Journal of Applied and Natural Science
16(2), 805 - 811 (2024)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

A case-control study unravelling the prognostic significance of oxidative markers in polycystic ovary syndrome (PCOS) patients

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Article Info
https://doi.org/10.31018/jans.v16i2.5618
Received: March 22, 2024
Revised: May 29, 2024
Accepted: June 03, 2024

How to Cite
Mishra, M. *et al.* (2024). A case–control study unravelling the prognostic significance of oxidative markers in polycystic ovary syndrome (PCOS) patients. *Journal of Applied and Natural Science*, 16(2), 805 - 811. https://doi.org/10.31018/jans.v16i2.5618

Abstract
Polycystic ovary syndrome (PCOS) is one of the most common endocrinological disorders in female. Reproductive organ is a metabolically active organ; hence reactive oxygen species (ROS) are inevitably generated during the physiological process. Studies have suggested that OS may affect female fertility by reproductive impairment, leading to alter ovulation patterns, oocyte maturation and steroidogenesis in women with PCOS. Thus, the present study aimed to assess the oxidative status of diagnosed PCOS women. Based on specific inclusion and exclusion criteria, 100 Subjects (Group – 1 PCOS N=50 , Group-2 Controls N=50) were enrolled with their written informed consent. Blood samples were collected in aseptic conditions for the estimation of hormonal parameters (Testosterone) and oxidative stress markers (MDA, SOD, Catalase, GSH, GSSG). The statistical analysis of data indicated significant elevated level of MDA, SOD, GSSG (5.21 vs 1.52 ; 248.15 vs166.15; 11.38 vs 4.37), while decreased level of catalase and GSH (37.57 vs 78.2; 84.09 vs 121.7) was observed in PCOS cases when compared to controls. The linear regression model showed significant R^2 values for MDA, SOD and GSSG . Further ROC curve was plotted for MDA, SOD and GSSG to estimate the sensitivity of these predictive markers. OS damages oocyte and follicle growth in females, damaging the endometrium and affecting endocrine function. Significantly higher levels of MDA, SOD & GSSG were reported in cases, and catalase, GSH levels were decreased. Linear regression and ROC curve analysis indicated that these MDA, SOD, GSSG may act as significant predictive markers of OS playing a pivotal role in the pathophysiology underlying PCOS and as prognostic tools to reduce the severity of the disease.

Keywords: Antioxidant , Oxidative stress , Polycystic ovary syndrome, Reduced Glutathione

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a global health concern for women of reproductive age (Abudawood *et al.,* 2021) and it is a multifactorial and polygenic condition (Mortada and Williams, 2015). Approximately 10% of women are affected by infertility, among which 6.5-8% of women of reproductive age are affected by polycystic ovary syndrome (PCOS), which constitutes the most prevalent cause of infertility among females. (Skrgatic *et al* , 2012). PCOS is marked by hyperandrogenism, anovulation, menstrual abnormalities (e.g., oligomenorrhea or amenorrhea) and polycystic ovaries. (Coskun *et al* , 2013). Because its pathogenesis has not been fully elucidated, PCOS has gradually become a research hotspot in recent years.

Most patients also have endocrine and metabolic disorders such as insulin resistance, obesity and compensatory hyperinsulinemia. Hyperandrogenemia and insulin resistance, as PCOS's pathophysiological basis, play an important role in its occurrence development. Obesity/ overweight can make PCOS symptoms worse by amplifying its various features (Li *et al.*, 2022). Diagnosis is based upon the presence of two of the following three criteria (Rotterdam criteria established in 2003): Oligo or anovulation, Hyperandrogenism (clinical and/or biochemical), Polycystic ovaries (Konar, 2020). Since reproductive and developmental processes accompany

dynamic changes in metabolism and energy consumption, byproducts are also generated on an extraordinary scale. The main source of ROS *in vivo* is oxidative phosphorylation of mitochondria, and the secondary sources are cytochrome P450, peroxisome, xanthine oxidase and activated inflammatory cells (Allen and Tresini, 2000)

Oxidative stress occurs due to an imbalance between the formation of antioxidant defenses and reactive oxygen species (ROS), leading to cellular damage. (Kaltsas *et al*, 2023) Free radicals are atoms or molecules that are present as unpaired electrons and circulate in the body, mainly damaging macromolecules, including lipids, proteins, and carbohydrates and affecting the cells' genetic integrity (DNA, RNA). The body has a distinctive system for defeating the damage obtained from free- radicals called the antioxidant defense system (Valko *et al.*, 2007) . Antioxidants are a class of molecules of two types: either enzymatic, like superoxide dismutase, catalase or non-enzymatic, such as reduced glutathione and oxidized glutathione. These antioxidants have been reported to have an important role in the female reproductive system (Agarwal *et al*., 2005)

In an ordinary situation, there is a balance between the production of free radicals and the antioxidant defense system in a healthy person. However, if, under any circumstance, this balance is impaired, it leads to a condition known as oxidative stress (Valko *et al.*, 2007). MDA (Malondialdehyde) is an important product of lipid peroxidation reactions and has been widely employed as biomarkers of OS (Senoner and Dichtl, 2019).

There are several antioxidants which include superoxide dismutase (SOD), catalase (CAT) and glutathione which is present in two forms reduced gluthaione (GSH) and oxidized glutathione oxidised (GSSG) . All of them can scavenge oxidative active molecules and maintain the oxidant/antioxidant balance. Excessive oxidative active molecules can affect the function of biological molecules by modifying the protein molecules, causing lipid peroxidation and DNA damage. At the same time, when the body's antioxidant defense function is not enough to remove many oxidized active molecules, the imbalance between oxidant and antioxidant levels will eventually lead to OS, resulting in cell damage and causing a variety of biological processes (Forman and Zhang, 2021).

PCOS is one of the several pathological conditions arising from the disparity between ROS production and elimination. The ROS category comprises the superoxide radical, hydrogen peroxide, and hydroxyl radical. Some ROS can act as signaling molecules to cells. Due to their unstable and highly reactive nature, peroxides and free radicals have the potential to damage various cellular components, with DNA damage having particularly concerning long-term consequences. (Sengupta *et al* , 2024 ; Darbandi *et al* , 2018 ; Alahmar and Sengupta , 2021) . OS is believed to be a potential triggering factor in the pathophysiology of PCOS. Moreover, the relationship between OS and the development of PCOS is not always straightforward, as several clinical symptoms of PCOS, such as HA, obesity, and IR, may contribute to the emergence of both local and systemic OS. This, in turn, can potentially exacerbate these metabolic abnormalities. Additionally, elevated levels of LH can function as H_2O_2, which can contribute towards a skewed redox balance (Shkolnik *et al.,* 2011). Concomitantly, around 50-70% of women with PCOS are insulin resistant, which may contribute towards increased oxidative stress (OS) via hyperglycemia and higher levels of free fatty acids, which in turn produce ROS via hyper-activated electron transport chain (Zuo *et al*., 2016). Excess ROS can lead to compromised oocyte competence due to mitochondrial dysfunction, reduced ATP production, oocyte aging and leading to infertility in women with PCOS (Dumesic *et al.,* 2015). Numerous studies indicate that individuals with PCOS tend to exhibit higher OS levels compared to control groups. Nonetheless, outcomes frequently differ, primarily because of diverse markers and disparities in how even the same marker is evaluated, which is contingent on the origin and research approach (Herman *et al.,* 2020).Thus, the present study aimed to understand the prognostic role of oxidative stress markers in PCOS.

MATERIALS AND METHODS

A case-control study was conducted with 100 women participants (50 diagnosed PCOS cases and 50 healthy controls) within the age group of 18-40 years. The ascertainment of PCOS diagnosis was based on Rotterdam criteria, defined by any of the following two characteristics: oligo and/or anovulation, clinical and/or biochemical signs of hyperandrogenism and polycystic ovary morphology. Written informed consent was obtained from all the participants. Subjects with regular menstrual cycles and normal serum androgen concentrations were considered healthy controls. Subjects with a history of liver diseases, cardiovascular diseases, infectious diseases and other endocrine disorders were excluded from the study. A data collection proforma was used to collect each subject's demographic, anthropometric and clinical information.

Ethical committee approval

The study was approved by the MGM Institute of Health Sciences' Ethics Committee for human participant enrolment and blood sample collection.

Sample collection

Under aseptic conditions, 4 ml of venous blood was drawn from the subjects. The blood was collected in a

plain-vial which was further subjected to centrifugation at 3000 rpm. Serum was separated and further used for the estimation of the hormonal assay (Testosterone) and oxidant-antioxidant parameters (MDA, SOD, catalase, GSH, GSSG).

Laboratory investigation

Hormonal parameters such as serum testosterone were estimated by the ELCIA method on MAGLUMI autoanalyzer. The oxidative stress marker, such as MDA was estimated by KEI Sathos method and antioxidants such as SOD, catalase, GSH, GSSG were estimated by the commercially available Colorimetric kit method (Abbkine Assay kit).

Statistical analysis

IBM SPSS Statistics software, version 25 was used for statistical analysis. All the data were presented as mean ± Standard deviation. An unpaired t-test was used to compare the study parameters between the Cases and controls. Pearson's correlation coefficient was employed to determine the relationship between the variables in Cases. Regression analysis was done to find independent forecasters for oxidant-antioxidant in PCOS Cases. Further, the ROC curve was plotted to estimate the sensitivity of the variable. A P-value less than 0.05 was deemed statistically significant.

RESULTS AND DISCUSSION

The results of the statistical analysis for a total of 100 subjects enrolled in this case-control study are summarized in Table 1, 2 and Fig. 1). Results indicated significantly elevated levels of MDA, SOD, and GSSG, alongside a significant decrease in GSH and catalase in the Cases (Table 1). Regression model indicated that MDA, SOD and GSSG may act as significant predictive markers for PCOS (Table 2). Additionally, ROC curve showed the sensitivity of these markers (MDA, SOD and GSSG) in predicting PCOS (Figure 1).

In humans, oxidative stress-induced reproductive impairments lead to altered ovulation patterns, oocyte maturation and steroidogenesis, which accelerates the natural process of apoptosis in granulosa cells. These conditions can lead to the development of PCOS and may further lead to infertility (Kaltsas *et al.*, 2023). The ovaries and uterus are particularly affected by ROS because they contain the highest amount of mitochondria in the body due to the need for ATP or energy in reproductive processes. (Bellver *et al* , 2007). The mitochondrial dysfunction has been addressed as a central phenomenon since mitochondria carry out a pivotal role in cell energy mechanisms, representing the main source of ROS as byproducts of nutrient translation (Zhang, 2019).

The OS imbalance in the ovaries' follicular environment can cause detrimental issues such as poor oocyte development, embryo development and overall fertility outcome (Miyamoto *et al.*, 2010)

Numerous studies have shown that markers of oxidative stress are greater than normal in the patients with PCOS. (Turan *et al*, 2015; Blair *et al*, 2013). Through oxidative stress is considered a potential inducement of PCOS pathogenesis (Murri *et al.*, 2013). It is still undetermined whether the abnormal oxidative stress levels of patients with PCOS derive from PCOS itself or if they are related to potential complications (such as obesity and insulin resistance).

Thus, the present study planned to understand the prognostic role of OS markers in PCOS patients. One

Table 1. Comparison of biochemical parameters in PCOS Cases and controls

Parameters	PCOS Cases	Controls	p-value
BMI (Kg/m^2)	26.85±3.45	25.13±2.73	0.05
Testosterone (ng/dl)	84.01±7.90	29.37±11.71	0.001
MDA (nmol/ml)	5.21±1.32	1.52±1.02	0.001
Catalase (nmol/min/ml)	37.57±8.72	78.27±8.91	0.001
SOD (U/ml)	248.15±15.92	166.15±12.54	0.001
GSH (ug/ml)	84.09±6.85	121.71±16.68	0.001
GSSG (nmol/ml)	11.38±3.68	4.37±1.63	0.001

Level of significance : < 0.05; LH - Luteinizing hormone; FSH – Follicular stimulating hormone; MDA – Malondialdehyde; SOD – Superoxide Dismutase; GSH – Reduced Glutathione; GSSG – Oxidized Glutathione

Table 2. Linear Regression between the dependent variable (Testosterone) and independent variables (Oxidative stress parameters)

Parameters	R	R Square	Significant-F change
MDA	0.469	0.220	0.001
Catalase	0.447	0.200	0.001
SOD	0.522	0.273	0.000
GSH	0.285	0.082	0.044
GSSG	0.537	0.289	0.000

MDA – Malondialdehyde; SOD – Superoxide Dismutase; GSH – Reduced Glutathione; GSSG – Oxidized Glutathione

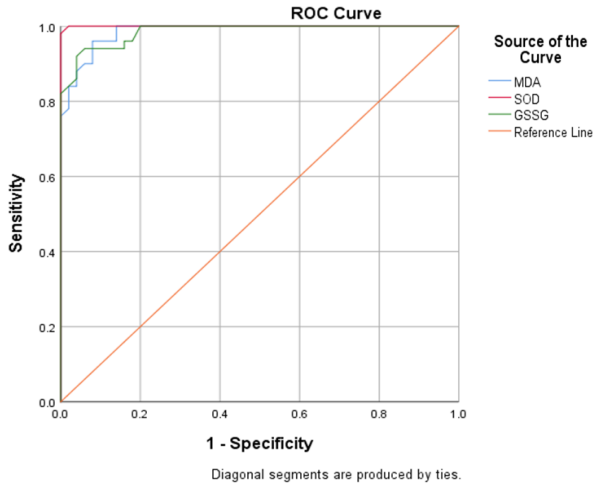

Fig. 1. *Receiver Operating Characteristic Curve (ROC) for the curve of MDA, SOD, GSSG predicting AUC-0.986, 1.000, 0.985 (PCOS), respectively*

hundred subjects between the age of 18-40 years were enrolled after imposing certain inclusion and exclusion criteria. Out of which 50 PCOS Cases as per Rotterdam criteria and 50 healthy controls with normal menstrual cycle and normal serum androgen levels were included. The oxidative stress markers between the cases and controls were compared. MDA, SOD, GSSG levels were significantly elevated, whereas catalase and GSH were significantly decreased in Cases when compared to controls.

MDA, a product of lipid peroxidation reactions, has been widely employed as a biomarker for oxidative stress (Abuja *et al.*, 2001). MDA is produced enzymatically by the breakdown of unstable hydroperoxides during peroxidation of unsaturated fatty acids (Gurdol *et al.,* 2008). Measurement of MDA levels in plasma or serum provides a convenient in vivo index of lipid peroxidation. It represents a non-invasive biomarker of oxidation stress often clinically employed to investigate radical-mediated physiological and pathological conditions. (Merendino *et al.*, 2003)

In the present study, MDA level were significantly higher in cases compared to controls (5.21±1.32 vs 1.52±1.02). The study's findings were similar to the results of the previous studies (Kuscu *et al.*, 2009; Fan *et al.*, 2012; Zhang et *al.,* 2008; Macut et al., 2011). Thus, MDA might act as an indicator of excess ROS formation.

Antioxidants play a great role in preventing cell damage due to oxidative stress. Superoxide dismutase is an enzyme that catalyzes the dismutation of superoxide anion into O_2 and H_2O_2. They are an important antioxidant defense against the toxicity of superoxide radicals in all cells exposed to O_2.

Moreover, the study reported a significant increase in SOD levels in PCOS cases compared to controls (248.15±15.92vs166.15±12.45). The results of the present study align with the findings of the prior studies conducted by various researchers (Sabuncu *et al.*, 2001; Kuscu *et al.*, 2009; Seleem *et al.*, 2014; Joo *et al.*,2010). Additionally, a meta-analysis study, including 558 PCOS Cases and 529 Controls, using serum samples, highlights a similar result regarding SOD levels in PCOS Cases compared to controls (Talat *et al.*, 2021).

The probable reasoning may be that in the follicular phase of the ovarian cycle when there is a development of follicles from primordial to graafian follicle, there is an increase in steroid production in the growing follicle. Due to this causes an increase in P450 enzyme activity that tends to result in oxidative stress. The ROS formation by pre-ovulatory follicles is considered an important inducer for ovulation. With increases in ROS formation, the level of SOD also increases (Behrman *et al.*, 2001). This increases in SOD continues till mid-luteal phase and decreases during the late luteal phase. (Shkolnik *et al.,* 2011)

ROS formation increases more in the corpus luteum stage. Increases in ROS trigger activation of TF (NF kappaβ), which activates cyclooxygenase and phospholipase A2 in the corpus luteum, which are key enzymes for PGF2 alpha, inhibits progesterone level or decrease in ovarian blood flow. A rapid decline in progesterone is needed for adequate follicle development in the next cycle. SOD activity is parallel with the change in progesterone conc. Complete disruption of the corpus luteum causes a substantial decrease of SOD in the regressed cell. (Shkolnik *et al.,* 2011)

Catalase is a common antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. It is widely found in aerobic cells containing cytochrome systems. It plays an important role in organs such as the liver, but its specific function in the genital tract is largely unknown.

The present study revealed a significant decrease in catalase levels in cases compared to controls (37.57±8.72vs78.27±8.91), per the findings reported by Rajwan and Alaa (2019) studied in the human sample. Similarly, another prospective study with 90 PCOS cases and 45 controls concluded with similar results. This decrease in catalase level may be due to hyperinsulinemia and dyslipidaemia, which actively reduce the antioxidant level while increasing oxidative stress (Uckan *et al.*, 2022).

Glutathione is the most abundant low molecular-weight thiol and plays an important role in antioxidant defense. Glutathione in reduced form in glutathione peroxidase reacts with hydrogen peroxide and lipid peroxide, oxidizing to disulfide. Regeneration of the active thiol form occurs with NADPH-dependent glutathione reductase. In addition to the regeneration of GSH from GSSG, the second process affecting the increase in glutathione is its neo-synthesis in the cell and is limited by the availa-

bility of its constituent amino acids, in particular sulfur-containing amino acid, cysteine (Fatim *et al.*, 2019).

Furthermore, the study determined a decrease in reduced glutathione (GSH) and an increase in oxidized glutathione (GSSG) levels (84.09±6.85vs121.71±16.68; 11.38±3.63vs4.37±1.63) in PCOS group as compared to Control group. In continuation with the above findings, similar results were reported by Seleem *et al*, 2014, Abudawood *et al.,* 2021, Maha *et al.,* 2018. GSH depletion might be responsible for exacerbating ROS formation in PCOS patients. (Dincer *et a.* , 2005)

A commonly known measure of oxidative stress is the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). The GSH/GSSG system is the main "redox buffer" that protects cellular structures from the damaging effects of free oxygen radicals. (Fatim *et al.*, 2019). Thus, in the present study, the determination of glutathione status (GSH/GSSG), which appears to be a good indicator of oxidative status in women with PCOS was estimated. The ratio of reduced glutathione to oxidized glutathione was decreased in PCOS Cases (7.38) compared to controls (27.8) after indicating the increased oxidative stress levels in PCOS.

Linear regression was carried out to determine the predictive significance of oxidative stress markers. The R square value is (0.220), (0.273) and (0.289), which explains that (22%) of the variation in PCOS is due to MDA, (27.3%) of the variation was due to SOD and (28.9%) of variation was due to GSSG. Thus, MDA, SOD and GSSG may be considered as predictive markers in PCOS. As oxidative stress plays an empirical role in exaggerating the clinical complications of PCOS.

Lastly, the ROC curve showed the sensitivity and specificity of these oxidative stress markers for PCOS. The AUC value 0.986 (95% CI:0.97-1.00; P = 0.000), 1.000 (95% CI:0.99-1.00; P=0.000), 0.985 ((95% CI:0.96-1.00; P = 0.000) of MDA, SOD and GSSG depicts as excellent prognostic utility of these markers for PCOS.

Although considerable progress has been made to address the role of oxidative stress in execrating the complexity of this multifactorial disease, due to certain limitations in the study (small sample size, not accounting variation in ethnicity or diagnostic criteria of subjects, unavailability of standardised method and unit of OS markers) the results were inconclusive. Thus, the present study further recommends more studies to explore the exact mechanism that links oxidative stress with PCOS.

Conclusion

Among women of reproductive age, PCOS is the leading cause of infertility associated with anovulation. The present study data indicated increased oxidant and a compensatory response of antioxidant status in women (18-40 years) with PCOS. This study has outlined oxidative markers' sensitivity and specificity, showing its prognostic significance. Mechanistically, the abnormal oxidative stress in PCOS can cause genetic instability and increase the risk of infertility. OS is also intertwinged with obesity, insulin resistance (IR), inflammation and hyperandrogenemia, which are the common characteristics and potential inducers of PCOS. Thus, evaluating oxidative stress markers alongside the conventional biochemical parameters may be a good prognostic approach for early diagnosis of PCOS to ensure a healthy status among females of reproductive age.

ACKNOWLEDGEMENTS

We would like to acknowledge the help of all the Physicians, other staff members, lab mates for their constant help throughout the study and the participants who volunteered in the present study.

Conflict of interest

The authors declare that they have no conflicts of interest.

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