Original Paper

Impaired Arginine Metabolism Coupled to a Defective Redox Conduit Contributes to Low Plasma Nitric Oxide in Polycystic Ovary Syndrome

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Key Words

Polycystic ovarian syndrome • Nitric oxide • Nitric Oxide Synthase • Hydrogen peroxide • Arginine bioavailability

Abstract

Background: Though oxidative stress is associated with Polycystic Ovary Syndrome (PCOS), the status of nitric oxide is still unclear. Nitric Oxide (NO) plays pivotal roles in many physiological functions which are compromised in PCOS. Our recent study reveals lowered T-regulatory cells (Tregs) in PCOS, and Treg generation is known to be regulated by NO levels. However concrete evidences are lacking on mechanisms modulating NO levels under PCOS.

Methods: This is a retrospective case-control cohort study, comprised of PCOS women (N=29) and normal menstruating women as controls (N=20). We analysed NOx (nitrite+nitrate) and hydrogen peroxide (H₂O₂) concentrations, transcript levels of endothelial nitric oxide synthase (eNOS)/inducible nitric oxide synthase (iNOS) and arginine modulators, hydrogen peroxide regulators in the cohort. **Results:** PCOS women showed reduced plasma NOx(nitrate+nitrite) and H₂O₂ compared to controls. We report reduction in transcript levels of *iNOS/NOS2* and *eNOS/NOS3* in PCOS peripheral blood. The transcripts involved in arginine bioavailability: Argininosuccinate lyase (*ASL*), Solute Carrier Family1, member 7 (*SLC7A1*) and Arginase 1 (*ARG1*) and Asymmetric Dimethyl Arginine (ADMA) metabolism: Protein arginine methyltransferase 1 (*PRMT1*) and Dimethylarginine dimethylaminohydrolase 2 (*DDAH2*) also showed differential expression. H₂O₂ concentration in PCOS women was also found to be reduced. The reduction can be attributed to increase in catalase levels as a consequence of the body's effort to alleviate the oxidative burden in the system. **Conclusion:** Our study advocates that PCOS women have lowered NO due to reduced *iNOS/eNOS* expression, low H₂O₂, high ADMA synthesis and reduced arginine bioavailability. An in-depth analysis of redox biology of PCOS to open up potential therapeutic strategies is highly recommended.

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Introduction

Polycystic ovarian syndrome (PCOS) is the leading cause of onset to the metabolic syndrome in adolescent women. The classic hallmark of the disorder is a combination of hyperandrogenemia, oligo/ or anovulation and polycystic ovaries. PCOS patients are also at a high risk of comorbidities such as insulin resistance (IR), Type 2 Diabetes Mellitus, oxidative stress, inflammation, cardiovascular disorders, obesity, dyslipidemia and infertility. PCOS is ranked as the most common cause of female infertility on a global scale. Further deteriorating the scenario PCOS patients have low IVF/ICSI success rates [1] and high incidence of ovarian hyperstimulation syndrome (OHSS) [2].

The high levels of androgen and advanced glycation end products (AGEs) in PCOS women induces oxidative stress and inflammation [3]. Recent reports showed strong involvement of oxidative stress [4, 5] and endothelial dysfunction [6-8] in PCOS pathogenesis. Both endothelial dysfunction and oxidative stress are prognostic indicators of cardio vascular disorders, pre-eclampsia, placenta-related defects and recurrent pregnancy loss [9-12]. Endothelial function is compromised under PCOS although results on brachial artery flow-mediated dilatation (FMD) are controversial as decrease in Turkish population [13] while increase in Indian population [14] in FMD are reported. Endothelial impairment is majorly attributed to endothelial nitric oxide (NO) synthesis, its bioavailability and oxidant-antioxidant mechanisms.

Nitric Oxide (NO) is a gaseous free radical molecule mediating various physiological functions like vasodilation, neuronal transmission and immune functions. It is synthesized by nitric oxide synthase (NOS) during the conversion of L-Arginine to citrulline using oxygen and NADPH as the cofactors. The enzyme nitric oxide synthase has 3 isoforms *viz.*, the neuronal form (nNOS or NOS1), the inducible form (iNOS or NOS2) and the endothelial derived form (eNOS or NOS3). NO plays an inevitable part in various human reproductive functions which have been reviewed in great detail by Rosselli [15]. The importance of NO in male reproduction encompasses ground-breaking discovery of electrical stimulation mediated NO production and release by isolated strips of corpus cavernosum penis [16]. NO plays inevitable part in penile erection [17] and regulation of sperm motility [18] due to presence of eNOS and bNOS (nNOS) [19]. Magnitude of role of NO in female reproduction is reflected by its unequivocal involvement in follicular maturation [20], ovulation [21], decidualization [22, 23], embryo implantation [24] and cervical secretion [25]. NO has been correlated with many human disorders like hypertension, diabetes, insulin resistance, obesity, stroke, neurodegenerative diseases, inflammatory disorders and infections. So, maintaining adequate amount of NO is a prerequisite for its proper functioning of the biological system. NO interacts with superoxide radicals ($O_2^{\cdot-}$) to generate toxic peroxynitrites ($ONOO^-$) adding to oxygen radical burden. Superoxide dismutase (SOD), catalyses the dismutation of superoxide anion ($O_2^{\cdot-}$) to produce hydrogen peroxide (H_2O_2), and sustain adequate NO pool in the biological system. Substantial evidences are lacking on the stature of NO and H_2O_2 in PCOS women. PCOS women have increased oxidant xanthine oxidase (XO) activity and decreased lipid antioxidant paraoxonase 1 (PON1) activity, indicating that the patients are under high oxidative stress. Under prediabetic conditions like insulin resistance, endothelial function is found to deteriorate with decrease in NO bioavailability [26]. Contradictory reports exist about the status of NOx under PCOS. Some researchers reported decreased NO metabolites in PCOS [27, 28]. Another noteworthy study performed in PCOS women has proved that the pregnancy rate in PCOS women can be increased through NO administration [29]. These scanty reports do not clarify the stature of NO and H_2O_2 in PCOS women. Therefore, exploring the status and mechanism of NO generation in PCOS is indispensable to develop novel therapeutic approaches. In-depth understanding of this aspect will contribute to improve PCOS pathology and related anomalies such as reduced uterine receptivity.

The aim of our study was to assess the status of NOS activity, factors involved in NO generation and its bioavailability modulators in peripheral blood of PCOS women. We measured the plasma levels of NO_x (nitrate+nitrite), nitrite and hydrogen peroxide (H₂O₂) in PCOS women and control subjects. We also analysed the causatory factors underlying mechanism behind the altered NO levels. To reveal the mechanism behind the NO regulation PCOS women, we conducted relative expression study of transcripts involved in NO and H₂O₂ pathways viz., iNOS (*NOS2*), eNOS (*NOS3*), Superoxide dismutase 2 (*SOD2*, enzyme for H₂O₂ synthesis), Argininosuccinate lyase (ASL, NO activator), *SLC7A1* (Cationic arginine transporter), Arginase (*ARG1*, competitive inhibitor of nitric oxide synthase), Dimethylarginine dimethylaminohydrolase 2 (DDAH2, activator of nitric oxide synthase), and Protein arginine methyltransferase 1 (PRMT1, inhibitor of nitric oxide synthase) in peripheral blood of PCOS women.

Materials and Methods

Subjects

We conducted a retrospective case-control study including 29 PCOS patients and 20 healthy donors and collected peripheral blood on day 2-4 of follicular phase. All the patients were enrolled at SAMAD IVF clinic, Thiruvananthapuram for infertility and pregnancy related complications and were diagnosed for PCOS. The diagnosis was based on the Rotterdam European Society for Human Reproduction and Embryology (ESHRE)/American Society for Reproductive Medicine (ASRM)-sponsored PCOS Consensus Workshop Group [30]. Regular cycling women were recruited as control subjects. The study was approved by the Institute Ethical Review Board (IHEC/01/2011/08, IHEC/02/2009/14) and SAMAD ethical committee. Informed prior consent was attained from the subjects according to the declaration of Helsinki [31].

Measurement of Nitric Oxide products (Nitrate plus nitrite) in plasma

Plasma samples were centrifuged at 1500 rpm for 20 minutes to remove protein content. The supernatant was further deproteinized by ultrafiltration through 10kDa cut-off filter at 4,000 rpm for 1 hour. The filtrate was used for further analysis. Plasma nitrate and nitrite concentrations were determined using Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemicals, MI, USA) according to manufacturer's instructions. The assay involved reduction of serum nitrate to nitrite by nitrate reductase enzyme, followed by addition of Greiss reagent for converting nitrite to Azo product. Absorbance of the final product was measured at 540 nm using Tecan Infinite M2000 (Tecan Group, Switzerland).

Measurement of Hydrogen Peroxide (H₂O₂)

Plasma H₂O₂ was measured using the Amplex Red (Invitrogen) according to manufacturer's instructions. In the presence of horseradish peroxidase, Amplex Red reacts with H₂O₂ to form resorufin and O₂. Resorufin is a stable and highly fluorescent compound whose wavelength spectra excitation/emission are 570/585 nm. 50 µL of the Amplex® Red reagent/ H₂O₂ working solution was added to microplate well containing the standards, controls, and samples. The plate was incubated for 30 minutes at room temperature and the absorbance was measured in excitation range of 530–560 nm and fluorescence emission detection at ~590 nm. The concentration was measured from a standard curve with 20 mM H₂O₂.

Total RNA extraction, First strand cDNA synthesis, Quantitative Real Time PCR

Total RNA was extracted from the whole blood samples using miRNeasy mini kit (Qiagen) according to manufacturer's protocol. The red blood cells from the peripheral blood were lysed and the leukocytes were taken for RNA extraction. The RNA samples were quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). 100ng of total RNA was reverse transcribed with SuperScript® VILO™ cDNA Synthesis Kit and q-PCR amplification was performed with Power SYBR® Green PCR Master Mix (Life Technologies, CA). Oligonucleotide primers for transcript real time were designed by the Primer Express program. The primers used were: *ARG1*- F (TCC ACC CTG ATC TTG GAG TCA T), *ARG1*- R (GTT GTC CAT GCA AGT TTC CAC TT); *ASL* - F (GCC ACT GGC GTC ATC TCT AC), *ASL* - R (GGA CCA GGT AAT AGG CAGG GGT); *NOS2* - F (TCA CTC AGC TGT GCA TCG AC), *NOS2* - R (CCT CAA GCA CAA GGT CAG GT); *NOS3* - F (ATC TTC AGC CCC AAA CGG AG), *NOS3* - R (CTG GAA CAT CTT CCG CCT GT); *DDAH2* - F (CTC TGG ATC TGG CCA AAG CT), *DDAH2* - R (GAC TCC TCA GGT GGC AGT TC); *PRMT1* - F (TCA CAC GCT GCC ACA AGA G), *PRMT1* - R (ACG GTC AGG TAG TCC TCC ATG T); *SLC7A1* - F (TCA CCG GCT GGA ACT TAA TC), *SLC7A1* - R (CCG ATG GGT CTG CCT ATC A); *SOD2*-F (GCT GCA CCA CAG CAA GCA), *SOD2*-R (TCG GTG ACG TTC AGG TTG TTC). The

relative expression was calculated using $\Delta\Delta C_t$ method with *18S rRNA* as the endogenous control. The run was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems, CA) under standard cycling conditions.

SDS- PAGE and Western Blotting

Peripheral Blood Mononuclear Cells (PBMCs) were extracted from whole blood by standard Ficoll-Hypaque density centrifugation. Protein extract was prepared by lysing the cells in ice-cold buffer (50mM HEPES pH 7.2, 150mM NaCl, 5M EDTA, 0.5% NP-40) containing protease inhibitor cocktail. For plasma protein extraction, the human plasma were subjected to albumin depletion and the pellets collected according to depletion of albumin component method [32]. 20 μ g of proteins were separated by SDS-PAGE under denaturing and reducing conditions and then proteins were transferred to PVDF membranes.

Nonspecific binding of the antibodies to the membrane was blocked by 2-hour incubation with 1% (w/v) bovine serum albumin /0.1% (v/v) Tween 20 in Phosphate Buffered Saline (PBS). Membranes were incubated for 2 hours at room temperature with specific antibodies against SOD2, SOD3, catalase and Histone H2B [rabbit polyclonal (1:1000 dilution, Santa Cruz). Anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (dilution 1:2000, Sigma) was used as the secondary antibodies to detect the signals. The signals were then measured either by ECL detection solution (GE healthcare) or by colour development using 3, 3' diaminobenzidine tetrahydrochloride dehydrate (DAB) as an electron donor, H_2O_2 as the enzyme substrate and $NiCl_2$ as metal enhancer. A silver stained gel served as the reference control for plasma SOD3. Protein bands were visualized and images were captured on VersaDoc imaging system (BioRad).

Statistical analysis

Statistical evaluation was carried out with GraphPad Prism software. Data obtained from the study groups were compared by the parametric Student's t-test. The correlation analysis among the variables was performed by Pearson's test; a p-value ≤ 0.05 was considered as significant. All the results are expressed as mean $\pm$ SD.

Results

The study was conducted in a group comprising PCOS (N=29) and age-weight matched control (N=20) women. Other characteristics of the study group are summarised in Table 1. All values are expressed as mean $\pm$ standard deviation.

PCOS women have decreased NOx /iNOS/eNOS:

Plasma NOx end products (nitrate plus nitrite) concentration of PCOS women was significantly lower ($19.58 \mu M \pm 1.165$) compared to control ($25.81 \mu M \pm 2.641$) ($t=2.45$, $p=0.018$) (Fig. 1a). The mean difference between the groups was 6.233 ± 2.545 , (95% CI 1.104 to 11.36, $R^2=0.118$). Fig. 1b represents the dotplot showing the nitrate plus nitrite levels in individual subjects. Nitrite levels are also reduced in PCOS women (1.549 ± 0.1578) compared to control ($2.444 \mu M \pm 0.3573$) ($t=2.6$, $p=0.0126$), (Fig. 1c & d) with a mean difference of $0.895 \mu M \pm 0.344$, 95% CI - 0.201 to 1.59, $R^2=0.1306$).

Real time PCR analysis was performed to assess the transcript expression in peripheral blood of PCOS (N=15) and the control (N=13) women. We found the enzymes involved in NO synthesis, inducible NOS (iNOS, *NOS2*), was

Table 1. The table represent the biochemical and physiological characteristics of PCOS (N=22), Control (N=19) group. All values are expressed as mean $\pm$ standard deviation. P value represents the significance as calculated by student's t-test between the two groups

Biochemical and physiological characteristics of study group			
Parameters	PCOS	Normal	P value
Age(Yr)	28 $\pm$ 2.52	28.16 $\pm$ 2.01	0.83
BMI(Kg/M ²)	24.96 $\pm$ 3.22	23.44 $\pm$ 2.03	0.082
LH(IU/litre)	11.5 $\pm$ 2.67	6.81 $\pm$ 1.26	2.32E-08
FSH(IU/litre)	5.44 $\pm$ 1.14	7.12 $\pm$ 1.40	0.000134
Total testosterone(ng/mL)	46.6 $\pm$ 17.72	26.44 $\pm$ 8.19	5.13E-05
Blood Pressure, systolic	115.91 $\pm$ 9.08	116.84 $\pm$ 5.58	0.6997
Blood pressure, diastolic	72.73 $\pm$ 6.31	74.73 $\pm$ 5.89	0.3008

down regulated in patients with a fold change of -6.25 and ($p=0.001$) (Fig. 1e & 1f) and endothelial NOS (eNOS, *NOS3*), was down regulated in patients with a fold change of -6.53 ($p<0.0003$) (Fig. 1g & h).

Differential expression of enzymes involved in arginine bioavailability:

Arginine is the substrate for NOS during NO synthesis, thus the bioavailability of this amino acid is crucial to NOS action. On RT-PCR analysis, the transcript levels of

Fig. 1. Estimation of NO/iNOS/eNOS in PCOS women. a) Bar graph representation of concentration (μM) of NO end products (Nitrite plus nitrate) in plasma of PCOS women (left) v/s control women (right). b) Dotplot representation of concentration (μM) of NO end products (Nitrite plus nitrate) in plasma of PCOS women (left) v/s control women (right). c) bar-graph of nitrite concentration in plasma of PCOS women (left) v/s control women (right). d) Dotplot representation of concentration (μM) of nitrite in plasma of PCOS women (N=29) (left) v/s control women (N=19) (right). e) Bar graph showing real time expression analysis of iNOS (*NOS2*) transcript in peripheral blood of PCOS women. f) Dotplot representation of $2^{-\Delta\text{Ct}}$ values of iNOS (*NOS2*) in PCOS women (left) v/s control women (right). g) Bar graph showing real time expression analysis of eNOS (*NOS3*) transcript in peripheral blood of PCOS women. h) Dotplot representation of $2^{-\Delta\text{Ct}}$ values of eNOS (*NOS3*) in PCOS women (left) v/s control women (right). Fold change (FC), log2RQ was calculated according to $\Delta\Delta\text{Ct}$ method with *18SrRNA* as the endogenous control. The $2^{-\Delta\text{Ct}}$ values are plotted in Y axis, h) Dotplot representation of $2^{-\Delta\text{Ct}}$ values of eNOS (*NOS3*) in PCOS women (left) v/s control women (right). Fold change (FC), log2RQ for (e) & (g) was calculated according to $\Delta\Delta\text{Ct}$ method with *18SrRNA* as the endogenous control. The $2^{-\Delta\text{Ct}}$ values are plotted in Y axis. P value (<0.05) was calculated using Student's T test on GraphPad Prism 6.0 software.

Bar graph showing real time expression analysis of eNOS (*NOS3*) transcript in peripheral blood of PCOS women. Fold change (FC), log2RQ was calculated according to $\Delta\Delta\text{Ct}$ method with *18SrRNA* as the endogenous control. The $2^{-\Delta\text{Ct}}$ values are plotted in Y axis, h) Dotplot representation of $2^{-\Delta\text{Ct}}$ values of eNOS (*NOS3*) in PCOS women (left) v/s control women (right). Fold change (FC), log2RQ for (e) & (g) was calculated according to $\Delta\Delta\text{Ct}$ method with *18SrRNA* as the endogenous control. The $2^{-\Delta\text{Ct}}$ values are plotted in Y axis. P value (<0.05) was calculated using Student's T test on GraphPad Prism 6.0 software.

Fig. 2. Differential expression of transcripts in Arginine synthesis/transport/degradation. Bar graph representation of real time PCR analysis of a) *ASL*, c) *SLC7A1* and e) *ARG1* in peripheral blood of Control and PCOS women. Bars represent $2^{-\Delta Ct}$ of the transcripts in control (right) and PCOS (left) women. Fold change (FC), log2RQ was calculated according to $\Delta\Delta Ct$ method with 18SrRNA as the endogenous control. The $2^{-\Delta Ct}$ values are plotted in Y axis. P value (<0.05) was calculated using Student's

T test on GraphPad Prism 6.0 software. b, d and f) Dotplot representation of $2^{-\Delta Ct}$ values *ASL*, *SLC7A1* and *ARG1* respectively of PCOS women (left) v/s control women (right).

Argininosuccinate lyase (*ASL*), the enzyme involved in breakdown of arginosuccinate to arginine, showed a significant decrease with a fold change of -1.514 ($p < 0.0002$) (Fig. 2a). Patient variation is represented as a dotplot in Fig. 2b. Cationic arginine is transported via its transporter gene called solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (*SLC7A1*). Thus, this would be important in the bioavailability of arginine for NOS action. Our results revealed a reduction of *SLC7A1* levels in PCOS patients with a fold change of -3.608 ($p < 0.0001$) (Fig. 2c). Subject wise individual copy numbers are represented in Fig. 2d. The arginine degrading enzyme, Arginase 1 (*ARG1*) was seen to be significantly upregulated in PCOS patients with a fold change of 3.30 ($p < 0.0001$) (Fig. 2 e & f). The fold change was expressed as log2RQ and the p values of <0.05 were considered to be significant.

ADMA biosynthesis regulators exhibit aberrant expression:

NOS activity is known to be regulated by Asymmetric dimethylarginine (ADMA), a competitive inhibitor of NOS. Therefore, we analysed the expression levels of transcripts involved in ADMA biosynthesis and degradation. Our results showed an increase in Protein arginine methyltransferase 1 (*PRMT1*) involved in ADMA biosynthesis with a fold change of 5.916 ($p < 0.0001$) (Fig. 3 a & b). Dimethylarginine dimethylaminohydrolase 2 (*DDAH2*), the enzyme which degrades methylarginines (especially ADMA and NG-monomethyl-L-arginine), was significantly down regulated in our real-time expression analysis with a fold change of -2.143 ($p < 0.001$) (Fig. 3 c & d) contributing to reduced degradation of ADMA. The fold change was expressed as log2RQ and the P values of <0.05 were considered to be significant.

Fig. 3. Differential expression of transcripts in ADMA biosynthesis/degradation. Bar graph representation of real time PCR analysis of a) *PRMT1* and c) *DDAH2* in peripheral blood of Control and PCOS women. Bars represent $2^{-\Delta Ct}$ of the transcripts in control (right) and PCOS (left) women. Fold change (FC), log2RQ was calculated according to $\Delta\Delta Ct$ method with *18SrRNA* as the endogenous control. The $2^{-\Delta Ct}$ values are plotted in Y axis. P value (<0.05) was calculated using Student's T test on GraphPad Prism 6.0 software. b) and d) Dotplot representation of $2^{-\Delta Ct}$ values *PRMT1* and *DDAH2* respectively of PCOS women (left) v/s control women (right).

Fig. 4. Estimation of H_2O_2 /SOD2/3 & catalase in PCOS women. a) Bar graph representation of concentration (μM) of H_2O_2 in plasma of PCOS women (left) v/s control women (right). b) Dotplot representation of concentration (μM) of H_2O_2 in plasma of PCOS women (left) v/s control women (right). The estimation was performed with Amplex Red reagent and standard curve was generated using 20nM H_2O_2 . P value was calculated using Student's T test on GraphPad Prism 6.0. c) Bar graph representation of real time PCR analysis of *SOD2* in peripheral blood of Control and PCOS women. Bars represent $2^{-\Delta Ct}$ of the transcripts in control (right) and PCOS (left) women. Fold change (FC), log2RQ was calculated according to $\Delta\Delta Ct$ method with *18SrRNA* as the endogenous control. The $2^{-\Delta Ct}$ values are plotted in Y axis. P value (<0.05) was calculated using Student's T test on GraphPad Prism 6.0 software. d) Dotplot representation of $2^{-\Delta Ct}$ values *SOD2* of PCOS women (left) v/s control women (right). e) Total PBMC extract of control (Lanes 2-5) and PCOS (Lanes 6-10) subjects were run on a single gel for direct comparison by western blotting analysis and developed against *SOD2* (sc-30080, Santacruz) and Catalase (sc-50508, Santacruz). Histone H2B (sc-8651, Santacruz) was used as the endogenous control for comparison. f) Plasma proteins of control (Lanes 2-5) and PCOS (Lanes 6-10) subjects were developed against *SOD3* (sc-67089, Santacruz) and Catalase (sc-50508, Santacruz). A silver stained gel was used as endogenous control.

Impaired H₂O₂ levels characterize PCOS patients:

Our data indicated that the level of H₂O₂ in plasma of PCOS women (3.74±3.14 μM) was significantly reduced in comparison with the controls (5.588±0.94 μM), (t =2.462, p=0.0184). The mean difference between the groups was -1.844 ± 0.7492 (95% CI- -3.360 to -0.3289, R²= 0.1345) (Fig. 4 a & b). Since H₂O₂ is the product of SOD, we analysed the transcript and protein expression of SOD2 in whole blood. Surprisingly, our results reveal a significant (p<0.05) upregulation of SOD2 in PCOS women with a fold change (log2RQ) of 2.31(p<0.0001) when compared to control women (Fig. 4 c & d). The increased expression of SOD2 in whole blood of PCOS women was consistent in the protein level also (Fig. 4e). In addition, our results on differential expression of SOD3, the extracellular SOD (ecSOD) which is responsible for H₂O₂ generation in plasma, revealed mildly higher expression in PCOS (Fig. 4f). Interestingly, the protein expression of catalase, the enzyme breaking down H₂O₂ was found to be substantially increased in peripheral blood of PCOS women (Fig. 4e) thereby explaining the lowered H₂O₂ status in PCOS women.

Discussion

Reactive oxygen species mediate many functions in reproduction and are implicated in infertility, recurrent abortion, pre-eclampsia, CVDs and PCOS [33]. In the present study we focused our efforts in deciphering the regulators of NO synthesis in PCOS women. We found a significant reduction in circulating level of NO end product (NOx) in PCOS women compared to controls. Previous studies have reported reduction in NO in Turkish population [27] while Gao et al. [34] showed increased levels of NO in non-obese Chinese PCOS women. Meanwhile a couple of other studies reported no significant difference in NO levels among PCOS patients and controls [35, 36]. Further insights on NO levels can be achieved by assessing the mechanism which maintain circulating NOx homeostasis in PCOS women. Decreased expression of eNOS and iNOS can be an explanation for our observed reduction in NOx metabolites in PCOS women. Earlier reports had shown that iNOS derived NO regulates mouse oocyte maturation [37] as well as NO-Treg generation [38]. We have recently reported lowered Tregs in PCOS [39] and thus our observed low iNOS expression assumes larger significance as it would add to the burden of reduced FOXP3 expression further compromising Treg generation. eNOS expression has been shown in PBMC especially in neutrophils and monocytes [40, 41]. Decreased expression of eNOS can further augment the NOx deficiency due to reduced iNOS in PCOS women. Increased expression of eNOS and iNOS is required for tissue remodelling, vasoregulation and immunosuppression at the time of embryo implantation and marks a key factor for a successful pregnancy [42]. Therefore, our observed low NO in PCOS would be one of the causative factors compromising fertility in PCOS women.

In addition, NO synthesis necessitates uptake of the semi-essential, cationic amino acid L-arginine, hence serving as the key substrate level the regulation point. A pilot study involving metabolic profiling had shown that levels of both arginine [43] and citrulline are significantly reduced in PCOS women. The modulation of arginine bioavailability can occur at degradation, synthesis or transport level. The over expression of Arginase 1 (ARG1), the enzyme involved in arginine breakdown to ornithine and urea can deplete the substrate pool of L-arginine that would otherwise be available to NO synthase. Thus, the increased Arginase 1 observed in PCOS patients would limit arginine bioavailability for NOS action. On the contrary, Argininosuccinate lyase (ASL) can replenish the arginine substrate pool by reversibly breaking down argininosuccinate (ASA). Loss of ASL would lead to abridged NO synthesis, due to both impaired endogenous arginine synthesis and an inability to employ extracellular arginine for NO production [44]. Consequently, the decrease in ASL transcript

in our study group can also add to the plunge of NO. Transport of L-arginine mediated by the activity of high-affinity cationic amino acid transporters 1 (hCAT-1) and 2 (hCAT-2). hCAT-1 is encoded by *SLC7A1* gene whose promoter activity is highly enhanced by insulin [45]. The insulin insensitivity in PCOS women can be a strong ground for decreasing *SLC7A1* transcript and arginine transport in PCOS women in our study. Since NO is a short lived free radical, usually therapeutic interventions for NO deficiency is achieved by L -arginine supplementation. Long-standing L-arginine therapy was reported to improve gonadal dysfunction under PCOS [46]. The aberrant arginine pathway hence implicated by our study in PCOS women can have significant clinical implications.

Asymmetrical dimethylarginine (ADMA), guanidine substituted substrate analogue of L arginine impairs NO synthesis by competing for endogenous nitric oxide synthase. ADMA belongs to group of methylarginines derived from the degradation of methylated proteins. In addition to higher symmetric dimethylarginine (SDMA) [47], ADMA levels have been shown to be significantly higher in PCOS women [48-51]. DDAH2 enzyme plays a pivotal role in clearance of ADMA in endothelial cells [4]. The reduction of *DDAH2* in our study can create alterations in DDAH/ADMA/NOS/NO pathway, accumulating ADMA and in this manner inhibiting NOS and NO synthesis. Higher expression of *PRMT1*, (enzyme deriving methyl group from S-adenosylmethionine for ADMA synthesis) also can direct the metabolic pathway to enhanced synthesis of large amounts of ADMA.

Another very important finding from the study was the observed low circulating levels of H_2O_2 in PCOS women. Short exposure of H_2O_2 on endothelial cells can enhance NO synthesis whereas excess exposure to H_2O_2 reduces $NO^\bullet$ Bioavailability [52]. Our data connoted that H_2O_2 levels in PCOS could play a role in regulating NO synthesis. H_2O_2 is converted to water by glutathione peroxidase (GPx) or catalase. The reduced H_2O_2 levels in PCOS reported here could be an impact of efficient clearance mechanism of H_2O_2 by elevated catalase expression. This specific result is corroborated by reports of Bausenwein et al., showing increased activity of catalase in follicular fluid of PCOS women [53].

Fig. 5. Cartoon depicting the differential Nitric Oxide metabolic pathway in PCOS. Under PCOS condition, the level of $NO^\bullet$ is reduced. The reduction in $NO^\bullet$ can be attributed to two factors. 1. Arginine bioavailability: Arginine bioavailability may be limited as the transcripts involved in arginine synthesis (ASL) and transport (SLC7A1) are under expressed while degradation (ARG1) is over expressed. 2. Nitric oxide synthase (NOS) concentration/activity: Transcripts of *NOS2* and

NOS3 are under expressed in PCOS. The activity of NOS may be inhibited by low levels of hydrogen peroxide (attributed to high catalase levels and high ADMA (resulting from over expression of *PRMT1* involved in ADMA synthesis and under expression of *DDAH1* involved in ADMA degradation)). The low $NO^\bullet$ can lead to compromised endothelial and immune functions in PCOS patients.

Our microarray expression profiling (data not shown) and real time PCR experiment in peripheral blood of PCOS women has revealed an increased expression of *SOD2*, the principal enzyme involved in H_2O_2 synthesis. SOD activity in PCOS serum has been reported with contradicting results [5, 12, 54-56]. The higher expression of SOD implies higher antioxidant activity in PCOS to combat higher intrinsic reactive oxygen species induced oxidative load [57]. Thus, our results showing higher expression of SOD2 as well as SOD3 expression represent the body's local defence mechanism to alleviate higher levels of ROS produced in PCOS. We presume that the increased expression of antioxidant enzymes as SOD2/3 and catalase are responsible for the reduction of H_2O_2 under PCOS.

The reduction of NOx in peripheral blood can have extensive implications in PCOS. Niedbala et al. have reported $NO^\bullet$ to induce $CD4^+CD25^+Foxp3^-$ regulatory T cells ($NO^\bullet$ -Tregs) with suppressive properties in peripheral blood [38]. In our previous study we showed that peripheral pool of PCOS women contained low T-regulatory cells (Tregs) [39]. This is a strong implication that lowered NOS activity on account of decreased *iNOS/eNOS* expression in PBMC of PCOS patients contributes to decreased $NO^\bullet$ -Tregs and exacerbates Treg depletion due to lowered FOXP3. Low NOx lead to compromised uterine receptivity in PCOS women and this is substantiated by reports of increased uterine iNOS at the time of implantation [58]. High levels of iNOS at the time of implantation can be a strong indication of higher number of Tregs at implantation for enabling immune tolerance. Our observations and implications are represented in Fig. 5. NO is an important factor determining IVF success rates [59]. The report is of utmost importance as a recent study has reported that the IVF success rates in obese PCOS women is low on account of higher miscarriage risk [1]. Hence, NOx insufficiency in PCOS women has to be addressed at an early level. Therapeutic interventions can be achieved through L-arginine supplementation, since NO is short lived. Contemporary and alternative approach would suggest PCOS women to incorporate L-arginine rich foods (peanuts and walnuts, meats, seafood and legumes such as soybean and chickpeas) in their diet.

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Disclosure Statement

The authors declare no competing financial interests.

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