QSOX1 Regulates Trophoblastic Apoptosis in Preeclampsia Through Hydrogen Peroxide Production

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Abstract

Objective: Oxidative stress plays a significant role in the pathogenesis of preeclampsia

(PE), by inducing trophoblast cell death and consequent placental dysfunction. Quiescin sulfhydryl oxidase 1 (QSOX1) is upregulated in many types of cancer cells; it promotes disulfide bond formation as well as hydrogen peroxide (H_2O_2) production. The aims of present study are to investigate the expression pattern of QSOX1 in placentae of pregnancies complicated by PE and the role of QSOX1 in the regulation of trophoblastic function, thus providing in-depth understanding of the putative involvement of QSOX1 in the development of PE.

Methods: Human term placenta from normal pregnancies and from pregnancies complicated by PE were collected to measure QSOX1 expression and H_2O_2 levels. Down-regulation of QSOX1 in HTR-8/Svneo cells was achieved by siRNA interference. An *in vitro* cellular PE model was generated by hypoxic incubation. Protein expression levels were assessed by Western blotting, and H_2O_2 levels were determined in the cell culture medium as well as in the cell lysate. Trophoblast apoptosis was evaluated by TUNEL staining.

Results: QSOX1 was overexpressed in the PE placenta. Inhibition of QSOX1 expression in HTR-8/Svneo cells attenuated cell apoptosis and intracellular H_2O_2 levels. Hypoxia induced QSOX1 expression in HTR-8/Svneo cells and led to apoptosis of HTR-8/Svneo cells; 'knock-down of QSOX1 rescued hypoxia induced trophoblast apoptosis.

Conclusions: Hypoxia induced up-regulation of QSOX1 and a consequent elevation in intracellular H_2O_2 increased apoptosis in placentae of pregnancies complicated by PE.

Keywords: apoptosis, trophoblast, hydrogen peroxide, preeclampsia, QSOX1

Introduction

Preeclampsia (PE) is a pregnancy-specific disorder and one of the main causes of maternal and perinatal mortality [1]. It is diagnosed by newly increased blood pressure and proteinuria after 20 weeks of gestation [1,2]. Although the exact pathogenic mechanism of PE remains unclear, accumulating evidence indicate that PE is associated with abnormal placentation, endothelial dysfunction and an inflammatory response to compromised vascular remodeling, uteroplacental hypoperfusion and oxidative stress [3-8]. In addition, cytotoxic and apoptotic factors are also involved in the regulation of trophoblastic function and thus contribute to PE development [9]. Compared to normotensive pregnant woman, increased apoptosis of trophoblast cells has been observed in preeclamptic placentas [10,11], but the underlying mechanisms the elevated trophoblast apoptosis in PE placenta have yet been fully elucidated.

Quiescin sulfhydryl oxidase 1 (QSOX1) is a member of the QSOX family that belongs to a class of flavin adenine dinucleotide (FAD)-dependent thiol oxidases. It promotes the formation of disulfide bonds in peptide and protein, and reduces molecular oxygen(O_2) to hydrogen peroxide(H_2O_2) through the reaction: $2R\text{-}SH+O_2 \rightarrow R\text{-}S\text{-}S\text{-}R+H_2O_2$ [12]. It is reported that plasma levels of H_2O_2 are higher in women with PE than in normal pregnant controls [13, 14], and H_2O_2 is a known oxidative stress inducer that promotes apoptosis in primary cultured trophoblasts [15]. However, the role of QSOX1 in the regulation of H_2O_2 production in trophoblast cells remains to be addressed.

QSOX1 is highly expressed in various cancer tissues such as breast cancer, pancreatic cancer and prostate cancer [12]; this has been attributed to hypoxia [16]. Consistently, H_2O_2 is abundantly produced by cancer cells and promotes tumorigenesis by modifying the microenvironment [12,17]. Intriguingly, placentae from pregnancies complicated by PE are associated with hypoxia and elevated oxidative stress [3-6]. Moreover, QSOX1 has been found to be highly expressed in term placenta of both normal and preeclampsia pregnancies, implying that it may play a critical role in placental development [18]. However, the potential role of QSOX1 in trophoblast function and in the development of PE remains unknown. The present study investigated the expression patterns of QSOX1 in placentae of PE pregnancies, and the role of QSOX1 in modulating H_2O_2 generation and consequent apoptosis in trophoblast cells.

Material and Methods

Sample collection

Pregnant women undergoing caesarean section in the Department of Obstetrics at The First Affiliated Hospital of Chongqing Medical University were recruited; there were 20 PE complicated and 20 normotensive controls. PE was defined as de-novo hypertension (>140/90 mmHg) and proteinuria (>300 mg/day) after the 20th week of gestation. Women with pre-existing chronic hypertension, diabetes, renal or liver diseases, and gestational hypertension without proteinuria were excluded. Placental samples were collected and processed as previously reported [19], after informed

consent had been obtained. All procedures were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Cell culture

The HTR-8/Svneo human trophoblast cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai), and cultured in RPMI 1640 medium (Gibco, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) (Gemini bio-products, USA) as well as penicillin-streptomycin solution (Beyotime，Jiangsu, China) at 37°C in 5% CO_2. For hypoxia treatment, HTR-8/Svneo cells were placed in a hypoxic chamber with 1% O_2 and 5% CO_2 at 37°C. For catalase treatment, HTR-8/Svneo cells were cultured with 600 U/ml catalase (CAT) (Sigma, C9322-1G, Germany) for 24 hours [20].

siRNA interference

siRNA transfection was performed as previous established in our laboratory [21]. In brief, 1×10^5 HTR-8/Svneo cells were plated into each well in the 6-well plate. After culture for 24 hours, 50pmol/ml QSOX1-specific (siQSOX1) or 50pmol/ml scrambled siRNA (mock) oligonucleotides (GenePharma, Shanghai, China) were transfected to HTR-8/Svneo cells in the presence of Lipofectamine 2000 (Invitrogen, USA) for 24 hours according to the manufacturer's instructions.

siQSOX1-1sense: 5'-GCUGAACACAGAGGCCAAUTT-3',

antisense: 5'-AUUGGCCUCUGUGUUCAGCTT;

siQSOX1-2sense: 5'-GCUCCCACUGUUUGGAAAUTT-3',

antisense: 5’-AUUUCCAAACAGUGGGAGCTT;

siQSOX1-3sence: 5’-GCACUACAUCCUGCGGAUATT-3’;

anti-sence: 5’-UAUCCGCAGGAUGUAGUGCTT-3’;

Mock sence: 5’-UUCUCCGAACGUGUCACGUTT-3’,

anti-sence: 5’-ACGUGACACGUUCGGAGAATT-3’.

Quantitative real-time PCR

Total RNA was extracted from placentae by the use of TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s instructions, and then RNA concentration was detected by Nanodrop (Thermo Fisher Scientific, Paisley, UK). 1mg RNA were reverse transcribed to cDNA by using a RNA PCR kit (Takara, Dalian, China) according to the manufacturer’s guidelines. Quantitative real-time PCR was performed by using a qPCR SYBR Green Master (Roche, Mannheim, Germany) in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). QSOX1 transcript expression was normalized to β-actin. The primer sequences used are listed as follows:

QSOX1 forward- GCTTCTCTTACCTGGACATCAG;

QSOX1 reverse- CCTTATCTTGGCCTGGAAGTAG;

β-actin forward-TGGCACCCAGCACAATGAA;

β-actin reverse-CTAAGTCATAGTCCGCCTAGAAGCA

Western blotting

Western blotting was performed as previously described [22]. Cells and tissues were lysed or homogenized with ice-cold RIPA lysis buffer (Beyotime，Shanghai, China)

containing PMSF (100mM,Beyotime，Shanghai, China). The protein concentration was determined using the BCA Protein Assay Kit (Beyotime，Shanghai, China). 20 μg total protein of each sample was resolved on a polyacrylamide gel, and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and blocked with 5 % non-fat dried milk powder (Bio-Rad, CA, USA). Membranes were then incubated overnight at 4 °C with the primary antibodies anti-QSOX1(1:1000; Santa Cruz, California, USA)，anti-cleaved caspase 3 (1:1000;abcam, Cambridge, UK), anti-cleaved caspase 9 (1:500; Wanleibio， Shenyang, China) and anti-β-actin(1:1000;Santa Cruz, California,USA). Membranes were then washed and incubated with secondary antibody linked to HRP-linked secondary antibody (1:5000) purchased from ZSGB-BIO (Beijing, China). Images were acquired using ChemiDoc (Bio-Rad, CA, USA). Reagents for Western blot detection by enhanced chemiluminescence (ECL) were purchased from Millipore (Millipore, Massachusetts, USA) and the density of the bands was measured with Bio-Rad Image Lab Software.

H_2O_2 Measurement

Placental tissues were washed and homogenized with ice-cold PBS buffer，and then centrifuged at 13000 RPM for 20 min at 4°C. HTR-8/Svneo cells were washed and crushed with ice-cold PBS buffer using Scientz-IID ultrasonic cell disruptor （Ningbo，China), and then centrifuged at 13000 RPM for 20 min at 4°C. The supernatants or cell medium were then collected to measure H_2O_2 by using a human H_2O_2 ELISA kit (Mlbio, Shanghai, China) in accord with the manufacturer's instructions.

TUNEL assay

Cell apoptosis was measured by TUNEL staining (Beyotime, Jiangsu, China) as previously reported [23]. Briefly, cells were incubated with TUNEL reagents and 40, 6-diamidino-2-phenylindole (DAPI) solution (Beyotime, Jiangsu, China), according to the manufacturer's instructions, then observed by the EVOS FL auto imaging system (Life Technologies, Carlsbad, California, USA).

Statistics

Data were expressed as mean ± SEM, and comparisons between different groups were analyzed by independent t-test or ANOVA as appropriate, with $p < 0.05$ considered as statistically significant. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). All experiments presented in figures were repeated at least three times.

Results

PE placentae were associated with elevated QSOX1 expression and H_2O_2 production

QSOX1 mRNA levels in normal and PE placentae were determined by qRT-PCR; QSOX1 mRNA was elevated by 30.7％ in the PE group compare to the normal group ($p < 0.05$, n=20) (Fig, 1a). Consistent with this, placental QSOX1 protein expression increased 47.7％ in the PE group as compared to the normal pregnant control group (p

<0.05, n=20) (Fig.1b). Moreover, H_2O_2 levels in placental tissue were increased by 75% in the PE group, as compared to normal controls ($p < 0.05$, n=20) (Fig.1c).

QSOX1 regulated H_2O_2 production in HTR8/SVneo trophoblast cells

To investigate the regulatory effect of QSOX1 on H_2O_2 generation by trophoblast cells, QSOX1 deficient trophoblast cells were established by siRNA interference. 3 QSOX1 siRNAs were transfected to HTR-8/SVneo cells, and siQSOX1-1 and siQSOX1-3 suppressed 35.6% and 72.5% of the QSOX1 protein expression in HTR-8/SVneo cells respectively, while siQSOX1-2 failed to effectively down-regulate QSOX1 expression levels (Fig. 2a). Although H_2O_2 released into medium did not differ among groups (Fig. 2b), the intracellular H_2O_2 level was significantly reduced in QSOX1 deficient trophoblast cells, as compared to the control and mock cells ($p < 0.05$) (Fig. 2c).

Inhibition of QSOX1 suppressed caspases activation in trophoblast cells

To investigate whether QSOX1 derived H_2O_2 production was involved in the regulation of apoptosis in HTR8/SVneo cells, caspases were measured by Western blotting. In the presence of siQSOX1, cleaved caspase-9 ($p < 0.01$) and cleaved caspase-3 ($p < 0.0001$) were both significantly reduced compared to the control and mock cells (Fig.2d). The upregulated QSOX1 in placenta from pregnancies complicated by PE might thus be responsible for increased trophoblast apoptosis that has been reported previously [10, 11].

QSOX1 deficiency rescued hypoxia induced trophoblastic apoptosis

An *in vitro* model of PE was generated by subjecting HTR8/SVneo cells to hypoxia as previously discribed [3,4]. HTR8/SVneo cells transfected with siQSOX1 or control siRNA and treated with CAT were incubated in 1% O_2 for 12 hours. The Western blotting results showed that QSOX1 expression was induced by hypoxia in a time-dependent manner ($p<0.0001$) (Fig.2e). Furthermore, both cleaved caspase-9 ($p<0.001$) and cleaved caspase-3 ($p<0.01$) protein levels were significantly suppressed by siQSOX1 transfection (Fig.2f); a similar result was observed in CAT-treated cells (Fig.2f). TUNEL staining demonstrated that hypoxia significantly induced apoptosis in HTR8/SVneo cells, however, either siQSOX1 transfection or CAT treatment markedly reduced hypoxia-induced apoptosis of HTR-8/SVneo cells (Fig. 2j-h).

4. Discussion

Oxidative stress occurs when the generation of free radicals exceeds the scavenging capacity of antioxidants. Free radical-induced damage of cells, lipids, proteins and consequent apoptosis has been linked to the etiology of various diseases including PE. An imbalance between reactive oxygen species (ROS) and antioxidants plays an important role in the pathophysiology of PE [5, 6,30]. The most common free radical species include superoxide, peroxide and reactive nitrogen species. Whilst the role of nitric oxide in PE has been intensively studied [24], the involvement and importance of H_2O_2 in the development of PE remains largely unknown.

H_2O_2 is stable peroxide radical that has been found to contribute to ischemia/ reperfusion-induced oxidative stress in the placenta [25]. Aris et al. have shown that serum levels of H_2O_2 increase in women with PE at term [26]. In the present study, we focused on H_2O_2 production in placenta, and found increased levels of H_2O_2 in PE term placenta. Excessive H_2O_2 produced by trophoblast cells might be an important initiator of disturbed trophoblast cell function and result in the development of PE.

In PE, the accumulation of ROS can be caused by either up-regulation of ROS generating enzymes, such as NADPH oxidase, uncoupled eNOS, and xanthine oxidase, or a down-regulation of antioxidants. For example, there is increasing evidence that serum SOD and catalase are reduced in PE [27,28], whereas H_2O_2 generating enzyme xanthine oxidase has been shown to decrease ~~TCL1~~ trophoblast cell viability and increase apoptosis [29].

QSOX1 inserts disulfide bonds into unfolded, reduced proteins with the concomitant reduction of oxygen to H_2O_2. Most studies of QSOX1 have focused on its disulfide bond formation capability and downstream effects in promoting cancer cell migration and tumorigenesis [16,31], suggesting that QSOX1 plays an important role in functional regulation of matrix metalloproteinases [32]. Little is known about the effects of QSOX1-derived H_2O_2 on cellular function, although it has been assumed that tumor cells may take advantage of oxidative environments. The present study is the

first to report that inhibition of QSOX1 expression in trophoblast cells significantly reduced intracellular H_2O_2, implicating QSOX1 as one of major mediators of H_2O_2. QSOX1 could be a potential interventional target for influencing H_2O_2-related oxidative stress in trophoblast cells.

Many studies have reported that excessive H_2O_2 is cytotoxic, and directly affects the viability and function of human extravillous trophoblast cells, ultimately triggering apoptosis or necrosis of trophoblast cells [15,29,33]. Furthermore, exogenous H_2O_2 induces apoptosis in term placental villous explants [34]. Our data demonstrated that deficient QSOX1 expression significantly suppressed apoptotic signaling pathways in trophoblast, and thus imply that overexpression of QSOX1 in PE placenta may lead to trophoblast apoptosis through and upregulation in the generation of H_2O_2. To further validate the putative regulatory mechanism, an *in vitro* PE model was established through hypoxia, and hypoxia not only upregulated QSOX1, but also elevated apoptosis in HTR8/SVneo cells. Most importantly, interference of QSOX1 effectively blocked hypoxia-induced trophoblast apoptosis. QSOX1 expression thus correlated with trophoblast apoptosis.

In conclusion, this study reported that QSOX1 is up-regulated in PE, probably due to hypoxia, and induces the apoptosis of trophoblast cells through H_2O_2 generation. Although *in vivo* studies have shown that antioxidant administration can alleviate PE [35], suppression of H_2O_2-related oxidative stress through modulation of QSOX1

expression or activity – to manage PE, requires further validation, initially in more reliable animal models of PE.

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Conflict of interest

None.

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Figure legend

Fig.1. QSOX1 expression and H_2O_2 levels in placentas from PE and normotensive pregnancies.

(a) mRNA levels of QSOX1 in placentas from PE and control groups. n=20, *$p < 0.05$; (b) Western blotting of QSOX1 in placentas from PE and control groups. n=20, *$p < 0.05$; (c) H_2O_2 in placenta tissue from PE and controls. n=20, *$p < 0.05$.

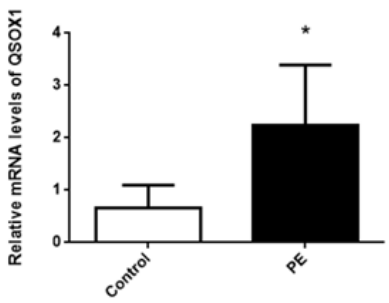
Fig.2. Suppressed QSOX1 HTR-8/Svneo cell expression attenuated cell apoptosis and was accompanied by an obvious decrease in H_2O_2 levels.

(a) QSOX1 peotein levels in HTR-8/SVneo cells after transfecting siRNAs of QSOX1 or scrambled control (Mock), *p < 0.05, ***p < 0.001; (b) H_2O_2 levels in culture medium of HTR-8/SVneo cells after transfection with siQSOX1, scrambled control (Mock) or untreated control; (c) intracellular H_2O_2 levels of HTR-8/SVneo cells transfected with siQSOX1, scrambled control (Mock) or untreated (Control), *p < 0.05; (d) Western blotting of QSOX1 and caspases in HTR-8/SVneo cells transfected with siQSOX1， scrambled control (Mock) or untreated (Control),**p

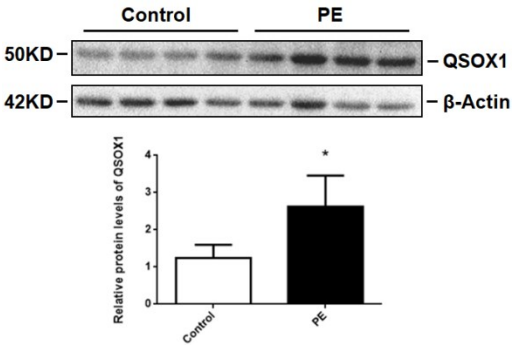
< 0.01, ****p < 0.0001; (e) QSOX1 expression of HTR8/SVneo cells was determined by Western blotting after 0,6,12 and 24 hours of hypoxia （1％O_2）, ****p < 0.0001; (f) QSOX1 and caspases expression levels in CAT （600U/ml，24h）treated and HTR-8/SVneo cells transfected with siQSOX1 siRNA，scrambled control (Mock) or untreated (Control) after 12 hours of hypoxia（1％O_2) treatment, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001;（j）TUNEL assay on normoxia and hypoxia treated HTR-8/SVneo cells, apoptotic cells were stained by TdT-mediated dUTP (red), nuclei were counterstained by DAPI（blue）, scale bar=100cm;（h） apoptotic ratio of HTR8/SVneo cells calculated by dividing TUNEL-positive cells to total cell number. *p < 0.05, ***p < 0.001, ****p < 0.0001.

Fig.1

（a）



（b）



（c）

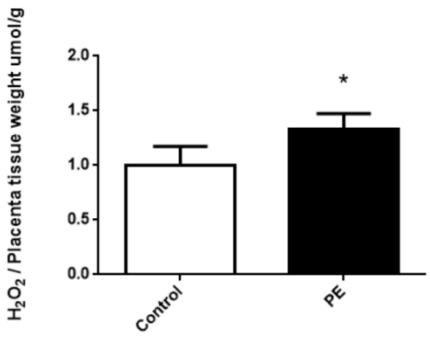
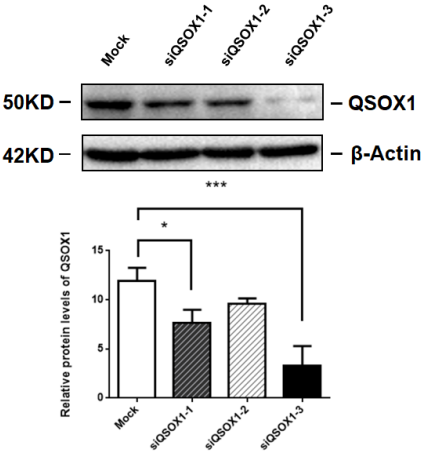

H_2O_2 / Placenta tissue weight umol/g
2.0
1.5
1.0
0.5
0.0
*
Control
PE

Fig.2

（a）


Mock
siQSOX1-1
siQSOX1-2
siQSOX1-3
50KD –
– QSOX1
42KD –
– β-Actin
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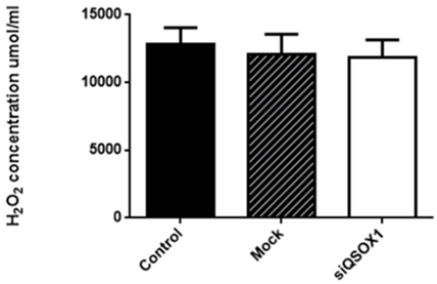
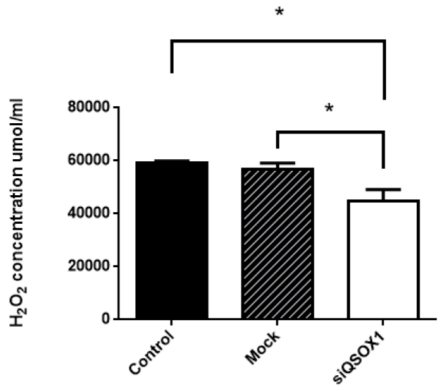
*
Relative protein levels of QSOX1
15
10
5
0
Mock
siQSOX1-1
siQSOX1-2
siQSOX1-3

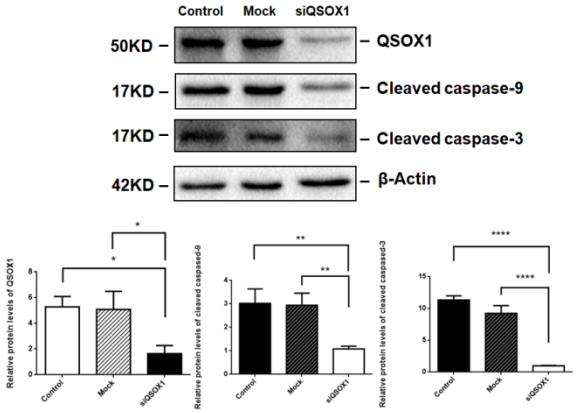
（b）


H_2O_2 concentration umol/ml
15000
10000
5000
0
Control
Mock
siQSOX1

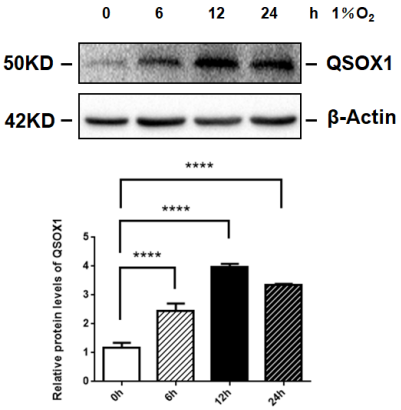
(c)


H_2O_2 concentration umol/ml
80000
60000
40000
20000
0
Control
Mock
siQSOX1

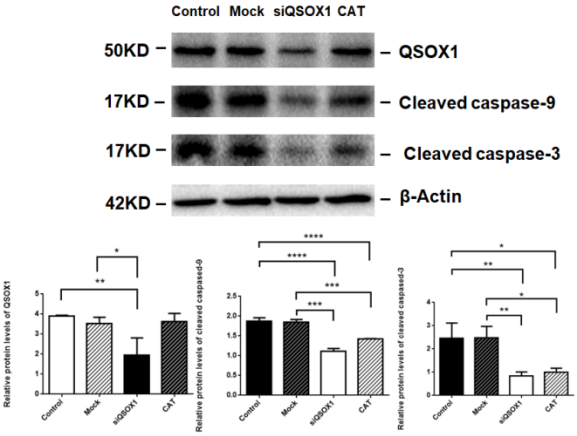
(d)


Control
Mock
siQSOX1
50KD
QSOX1
17KD
Cleaved caspase-9
17KD
Cleaved caspase-3
42KD
β-Actin

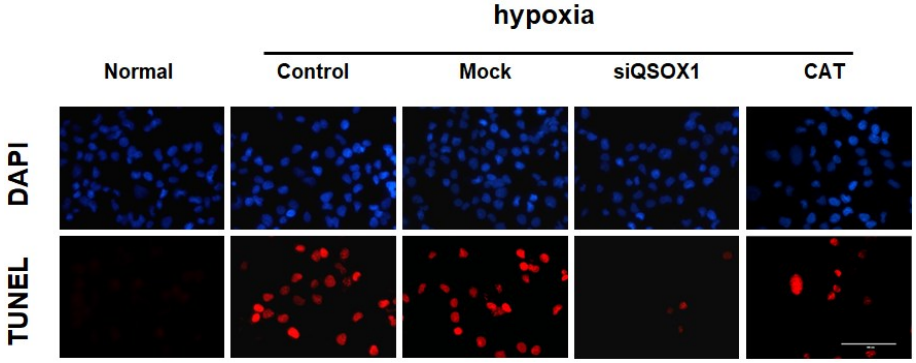
(e)


0
6
12
24
h
1%O_2
50KD
QSOX1
42KD
β-Actin
Relative protein levels of QSOX1
0h
6h
12h
24h

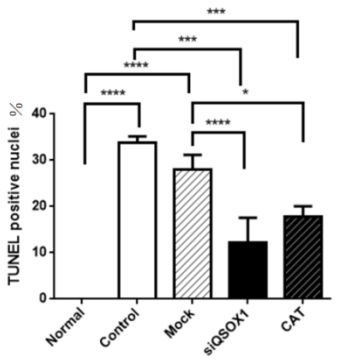
(f)


Control
Mock
siQSOX1
CAT
50KD
QSOX1
17KD
Cleaved caspase-9
17KD
Cleaved caspase-3
42KD
β-Actin

(j)


hypoxia
Normal
Control
Mock
siQSOX1
CAT
DAPI
TUNEL

(h)


TUNEL positive nuclei %
40
30
20
10
0
Normal
Control
Mock
siQSOX1
CAT
****
****
****
***
***
*