

## Original Article

# Protective effect of *Portulaca oleracea* extracts on hypoxic nerve tissue and its mechanism

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The aim of this study was to investigate whether *Portulaca oleracea* (PO) extracts have hypoxic neuroprotective effects and if so, by what mechanism. After being orally administrated with the PO extracts or distilled water for seven days, adult male BALB/c mice were adapted to a normobaric low oxygen environment (10% oxygen and 90% nitrogen) for different time and then were sacrificed. The mouse cortices were used for histological analysis by hematoxylin and eosin (H-E staining). The activities of pyruvate kinase (PK), phosphofructokinase (PFK), lactic acid (LD) and the level of lactate dehydrogenase (LDH) and ATP were detected, and the mRNA and protein levels of EPO in the cortices were analyzed. PC-12 cells and primarily cultured nerve cells were used for 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. The degree of LDH in the cell culture medium was tested. The results showed that the PO extracts enhanced the EPO mRNA and protein expression in the mouse cortices. Compared to the control group, the mouse in the group treated with the PO extracts by 1 g/d had significantly higher activities of PF, PFK, LDH and higher levels of ATP in the cortices, especially under the hypoxic environment for 24 hours. Histological analysis indicated that the extracts lessened the inflammation damage of the mouse brain. MTT assay results showed the PO extracts or the herb-containing serum raised the viability of the cells under the tested hypoxic conditions and decreased the degree of LDH in the culture medium in a dose-dependent manner. We thus demonstrated that the PO extracts had protective effects on hypoxic nerve tissue.

**Key Words:** *Portulaca oleracea*, hypoxia, erythropoietin, nerve cells, extract

## Introduction

*Portulaca oleracea* L. (PO) is a warm-climate annual and has a cosmopolitan distribution. It is an edible plant and is usually cut into small pieces and eaten with salt.<sup>3</sup> In the United Arab Emirates and Oman, a cultivated variety of PO is used as a vegetable.<sup>1</sup> It is also eaten as vegetable in some provinces of China.<sup>2</sup> It has been reported to be rich in  $\alpha$ -linolenic acid and  $\beta$ -carotene and has been reported to be a health food for patients with cardiovascular diseases.<sup>4</sup>

PO has many folkloric uses. It is used in the Arabian peninsula as antiseptic, anti-scorbutic, antispasmodic,<sup>1</sup> In China, it is used as an anti-bacterial and anti-viral agent, and for the treatment of viral hepatitis and in diabetes management.<sup>2</sup> Many studies have shown that this plant exhibits a wide range of pharmacological effects. Its muscle relaxant activity<sup>5-13</sup> has been extensively studied in Nigeria and Scotland. Other effects such as its antibacterial,<sup>14</sup> analgesic,<sup>2</sup> anti-inflammatory,<sup>15</sup> and wound-healing<sup>16</sup> activities have been reported. In our previous studies, we found that one of the PO extracts lengthened the survival time of hypoxic mice exposed to normal pressure closed bottles<sup>17</sup>. Brain is usually the most sensitive tissue to hypoxia in the body, so that neuroprotective substances have much potential for clinical use, and the neuroprotective effects of the PO extracts have not been investigated so far.

In the present study, we tried to clarify the neuroprotective

effect of the PO extracts and explore the possible mechanisms by in vivo and in vitro experiments.

## Materials and methods

PO extracts were obtained from Department of Traditional Chinese Medicine, Changhai Hospital (Shanghai, China). The sources for other materials were given below.

## Animals and treatments

All experimental procedures involving animals received the approval from the Animal Care and Use Committee of the Second Military Medicine University. Guidelines and Policy on using and caring of the laboratory animals were followed at all time. Male BALB/c mice (23-25 g) were purchased from the Shanghai-BK Ltd. Co and were bred in a temperature-controlled ((24±1) °C), (55 ± 5) % humidity room with a 12-hour light and 12-hour dark cycle. After adaptation for three days, the mice were divided into control group, 12-hour hypoxia exposure group, 24-hour hypoxia exposure group and 36-hour hypoxia exposure group.

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Each group was sub-divided into distilled water group, low-dose PO group, medium-dose PO group and high-dose PO group, and the mice were orally administrated with distilled water and 0.25, 0.5, 1.0 g/ml (crude drug) PO, one milliliter every day for seven days, respectively. One hour after the last drug administration, mice in the hypoxia exposure groups were adopted normobaric low oxygen environment (10% oxygen and 90% nitrogen) for 12, 24 or 36 hours. Mice in the control group were sacrificed one hour after the last drug administration, and mice in the hypoxia exposure groups were sacrificed immediately after hypoxia administration, and the mouse cortices were collected for analysis.

#### **Preparation of herb-containing serum**

According to the literature,<sup>18</sup> the SD rats were used for the preparation of herb-containing serum. Being orally administrated with the PO extracts in a dose of 3 g/ml for 2 ml, twice a day for five days, the rats were anesthetized with diethylether and sacrificed one hour after the last drug administration. The blood was collected and stored at 4 °C for 24 hours, then centrifuged for 10 min at 1000 r/m. The serum was collected and inactivated at 56 °C for half an hour, and stored for future use.

#### **Histopathological evaluation of cerebral cortex**

Histological evaluation was performed on 4% paraformaldehyde-fixed and paraffin-embedded sections of cortices. Slices of 5 µm thick were stained with H-E staining to assess inflammation damage. three slices were chosen randomly in each mouse and the damage were valued according the following scores of criteria<sup>19</sup>: normal (1); gliosis (2); spot degeneration (3); spot necrosis (4); focal degeneration (4); focal necrosis (5); piece degeneration (5); piece necrosis (6); diffused degeneration (6) and diffused necrosis (7).

#### **Enzymatic activity assay**

The activities of pyruvate kinase (PK), phosphofructokinase (PFK), lactic acid (LD), the level of lactate dehydrogenase (LDH) and adenosine triphosphate (ATP) were detected by the common methods and ATP kits (Sigma company).

#### **RT-PCR for EPO gene expression**

Total RNA was extracted from mouse cortices with the TRIzol reagent according to the recommendation of the manufacturer. Reverse transcription (RT) of RNA to cDNA was synthesized from equal amount of total RNA by using a TaKaRa mRNA selective PCR Kit. The mixture was incubated at 40 °C for 30 min, followed by incubation at 85 °C for seven min. Subsequently, PCR was carried out with five units of Taq DNA polymerase and five pM of primers for EPO. Primers were as follows. EPO sense: 5'-TCCTTGCTACTGATTCCTCTGGG-3', antisense: 5'-GT ATCCACTGTGAGTGTTCG-3'; β-actin sense: 5'-CTAG GCACCAAGGTGGTAT-3', antisense: 5'-CAAACATGA TCTGGGTCATC-3'. β-actin was used as internal standard. All reagents were added according to the protocol of the kit. PCR was performed in a PCR system starting with a five min incubation step at 99 °C, followed by a three-step temperature cycle (for EPO 30 s at

94 °C, 30 s at 60 °C, and one min at 72 °C; for β-actin 30 s at 94 °C, 30 s at 55 °C, and one min at 72 °C). This cycle was repeated 30 times and concluded with 10 min incubation step at 72 °C to complete polymerization. PCR products were electrophoresed on a 2% agarose gel and photographed after staining with ethidium bromide.

#### **Western blotting**

Expression of EPO protein was investigated by Western blotting. The protein was lysated in Phosphosate extraction buffer and the protein concentration was estimated by a BCA protein assay kit, and equal amount of proteins (40 µg) were resolved by using SDS-PAGE, after which the gels were transferred to nitrocellulose membranes. The blots were blocked with 5% BSA, 0.1% Tween 20 in Tris-NaCl buffer for one hour, and incubated overnight at 4 °C with the first antibody (Santa Cruz Biotechnology Inc) at a dilution of 1:500. After extensive washing, the blots were incubated with the secondary horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology Inc, 1:1000) for 2 hours at 37 °C. Immunoreactive bands were visualized by using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL). Levels of protein expression were estimated quantitatively by densitometric scanning by using a Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA), and standardized by dividing the expression level of each protein by the level of GAPDH expression. The relative intensity was then calculated by using the formula: Relative intensity = density of treated group / density of control group.

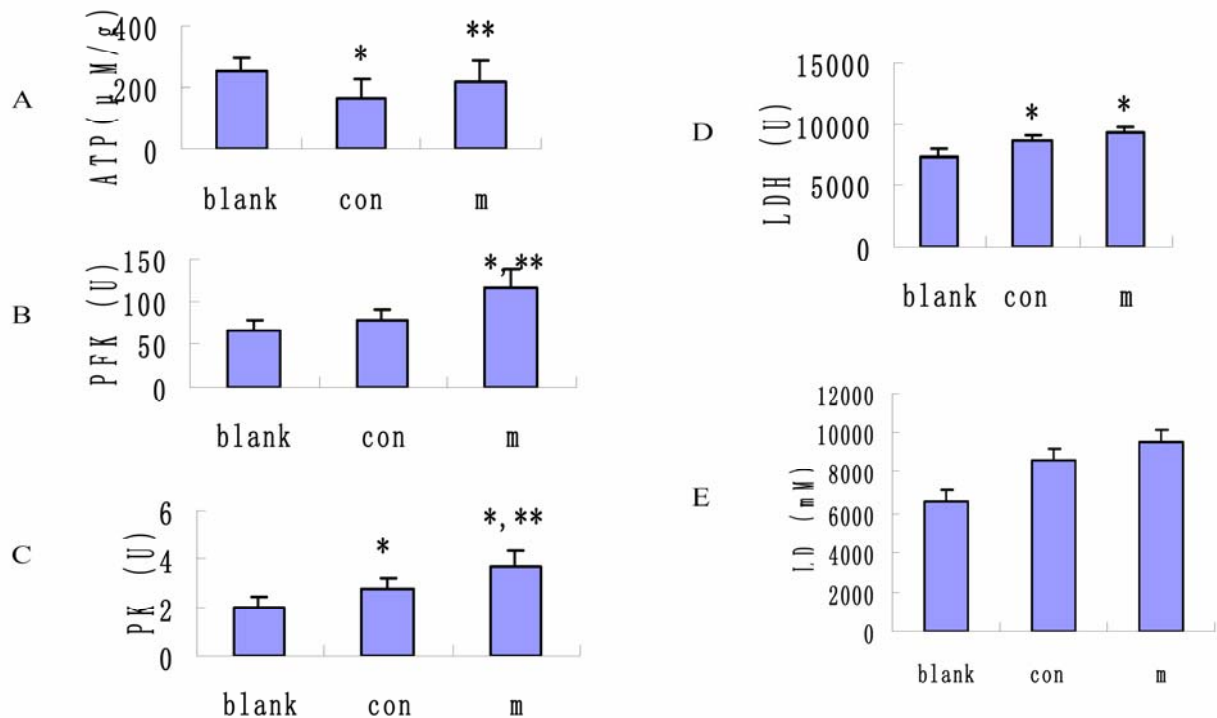
#### **Cell culture**

The PC-12 cells were purchased from the Institute of Biochemistry and Cell Biology ( Shanghai Institutes for Biological Science ) and grown in the Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS) and fresh medium were changed once every three days. The primarily cultured nerve cells were plated into non-tissue culture 12-well plates at a density of about  $1 \times 10^6$  cells/ml. The neurosphere medium consisted of neurobasal and B27 supplement (Gibco) was used after the cells were cultured in the plant liquid (80% DMEM, 10% FBS, and 10% horse serum) for 24 hours. Fresh medium was partially changed once every three days. The cells were incubated at 37 °C in a humid atmosphere containing 5% CO<sub>2</sub>.

**Table1.** the total value of pathological assessment of the mouse brain after being orally administrated with different doses (0.25, 0.5, 1g/day for 7days) of the PO extracts under hypoxic condition for different time (12h, 24h or 36h) (n=6)

	12h	24h	36h
control	36	51	60
PO extracts (0.25g/day)	35	49	56
PO extracts (0.5g/day)	34	48	53
PO extracts (1g/day)	33	40	45*

\*  $p < 0.05$  vs control group under the hypoxia condition for the same time



**Figure 1.** Effect of the PO extracts on the ATP, LD, LDH, PFK, PK of hypoxic mouse brain cortices.

After being orally administrated with 1g/day of the PO extracts or distilled water, the mice were exposed to hypoxic environment for 24h and were sacrificed. The level of ATP(A), LD(E) and the activities of PFK(B), PK(C), LDH(D), were tested. Data were shown as means $\pm$ S.E.M (n=10), \*  $p < 0.05$  vs blank group (nonhypoxic control group), \*\*  $p < 0.05$  vs control group orally administrated with distilled water.

### MTT assay

The MTT assay was used to investigate whether the PO extracts could protect the PC-12 cells or primarily cultured nerve cells from the hypoxic insult. The PC-12 cells were plated at a density of  $1 \times 10^4$ /well in a 96-well plate. Two or three days later, the cells were cultured in control medium, or in medium plus different volume of the water solution of the PO extracts (the end concentrations of the PO extract were 0%, 5%, 10%, 20%). The cells were adapted in the incubator filled with the hypoxic or anemia gas (5% CO<sub>2</sub>, 3% O<sub>2</sub> and 92% N<sub>2</sub> or 5% CO<sub>2</sub>, 95% N<sub>2</sub>) or cultured in the medium coupled with CoCl<sub>2</sub> (end concentration of CoCl<sub>2</sub> was 200  $\mu\text{M}$  or 400  $\mu\text{M}$ ) at 37 °C for three, six or 12 hours. Cells viability were determined by using a modified MTT (3-(dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) assay. After hypoxic treatment, the medium was changed for the control medium, 20  $\mu\text{l}$  MTT (5 mg/ml of phosphate buffer saline) was added into each well and incubation was continued at 37 °C for four hours. Then, 100  $\mu\text{l}$  Dimethyl Sulfoxide (DMSO) was added to each well, and the plate was transferred to room temperature for one hour. Absorbance values at 490 nm were measured with an ELISA plate reader (BioRad), and the absorption value was calculated. Each assay was performed at least for four times and the average absorbance were calculated. Data were expressed as the percentage inhibition by ratio of each absorbance relative to the absorbance in the non-hypoxic control medium.

For the primarily cultured nerve cells from new born (one day after born) Sprage-Dawley rats, after being in

cubated for seven to nine days, the cell viability were tested before hypoxic treatment by the CCK-8. Then the cells were treated similarly as mentioned above and the cell viability after hypoxic treatment were also tested.

### Statistics

One way NAVOA was used to compare the differences among groups (SPSS11.0, Analytical Software). Value of  $p < 0.05$  was considered to be statistically significant.

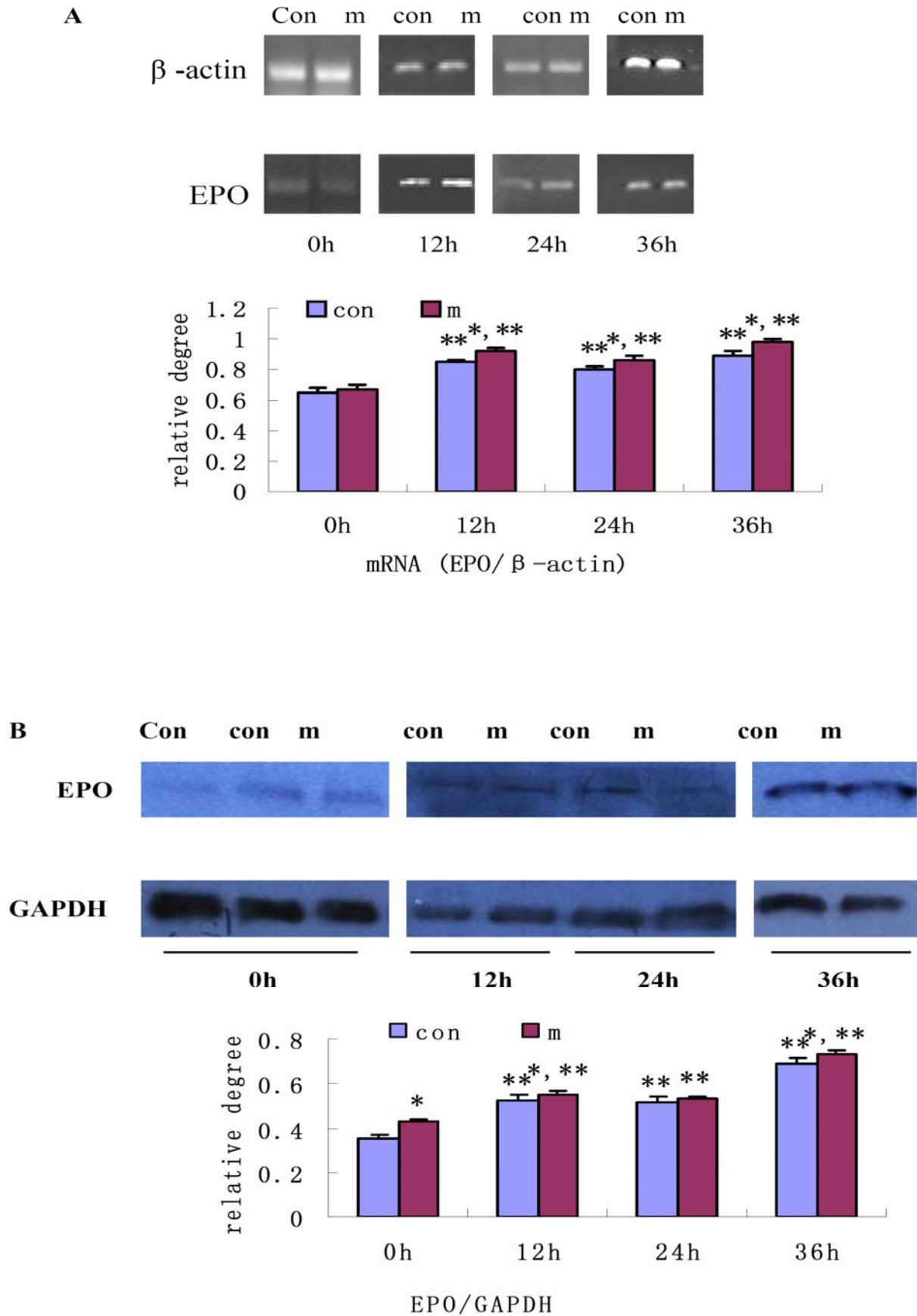
### Results

#### *Effect of the PO extracts on the hypoxic nerve tissue*

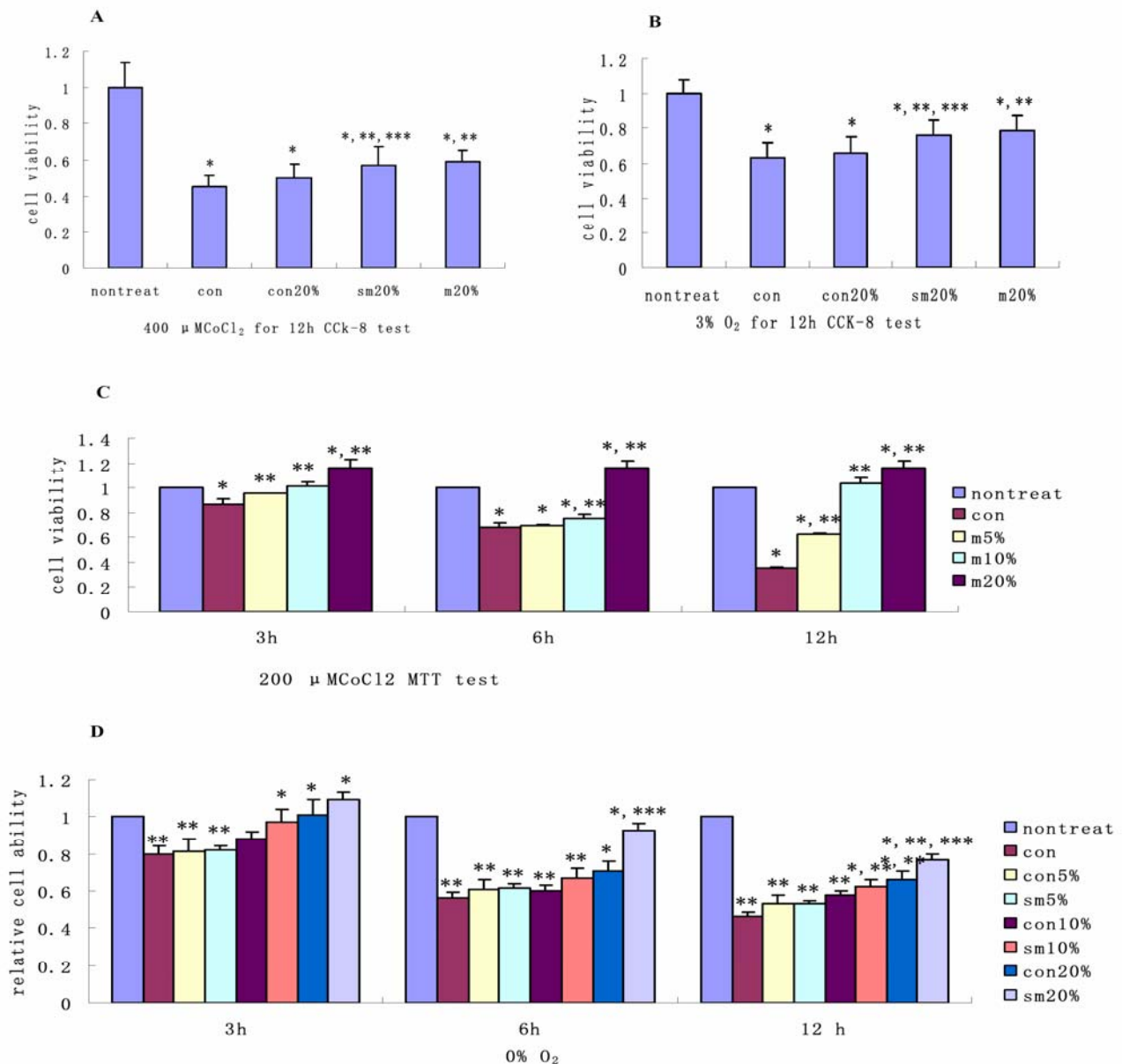
Hypoxia led to damage in mouse cortices. Compared to the untreated group, there was spot degeneration, spot necrosis and focal degeneration in mouse brains exposed to a hypoxic environment. Table 1 showed that the degrees of brain inflammation in the PO extract groups were lower than that in the control group. Mice in the 1g/d group suffered the lightest damage.

#### *Effect of the PO extracts on the activities of PFK, PK, LDH and the level of LD, ATP of hypoxic mouse brain*

After hypoxia treatment, the activities of PK, PFK, LDH and the level of LD in mouse brain increased, and the ATP level decreased. However, the PO extracts enhanced the increment of PFK, PK, LDH and lessened the decrement of ATP of the mouse cortices. The most significant enhancement occurred in the 1g/d group (Fig 1).



**Figure 2.** Effect of the PO extracts on the expression of EPO of the hypoxic mouse brain. Data were given as means $\pm$ S.E.M (n=4). A: EPO mRNA expression level; B: EPO protein expression level. \*  $p < 0.05$  vs con group under the same hypoxic condition; \*\*  $p < 0.05$  vs non-hypoxic group (0h group).



**Figure 3.** Effect of the PO extracts on the viability of cells in hypoxia environment or stimulated with CoCl<sub>2</sub>

Data were given as means $\pm$ S.E.M and were the present for at least four different tests. m5%, m10% and m20%: the end concentration of the PO extracts in the culture medium, sm5%, sm10% and sm20%: the end concentration of the herb-containing serum in the culture medium, con5%, con10% and con20%: the end concentration of the control serum in the culture medium. con: the control group. A and B: the protective effect on primarily cultured nerve cells in low oxygen environment, \*  $p < 0.05$  vs nontreated group, \*\*  $p < 0.05$  vs con group; \*\*\*  $p < 0.05$  vs con20% group. C: the protective effect of water solution of the PO extracts on the PC-12 cells stimulated with CoCl<sub>2</sub>. \*  $p < 0.05$  vs nontreat group, \*\*  $p < 0.05$  vs con group simulated for same time. D: the protective effect of herb-containing serum on the PC-12 cells under hypoxic conditions. \*  $p < 0.05$  vs con group exposed to hypoxia for same time, \*\*  $p < 0.05$  vs nontreat group, \*\*\*  $p < 0.05$  vs the group treated with the equal volume of control serum and exposed to hypoxia environment for the same time.

#### Analysis of the effect of the PO extracts on the expression of EPO

Hypoxia enhanced the expression of EPO mRNA and protein in mouse cortices. The expression levels of EPO mRNA and the protein were higher in the PO extracts group than those in the control group (Fig 2).

#### Effect on cell viability under the hypoxia and the CoCl<sub>2</sub> stimulation

To confirm the protective effect of the PO extracts, we used the PC-12 cells and the primarily cultured nerve cells for *in vitro* experiment. Compared with the control

group with the same treatment, the water solution of the PO extracts or the herb-containing serum increased the cell viability under all the conditions measured. This was in congruence with the *in vivo* experiment. Additionally, the level of the LDH in the culture medium were lower in the groups treated with the water solution of the PO extracts or the herb-containing serum than those of the control group with the same treatment (part data were shown in Fig 3 and Table 2).

#### Discussion

Hypoxia is a pervasive physiological stimulus that is

**Table 2.** Effect of the PO extracts on the level of LDH in the culture medium of PC-12 cells stimulated with 200 $\mu$ M CoCl<sub>2</sub> (means $\pm$ S.E.M, n=6~8)

Group	0h	3h	6h	12h
Con <sup>†</sup>	76.15 $\pm$ 7.08	92.45 $\pm$ 6.35	157.49 $\pm$ 10.32	213.56 $\pm$ 9.76
m5% <sup>‡</sup>	76.15 $\pm$ 7.08	91.38 $\pm$ 6.29	139.65 $\pm$ 8.74	205.87 $\pm$ 13.58
m10% <sup>‡</sup>	76.15 $\pm$ 7.08	90.98 $\pm$ 7.24	129.63 $\pm$ 9.84*	188.73 $\pm$ 9.92*
m20% <sup>‡</sup>	76.15 $\pm$ 7.08	90.05 $\pm$ 6.78	116.32 $\pm$ 6.59*	164.54 $\pm$ 14.21*

<sup>†</sup>con: control group. <sup>‡</sup>m5%、m10%、m20% were the end concentration of the water solution of the PO extracts in the culture medium (v/v). \*  $p < 0.05$  vs con exposed to hypoxia for the same time

encountered under many different circumstances. The ability of tissues to resist hypoxia depends on the glycogen content, while neurons contains little glycogen and its supporting cells, the astrocytes, contain only sufficient for some 15 seconds' anaerobic ATP production. So, neurons are notoriously sensitive to hypoxia with irreversible changes occurring within minutes. Continuous hypoxia does harm to, or even destroys nerve cells. The present study demonstrated that damage to mouse nerve tissues worsened with prolonged duration of hypoxia. In vitro experiments showed that cell viability decreased when the cells were exposed to a low oxygen environment or when CoCl<sub>2</sub> was added in the culture medium. However, administration of PO extracts lessened the damage of the hypoxic mouse brain and increased cell viability under hypoxic or CoCl<sub>2</sub> conditions. These results indicated that the PO extracts had neuroprotective effect on hypoxic mouse cortices.

It's well known that ATP act as the energy currency of the organism. In hypoxia it is the synthesis particularly of ATP that is impaired. So, increase the ATP level will be benefit for the function of the hypoxic brain. This study showed that the PO extracts lessened the decrement of ATP in mouse cortices in an hypoxia environment, in accordance with the lesser injury of the cortex and increased cell viability when PO extracts were administered. These results indicated that the neuroprotective effect of the PO extracts is possible by providing more ATP.

Glycolysis is a widely used strategy for hypoxia adaptation. Compared with the tricarboxylic acid cycle, the energy yield of glycolysis is small. But it does have the function of producing ATP anaerobically. The PFK and PK are both limited enzymes which regulate the glycolysis process. In hypoxia glycolysis is accelerated due to the high activity of PFK and PK to promote the production of ATP by the breakdown of phosphoenolpyruvate. Glycolysis would cease entirely if NADH accumulated and /or no NAD in the cytoplasm. The LDH restores the NAD by reduction of pyruvate with NADH yielding lactate and NAD to sustain the glycolysis. So, enhancing the activities of these enzymes will offer more ATP to sustain the cell function. The results in our present studies showed that, compared with the control group, the PO extracts increased the activities of PFK、PK、LDH in the cortices of hypoxic mice, indicating that the PO extracts might enhance the glycolysis to provide more ATP to sustain the cell function.

In our previous experiments we showed that the PO extracts increased plasma EPO level of hypoxic mice. EPO mRNA is constitutively expressed at comparable levels in

the brain of mice,<sup>24</sup> and at the protein level immunoreactive EPO was found in the cortices and hippocampus of normal human and mouse brain.<sup>25,26</sup> It is reported that EPO in the central nervous system functions as a trophic factor to protect the nervous system under a stressful condition. Neuroprotective effects of systemically administered EPO have been shown in animal models of focal cerebral ischemia, traumatic brain injury, subarachnoid hemorrhage and spinal cord injury.<sup>20-23</sup> So, we tried to find out whether the PO extracts could affect the EPO expression pattern of the mouse cortices. The results of our study showed that administration of the PO extracts increased the EPO expression in hypoxic mouse cortices. In line with the result histological analysis showed that the PO extracts lessened the damage of the hypoxic mouse cortices. These results suggested that the PO extracts might enhance the expression of EPO to protect the hypoxic nerve cells/tissues. Whether PO extract exerts its protective effect on hypoxic brain cells/tissue by enhancing the expression of EPO warrants further study. It has been generally accepted that HIF-1(hypoxia inducible factor-1) is the central factor regulating the adaptation reaction of the organism exposed to hypoxia. HIF-1 level increases soon after hypoxic insult and it can regulate expression of many downstream genes. PFK, PK and EPO are among the downstream genes regulated by HIF-1. All these data suggest that PO extracts protect nerve tissues from hypoxic damage probably by enhancing the expression of HIF-1. Further experiments are needed to confirm this assumption.

In conclusion, PO extracts can enhance glycolysis and EPO expression to protect nerve tissue/cells from hypoxic insult.

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