Evaluation of Oxidative Stress and hsCRP in Polycystic Ovarian Syndrome in a Tertiary Care Hospital

N. Unni C. Sumithra · R. Lakshman Lakshmi ·
N. Leela Menon · K. N. Subhakumari ·
V. S. Sheejamol

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Abstract PCOS is a heterogeneous endocrine disorder with diverse clinical presentation. Oxidative stress plays a key role in the pathophysiology of this disease. Serum high sensitive C-reactive protein (hsCRP), a marker of chronic low grade inflammation, is indicative of future development of cardiovascular disease. Our aim is to evaluate the oxidant status and hsCRP levels in PCOS. The study involved 61 cases and 61 controls in the age group of 18–40 years diagnosed with PCOS. Erythrocyte malondialdehyde (MDA), superoxide dismutase (SOD), serum hsCRP, gonadotrophins, thyroid stimulating hormone, prolactin, glycemic status and lipid profile were estimated. Erythrocyte MDA ($p < 0.001$), SOD ($p = 0.007$) and

serum hsCRP ($p < 0.001$) were significantly elevated in PCOS patients than controls. Oxidative stress is present in women with PCOS along with elevated hsCRP.

Keywords PCOS · Oxidative stress · hsCRP · Cardiovascular diseases · Type 2 DM · Free radicals

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder [1] that affects 5–10 % of women of reproductive age [2]. This condition was first described by Irvin Stein and Michael Leventhal in 1935 and hence it is also known as the Stein–Leventhal syndrome [3]. The early manifestations of this condition are menstrual abnormalities (oligomenorrhoea or amenorrhoea), hirsutism, insulin resistance (IR) and infertility [1]. Long term complications include cardiovascular disease (CVD), type 2 diabetes mellitus (Type 2 DM) [1, 4, 5], endometrial and breast cancer [6].

Increasing evidence show the high prevalence of cardiovascular disorders in PCOS. It has been proved that myocardial infarction is several times more in women with PCOS than normal age matched controls [7]. There has also been reports about the significant difference in the distribution of carotid plaques in these patients [8]. The high risk for CVD in PCOS patients might be due to the presence of associated features like obesity, diabetes and dyslipidemia which are all potent cardiovascular risk factors [9].

Oxidative stress occurs due to the imbalance between oxidants and antioxidants present in the body [10]. The most damaging radicals in the biological systems known are the oxygen radicals and hence free radicals that cause oxidative stress are also called reactive oxygen species

N. U. C. Sumithra (✉) · N. Leela Menon · K. N. Subhakumari
Department of Biochemistry, Amrita School of Medicine,
Amrita Institute of Medical Sciences and Research Centre,
Amrita Vishwavidyapeetham, Ponekkara PO,
Kochi 682041, Kerala, India
e-mail: sumithra.unni234@gmail.com

N. Leela Menon
e-mail: nleelamenon@aims.amrita.edu

K. N. Subhakumari
e-mail: subhakumarikn@aims.amrita.edu

R. L. Lakshmi
Department of Obstetrics and Gynecology, Amrita School of
Medicine, Amrita Institute of Medical Sciences and Research
Centre, Amrita Vishwavidyapeetham, Ponekkara PO,
Kochi 682041, Kerala, India
e-mail: nanolakshmi@gmail.com

V. S. Sheejamol
Department of Biostatistics, Amrita School of Medicine, Amrita
Institute of Medical Sciences and Research Centre, Amrita
Vishwavidyapeetham, Ponekkara PO, Kochi 682041, Kerala,
India
e-mail: sheejamolvs@aims.amrita.edu

(ROS). Reactive oxygen species include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$), hydroperoxyl ($HOO^{\bullet}$), lipid peroxide radical ($ROO^{\bullet}$), ozone (O_3) and singlet oxygen [11]. Proteins, carbohydrates, nucleic acids and lipids are targets of various ROS and these are oxidized to give a diverse array of products [12]. Cell membranes which are rich sources of polyunsaturated fatty acids (PUFA) are readily attacked by oxidising radicals. This process is known as lipid peroxidation and malondialdehyde (MDA) is one of the important end products of this process. It correlates with the extent of lipid peroxidation and thus is one of the most common biomarker used to assess the oxidant status [10]. Antioxidant enzymes help in maintaining the redox balance of the cell during periods of oxidative stress. Among the various antioxidant mechanisms in the body, superoxide dismutase (SOD) which converts the superoxide anion radical into hydrogen peroxide is thought to be one of the major enzymes that protect cells from ROS [12]. The role of ROS in the pathogenesis of atherosclerosis and myocardial ischemia is well established. Studies have shown significant correlation between lipid hydroperoxides levels in plasma and arterial wall in atherosclerotic patients [13, 14].

Recently several biochemical markers have been introduced to predict the vascular events in PCOS. These include serum high sensitive C-reactive protein (hsCRP), homocysteine, sialic acid and fibrinogen [15–18]. Of these, the role of hsCRP in PCOS is gaining increasing interest [9]. PCOS has been described as a state of chronic low-grade inflammation mainly characterized by a modest rise in serum C-reactive protein (CRP) compared to the weight matched controls [19]. Low grade chronic inflammation is an independent marker of CVD and is demonstrated by persistently elevated serum hsCRP. CRP selectively binds to the oxidised LDL molecules and is deposited in the atheromatous plaques and contributes to the pathogenesis and progression of the atheroma due to its proinflammatory property [20].

The aim of this study was to estimate the levels of hsCRP and oxidative stress in PCOS patients when compared to healthy controls.

Materials and Methods

This was a case control study which included 61 cases and 61 controls. The study was conducted over a period of 2 years from October 2010 to October 2012 and included 61 patients in the age group of 18–40 years with established PCOS, attending the Gynaecology OP of Amrita Institute of Medical Sciences, Kerala. 61 cases included both lean and obese PCOS. Lean (normal weight) group included 27 subjects with BMI 18.5–22.9. Obese group included 34 subjects with BMI ≥ 25 . Controls were

apparently normal healthy age matched subjects with normal BMI (18.5–22.9) presenting for routine health check up in the comprehensive health check up clinic of AIMS. The study was conducted as per the approval and guidelines of the ethical committee of AIMS-School of Medicine and with the informed, written consent of the participants.

Subject Selection

PCOS was diagnosed based on the Rotterdam ESHRE revised consensus 2003 [21]. As per the criteria any two of the following features should be present to diagnose PCOS:

- chronic oligo/anovulation,
- clinical and/or biochemical evidence of hyperandrogenism and
- appearance of polycystic ovaries on ultrasound; after excluding other etiologies for anovulation and hyperandrogenism.

Inclusion Criteria

Patients with complaints of any of the two of the following were included

- Oligomenorrhoea/amenorrhoea and inability to conceive
- Hirsutism
- Polycystic ovaries in Ultrasonography.

None of the patients received any hormonal contraceptives, aspirin, statins, vitamin supplements or any other significant drug therapy. Age matched healthy women with regular 28–32 day menstrual cycles, absence of hirsutism and infertility were the controls. None of the patients had history of any acute or chronic infections or illnesses.

Exclusion Criteria

Women with the following conditions were excluded from the study: hyperprolactinemia, androgen secreting neoplasm, cushing's syndrome, congenital adrenal hyperplasia and thyroid dysfunction.

Measurements

Venous blood samples were collected with aseptic precautions in vacutainers without anticoagulant to measure serum hsCRP, luteinising hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), prolactin, total cholesterol, HDL, LDL, VLDL, triglycerides and insulin. Samples for fasting plasma glucose and 2-h OGTT (Oral glucose tolerance test) were collected in vacutainers containing fluoride. hsCRP was measured

using immunoturbidimetry in Beckman Coulter Olympus AU 2700. Serum LH, FSH, TSH, prolactin and insulin were estimated by chemiluminescent microparticle immunoassay in Abbott Architect i 2000_{SR}. Fasting plasma glucose and 2-h OGTT were measured using the principle of colorimetry in Beckman Coulter Olympus AU 2700. Total cholesterol, LDL, HDL and Triglycerides were measured in Beckman Coulter Olympus AU 2700 enzymatically.

Total cholesterol was estimated enzymatically utilising cholesterol esterase, cholesterol oxidase and peroxidase. Cholesterol esters present in the serum is converted to cholesterol by the enzyme cholesterol esterase which is acted upon by cholesterol oxidase to form cholesten-3-one and hydrogen peroxide. Hydrogen peroxide then reacts with phenol and 4-amino antipyrine in the presence of peroxidase to form quinonimine, a pink coloured product which is proportional to the concentration of cholesterol present in the sample and is estimated colorimetrically.

HDL cholesterol was also estimated by the enzymatic method by utilising the enzymes cholesterol esterase and oxidase modified with polyethylene glycol (PEG). HDL cholesterol esters present in the serum is converted to HDL cholesterol by the PEG cholesterol esterase enzyme which is acted upon by PEG cholesterol oxidase to form cholesten-3-one and hydrogen peroxide. Hydrogen peroxide then reacts with phenol and 4-amino antipyrine in the presence of peroxidase to form a purple blue pigment which is estimated colorimetrically.

LDL cholesterol assay also utilises enzymatic method but in the presence of a sugar compound and a non ionic detergent in the reaction medium. LDL cholesterol esters present in the serum is converted to LDL cholesterol by the enzyme cholesterol esterase (in the presence of a detergent) which is acted upon by cholesterol oxidase to form cholesten-3-one and hydrogen peroxide. Hydrogen peroxide then reacts with phenol and 4-amino antipyrine in the presence of peroxidase to form a purple blue pigment is produced at the end of the reaction which is measured colorimetrically.

Triglycerides were estimated based on a series of coupled enzymatic reactions utilising microbial lipases, glycerol kinase and glycerol phosphate oxidase to produce hydrogen peroxide which was oxidatively coupled with *p*-chlorophenol and 4-aminoantipyrine catalysed by peroxidase to give a red dye with an absorbance maximum at 500 nm. The increase in absorbance at 520 nm is proportional to the triglyceride content of the sample.

VLDL was calculated according to the Friedewald equation (Triglycerides (mg/dl)/5).

Venous blood samples were also collected in EDTA containing vacutainers for assessing the parameters of oxidative stress; namely erythrocyte MDA and SOD. These blood samples were centrifuged at 1,500×*g* for 15 min and

the plasma was separated. 2 ml of blood cells was washed with 4 ml cold normal saline (0.9 % NaCl), centrifuged at 3,000×*g* for 15 min and supernatant discarded. This was repeated twice. The washed packed cells were hemolysed with 30 ml ice cold distilled water. This hemolysate was used to estimate the oxidative stress parameters namely MDA and SOD. MDA was determined by the method of Sinnhuber et al. [19]. SOD was estimated by the inhibition of auto oxidation of pyrogallol according to the procedure described by Marklund and Marklund [18].

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20 Windows (SPSS Inc., Chicago, USA). For all the continuous variables the results are given in mean ± standard deviation. To compare the means of continuous parameters between two groups, those are following normal distribution, Student's independent samples *t* test was performed. Log transformations were applied for those variables that were not following normal distribution and homogeneity of variance (TSH, fasting plasma glucose, fasting insulin and MDA). analysis of covariance (ANCOVA) was used for adjusting the effect of BMI. Pearson's correlation was used to find out the correlation between two parameters that were following normal distribution. Probability value *p* < 0.05 was considered for statistical significance.

Results

Age, BMI and biochemical parameters of the patients are given in Table 1. Mean age of the PCOS patients and controls does not show significant difference. BMI is significantly increased in cases than controls. Significantly elevated LH, FSH, LH:FSH ratio, 2 h OGTT, fasting insulin, total cholesterol, LDL, triglycerides and VLDL are seen in PCOS patients when compared to controls. TSH, prolactin, HDL and fasting plasma glucose levels do not show statistically significant difference. Erythrocyte MDA, SOD and hsCRP are significantly increased in PCOS patients than controls. After adjusting the effects of BMI, hsCRP (Mean ± SE; 3.292 ± 0.192 vs 1.456 ± 0.192, *p* < 0.001), MDA (Mean ± SE; 0.121602 ± 1.11064 vs 0.05787 ± 1.11064, *p* < 0.001) and SOD (Mean ± SE; 16.873 ± 1.34 vs 12.635 ± 1.34, *p* = 0.033) levels showed significant increase in cases when compared to controls. Correlation analysis reveals significant positive correlation of hsCRP with LH, LH:FSH ratio, MDA and BMI in cases (Table 2). Multiple regression analysis shows that BMI significantly predicted hsCRP levels in PCOS (Table 3).

Table 1 Comparison of age, BMI and biochemical parameters in PCOS patients and controls

Parameter	Controls Mean $\pm$ SD	Cases Mean $\pm$ SD	<i>p</i> value
Age (years)	27.92 $\pm$ 5.47	26.98 $\pm$ 7.11	0.418
BMI (kg/m ²)	20.85 $\pm$ 1.55	23.51 $\pm$ 4.31	<0.001
LH (mIU/ml)	3.95 $\pm$ 1.27	9.07 $\pm$ 5.71	<0.001
FSH (mIU/ml)	5.01 $\pm$ 1.44	4.03 $\pm$ 1.65	0.001
LH:FSH ratio	0.8485 $\pm$ 0.35324	2.35 $\pm$ 1.51	<0.001
TSH (μ IU/ml)	1.73 $\pm$ 2.2	1.96 $\pm$ 1.87	0.341
Prolactin (ng/ml)	14.08 $\pm$ 5.38	15.33 $\pm$ 8.99	0.356
Fasting plasma glucose (mg/dl)	91.93 $\pm$ 1.10	96.28 $\pm$ 1.23	0.119
2 h OGTT (mg/dl)	111.53 $\pm$ 16.25	124.68 $\pm$ 45.79	0.038
Fasting insulin (μ IU/ml)	5.73 $\pm$ 1.56	7.35 $\pm$ 2.17	0.032
Total cholesterol (mg/dl)	174.95 $\pm$ 13.35	221.36 $\pm$ 42.97	<0.001
HDL (mg/dl)	48.56 $\pm$ 5.6	47.96 $\pm$ 11.84	0.719
LDL (mg/dl)	108.42 $\pm$ 11.39	134.22 $\pm$ 28.58	<0.001
Triglycerides (mg/dl)	107.94 $\pm$ 30.42	136.37 $\pm$ 49.45	<0.001
VLDL (mg/dl)	21.23 $\pm$ 14.21	40.30 $\pm$ 30.04	<0.001
hsCRP (mg/l)	0.4016 $\pm$ 0.22825	4.35 $\pm$ 4.16	<0.001
MDA (nmol/ml)	0.045018 $\pm$ 2.84	0.156062 $\pm$ 2.54	<0.001
SOD (U/gmHb)	12.28 $\pm$ 9.11	17.23 $\pm$ 10.88	0.007

Table 2 Correlations among PCOS cases

Variables	Cases Pearson correlation (<i>r</i>)	<i>p</i> value
CRP and LH	0.495	<0.001
CRP and LH/FSH	0.387	0.002
CRP and BMI	0.928	<0.001
CRP and MDA	0.809	<0.001
MDA and BMI	0.946	<0.001

Table 3 Multiple regression analysis for hsCRP

	β	SE	<i>p</i>
BMI	0.842	0.056	<0.001
LH	0.092	0.076	0.232
LH:FSH	−0.215	0.270	0.431
VLDL	0.013	0.007	0.069

Discussion

Oxidative stress has been implicated in a number of pathologies like CVD, neurological diseases, malignancies, diabetes, inflammatory conditions and aging [22]. Free radicals are highly reactive molecular species with at least one unpaired electron in the outermost shell and are capable of independent existence. They persist for only a very short time before they collide with another molecule

and either abstract or donate an electron in order to achieve stability. In doing so they generate another free radical from the molecule with which they collided. Free radicals also impair cells and tissue properties related to human fertility [10].

Recently several studies conducted in PCOS have demonstrated increased oxidative stress in these patients. Cause of oxidative stress in PCOS is not fully understood and multiple reasons have been postulated like hyperglycaemia, IR and chronic inflammation. Oxidative stress and chronic inflammation are closely inter-related. A vicious cycle exists whereby inflammation induces generation of ROS, while oxidative stress promotes and aggravates inflammation [23].

Inflammation is considered as a key feature of atherosclerotic plaques, and a strong relationship exists between systemic inflammatory activity and the occurrence of atherothrombotic events like myocardial infarction [24]. CRP, one of the markers of inflammation, is produced from the hepatocytes under the influence of proinflammatory cytokines like Tumor necrosis factor- α (TNF α) and interleukins 1 and 6 [25]. A powerful predictive relationship exists between elevated CRP production and cardiovascular diseases [20, 26]. High BMI is yet another factor that contributes to the development of inflammation. Adipocytes secrete about 25 % of IL-6 which can stimulate the release of CRP from the liver [27]. A higher prevalence of low grade systemic inflammation was observed in overweight

and obese individuals compared with normal weight persons [28].

In our study we observed, significantly high MDA ($p < 0.001$) and SOD levels ($p = 0.007$) (Table 1) in patients when compared to controls. Elevated MDA suggests that increased lipid peroxidation was present in PCOS patients. Raised SOD might be an over expression of this enzyme as a compensatory adaptive response for the increased demand for dismutation of superoxide radical. Our findings are in accordance with the studies by Sabuncu et al. [13], Kuscü et al. [29], Azzawie et al. [30] and Mohan et al. [31] where they reported the presence of oxidative stress in PCOS patients.

Levels of serum hsCRP are significantly increased in the patients than controls ($p < 0.001$). Kelly et al. [32] first proposed that chronic low grade inflammation indicated by an increased CRP levels predicted the risk of coronary heart disease and Type 2 DM in PCOS women. In a study done by Boulman et al. [15] in PCOS patients, hsCRP levels were divided into three groups <1 , $1-3$ and >3 mg/l corresponding to low, moderate and high risk groups for future CV events. It was seen that out of the total 116 patients, 46.5 % patients had hsCRP levels >3 mg/l. Tosi et al. [33] hypothesised that adiposity was a main factor determining elevated CRP in PCOS patients.

The significant increase in the hsCRP levels in PCOS patients when compared to controls persisted even after adjusting for the BMI. Multiple regression analysis revealed that only BMI independently predicted CRP levels which indicate that the rise in hsCRP may be due to the difference seen in BMI initially. But as this difference persisted even after adjusting for BMI it shows that BMI alone may not be the contributing factor for increasing hsCRP levels; the disease itself or some other factors might have contributed to the same. Correlation analysis in our study showed that hsCRP levels were positively correlated with MDA ($r = 0.809$, $p < 0.001$) and BMI ($r = 0.928$, $p < 0.001$) among the cases which shows the close inter relation of chronic inflammation with oxidative stress and adiposity. In controls hsCRP levels showed significant positive correlation with MDA but not with BMI ($r = 0.070$, $p = 0.591$). hsCRP levels also positively correlated with LH, LH:FSH ratio in cases which indicates that hormonal factors can also contribute to elevation of hsCRP.

Conclusion

To conclude, our data suggest that PCOS patients have oxidative stress and elevated hsCRP which indicate that these women are at high risk for developing cardiovascular

diseases. Our results also suggest that PCOS alone may not be the cause for the increased oxidant status and hsCRP; adiposity also might play a role in this. Hence further long term serial studies on a large population are required in this field to arrive at a definite conclusion.

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