Original Article

Gene Expression and Plasma Level of CuZn and Mn Superoxide Dismutase in Iraqi Women with Polycystic Ovary Syndrome

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Abstract

Background: Polycystic ovarian syndrome (PCOS) is an endocrinopathy disorder that affects women worldwide and is linked to an etiological factor as well as pathophysiology. **Objective:** The purpose of this study was to determine the association between superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2) gene expressions and SOD enzyme activity in PCOS patients. In this study, 75 women were diagnosed with PCOS by Rotterdam criteria, and control healthy women with normal menstrual cycles and no signs of hyperandrogenism were included. Patients were separated into two subgroups according to their administration of metformin drug. **Materials and Methods:** CuZn SOD and MnSOD enzymes activity was determined based on the ability of the enzyme to inhibit the autoxidation of pyrogallol, and total oxidant status (TOS) was examined in the plasma using Erel method. mRNA level of SOD1 and SOD2 was evaluated in the blood sample via qPCR. **Results:** SOD enzyme activity was significantly higher in the patients' group than in the controls ($P < 0.0001$), along with a significant increase in *SOD2* gene expression ($P < 0.01$). In patients treated with metformin, gene expression of SOD2 was significantly increased ($P \leq 0.05$) comparing with patients without treatment, with increased enzyme activity (not significant). However, the SOD1 activity was significantly decreased ($P < 0.01$) with increased SOD1 expression in patients treated with metformin. In addition, TOS was increased in the patients' group than in the controls and decreased in patients treated with metformin than in untreated patients with metformin. **Conclusion:** The results revealed a significant association between PCOS and a higher level of enzyme activity and expression. Treatment with metformin drug was related to a higher level of activity and expression of SOD2, while lowering the expression of SOD1, which suggests that oxidative stress might be involved in the development of this syndrome.

Keywords: Polycystic ovarian syndrome, SOD1, SOD2, total antioxidant status

Introduction

Polycystic ovary syndrome (PCOS) is an endocrinology, reproductive metabolic disorder that affects 5%–15% of women in the world. It is thought to be the main cause of anovulatory infertility. PCOS is a syndrome and chronic systemic disease associated with hyperandrogenemia, hyperlipidemia, insulin resistance (IR), increased risks of type 2 diabetes, endometrial cancer, chronic inflammation, cardiovascular disease, and increased oxidative stress.[1] Although the pathophysiology of PCOS is undetermined, research suggests that it has a complex, multivariate etiology caused by the combinations of intrauterine and various genetic and environmental variables.[2] PCOS is more frequent in obese and overweight women, which can result in dyslipidemia and myocardial infarction[3]; it also causes an increase in the androgen production, which impairs metabolic and reproductive function.[4] Syndromes involving the hypothalamus, pituitary glands, and ovaries are caused by excessive androgen production. The fundamental cause of PCOS is an increase in gonadotropin hormone, which leads to an increase in the luteinizing hormone that encourages the ovaries to release more androgens (usually testosterone).

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This syndrome was also linked to chronic inflammation and oxidative stress. It has been found that circulating oxidative indicators are much higher in women with PCOS compared to normal women and are thought to be capable of inducing PCOS pathogenesis.[1] The earliest and most effective enzyme in the antioxidant defense mechanism is superoxide dismutase (SOD). Its function is to dismutase the superoxide anions to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), which are then removed by catalase (CAT) or glutathione peroxidase (GPx) and by regulating intracellular reactive oxygen species (ROS). SOD is found in humans in three types: Copper/zinc superoxide dismutase (CuZn SOD) enzyme, MnSOD enzyme, and extracellular SOD enzyme, respectively.[5] CuZn SOD found in the cytosol is encoded by the superoxide dismutase 1 (*SOD1*) gene, and manganese superoxide dismutase (MnSOD) found in mitochondria is encoded by superoxide dismutase 2 (*SOD2*) gene. SOD enzyme activity was increased as a result of oxidative stress and elevation of oxidants.[6]

Based on our knowledge, this is the first study dealing with CuZn SOD and MnSOD expression in the blood samples from PCOS women; other studies evaluated SOD enzyme expression in follicular fluid[7] and placental tissue samples.[8] Biguanides, insulin-sensitizing drugs, are used to treat PCOS because the condition is linked to IR, affecting PCOS development. Metformin (N, N′ dimethylbiguanide) becomes the common drug recognized and utilized in this context. Metformin has been demonstrated to restore sexual cycles and prolong early pregnancy in PCOS women. Metformin can also link with several pathways (like prostaglandin and nitric oxide) and act as a ROS scavenger.[9] However, metformin is being utilized in clinical trials despite a lack of knowledge of the underlying process.

The current study aimed to estimate the association of SOD1 and SOD2 activity and gene expression in the patients diagnosed with PCOS disease and the influence of metformin drug on their levels.

Materials and Methods

Sample collection

This study was conducted as a case and control study; samples were collected from AL-Sadiq and Al-Mutahida gynecological private clinic in Hillah City, Iraq, during the period from November 2019 to October 2021. Samples included 75 women diagnosed with PCOS depending on the revised 2003 consensus on PCOS diagnostic criteria and long-term health risk. Patients were separated into two subgroups according to their treatment with metformin drug. Healthy women with regular menstrual cycles and no symptoms of hyperandrogenism were included in the control group. This research also excluded hyperprolactinemia because it causes a woman's menstrual cycle to stop.[10]

Sample preparation

Intravenous blood samples (5 mL) were collected into heparin tubes throughout the early follicular period of a menstrual cycle (days 2–3). The plasma of the collected blood samples was obtained by the centrifugation of the blood sample at 1000× *g* for 10 min at 4°C, the buffy coat was separated carefully, and the plasma was frozen at −20°C until the time of analysis. For RNA extraction, 250 µL of blood was added to 750 µL of TRIzol reagent (Solarbio CO., Beijing, China).

Determination of superoxide-dismutase activity through the inhibition of pyrogallol autoxidation

CuZn SOD enzyme activity was investigated in the plasma of patients and controls by using the method given in reference, [11] which is based on the enzyme's ability to inhibit the pyrogallol autoxidation. MnSOD activity was achieved by the addition of sodium cyanide (0.1 M NaCN) in order to inhibit the CuZn SOD isoform.[11,12]

Determination of total oxidant status

The total oxidant status (TOS) was determined using the method suggested in references [13,14], which is based on the oxidizing of ferrous ion-o-dianisidine complex to ferric ion by the oxidants present in the plasma. Glycerol molecules, which are plentiful in the reaction media, help enhance the oxidation process. In an acidic media, the ferric ion forms a colorful complex with xylenol orange. Color intensity was assessed spectrophotometrically and is related to the total number of oxidant molecules present in the sample.[13,14]

Assessment of *SOD1* and *SOD2* gene expression

Blood samples were collected from patients and stored in Trizol. Total RNA was extracted from the sample using the TRIzol reagent protocol. RNA was eluted in 100 µL RNAse-free water and kept at −80°C. PCR amplification was performed with SYBR Green PCR Mix, GoTaq 1-Step RT-qPCR System (Promega, USA). The primers were ordered and synthesized by Macrogen Company (Macrogen, Korea).

The sequences of the primers for *SOD1* and *SOD2* as described in reference [15] are as follow:

SOD1-F	5′-CGAGCAGAAGGAAAGTAATG-3′
SOD1-R	5′-TAGCAGGATAACAGATGAGT-3′
SOD2-F	5′-AGTTCAATGGTGGTGGTCATA-3′
SOD2-R	5′-CAATCCCCAGCAGTGGAATAA-3′

The primers for *β-actin* as a housekeeping gene are as described in reference [16] as below:

β-actin-F	5′-CCCTGGACTTCGAGCAAGAG-3′
β-actin-R	5′-TCACACTTCATGATGGAGTTG-3′

Thermal cycling conditions to cDNA quantification assays were established according to the Mic qPCR cycler

detection system (Bio Molecular System, Australia). The final volume of the reaction was 10 µL. The conditions of amplification reactions were as follow: 95°C for 20 s, denaturation; annealing at 52°C, 60°C, and 62°C for *SOD1*, *SOD2*, *and B-actin*, respectively, for 20 s for 40 cycles; and extension at 72°C for 20 s. The analysis of relative gene expression data was performed according to the fold change.

Statistical analysis

The majority of experiments were statistically assessed using one-way analysis of variance (ANOVA) and the t-test. The studies were done in triplicate, and the error bars represent the standard deviation of the means. For analysis, the GraphPad Prism7 and Excel software were employed.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 224 (18/1/2021) to get this approval.

Results

Determination of superoxide dismutase enzyme activity

CuZn SOD and MnSOD activity were examined in the patients and controls groups. Mean and standard deviation were shown in Table 1 for SOD1 and SOD2 enzymes. The results showed that CuZn SOD activity (U/mL) was significantly elevated in the patients' group than in the controls ($P = 0.0001$) but decreased ($P = 0.0196$) in patients who received metformin [Figure 1]. On the other hand, MnSOD levels in patients were substantially greater than in the controls ($P = 0.0001$) and elevated (although not significantly) in metformin-treated patients [Table 1].

Determination of total oxidant status

TOS was investigated in the plasma for patients (treated and untreated with metformin) and control groups [Figure 2]. The results represented the comparison between all patients and control group and between both types of patients. The results showed that the TOS values were significantly ($P = 0.0001$) higher in the plasma of the patients' group than in the controls. However, the values were significantly ($P = 0.0308$) decreased in patients treated with metformin [Table 2].

Gene expression of *SOD1* and *SOD2* in polycystic ovarian syndrome patients

The association between *SOD1, SOD2* gene expression and PCOS disease has been thoroughly investigated. The expression of *SOD2* gene was significantly increased (4.54 ± 6.37) ($P = 0.0118$) in women with PCOS compared to the controls, and it was higher (4.74 ± 2.346) in patients taking metformin. *SOD1* expression was increased in patients with PCOS treated and without the treatment of metformin drug (not significant) as in Table 3.

Discussion

In the present study, *SOD1* and *SOD2* genes were chosen because of their importance in maintaining cellular

Table 1: Means and standard deviation of SOD activity in the plasma for patient and control groups

Enzymes	Controls	All patients	*P* value	Patients without metformin	Patients with metformin	*P* value
CuZn SOD (U\mL)	29.68 ± 1.416	$54.77 \pm 4.796^{****}$	0.0001	64.58 ± 6.363	$45.47 \pm 4.835^{*}$	0.0196
MnSOD (U\mL)	1.931 ± 0.1801	$6.106 \pm 0.2102^{****}$	0.0001	5.818 ± 0.2684	6.153 ± 0.2727	0.3872

*Significant difference ($P \leq 0.05$)

**Significant difference ($P \leq 0.01$)

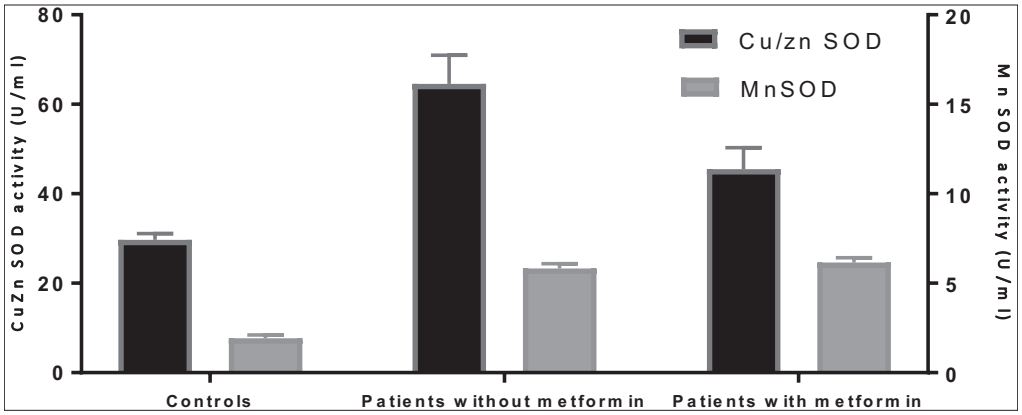

Figure 1: SOD activity in the plasma of patients (treated and untreated with metformin) and controls groups. The left scale plotted for CuZn SOD values, whereas the right scale used for MnSOD activities. Bars represent standard deviation

function and hemostasis. For example, the redox state is a major regulator of the cell activity in response to external incentives of white blood cells.[17] Although both SOD enzymes (CuZn SOD and MnSOD) participate in the same metabolic pathway, their locations and transcriptional regulation are different.[18] SOD1 expression is steady, and its products regulate the turnover of reactive species in the cytoplasm, allowing cells to maintain homeostasis, whereas SOD2 expression is sensitive to a wide range of internal and external factors and acts as mitochondria's principal defense against oxidative stress.[19]

To evaluate the involvement of oxidative stress in the disease development, oxidative stress biomarkers such as total antioxidant capacity, SOD, glutathione peroxidase, and reduced glutathione should be examined. The assessment of oxidative stress and antioxidant biomarkers has been proposed as valuable techniques for evaluating the risk of oxidative damage and related illnesses, as well as aiding in the prevention and management of oxidative disorders.[20] The findings of the CuZn SOD activity and MnSOD in PCOS patients' and control groups are shown in Table 1. These results suggested a very significant ($P = 0.01$) increase in the plasma enzyme activity in PCOS patients. Several studies have found an increase in SOD activity in PCOS women, which has been linked to a compensatory response of defense systems to increased oxidative stress.[21-23] In terms of metformin's influence on the preceding criterion, as this medication is vital in the treatment of PCOS patients, when patients were compared, this drug lowered the levels of SOD activity. Metformin does not scavenge O_2 radicals or H_2O_2, but it interacts with OH radicals and lowers oxidative stress, according to studies on its scavenging ability against ROS [hydroxyl (OH), hydrogen peroxide (H_2O_2), and superoxide (O_2) radicals].[24] In the same way,[25] we found that metformin-treated group had lower enzyme activity than the untreated subjects, but the data were not at significant levels.

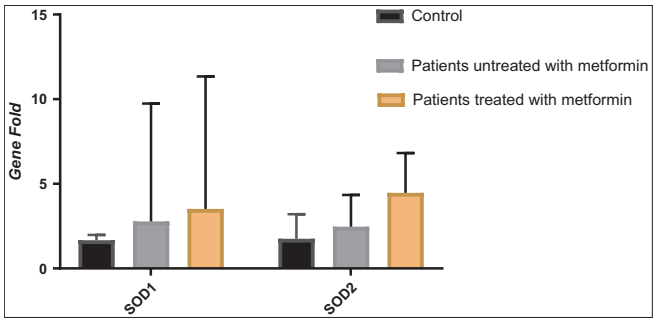

Figure 2: The expression of the *SOD1* and *SOD2* genes in the blood samples of controls and PCOS patients' groups. The values represent the means and standard deviations of comparative real-time PCR results obtained from studied samples

We performed qRT-PCR analysis on blood samples from patients and control to estimate mRNA levels of *SOD1 SOD2*. Figure 1 revealed a significant increase in the *SOD2* gene and an increase in *SOD1* expression (not significant).

The upregulation of these genes discovered in this study is most likely due to ROS accumulation, i.e., elevated TOS as shown in Table 2, which increased levels of antioxidant enzymes that act against ROS to counterbalance them and prevent their diffusion.[26] On the other hand, increasing changes in oxidative damage and cellular homeostasis commonly results in the activation or silence of genes coding antioxidant enzymes and cellular proteins.[27]

As stated before, there are no previous studies dealing with the expression of these genes in blood samples from PCOS women, with the exception of a research on *SOD1* expression in women with endometriosis (Donabela *et al.*, 2015), which found a higher level of *SOD1* in patients than in the controls.

There was a significant increase in *SOD2* expression and higher *SOD1* mRNA level (not significant) in patients treated with metformin [Table 2]. These results were agreed with Buldak *et al.* study that demonstrated the influence of metformin on increased mRNA expression level of SOD.[28]

Table 2: Means and standard deviation of TOS in the plasma for patient and control groups

Enzymes	Controls	All patients	*P* value	Patients without metformin	Patients with metformin	*P* value
TOS (µmol/L)	26.81 ± 0.791	35.05 ± 1.57****	0.0001	37.32 ± 2.122	30.98 ± 1.82*	0.0308

*Significant difference ($P \leq 0.05$)
**Significant difference ($P \leq 0.01$)

Table 3: Means and standard deviations of *SOD1* and *SOD2* expression in PCOS patients treated with metformin, without treatment, and control subjects

Genes	Controls	All patients	*P* value	Patients without metformin	Patients with metformin	*P* value
SOD1 (gene fold)	1.67 ± 0.310	3.20 ± 1.116	0.1939	2.78 ± 6.965	3.52 ± 7.823	0.7760
SOD2 (gene fold)	1.75 ± 1.46	4.54 ± 6.37*	0.0118	2.57 ± 1.872	4.74 ± 2.346*	0.0135

*Significant difference ($P \leq 0.05$)

Metformin's principal mode of action is based on the stimulation of AMP-dependent kinase (AMPK). AMPK is a serine-threonine kinase that plays an important role in cellular metabolism, fatty acid oxidation, energy outflow, and maintenance.[29] Metformin also can block mitochondrial complex I,[30] causing a decrease in the production of ROS and mitochondrial oxidative capacity.[31] Moreover, many studies have reported that metformin has the ability to reduce intracellular ROS.[32-35]

Some research indicates that the AMPK pathway reduces ROS levels by stimulating the forkhead transcription factor 3 (FOXO3).[36] FOXO is an O class protein with a winged-helix structure in its DNA binding domain (Calnan and Brunet, 2008). It modulates ROS detoxification by upregulating mitochondrial SOD2 and directly boosting transcription of the SOD2 gene, which encodes mitochondrial MnSOD.[37] Several studies reported that metformin activates FOXO3 *via* the AMPK signaling pathway, which led to the induction of FOXO3 antioxidative goal genes *SOD2*.[14,38,39]

Conclusion

In conclusion, the results revealed a significant association between PCOS disease and higher enzyme activity and expression levels. Treatment with metformin drug was significantly related with a higher level of activity and expression of SOD2, while lowering the expression of SOD1. These findings can open a new viewpoint in understanding the pathogenesis of PCOS, suggesting that oxidative stress might be involved in developing this syndrome.

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Conflicts of interest

There are no conflicts of interest.

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