had a higher serum PAB value compared to healthy subjects.

Furthermore, our results demonstrated that, regardless of the type of drug, treatment of PCOS could be reduced the serum PAB values. These findings are supported by the previous studies. Daneasa et al. [32] studied the effects of spironolactone on glucose metabolism and oxidative stress parameters in oestradiol valerate induced PCOS rat model. They found that the spironolactone and dimethylsulfoxide (DMSO) treatment improved antioxidant capacity and had a beneficial effect on metabolic deregulation in PCOS. They also showed that administration of DMSO has an unexpected hypoglycaemic effect and improved oxidative stress parameters [32]. Similar results have been reported with regards the reduction effects of spironolactone on oxidative stress [33–35]. It has been suggested that by inhibition of aldosterone actions, spironolactone partly corrected angiotensin II-induced abnormalities. These effects were associated with reduced vascular NADPH oxidase activity and decreased plasma markers of oxidative stress [32].

It has been shown that the intake of OCPs increases the lipid peroxidation and can be served as a potential cardiovascular risk factor in women who are treated with these drugs [36, 37]. After OCPs treatment a free radical mediated-process combined with an increase of plasma lipid peroxidation parameters such as conjugated dienes, lipid peroxides, thiobarbituric acid reactive substances, and a decrease in plasma and platelet long chain polyunsaturated fatty acids, particularly (n-3) might occur. Furthermore, it has been found that OCPs induced a severe decrease in urokinase plasminogen activator activity which might further contribute to the platelet hyperactivity. These results suggested that, in addition to increase in clotting factors, platelet hyperactivity, possibly through a stimulated free radical-induced arachidonic acid metabolism, might be involved in the high thrombogenic risk observed in OCPs users [38]. According to our results, the difference between serum PAB value in patients treated with OCPs and new cases was not significant.

The potential role of medroxyprogesterone on reduction of oxidative stress markers has been studied recently [39, 40]. By inhibiting cell apoptosis and decreasing damage due to oxidative stress, treatment with medroxyprogesterone could improve acute brain injury after subarachnoid hemorrhage. Besides, it has been suggested that medroxyprogesterone ameliorates sepsis syndrome by

reduction of the inflammatory cytokines IL-6 and TNF- α , and by renewal of antioxidant enzyme activities in some tissues. The possible mechanism for these effects was related to the mitochondrial pathway [39, 40]. In line with previous studies, our results clearly showed that patients treated with progesterone had a lower serum PAB values compared to new cases, although it was not significant.

On the other hands, incremental effect of clomiphene citrate on oxidative stress biomarkers has been studied [41, 42]. However, in the present study, we could not find any significant difference between patients treated with clomiphene and new cases for serum PAB values.

There are more evidence about the association of metformin and oxidative status in different situations. Anedda et al. [43] found that metformin treatment leads to higher level of oxidative stress biomarkers since it increases the levels of reactive oxygen species (ROS) and lowers the aconitase activity. On the other hands, Chakraborty et al. [44] found that in type 2 diabetic patients, metformin treatment returns the antioxidant status, enzymatic activity and inflammatory parameters. Besides they showed that metformin can improves the status of oxidative and nitrosative stress. In another study, Yilmaz et al. [45] evaluated the effects of rosiglitazone and metformin on oxidative stress and homocysteine levels in lean PCOS patients. They found that lean PCOS patients have higher oxidative stress and plasma homocysteine levels and rosiglitazone seemed to decrease elevated oxidative stress when compared with metformin treatment in lean PCOS patients. However, in a recent study conducted by Kocer et al. [46], it has been reported that Metformin seemed to decrease oxidative stress and improve insulin resistance, dyslipidemia and endothelial dysfunction in PCOS patients. Although our results, with regards to metformin, were not significant, but we showed that patients who are treated with metformin had lower serum PAB values when compared with new cases. Furthermore, with regards to patients who are treated with a combined regimen, we found that a combination of medroxyprogesterone and spironolactone has a higher effect on reduction of serum PAB values compared to metformin, medroxyprogesterone and spironolactone and to metformin and medroxyprogesterone.

In summary, we found that serum PAB value is increased in PCOS patients and treatment of PCOS may reduce the PAB value. Furthermore, we showed that serum PAB value is highly correlated to clinical and laboratory outcomes of PCOS patients and treatment of PCOS, correct both disease manifestations and serum PAB values. We also showed that drugs regimen containing spironolactone have a strongest effect on reduction of PAB values. It can be concluded that determination of serum PAB value might be a diagnostic tool for screening and monitoring of PCOS patients along with other markers.

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Author Contribution N. Heshmati: Data collection; S. Shahgheibi: Project development, manuscript editing; B. Nikkhoo: Data collection; S. Amini: Project development; M. Abdi: Project development data management, data analysis, manuscript writing.

Compliance with Ethical Standards

Conflict of interest None.

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