

Impaction of factors associated with oxidative stress on the pathogenesis of gestational hypertension and preeclampsia

A Chinese patients based study

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Abstract

We aimed to investigate the effect of Kelch-like ECH-associated protein 1/NF-E2 p45-related factor 2 (Keap1/Nrf2) pathway on the biological function of trophoblast cells in oxidative stress model at the cellular level, and analyzed the expression level and clinical significance of Keap1/Nrf2 pathway and related antioxidant factors in placental tissues of Preeclampsia (PE) patients at clinical level. In present study, we found that under hypoxia/reoxygenation conditions, the activity of oxidative stress-related enzymes (CAT, GSH-Px, SOD) in HTR8/SVneo cells was significantly lower than that before treatment ($P < .01$). The activities of CAT, GSH-Px and SOD in HTR8/SVneo cells in SiRNA+H/R group decreased significantly ($P < .01$), indicating the important defense effect of Keap1/Nrf2 signaling pathway in oxidative stress. As a control group of Nrf2 SiRNA+H/R group, Si-NC+H/R group had CAT, GSH-Px and SOD activities decreasing, which was similar to that in H/R group. Moreover, the activities of oxidative stress-related active enzymes in patients with PE were further confirmed by detecting and comparing the activities of CAT, GSH-Px and SOD in placental tissues. The results showed that the activity of SOD ($P < .001$), GSH-Px ($P < .01$) and CAT ($P < .01$) in placental tissues of patients with PE were significant different from those of normal placental tissues. The expression level of Keap1 in placenta of patients with PE was slightly lower than that of normal placenta. While the expression of Nrf2 in placenta of patients with PE was significantly higher than that of normal placenta. HO-1 expression in placenta of patients with PE was significantly higher than that of normal placenta. These results implicate the importance of Keap-1/Nrf2 pathway in PE.

Abbreviations: DCFH = dichlorofluorescein, Keap1 = Kelch-like ECH-associated protein 1, Nrf2 = NF-E2 p45-related factor 2, PE = Preeclampsia, ROS = reactive oxygen species.

Keywords: Kelch-like ECH-associated protein 1/NF-E2 p45-related factor 2, oxidative stress, preeclampsia

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1. Introduction

Hypertensive disorders of pregnancy constitute an enigmatic and clinically challenging group of pregnancy complications that are responsible for a substantial burden of illness in both industrialized and less industrialized countries.^[1–3] Preeclampsia (PE) is 1 of the leading causes of maternal mortality and morbidity and a life-threatening pregnancy complication for both mother and embryos. It is characterized by new-onset hypertension and proteinuria after 20 weeks of gestation, and its incidence is about 2% to 9.4%.^[4–6] Various factors have been proposed as risk factors associated with PE including age, obesity, chronic hypertension, renal disease and diabetes mellitus.^[7–9] Despite the involvement of multiple risk factors in PE, the pathogenesis of PE development remains unclear.^[10,11] The development process of PE can be divided into the following 2 stages: the first stage is the invasion of the embryonic trophoblast into the myometrium of the mother, resulting in insufficient remodeling of the uterine spiral artery, which causes the placenta to fall into an ischemic and anoxic environment and multiple release of Inflammatory cytokines to the placenta. In the second stage, the endothelial dysfunction caused by the systemic inflammatory response and the increase in blood pressure caused by decreased renal function are the main features, eventually leading to clinical syndrome characterized by maternal hypertension and proteinuria. The only effective way to relieve maternal symptoms is delivery of the placenta and fetus.^[12–14]

One pathophysiologic mechanism associated with PE is oxidative stress, which is defined as the homeostatic imbalance within the reduction–oxidation (redox) environment that involves a dysregulation between oxidants and antioxidants.^[15–17] Oxidative stress arises during pregnancy either from an excessive production by the placental metabolism of reactive oxygen species (ROS) or from an inadequate supply of antioxidant substances.^[18–21] Endogenous ROS play a critical role in maintaining biological homeostasis and have been identified as signaling molecules in physiological and pathophysiological processes. Specifically, ROS can activate redox-sensitive transcription factors to regulate inflammatory cytokines or activate protein kinases to promote cell proliferation.

Transcription factor NF-E2 p45-related factor 2 (NF-E2 p45-related factor 2 [Nrf2], gene name NFE2L2) regulates the expression of a large network of genes encoding inducible cytoprotective proteins that allow mammalian cells and organisms to adapt and survive under various conditions including oxidative stress. Together with its principal negative regulator, Kelch-like ECH-associated protein 1 (Keap1), Nrf2 forms a molecular effector and sensor system that robustly responds to perturbations of the cellular redox balance and orchestrates a comprehensive defense program, which in turn restores homeostasis.^[22,23] The Nrf2 status affects the production of ROS by the 2 major ROS producing systems, mitochondria and NADPH oxidase. Among the Nrf2 transcriptional targets are proteins with critical roles in the generation and utilization of reducing equivalents such as NADPH and reduced glutathione (GSH), as well as thioredoxin, thioredoxin reductase, peroxiredoxin, and sulfiredoxin, which collectively provide compartmentalized redox sensing of ROS to maintain redox balance under homeostatic conditions.^[24,25]

In present study, we investigated the effect of Keap1/Nrf2 pathway on the biological function of trophoblast cells in oxidative stress model at the cellular level, and analyzed the expression level and clinical significance of Keap1/Nrf2 pathway and related antioxidant factors in placental tissues of PE patients at clinical level. The aim is to clarify the role of Keap1/Nrf2 pathway in the regulation of oxidative stress in PE patients, and provide a theoretical basis for follow-up research and drug development.

2. Materials and methods

2.1. Patients

Participants were recruited after written informed consent under a protocol approved by the Committee on Human Research at the Department of Obstetrics and Gynecology, Yancheng Maternity and Child Health Care Hospital. The number issued by the local ethics committee of Yancheng Maternity and Child Health Care Hospital was YC195892. All samples in our study were from placenta of single pregnancy. Placental tissues from normal pregnancy (n=20), and PE patients (n=20) were obtained from individuals at Department of Obstetrics and Gynecology, Yancheng Maternity and Child Health Care Hospital. Several aliquots of tissue were collected randomly from the maternal side of the placenta. The tissues were snap frozen in liquid nitrogen and stored at -80°C before use. We followed the previous published guidelines for placental examination and protocol for sampling.^[26]

2.2. Cell culture HTR8/SVneo cell hypoxia reoxygenation model

The trophoblastic oxidative stress model uses a human chorionic trophoblast cell line HTR8/SVneo in early pregnancy, which is from Dr. Laurent (Dr. Laurent's lab, UCSD). Cell culture conditions: Cultured in a 37 ° C, 5% CO₂ incubator, Gibco RPMI 1640 medium, 10% fetal bovine serum, 1% penicillin/streptomycin double antibody was required. HTR8/SVneo cells were cultured in the above cell culture manner, and the cells were cultured in a 3-gas incubator (O₂, CO₂, N₂) to detect oxidative stress related indicators. HTR8/SVneo cell hypoxia/reoxygenation (H/R) is a PE cell model, which is the most commonly used through culture with mixed gas. In this study, HTR8/SVneo cells were cultured in 2% CO₂ for 8 hours as hypoxia, and then cultured in 20% O₂ for 16 hours to reoxygenate.

2.3. Construction of Nrf2 siRNA interferes with lentiviral vector and transfects HTR8/SVneo cells

Due to the lack of a specific Keap1/Nrf2 signaling pathway inhibitor, viral transfection induces Nrf2 gene silencing in HTR8/SVneo cells to inhibit Nrf2 expression: selection of small interfering RNA (siRNA) interfering with Nrf2. The viral vector was then transfected into HTR8/SVneo cells.

2.4. Preparation of dichlorofluorescein (DCFH)

DCFDA was hydrolyzed to dichlorofluorescein (DCFH) according to a reported method. Briefly, 0.5 mL DCFDA in water (1 mmol L⁻¹) was added to 2 mL of 0.01 N NaOH and allowed to stand at room temperature for 30 min. The hydrolysate was neutralized with 10 mL PBS (25 mmol L⁻¹), pH 7.2, and was always freshly prepared prior to use.

2.5. Oxidation of DCFH

100-μL aliquot of DCFH (40 μmol L⁻¹) was added to 100 μL PBS containing Cu²⁺ or Cu⁺ and fluorescence development was monitored in an automated plate reader at 37°C (Wallac). Excitation wavelength was set at 485 nm and emission was recorded at 535 nm. At the indicated time points, 20 μL of the respective copper chelator or PBS (for control) was added to give the final concentrations.

2.6. Antioxidant status in placental tissues

Intracellular antioxidant enzyme concentrations were measured as a proxy for oxidative stress in fetal membranes using a Milliplex Human Oxidative Stress Magnetic Bead Panel (Catalog no. HOXSTMAG-18K, EMD Millipore, Merck, Barmstadt, Germany). Catalase (CAT), Glutathione peroxidase (GSH-Px) and superoxide dismutases (SOD) were measured in tissue homogenates. Samples were run in duplicates, and the manufacturer's instructions were followed for conducting this assay after reconstituting tissue lysates and diluting them in the MAPAssay Buffer. The plates were analyzed using the Luminex system. Data were normalized to total protein concentration prior to analysis and reported as median fluorescence intensity (MFI).

2.7. RNA extraction and real-time reverse transcriptase-PCR

Total RNA of tissue samples was isolated using TRIzol (Life Technologies, Inc., Rockville, MD) according to the manufac-

turer’s instructions. cDNA was generated from 1 ug of each RNA sample and a reverse transcribed using a transcription kit (Takara, Kyoto, Japan). Real-time quantitative reverse transcriptase-PCR (RT-PCR) was done in the 7300 Real Time PCR System (Applied Biosystems).

2.8. Western blotting analysis

Denatured protein samples were resolved on SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA). After blocking with non-fat milk, membrane was incubated overnight at 4°C with antibodies including Keap-1, Nrf2, HO-1 and β-actin (Abcam) followed by incubation with the antirabbit HRP-conjugated secondary antibodies (Santa Cruz, Billerica, MA). Chemiluminescence detection was performed using ECL advance Western blotting detection reagents (GE healthcare, Little Chalfont, Buckinghamshire). The relative expression was quantified by image software.

2.9. Statistical analysis

The χ^2 test or Fisher exact test were used to compare qualitative variables, while continuous variables were compared using Student *t*-test or Mann-Whitney test for variables with an abnormal distribution. Receiver operating characteristic curve analysis was used to determine the optimal cut-offs of continuous variables. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc.) and GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc.). A value of $P < .05$ was considered to be statistically significant in all the analyses.

3. Results

3.1. Identification of Nrf2 transfection efficiency in HTR8/SVneo cells

The lentiviral vector was transfected into HTR8/SVneo cells, and the optimal infection value of lentivirus with a titer of 1×10^7 TU/mL was 40; the transfection efficiency was 90% after stable transfection. The expression of Nrf2 mRNA after transfection was detected by qRT-PCR. The expression of Nrf2 mRNA in Si-Nrf2 group was significantly lower than that in Si-NC group ($P < .001$, Fig. 1A, B). The expression of Nrf2 protein was detected by Western blotting. The Si-Nrf2 group was significantly lower than the Si-NC group, and the difference was statistically significant ($P < .001$, Figure 1C). We applied DCFH that were

commonly used to detect ROS levels. The ROS level in hypoxia/reoxygenation (H/R) group of HTR8/SVneo cells was significantly higher than that in Non-treated group ($P < .01$, Figure 1D), indicating that hypoxia/reoxygenation had a significant effect on intracellular oxidative stress levels; ROS levels in Nrf2 SiRNA+H/R group were significantly higher than those in H/R group ($P < .01$), indicating that Nrf2 participates in and regulates oxidative stress levels in HTR8/SVneo cells; Si-NC+H/R group In the Nrf2 SiRNA+H/R group, there was no significant difference in ROS levels compared with the H/R group.

3.2. Detection, comparison of CAT, GSH-Px and SOD activities in different groups of HTR8/SVneo cells and the effect of Nrf2 on oxidative stress-related active enzymes

Under hypoxia/reoxygenation conditions, the activity of oxidative stress-related enzymes (CAT, GSH-Px, SOD) in HTR8/SVneo cells was significantly lower than that before treatment ($P < .01$). The activities of CAT, GSH-Px and SOD in HTR8/SVneo cells in SiRNA+H/R group decreased significantly ($P < .01$), indicating the important defense effect of Keap1/Nrf2 signaling pathway in oxidative stress. As a control group of Nrf2 SiRNA+H/R group, Si-NC+H/R group had CAT, GSH-Px and SOD activities decreasing, which was similar to that in H/R group ($P > .05$, Fig. 2).

3.3. Detection and comparison of expression levels of anti-oxidation related genes Keap1, Nrf2 and HO-1 in HTR8/SVneo cells under hypoxia-reoxygenation

HTR8/SVneo cells were cultured and isolated in normal control group and H/R group. qRT-PCR was used to detect the expression changes of antioxidant genes Keap1, Nrf2 and HO-1 at mRNA level. The results showed that the expression of Keap1 mRNA in HTR8/SVneo cells was slightly lower than that in normal controls after hypoxia-reoxygenation ($P < .05$, Fig. 3A). The expression of Nrf2 mRNA in HTR8/SVneo cells was significantly higher than that in the normal control group ($P < .001$, Fig. 3B); HO-1 mRNA expression levels were significantly higher in HTR8/SVneo cells after hypoxia-reoxygenation than in normal cells ($P < .001$, Fig. 3C). The expression of Keap1 protein in HTR8/SVneo cells was significantly lower than that in the normal control group. The expression of Nrf2 protein in HTR8/SVneo cells was significantly higher than that in normal cells after hypoxia-reoxygenation ($P < .001$, Fig. 3D); HO-1 protein expression level was also significantly higher than normal cells after hypoxia-reoxygenation ($P < .001$, Fig. 3D).

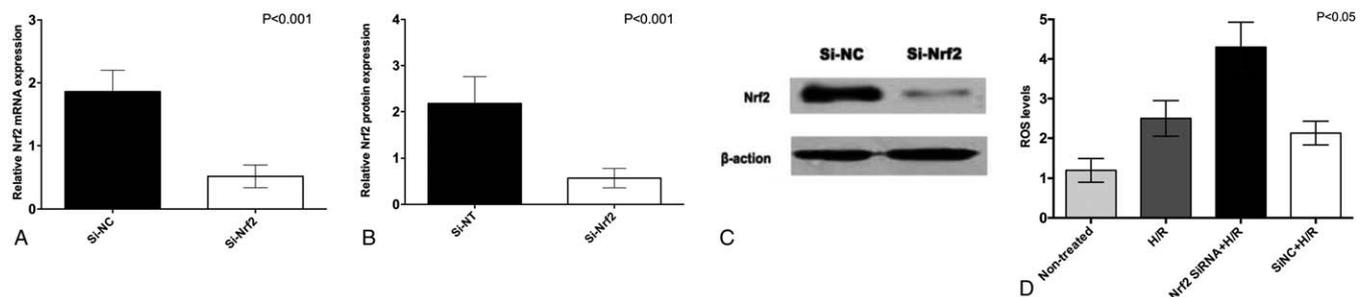


Figure 1. Nrf2 interference efficiency in HTR8/SVneo cells (normal group value is set to 1) detected by miRNA interference.

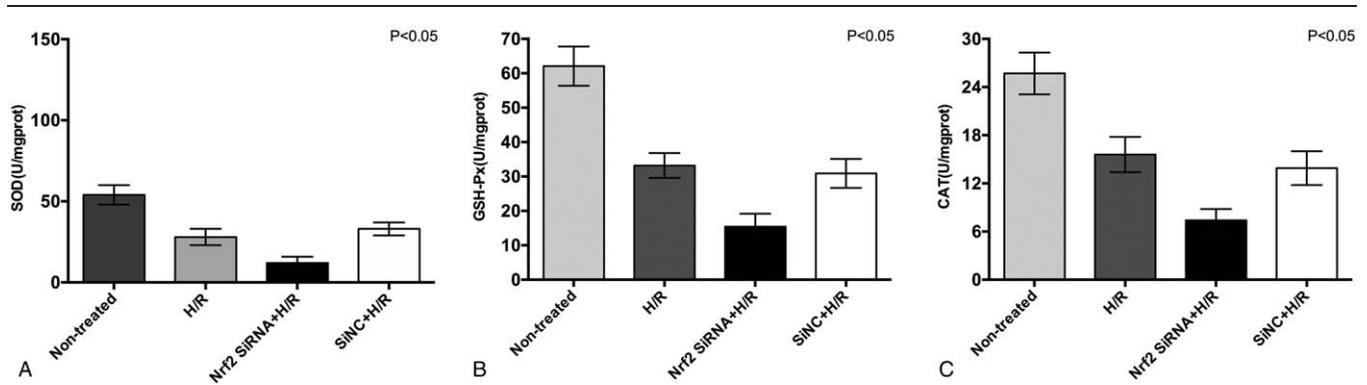


Figure 2. Comparison of SOD (A), GSH-Px (B) and CAT (C) enzyme activities using ELISA in different groups of HTR8/SVneo cells.

3.4. Comparison of clinical characteristics between normal group and PE group

There were no significant differences in age, BMI, and previous smoking history between the 2 groups of patients ($P > .05$). There were significant differences in the gestational age, systolic blood pressure, diastolic blood pressure and neonatal weight between the normal group and PE group (Table 1).

3.5. Detection and comparison of enzymes related to oxidative stress in placental tissues of patients in normal and PE group

The activities of oxidative stress-related active enzymes in patients with PE were further confirmed by detecting and comparing the activities of CAT, GSH-Px and SOD in placental tissues. The results showed that the activity of SOD ($P < .001$, Fig. 4A), GSH-Px ($P < .01$, Fig. 4B) and CAT ($P < .01$, Fig. 4C) in placental tissues of patients with PE were significant different from those of normal placental tissues.

3.6. Detection and comparison of mRNA expression levels of anti-oxidation related genes Keap1, Nrf2 and HO-1 in placental tissues of patients in normal and PE group

The mRNA expression levels of Keap1, Nrf2 and HO-1 in the placenta tissues of the 2 groups were detected by qRT-PCR. The results showed that the expression level of Keap1 mRNA in placenta of patients with PE was slightly lower than that of normal placenta ($P < .05$, Fig. 5A,B). The expression of Nrf2 mRNA in placenta of patients with PE was significantly higher

than that of normal placenta ($P < .001$, Fig. 5C,D). HO-1 mRNA expression in placenta of patients with PE was significantly higher than that of normal placenta ($P < .001$, Fig. 5E,F).

3.7. Detection and comparison of protein expression levels of anti-oxidation related genes Keap1, Nrf2 and HO-1 in placental tissues of patients in normal and PE group

The protein expression levels of Keap1, Nrf2 and HO-1 in the placenta tissues of the 2 groups were detected by western blot. The results showed that the expression level of Keap1 in placenta of patients with PE was slightly lower than that of normal placenta ($P < .05$, Fig. 6). The expression of Nrf2 and HO-1 in placenta of patients with PE were significantly higher than that of normal placenta. Tissue ($P < .001$, Fig. 6).

4. Discussion

Classically, PE is defined by de novo maternal hypertension ($>140/90$ mm Hg systolic/diastolic blood pressure) and proteinuria (>300 mg/24 h).^[27] In severe cases, the mother may develop comorbidities such as hepatic alterations (HELLP syndrome), edema, disseminated vascular coagulation (DIC), and eclampsia, particularly targeting the brain (cerebral edema).^[28,29] Over the last decade, substantial progress has been made in understanding the pathophysiology of PE.^[30–32] Oxidative damage in the placenta leads to inflammation, apoptosis, and the release of cellular debris into maternal circulation, along with several anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt1) and soluble Endoglin (sEng), cytokines, and oxidants.^[33,34] These placental-derived factors act on the maternal vascular endothelium, inducing oxidative stress and stimulating the production and secretion of pro-inflammatory cytokines, as well as vasoactive compounds. This results in a massive systemic endothelial dysfunction characterized by vascular inflammation and constriction. Indeed, oxidative stress appears to be the central component of both placental and endothelial dysfunction, the causative etiology of PE.^[35]

Oxidative stress has emerged as a putative pathogenic factor in the development of hypertension. NADPH oxidase, xanthine oxidase and mitochondrial dysfunction are involved in the increased production of superoxide anion.^[36] Moreover, more and more evidence showed that oxidative stress had an important role in hyperglycemia-induced tissue injury as well as in early events relevant for the development of Type 2 diabetes mellitus

Table 1
Demographics and clinical characteristics of all patients.

variables	Normal group	Preeclampsia group	P value
Case (n)	20	20	
Maternal age (yr)	30.2±3.8	29.3±4.6	>.05
Gestational age (wk, d)	38.6±1.6	35.3±3.1	<.05
BMI (kg/m ²)	25.4±3.2	27.1±2.1	>.05
Smoking (N)	2	5	>.05
Systolic pressure (mm Hg)	129.7±7.5	158.6±10.6	<.01
Diastolic pressure (mm Hg)	79.3±8.2	120.7±9.2	<.01
Birthweight (g)	3500.2±2.8	2601.7±8.5	<.01

BMI=body mass index.

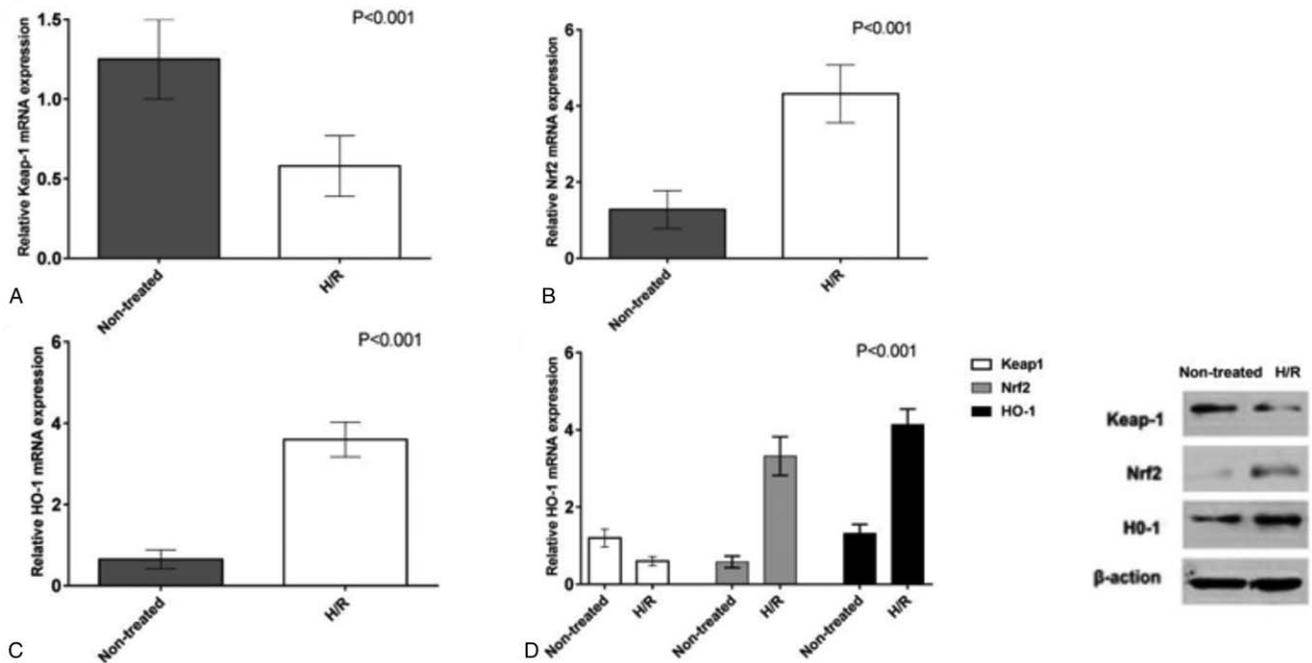


Figure 3. Detection and comparison of mRNA and protein expression levels of anti-oxidation genes Keap1, Nrf2 and HO-1 by qRT-PCR and western blot in HTR8/SVneo cells by hypoxia-reoxygenation.

(T2DM).^[37] Both primary hypertension and T2DM closely related with pathogenesis of PE development.

During the first stage of an oxidative stress, Nrf2 is activated via the disassociation of Nrf2 from its repressor protein in the cytoplasm, Keap-1, which contains cysteine residues. In detail, Keap-1 reacts with oxidative and electrophilic radicals leading to conformational changes and the release of Nrf2.^[38,39] Subsequently, the translocation of Nrf2 to the nucleus takes place and it binds to Antioxidant Response Element (ARE) resulting in the transcription of defensive genes.^[40] The activation of the transcription involves Nrf2 recognizing its own promoter and establishing an effective interaction with it and the newly formed and accumulated Nrf2 in the nucleus binds to promoters of other specific genes.^[41]

In present study, we found that under hypoxia/reoxygenation conditions, the activity of oxidative stress-related enzymes (CAT, GSH-Px, SOD) in HTR8/SVneo cells was significantly lower than that before treatment ($P < .01$). The activities of CAT, GSH-Px and SOD in HTR8/SVneo cells in SiRNA+H/R group decreased significantly ($P < .01$), indicating the important defense effect of Keap1/Nrf2 signaling pathway in oxidative stress. As a control group of Nrf2 SiRNA+H/R group, Si-NC+H/R group had CAT, GSH-Px and SOD activities decreasing, which was similar to that in H/R group. Moreover, The activities of oxidative stress-related active enzymes in patients with PE were further confirmed by detecting and comparing the activities of CAT, GSH-Px, and SOD in placental tissues. The results showed that the activity of SOD ($P < .001$), GSH-Px ($P < .01$) and CAT ($P < .01$) in placental

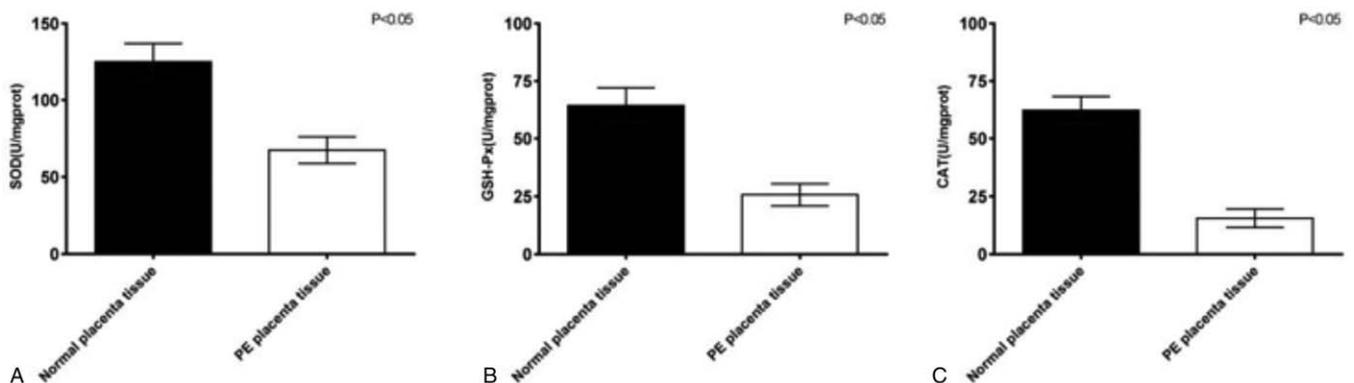


Figure 4. Comparison of SOD (A), GSH-Px (B) and CAT (C) enzyme activities using ELISA between normal placental tissue and placental tissue in preeclampsia patients.

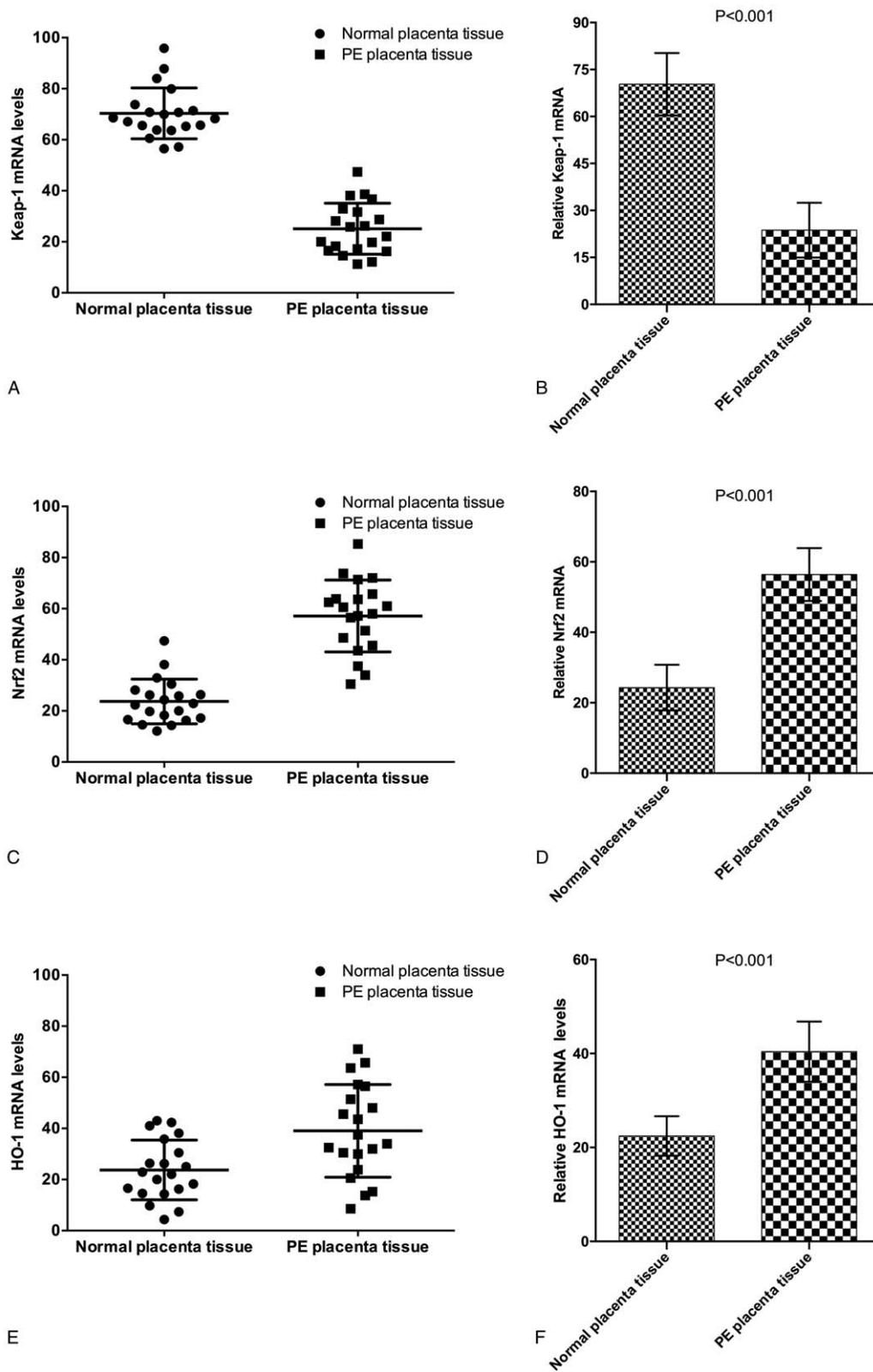


Figure 5. Detection and comparison of mRNA expression levels of anti-oxidation related genes Keap1 (A, B), Nrf2 (C, D) and HO-1 (E, F) in placental tissue of normal patients with that of preeclampsia patients by qRT-PCR.

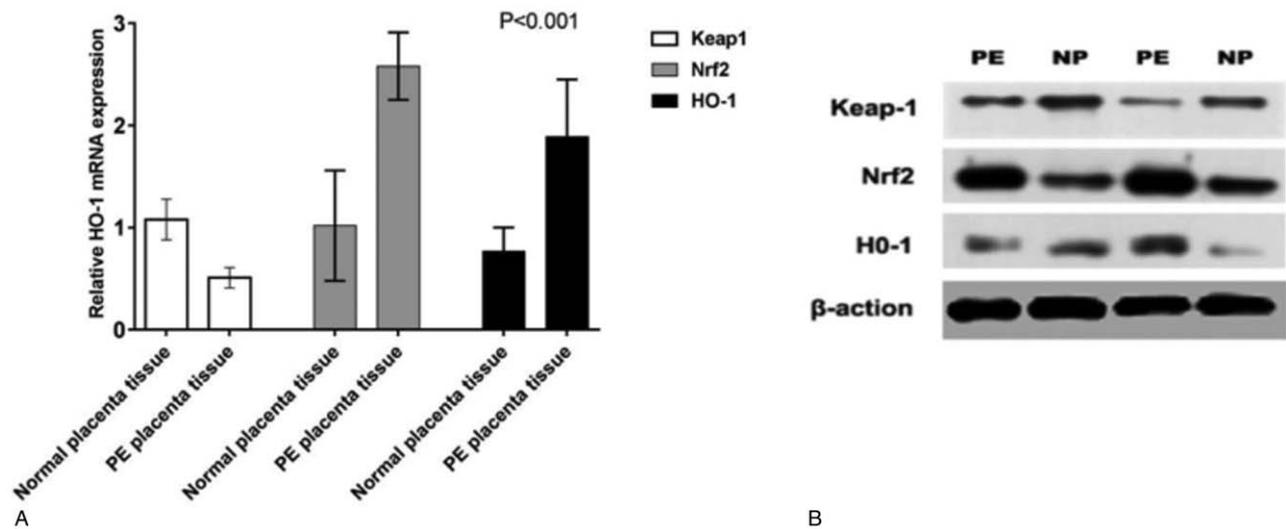


Figure 6. Detection and comparison of protein expression levels of anti-oxidation related genes Keap1, Nrf2 and HO-1 in placental tissue of normal patients with that of preeclampsia patients by western blot.

tissues of patients with PE were significant different from those of normal placental tissues. The expression level of Keap1 in placenta of patients with PE was slightly lower than that of normal placenta. While the expression of Nrf2 in placenta of patients with PE was significantly higher than that of normal placenta. HO-1 expression in placenta of patients with PE was significantly higher than that of normal placenta.

However, there are limitations of this study:

- (1) the sample size is small in this study, and further larger sample study is needed to confirm the present experimental results;
- (2) whether the decreased antioxidant enzymes have the effect on the Keap1/Nrf2 signaling pathway and the potential mechanism also needs future confirmation.

In summary, we found that the decreased antioxidant enzymes were observed under hypoxia/reoxygenation conditions in HTR8/SVneo cells. The levels of anti-oxidation related genes Keap1, Nrf2 and HO-1 in HTR8/SVneo cells also have significantly different. Moreover, the activities of oxidative stress-related active enzymes in placental tissues of patients with PE were significantly lower than those in the normal placental tissues. These results implicate the importance of Keap-1/Nrf2 pathway in PE.

Author contributions

Dongmei Qiu and Youguo Chen designed the research; Jufei Wu, Min Li, Li Wang, Xianggan Zhu and Youguo Chen conducted acquisition of data; Dongmei Qiu and Youguo Chen performed research and statistical analysis; Dongmei Qiu and Youguo Chen wrote the paper; Dongmei Qiu and Youguo Chen conducted a critical revision of the manuscript.

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