Reports of Biochemistry & Molecular Biology
Vol.12, No.1, Apr 2023

*Original article*

www.RBMB.net

# Aberrant Methylation of the *SOD1* GENE, its Expression and Enzyme Activity in the Placenta of Patients with Preeclampsia

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## Abstract

***Background:*** Oxidative stress is involved in the pathogenesis of preeclampsia (PE). Dysregulation of *SOD1* may be involved in the pathogenesis of PE. We examined and compared the methylation level of the promoter region (PMR) of the *SOD1*, gene expression, and enzyme activity of superoxide dismutase (SOD) in both placenta and maternal blood in PE women.

***Methods:*** A total of 140 blood samples and 40 placental tissue samples from PE and healthy pregnant controls were studied. The PMR of the *SOD1* (Methylight PCR method), the expression (Real-time PCR), and its enzyme activity were investigated and compared in two groups.

***Results:*** The PMR of the *SOD1* gene in the placental tissue of the patients was significantly increased compared to the control group (P= 0.008); this result was accompanied by a decrease in the expression of the gene and a decrease in the activity of the SOD enzyme. Meanwhile, the PMR of the *SOD1* gene did not significantly change in the blood samples of the patients (P= 0.95), while a significant decrease in the expression of *SOD1* (without a significant change in the SOD activity) was observed.

***Conclusions:*** The results showed significant changes in the PMR of the *SOD1* gene and gene expression in placenta tissue. The results highlight the role of the placenta in complications during pregnancy and also revealed epigenetics as an important regulatory pathway in the pathogenesis of preeclampsia.

**Keywords:** Preeclampsia, *SOD1*, Methylation, Gene expression, Oxidative stress.

## Introduction

Preeclampsia (PE) is one of the most common medical complications of pregnancy that affects 5-7% of all pregnancies and is one of the leading causes of death in the prenatal period. This is prevalent in young women and more early in pregnancy. The pathophysiology of preeclampsia is not fully understood; also, clinically effective biomarkers for the prediction and prevention of preeclampsia have not yet been identified. Nowadays, the diagnosis of preeclampsia is based on two criteria: a) high maternal blood pressure (systolic > 140 mm Hg and diastolic > 90 mm Hg; with two measurements at an interval of 6 hours), and b) proteinuria > 300 mg in the urine sample (1-3). The pathogenicity of preeclampsia is still a matter of debate; Genetic, immunologic, placental, and endothelial abnormalities are involved in the development of PE. Among all the factors and assumptions that have been raised so far, the hypoxia theory is well-accepted (4, 5). In normal pregnancy, antioxidant activity increases due to hypoxia. When the

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Received: 15 Dec, 2022; Accepted: 2 Jan, 2023



[ DOI: 10.61186/rbmb.12.1.112 ]

antioxidant response is decreased, a series of events occur that eventually lead to defects in placental implantation. Decreased antioxidant activity has been observed in preeclampsia, and unlike normal pregnant women, the enzymatic antioxidant system of preeclamptic women such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and non-enzymatic antioxidants such as glutathione, which are an adaptive mechanism to enhance maternal antioxidant defenses, is reduced (6, 7).

The antioxidant enzyme system includes a number of gene families, such as the SOD family [(EC 1.15.1.1), (EC 1.15.1.1), and EC 1.15.1.1)] (8). Superoxide dismutase is an antioxidant enzyme that prevents free radical-induced damage in the body by degrading superoxide anions ($2O2^- + 2H^+ \rightarrow O2 + H2O2$). Superoxide dismutase occurs in three forms in mammalian tissues. *SOD1,* the most abundant isoform of SOD, has copper and zinc coenzymes and is found in the cytosol and mitochondrial intermembrane space. SOD1 has the highest activity among the other SODs in the cell and is responsible for the removal of superoxide radicals generated in the cytoplasm and nucleus. Copper and zinc and various combinations of the oxidative defense system can be affected under oxidative stress conditions. SOD -Cu-Zn activities are sensitive to the level of copper in the tissue because this enzyme requires copper and zinc as catalytic cofactors (3, 9).

As a member of the antioxidant system, SOD1 has a key role in the pathogenesis of PE. However, the current data regarding the role of SOD in the pathogenesis of PA are not clear (3, 10). To further investigate and uncover the mechanism of gene regulation, we conducted a comprehensive study of SOD1 during the development of preeclampsia. In the present study, we examined SOD1 status in three aspects, the presence of SOD1 gene promoter methylated DNA (by Methylight PCR method), the level of gene expression, and the level of enzyme activity in both the placenta and maternal blood samples.

## Materials and Methods

### *Study design and samples*

The present case-control study was involved 140 pregnant women who were divided into two groups: preeclamptic and healthy pregnant women, all of whom delivered by cesarean section. Informed written consent was obtained from all study participants before the study.

The diagnosis of preeclampsia was based on maternal systolic blood pressure >140 mmHg and diastolic blood pressure >90 mmHg (with two measurements at an interval of 6 hours), and proteinuria >300mg in the urine sample (1-3). The final diagnosis of preeclampsia was made by a gynecologist. Preeclampsia was diagnosed after 20 weeks of gestation with hypertension and proteinuria. Exclusion criteria in both groups included chronic hypertension, renal and liver disease, asthma, diabetes, cardiovascular disease, autoimmune disease, and twin pregnancies. In addition, the case and control groups were age- and BMI-matched. Blood and placental tissue were collected from the delivered women. Placental tissue was taken to the genetics laboratory in liquid nitrogen. Blood samples were collected in two separate tubes, one EDTA and one sodium citrate tube. The samples were immediately transferred to the laboratory.

### *Methylation Analysis*

DNA was extracted from EDTA-treated whole blood and human placental tissue stored in liquid nitrogen by the phenol-chloroform method (11, 12). Subsequently, the concentration and the purity of total DNA were determined using a *Nanodrop* spectrophotometer (ND-1000 Spectrophotometer (*Saveen Werner*, Malmö, Sweden) (13). Bisulfite conversion and subsequent purification were performed according to the manufacturer's instructions (EZ DNA Methylation-Lightning Kit (catalog no. D5006), Zymo Research, Irvine, California, USA) (14). Primers and probes specific for the SOD1 (NM_000454) and



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ALU-C4 gene promoters were designed using Beacon Designer™ (version 8.13; www.premierbiosoft.com/molecular beacons; Premier Biosoft International, Palo Alto, CA, USA) (Table 1) (15). The methylation status of the *SOD1* gene was determined using the methylation-specific polymerase chain reaction. The sequences of primers and probes are listed in Table 2. Then, the methylation status of SOD1 genes in blood and placental tissue was quantitatively compared between cases and controls. The qPCR MethyLight data were analyzed using ABI 7500 SDS version 1.3.1 software. The methylation level of the promoter region (PMR) of DNA was calculated for all samples using the following formula: [(*SOD1*/*ALU*) sample/(*SOD1*/*ALU*) positive control] x 100 (16, 17).

**Table 1.** Primer and probe sequences used for MethyLight polymerase chain reaction of the SOD1 gene.

| Gene | | Sequence (5'-3') |
|---|---|---|
| ***SOD1*** | Forward primer | GATTCGAGGTTGTCGTAGGG |
| | Reverse primer | CCGCGACTACTTTATAAACCAA |
| | Probe | 6-FAM-CGCCTCGCCCACTCTAACCC-BHQ-1 |
| ***ALU-C4*** | Forward primer | GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA |
| | Reverse primer | ATTAACTAAACTAATCTTAAACTCCTAACCTCA |
| | Probe | 6-FAM-CCTACCTTAACCTCCC-BHQ-1 |

**Table 2.** Primer sequences used for q-PCR of the *SOD1* gene.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| ***SOD1*** | 5'CGAGCAGAAGGAAAGTAA3' | 5'TGGATAGAGGATTAAAGTGAG3' |
| ***GAPDH*** | 5'CTCTCTGCTCCTCCTGTTC3' | 5'ACGACCAAATCCGTTGAC3' |

### *Gene expression Analysis*

Total RNA was extracted from buffy coat and human placental tissue stored in liquid nitrogen according to the manufacturer's protocol (RX-BON 0012). The concentration and the purity of total RNA was determined using a *Nanodrop* spectrophotometer (ND-1000 Spectrophotometer (*Saveen Werner*, Malmö, Sweden). The RNA was stored in the -80 °C until cDNA synthesis. The cDNA was prepared from total RNA using the cDNA synthesis kit (BON YAKHTE, BN-0011.37, Tehran, Iran). Quantification of SOD1 mRNA expression was determined by real-time PCR technique. The primers for amplification of the *SOD1* transcript and the specific primer sequences for the housekeeping gene are mentioned in Table 2.

### *Biochemical analysis*

Serum and supernatant were prepared from blood and tissue samples of the study participants. The SOD enzyme activity was measured by the calorimetric method using SOD activity kit, (Kiazist, Iran, KSOD96). The enzyme was reported as U/mg pf protein.

### *Statistical analysis*

The statistical analysis was performed using SPSS version 16.0. Mann-Whitney U test, independent-samples t-test analysis of variance, and logistic regression was used to compare categorical and continuous variables. The $P< 0.05$ was considered to indicate a statistically significant difference. To check the expression level of the gene *SOD1* between PE patients and healthy controls, Real-time PCR data analysis



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was performed using the Relative Expression Software Tool (REST 2009).

## Results

This study included 140 participants, 70 PE and 70 healthy matched pregnant controls. In 20 pairs of participants, both placenta and blood samples were available; for the rest samples, we had only the blood sample. The mean age of the cases was 30.71±6.14 years old and the mean age of the controls was 28.36±6.37 years, which was not significantly different. The demographic data of the study population are shown in Table 3.

Statistical analysis of the PMR of the *SOD1* promoter in the placental tissue samples from patients (PMR=277.9±162.8) showed a statistically significant difference compared with the tissue samples from the control group (PMR=173.9±58.9, P= 0.01). However, the PMR level of the SOD1 gene in the blood samples of the preeclampsia patients (PMR=121.3±115.2) compared with the control group (PMR=118.9±85.5) was not a statistically significant difference (P= 0.88) (Table 4).

**Table 3.** Demographic features of studied women

| Preeclamptic Parameter | Patients Mean ± SD (n=70) | Controls Mean ± SD (n=70) | P value* |
|---|---|---|---|
| Age(years) | 30.71±6.14 | 28.36±6.37 | 0.259 |
| Gestational age (week) | 35.61±3.56 | 38.50±1.44 | <0.001 |
| BMI (kg/m$^2$) | 35.59±13.86 | 31.79±8.67 | 0.054 |
| Systolic blood pressure (mmHg) | 148.08±17.00 | 112.85±11.56 | <0.001 |
| Diastolic blood pressure (mmHg) | 91.66±16.17 | 70.55±10.52 | <0.001 |

*Statistical analysis was performed using Kolmogorov–Smirnov test (K-S test or KS test) and independent-sample t-test

**Table 4.** The percentage of methylated ratio (PMR) SOD1 gene methylation in blood and tissue samples.

| Parameter | | Frequency | PMR Mean± SD | Minimum (%) | Maximum (%) | P value |
|---|---|---|---|---|---|---|
| **Tissue** | Patients | 20 | 277.9±162.8 | 287 | 687 | 0.01 |
| | Controls | 20 | 173.9±58.9 | 100 | 287 | |
| **Blood** | Patients | 70 | 121.3±115.2 | 14.2 | 680 | 0.88 |
| | Controls | 70 | 118.9±85.5 | 9.4 | 387 | |

We also studied *SOD1* gene expression in blood and placental tissue of PE patients compared with healthy controls. The results showed that the level of SOD1 transcript in the blood was significantly decreased compared to the control group (P< 0.05). Also, there was a significant decrease in the tissues of the pre-eclampsia group compared to the control group (P< 0.05). The *SOD1* expression decreased by 0.186 in tissue and 0.143 in blood samples.

We next examined SOD enzyme activity in supernatants isolated from placental tissue samples and blood samples of both preeclampsia patients and healthy controls. Enzyme activity was reduced in placental tissue from preeclampsia patients (0.95±0.52 U/mg protein) compared with healthy subjects (2.06±1.92 U/mg) (P= 0.020). As for enzyme activity in serum, the mean SOD activity in the patients was 10.2±23.5 U/mg protein compared with 13.4±21.8 U/mg protein in the healthy subjects, which was not statistically significant (P= 0.40).



[ DOI: 10.61186/rbmb.12.1.112 ]

## Discussion

In the present study, we examined and compared the PMR of the *SOD1*, gene expression, and enzyme activity of SOD in both placenta and maternal blood in PE and healthy pregnant women. Our results showed significant changes in the PMR of the *SOD1* gene and gene expression in placenta tissue. On the other hand, the PMR of the *SOD1* gene did not significantly change in the blood samples of the patients, while a significant decrease in the expression of *SOD1* (without a substantial reduction in SOD activity) was observed.

Impaired oxidative stress signaling is strongly associated with preeclampsia (18). To date, genetic and epigenetic alterations have been identified that may mediate susceptibility to PE (19). In this study, we sought to gain detailed insights into the role of *SOD1* in the development of PE. Our study showed that there was a significant difference in the mean PMR of the *SOD1* gene between placental tissue samples from patients and healthy individuals. The hypermethylation of *SOD1* was correlated with decreased transcription of *SOD1* as well as decreased enzyme activity in the placental tissues of patients. Taking all these findings together, it can be concluded the hypermethylation of the *SOD1* promoter may be responsible to some extent for the decreased *SOD1* expression and enzyme activity in the placenta. Interestingly, in the placenta of women with preeclampsia, we simultaneously observed decreased enzyme activity, increased promoter methylation, and decreased transcript levels of *SOD1*. In contrast, no significant findings were detected in maternal blood. These results may shed more light on the pathogenesis of PE, especially with regard to the antioxidant aspect.

Consistent with placental tissue, the mRNA of *SOD1* was downregulated in maternal blood from PE cases. However, the methylation status of the *SOD1* promoter did not change significantly between blood samples of cases and controls. Moreover, despite the decreased expression of *SOD1*, the total activity of the enzyme SOD did not significantly change in the blood of mothers with preeclampsia. This finding suggests that decreased mRNA levels of *SOD1* do not alone lead to a change in total SOD enzyme activity, but that other mechanism needs to be fully investigated. In a similar study, Martinez-Fierro et al examined the expression levels of *SOD1* in blood samples from women with PE in the Mexican population and found no significant changes in *SOD1* gene expression (20). To our knowledge, the methylation status of *SOD1* in peripheral blood mononuclear cells (PBMCs) from PE women has not been studied previously, and our study is the first report in this field.

Promoter methylation is one of the most important forms of epigenetic regulation of gene expression (21). In this study, we found evidence for epigenetic regulation of *SOD1* by methylation in the placenta. However, another study reported decreased expression of *SOD1* transcript and protein, without significant changes in promoter methylation (22). Besides promoter methylation, other mechanisms of epigenetic control of gene expression, including noncoding RNAs, can also be postulated. For example, circular RNA fibronectin 1 (circFN1) may act as a miRNA sponge for miR-19a/b-3p and subsequently increase the activity of the enzyme SOD in the placental trophoblast of mothers with preeclampsia (23). Identification of the entire network of noncoding RNAs regulating *SOD1* and the oxidative stress pathway may open a new window to a deeper understanding of PE pathogenesis.

The *SOD1* gene does not act alone but works in pathways with different levels of interaction. These interacting molecules may also play a central role in the pathophysiology of PE. Accordingly, we have drawn putative interactions of *SOD1* using genemania (genemania.org) (Fig. 1). These interactions may be the subject of future studies to further delineate the underlying mechanisms of preeclampsia.



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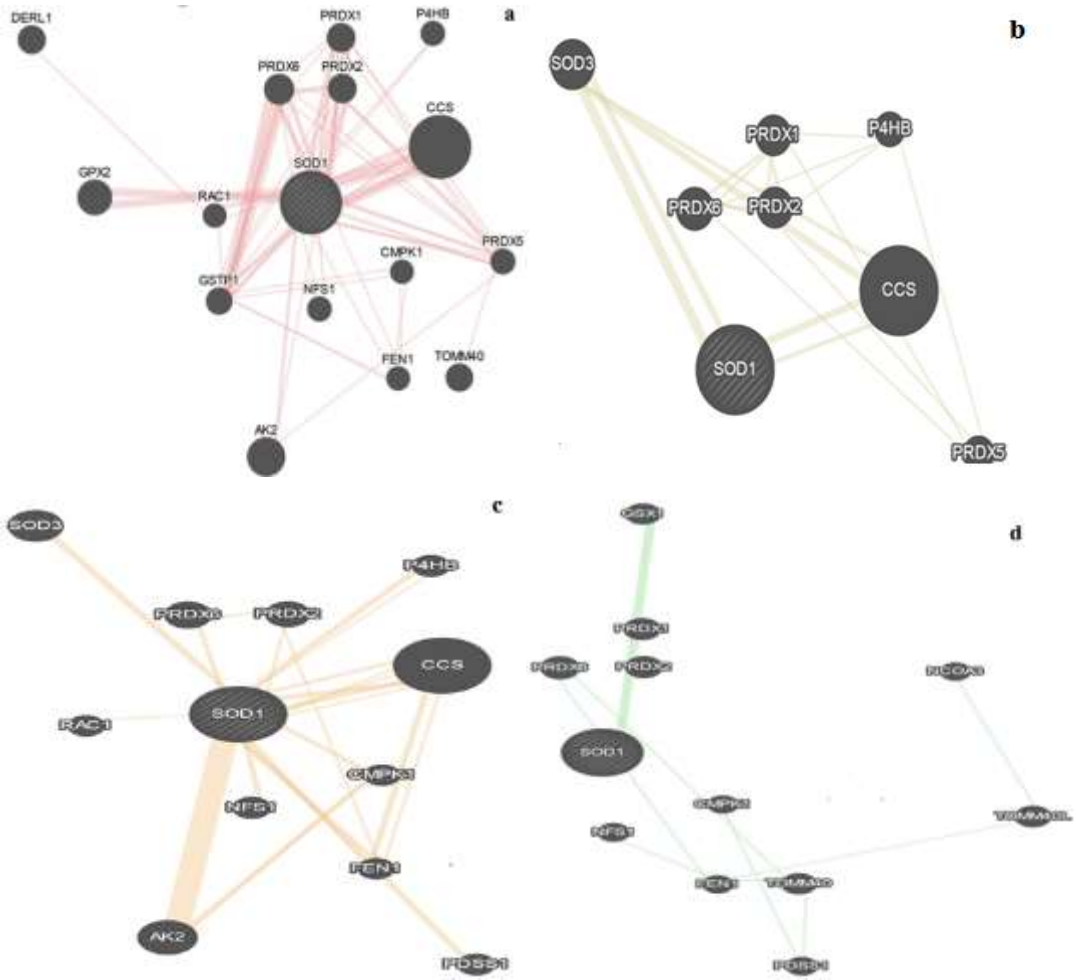


**Fig. 1.** Interactions of the SOD1 at different levels based on Genemania. a) Physical interaction; b) shared protein domains; c) Predicted interactions; d) Genetic interaction.

Here, we demonstrated the aberrations of *SOD1* at three levels of methylation, transcription, and enzyme activity in the placental tissues of pre-eclampsia women. According to our results, promoter methylation can be considered an important regulatory mechanism for *SOD1* gene expression. The results also highlighted the role of the placenta as a major target of complications during pregnancy and *SOD1* as one of the major contributors. Various aspects of *SOD1* regulation and oxidative stress need to be carefully investigated.

## Ethics approval

Procedures adopted in this study have been approved by the Ethics Committee of Kermanshah University of Medical Sciences (IR. KUMS. REC.1399.1078) and are according to the Declaration of Helsinki principles.

## Acknowledgments

The authors would like to thank the Vice Chancellor Office for Research of the Kermanshah University of Medical Sciences, Kermanshah, Iran.

## Funding

This project was funded by the Vice Chancellor Office for Research of the Kermanshah University of Medical Sciences, Kermanshah, Iran (Grant NO. 990949).

## Conflict of Interest

None.



[ DOI: 10.61186/rbmb.12.1.112 ]

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[ DOI: 10.61186/rbmb.12.1.112 ]

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[ DOI: 10.61186/rbmb.12.1.112 ]