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Original Article

Protective effects of erythropoietin on U251 cells under hypoxic condition

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Abstract: Objective: We aimed to investigate the effects of erythropoietin (EPO) on the proliferation and apoptosis of U251 cells under hypoxic condition and its mechanism, in order to provide some guidance for the treatment of hypoxic-ischemic encephalopathy (HIE). Methods: U251 cell viability was measured by CCK-8 assay; change in the expression of hypoxia-inducible factor (HIF)-1α mRNA was determined by qPCR; levels of U251 reactive oxygen species and apoptosis rate were examined by flow cytometry; expression differences in transcriptome in the $CoCl_2$ and $CoCl_2$+EPO groups were detected by high-throughput sequencing; and genes that were screened out for expression differences were verified by qPCR. Results: The results of our study showed that the optimal concentration of $CoCl_2$ for establishing cell hypoxia model was 400 μmol/mL, while the optimal concentration of EPO for inducing protective effects on U252 cells under hypoxia model was 75 IU/mL. It was found through flow cytometry that the apoptosis rate in the $CoCl_2$ group was higher than that in the $CoCl_2$+EPO group ($P<0.05$). Results of high-throughput sequencing exhibited that U251 cells under hypoxic condition treated with EPO could lead to up-regulations of CyclinH, BMI1, CCL2, TRPC1, SUMO1, and ZNF302 gene expressions, which was consistent with the results obtained by qPCR. Moreover, the mRNA expressions of GLUT1 in the $CoCl_2$+EPO group were greater compared with the $CoCl_2$ group ($P<0.05$). Conclusion: EPO can promote proliferation of U251 cells and inhibit apoptosis of U251 cells in the hypoxic environment, demonstrating that EPO has protective effects on glioma cells.

Keywords: Cobalt dichloride, erythropoietin, hypoxic-ischemic encephalopathy neonate, neuroprotection

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is a type of central nervous system (CNS) injury in neonatal brain tissues. The condition occurs when there is a full or partial oxygen deprivation and limited blood flow to the brain caused by fetal distress andperinatal asphyxia during the perinatal period. The incidence and mortality of HIE are high. Some studies have discovered that erythropoietin (EPO) has neuroprotective effects, as it can improve neural function in rats after brain hemorrhage [1]. EPO is a type of glycoprotein hormone containing sialic acid. It can bind to EPO receptor and serve some protective effects on the neural system, and in particular, it can improve the brain injury caused by neonatal HIE and long-term prognosis of nerve damage [2]. Impact of EPO on HIE is believed to be the result of actions of multiple genes [3]. However, there is still some debate about the role of EPO in HIE, and few studies on the transcriptional level were conducted. Therefore, in the present study, we created hypoxic U251 human glioma cell model using $CoCl_2$, and the model was assessed by indices such as levels of hypoxia-inducible factor (HIF)-1α mRNA expression and reactive oxygen species (ROS), in an effort to examine the effects of EPO on the proliferation and apoptosis of hypoxic U251 cells. Moreover, genes with transcriptional changes were screened out using high-throughput sequencing and verified by qPCR through measurement of mRNA expression, in order to elucidate the role of EPO in neonatal HIE and neuroprotection.

Table 1. Primer sequences for each gene

Gene	Forward primer	Reverse primer
GAPDH	5'-GGAAGACTCATGACCACAGT-3'	5'-GGCAGGTTGACCTAGACGGC-3'
HIF-1α	5'-GGCAGCAACGACACAGAAAC-3'	5'-TGCAGGGTCAGCACTACTTC-3'
CCNH	5'-CTCATCGACTTAAAGACCCGC-3'	5'-GTAATTCCAGCCCTGGAGGC-3'
BMI1	5'-TGACCAGAACAGATTGATCGC-3'	5'-GCTGCTGGGCATCGTAAGTA-3'
CCL2	5'-TGCAATCAATGCCTCAGTCA-3'	5'-GGGTCAGCACAGATCTCCTT-3'
SUMO1	5'-TCAACTGAGACTTGTGAGAT-3'	5'-ATCAGCAATCTCTGACTCTCA-3'
ZNF302	5'-ACAGATGCGACACATAGCAG-3'	5'-TTGAGAGGCTGCTGACTGAT-3'
TRPC1	5'-TGGTGGCAATCGACTCTGAC-3'	5'-AGACGAGTCCTGTCATGCAG-3'
Caspase 4	5'-CCGAATATGCAGACTGCATC-3'	5'-GCTGACTCGTCATCGCTGAC-3'
SEPT7	5'-GGCAGTATCGCTGACGTGTC-3'	5'-TGTGCAAGAGGTCTCTTAGT-3'
GLUT1	5'-TGGCATCAACGCTGTCTTCT-3'	5'-CTAGCGCGATGGTCATGAGC-3'

Note: HIF, hypoxia-inducible factor.

Materials and methods

Main instruments and reagents

Main instruments used in the study were as follows: inverted fluorescence microscope (Nikon Ti200), CO_2 incubator (Binder), thermostatic water bath (Grant), high temperature and low speed centrifuge (Eppendorf 5415R), low temperature and ultra-high speed centrifuge (Beckman), Elx800 universal microplate reader (BioTek), quantitative real-time PCR (BIO-RAD), and flow cytometer (BD).

Experimental materials and reagents included U251 glioma cells (Shanghai cell bank of the Chinese Academy of Sciences), cell culture reagents (minimum essential medium, D-PBS, 0.25% trypsin, dimethyl sulfoxide, standard fetal bovine serum, and antibiotics, all from Gibco), Annexin V-FITC/PI double staining kit (BD), EPO (SihuanPharm, China), and RNA reverse transcription kit (Toyobo). Other regents, if not specifically indicated, were purchased from Sigma, USA.

Establishment of hypoxic U251 cell model

The U251 cells in logarithmic phase were seeded in a 96-well plate and divided into six groups with five replications per group. Each group was added with $CoCl_2$ solution at the final concentrations of 0 μmol/mL, 50 μmol/mL, 100 μmol/mL, 200 μmol/mL, 400 μmol/mL, and 800 μmol/mL respectively. Each group was incubated at 37°C for 24 h, 48 h, and 72 h in a 5% CO_2 atmosphere. Cellviability was measured by CCK-8 assay, and mRNA expression of HIF-1α were measured by qPCR after treatment with different levels of $CoCl_2$ in each group for 48 h. Effects of $CoCl_2$ on levels of U251 ROS and apoptosis rate were examined using flow cytometry, and the optimal concentration of $CoCl_2$ (400 μmol/mL) for simulating hypoxic condition was selected.

Treatment of hypoxic U251 cell with EPO

After successful creation of hypoxic model, the U251 cells in logarithmic phase were seeded in a 96-well plate and divided into eight groups with five replications per group. Each group was added with EPO at concentrations of 0.0 IU/mL, 4.2 IU/mL, 9.4 IU/mL, 18.7 IU/mL, 37.5 IU/mL, 75.0 IU/mL, 150.0 IU/mL, and 300.0 IU/mL respectively and was placed at 37°C for 24 h, 48 h, and 72 h in a 5% CO_2 atmosphere. Cellviability was measured by CCK-8 assay, and the optimal concentration of EPO (75 IU/mL) was determined. In the $CoCl_2$+EPO group, 400 μmol/mL $CoCl_2$ and 75 IU/mL EPO were used, while in the $CoCl_2$ group, only 400 μmol/mL $CoCl_2$ was used.

U251 proliferation measured by CCK-8 assay

The U251 cells in logarithmic phase were seeded in a 96-well plate. After group-based treatment, 15 μL CCK-8 was added into each well followed by 1.5 h incubation after mixing. The absorbance at 450 nm in each well was measured using a microplate reader to calculate cell viability. Cell viability % = ((OD value in the group with EPO) - (blank OD value))/((OD value in the group without EPO) - (blank OD value)) * 100.

mRNA expression level of genes relating to U251 cell measured by qPCR

Well-grown U251 cells under different treatments were collected for RNA extraction, and cDNAs were synthesized using a reverse transcription kit with four replications per group. qPCR was conducted according to manufacturer's instruction. The reaction volume was 25 μL

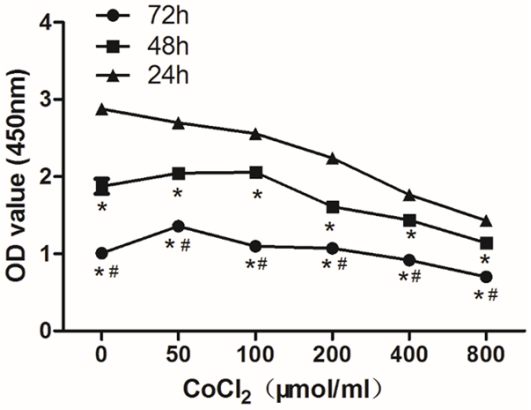

Figure 1. The absorbance values of the cells treated with different concentrations of $CoCl_2$ at different time points. OD, optical density; *P<0.05 vs. the group treated with the same $CoCl_2$ concentration for 24h; #P<0.05 vs. the group treated with the same $CoCl_2$ concentration for 48 h; the absorbance value at 450 nm in each group is displayed on the Y-axis.

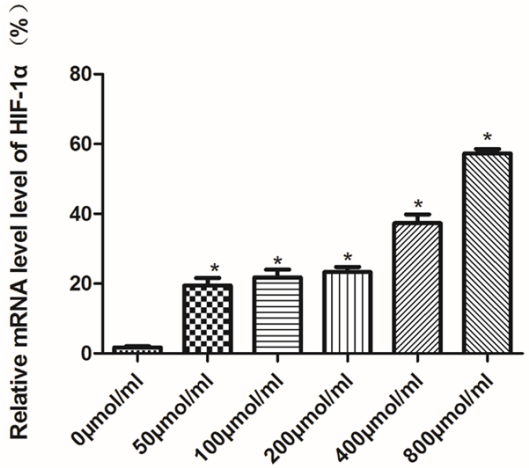

Figure 2. Effect of $CoCl_2$ onHIF-1α mRNA expression level. HIF, hypoxia-inducible factor; *P<0.05 vs. the control group treated with 0 μmol/mL $CoCl_2$ at the same time point.

including 8 μL SYBR premix, 2 μL cDNA template, 5 μL forward primer, 5 μL reverse primer, and 5 μL deionized water. Relative quantification (RQ) value, which represents the fold change of gene expression compared between the control and experimental groups, was then calculated ($RQ = 2^{-\Delta\Delta CT}$). Information of the primer sequences are listed in **Table 1**.

Effect of $CoCl_2$ on U251 ROS measured by flow cytometry

The cells were resuspended with solution of DCFH-DA probe, and changes in ROS level were observed. The results were analyzed by flow cytometry and CellQuest software. ROS level in each group was calculated.

Effect of $CoCl_2$ on U251 apoptosis rate

The cells were resuspended with solution of PI and FITC probe, and the apoptosis rate in each group was measured and analyzed by flow cytometry and CellQuest software.

Effect of EPO on U251 cell transcriptome in a hypoxic environment

Cells in the $CoCl_2$ and $CoCl_2$+EPO groups were chosen and treated by Sango Biotech, China. The experiment in each group was run in triplicate. Total RNA was extracted and measured using agarose gel electrophoresis, spectrophotometer (NanoPhotometer), fluorometer (Qubit 2.0), and bioanalyzer (Agilent 2100). After library construction and quality check, samples were sequenced, and the optical signal was converted to sequence peak through computer software to obtain the sequence information.

Statistical analysis

Statistical software SPSS 19.0 was applied for data analysis. Measurement data are presented as mean ± standard deviation. Comparisons across multiple groups were conducted by one-way ANOVA, while comparisons between two groups were performed by LSD-t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of $CoCl_2$ on U251 cell proliferation measured by CCK-8 assay

The cell absorbance value, measured by CCK-8 assay, indirectly exhibited the cell proliferation capability. Results showed that inhibition of cell growth at 24 h was not noticeable. In the groups of cells with hypoxic exposure for 24 h, 48 h, and 72 h, the cells hypoxic for 24 h had the highest survival rate, whereas the cells hypoxic for 72 h had the lowest survival rate (all P<0.05). Therefore, the cells treated with $CoCl_2$ for 48 h were chosen for the subsequent experiments (**Figure 1**).

HIF-1α mRNA expression level after treatment with CoCl2 measured by qPCR

The expression level in the control group without the addition of $CoCl_2$ was regarded as unit

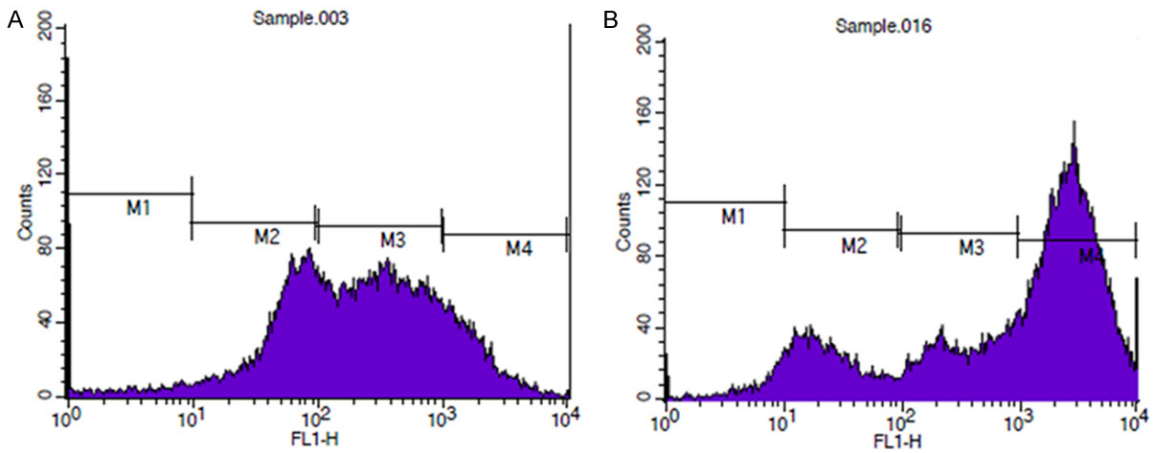

Figure 3. Effect of $CoCl_2$ on U251 ROS level. ROS, reactive oxygen species; A: ROS level in the group treated with 0 μmol/mL $CoCl_2$; B: ROS level in the group treated with 400 μmol/mL $CoCl_2$.

one, whereas the HIF-1α mRNA expression levels in the groups treated with 50 μmol/mL, 100 μmol/mL, 200 μmol/mL, 400 μmol/mL, and 800 μmol/mL $CoCl_2$ solution were 16.97±0.63, 20.29±0.41, 21.59±2.96, 35.26±1.21, and 56.8±3.63 respectively, which differed significantly from that in the control group (all $P<0.05$). The results revealed that the higher the concentration of $CoCl_2$ solution, the greater the mRNA expression of HIF-1α. Combined with the findings of the CCK-8 assay, 400 μmol/mL $CoCl_2$ for a reaction of 48 h was used for establishing the hypoxic cell model (**Figure 2**).

Effect of $CoCl_2$ on U251 ROS level measured by flow cytometry

Changes in the ROS level were examined after cells were resuspended with solution of DCFH-DA probe. The results of flow cytometry showed that the ROS level increased after 48 h of incubation with $CoCl_2$, and the group treated with 400 μmol/mL $CoCl_2$ had a much higher ROS level than the group treated with 0 μmol/mL $CoCl_2$ (55.832% vs. 13.755%, $P<0.05$) (**Figure 3**).

Effect of EPO on apoptosis rate of hypoxic U251 measured by flow cytometry

Annexin V-FITC/PI double staining assay was employed to examine the effects of EPO on the apoptosis rate of hypoxic U251 cells. The results displayed that the group treated with 400 μmol/mL $CoCl_2$ had a much higher apoptosis rate compared with the group treated with 0 μmol/mL $CoCl_2$ (9.176% vs. 3.186%, $P<0.05$). Also, the results showed that cell apoptosis occurred in both $CoC1_2$ and $CoC1_2$+EPO groups, while the apoptosis rate in the $CoC1_2$ group was greater (9.169% vs. 3.756%, $P<0.05$). This finding indicated that EPO can suppress U251 cell apoptosis under hypoxic condition (**Figure 4**).

Effect of EPO on cell proliferation of hypoxic U251 measured by CCK-8 assay

The results showed that the optimal concentration of EPO was 75 IU/mL. Therefore, $CoCl_2$ plus 75 IU/mL EPO were used for the subsequent experiments. It was found that EPO could promote U251 cell growth in a hypoxic environment (**Figure 5**).

Effect of EPO on transcriptome of hypoxic U251 measured by high-throughput sequencing

Quantitative analysis on gene level was conducted in both $CoC1_2$ and $CoC1_2$+EPO groups using HTSeq software. Compared with the $CoC1_2$ group, 558 genes were up-regulated, whereas 98 genes were down-regulated in the $CoC1_2$+EPO group (**Figure 6**).

Based on the sequencing results, six up-regulated genes were selected, which were CyclinH, BMI1, CCL2, TRPC1, SUMO1, and ZNF302 (**Table 2**).

Verification of mRNA expressions of CyclinH, BMI1, CCL2, TRPC1, SUMO1, and ZNF302 by qPCR

mRNA expressions of CyclinH, BMI1, CCL2, TRPC1, SUMO1, ZNF302 and GAPDH (internal

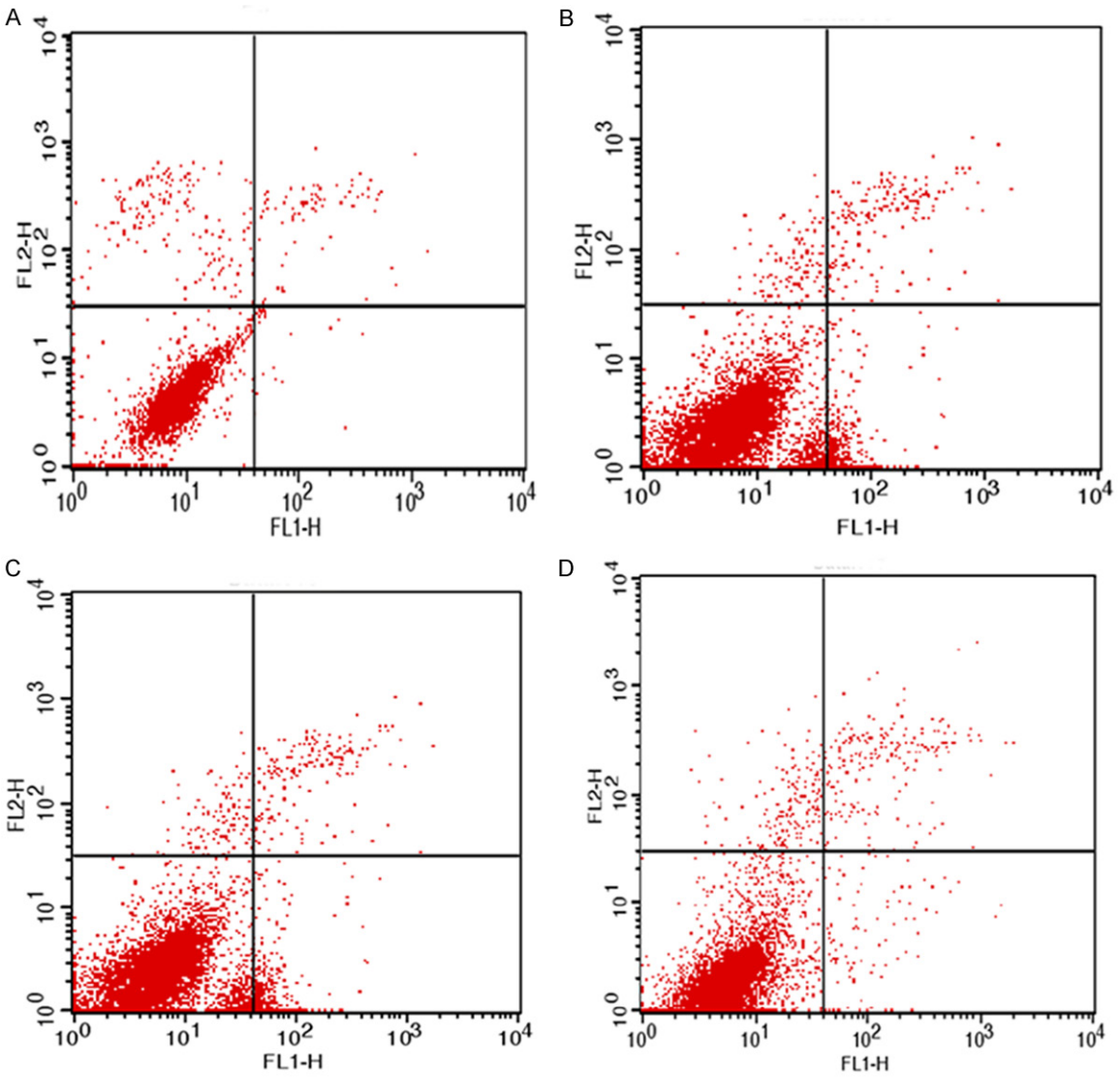

Figure 4. Effect of EPO on U251 cell apoptosis rate under hypoxic condition. EPO, erythropoietin; A: U251 apoptosis in the group treated with 0 μmol/mL $CoC1_2$; B: U251 apoptosis in the group treated with 400 μmol/mL $CoCl_2$; C: cell apoptosis in the $CoCl_2$ group; D: cell apoptosis in the $CoCl_2$+EPO group.

control gene) were measured by qPCR in both $CoC1_2$ and $CoC1_2$+EPO groups. The results showed that all these genes were up-regulated (all $P<0.05$, **Table 3**), which were consistent with the results of high-throughput sequencing.

mRNA expressions of GLUT1, caspase 4, and SEPT7 measured by qPCR

Some studies have reported that GLUT1, caspase 4, and SEPT7 also serve critical roles in HIE. Thus, we used qPCR for further investigation, and the results showed that GLUT1 was up-regulated, whereas caspase 4 and SEPT7 were down-regulated (all $P<0.05$, **Table 4**).

Discussion

HIE refers to a CNS injury in brain tissues due to a lack of oxygen and blood supply caused by fetal distress and perinatal asphyxia during the perinatal period. The condition is a severe complication in neonatal asphyxia, which has a high incidence and mortality in clinic and is one of the main causes of physical disability in children. Early intervention is an effective way for preventing nervous system sequelae of HIE. Chu et al. found that the use of EPO in treating neonatal mouse model of hippocampus damage could markedly improve neurobehavior of mice and avoid neuronal death, microglial activation, loss of mature oligodendrocyte and hip-

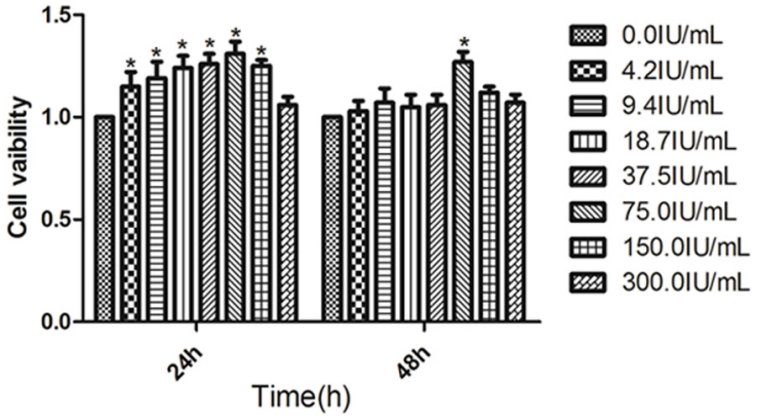

Figure 5. Effect of EPO with different concentrations on U251 cell proliferation under hypoxic condition. EPO, erythropoietin; *P<0.05 vs. the control group without EPO at the same time point.

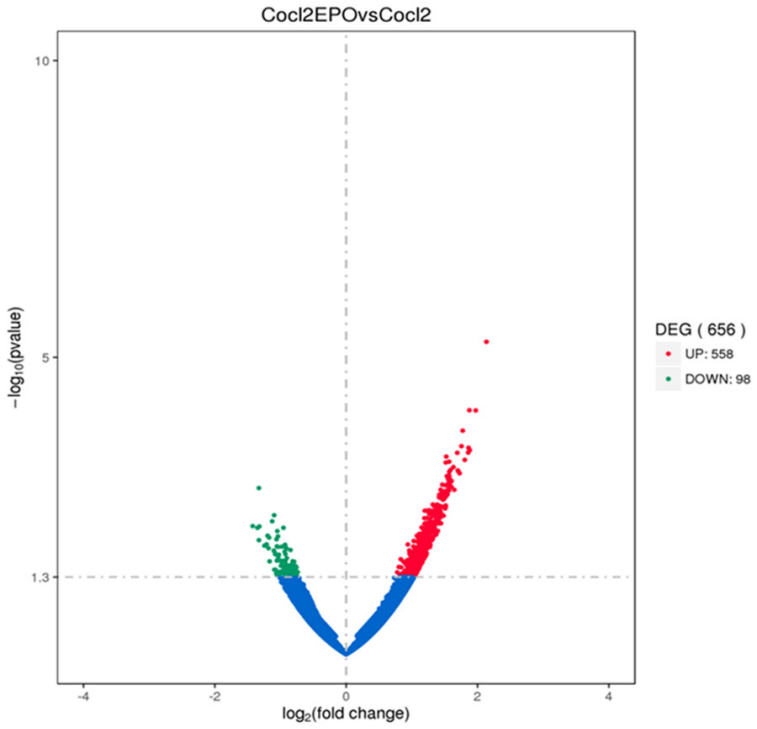

Figure 6. Volcano plot of genes with expression differences. Up-regulated genes are indicated by red dots; down-regulated genes are indicated by green dots; genes without significant differences in expression are indicated by blue dots; fold change in gene expression of different samples is displayed on X-axis (log_2 fold change), and the significance level of the expression differences is displayed on Y-axis (-log10 *P* value).

pocampal neuron induced by hippocampus damage, revealing that EPO may be an effective agent for treating neonatal HIE [4]. $CoCl_2$, a common chemical that can simulate hypoxic condition, can induce cells to generate ROS, inhibit cell proliferation, and promote cell apoptosis [5]. Therefore, in the present study, U251 cells were treated with different concentrations of $CoCl_2$, and the cell viability was measured by CCK-8 in each group at 24 h, 48 h, and 72 h respectively. Besides, HIF-1α mRNA expression in each group treated with different concentrations of $CoCl_2$ was detected by qPCR, and effects of $CoCl_2$ on U251 ROS level and cell apoptosis rate were measured by flow cytometry, in order to determine the optimal concentration of $CoCl_2$ for further investigating the protective effects of EPO on U251 glioma cell under hypoxic condition.

The results of this study indicated that EPO could promote U251 glioma cell proliferation under hypoxic condition and inhibit U251 cell apoptosis. However, the protective effects of EPO on U251 hypoxic glioma cells may be the result of regulations by multiple genes. Therefore, we verified and discussed gene expression levels in the $CoC1_2$+EPO group and $CoC1_2$ group.

CyclinH, ZNF302, BMI1, and SEPT7 are proteins closely related to cell proliferation and division [6]. Some studies have found that up-regulations of CyclinH, ZNF302, and BMI1 expressions can promote cell cycle shift and ensure smooth mitosis [7]. Moreover, increase in ZNF302 gene expression may facilitate transcription of DNA into RNA, while BMI1 gene can co-operate with c-Myc protein to repress the action of INK4a locus and promote cell proliferation [8]. Bmil gene defect can cause decreased self-renewal capability and cell proliferation of neural stem cell. SEPT7, as a

Table 2. Gene expressions of CyclinH, BMI1, CCL2, TRPC1, SUMO1, and ZNF302 measured by high-throughput sequencing

Gene	Log_2 fold Change	P	Up or down-regulation
CyclinH	1.037	0.024	Up
BMI1	1.174	0.008	Up
CCL2	0.956	0.040	Up
SUMO1	0.971	0.031	Up
ZNF302	2.136	0.027	Up
TRPC1	0.935	0.034	Up

Table 3. mRNA expressions of CyclinH, BMI1, CCL2, TRPC1, SUMO1, and ZNF302 measured by qPCR

Gene	RQ value in the $CoC1_2$+EPO group	RQ value in the $CoC1_2$ group	P
CyclinH	2.20±0.02	1.35±0.01	0.000
BMI1	2.01±0.27	1.27±0.13	0.005
CCL2	1.35±0.09	1.14±0.04	0.003
TRPC1	1.59±0.05	1.32±0.02	0.000
SUMO1	1.44±0.06	1.24±0.03	0.001
ZNF302	1.67±0.05	1.15±0.03	0.000

Note: RQ, relative quantification.

Table 4. mRNA expressions of GLUT1, caspase 4, and SEPT7 measured by qPCR

Gene	RQ value in the $CoC1_2$+EPO group	RQ value in the $CoC1_2$ group	P
Caspase 4	0.59±0.02	2.16±0.04	0.001
GLUT1	7.42±0.39	2.47±0.14	0.000
SEPT7	0.49±0.05	1.64±0.05	0.000

Note: RQ, relative quantification.

member of septin family, can keep cell in G1 phase from progressing into S phase and make the cell stay in G1/G0 phase [9]. The present study showed that after treating hypoxic U251 with EPO, there were up-regulations of CyclinH, ZNF302, and BMI1 and a down-regulation of SEPT7, suggesting that EPO can promote cell proliferation of U251 in a hypoxic environment. Some studies have reported that the proliferation of cardiomyocyte exposed to chronic hypoxia can increase to some extent after EPO treatment [10], which aligns with our study results.

Caspase-4 is an essential protein in inducing cell apoptosis [11, 12]. GLUT-1 and TRPC1 genes can stabilize saccharometabolism in brain cells and adjust Ca^{2+} level in cytoplasm, thereby enhancing brain cell's tolerance to hypoxia [13]. The present study showed that after hypoxic U251 cells were treated with EPO, GLUT-1 and TRPC1 were up-regulated, whereas caspase-4 was down-regulated, indicating that EPO can inhibit cell apoptosis of U251 under hypoxic condition. Some studies have found that the use of EPO, as adjuvant therapy for HIE, can help to reduce oxidative stress injury caused by hypoxia, thereby decreasing cell apoptosis [14]. In a study by Kushwah et al., it was found that EPO could improve the expression levels of GLUT-1 and TRPC1 in rats after brain hemorrhage and suppress neural cell apoptosis [15], which is in consistency with our study results.

Some researchers have documented that intramedullary injection of CCL2 can significantly induce activation of microglia in dorsal horn at the same side of spinal cord, indicating that after nerve damage, CCL2 in spinal cord participates in the interaction between neuron and microglia and helps microglia to devour accumulated metabolites and cellular debris [16]. SUMO-1 expression is part of the processing after eukaryotic gene translation, and the processing can decrease hypoxia-induced nerve cell damage by strengthening the stability of the protein structure [17, 18]. Our study has demonstrated that EPO can promote up-regulations of CCL2 and SUMO-1 gene expressions in U251 cells under hypoxic condition, thereby attenuating hypoxia-induced glial cell damage, which is consistent with other reports [19].

Although the protective effects of EPO on glioma cells were verified on the cellular and transcription level in the present study, adverse effects of EPO in short-term or long-term during treatment for HIE were not examined. High-throughput sequencing was used in our study to investigate the impact of EPO on U251 transcriptome. However, this technique still has some instabilities with possibilities of judgment error [20, 21]. Thus, we conducted qPCR based on the sequencing results for verification. In addition, Western blot for validating the changes in protein levels, investigation in the area of signaling pathway, and verification using transfections of eukaryotic overexpression vector or shRNA eukaryotic expression vector for each gene were not carried out.

In conclusion, EPO can promote U251 cell proliferation, suppress apoptosis, up-regulate transcription levels of CyclinH, BMI1, CCL2, TRPC1, SUMO1, ZNF302, and GLUT1, and down-regulate transcription levels of caspase 4 and SEPT7 in a hypoxic environment, which exhibitsneuroprotective effects.

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Disclosure of conflict of interest

None.

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