

Full Length Research Paper

Cytokines expression profiling as a window into hypoxia SH-SY5Y cells treated with N-butyl extract of *Potentilla anserina* L.

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Potentilla anserina L. is the root of Chinese cinquefoil herb of the Rosaceae family, and its ethanol extract improves the immunity and exhibits anti-fatigue and anti-hypoxia properties. This study aimed to explore the cytokines expression profiling and the effect of n-butanol extract of *P. anserina* L. (NP) on the hypoxia cells. The subjects were divided into three groups: control, hypoxia, and intervention (the group treated with the n-butyl extract of *P. anserina* L. under the condition of hypobaric hypoxia) groups. The results of the experiment showed that NP could down-regulate the expression of the proinflammatory cytokines of hypoxia SH-SY5Y cells and meantime up-regulate the expression of inhibitive cytokines of immune reaction. The cytokines expression profiling can be deduced as a window into hypoxia SH-SY5Y cells, and n-butyl extract of *P. anserina* L. can significantly protect the cell against the hypoxia induced damage set up *in vitro* by sodium dithionite.

Key words: Hypoxia, inflammatory cytokines, *Potentilla anserina* L., sodium dithionite.

INTRODUCTION

The prevention of altitude hypoxia and sickness has been an important research arousing the concern of scholars worldwide. *Potentilla anserina* L., commonly called monorchid herminium herb, belongs to the Rosaceae family and contains polysaccharides, amyllum, fatty acids, essential amino acids, and vitamins. It possesses a high medical and nutritional value (Hui et al., 2003). It has been used as a crude drug and as a Chinese herbal medicine in Tibet. Recent studies have shown that *P. anserina* L. strengthens the immunity, exhibits anti-oxidative activities, and anti-hypoxic properties (Frede et al., 2007). This provides a possibility for the prevention and cure of cerebral anoxia. The modern pharmacological study shows that the extract of *P. anserina* L. could strengthen the immunity, exhibits anti-fatigue and

other bioactivities, and remarkably improves the body's oxygen utilization (Frede et al., 2007). Previous studies have mainly focused on the effects of hypoxia on the expression of some individual cytokines, such as TNF- α , IL-6, vascular endothelial growth factor (VEGF), etc (Spooren et al., 2011; Rosenstein et al., 2003), but few has touched the network regulation of related cytokines. The present research applied n-butanol extract of *P. anserina* L. (NP) to sodium dithionite-induced SH-SY5Y cells hypoxic injury model, selected monocyte chemoattractant protein-1 (MCP-1), interleukin-1beta (IL-1 β), interferon-gamma (IFN- γ), VEGF, interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor- α (TNF- α) to study the effect of *P. anserina* L. on the expression of hypoxic injury inflammation-related cytokines, and then

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explored the anti-hypoxia mechanism of *P. anserina* L.

MATERIALS AND METHODS

Drugs

P. anserina L. was purchased from Qinghai Institute of Chinese Medicine and identified as the root of genus *Potentilla*. From the total alcohol extract of *P. anserina* L., we obtained its n-butanol strong liquid after the procedure of n-butanol extraction, concentration by reduced pressure, lyophilization, and drying (The crude drug in strong liquid per gram is 56.5 mg, and the ingredients are β -sitosterol, ursolic acid, euscaphic acid, tormentic acid, rosamultin, kaji-ichigoside F₁, 24-deoxy-sericoside, 2-oxo-pomolic acid- β -D-glucopyranosyl ester, β -daucostarin, adenosine, daidzin, puerarin, 3'-methoxypuerarin, and daidzein 8-C- β -glucoside, which are obtained from the Pharmaceutical Chemistry Laboratory of Logistics University of Chinese People's Armed Police Forces). Then, we dissolved the n-butanol strong liquid in 0.3% sodium carboxymethyl-cellulose (CMC-Na) and made up the required concentration of its solution with D-Hank's medium which was obtained by dissolving sodium chloride (8 g), potassium chloride (0.4 g), 12-hydrate disodium hydrogen phosphate (0.13 g), dipotassium hydrogen phosphate (0.06 g), sodium hydrogen carbonate (0.35 g), and phenolsulfonphthalein (0.02 g) into water distilled for three times (1 L).

Reagents

Fetal bovine serum was purchased from Hangzhou Sijiqing Institute of Biological Engineering and Materials; Dulbecco's Modified Eagle Medium (DMEM) high glucose medium, trypsin, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Beijing Dingguo Biological Technology Development Center; Lactate dehydrogenase (LDH) kit from Nanjing Jiancheng Bio-Engineering Company; Suspension kit from Millipore Company. 3111-type CO₂ incubator is produced by Thermoforma Company.

Establishment of hypoxia cell model

Human neuroblastoma cell strain SH-SY5Y was purchased from Shanghai Institute of Cells, Chinese Academy of Sciences. The hypoxia model was established by using sodium hydrosulfite, an oxygen scavenger: adding 100 μ l serum-free culture media and 100 μ l sodium dithionite solution by D-Hank's (2 and 4 mmol/L), cultivating, respectively for 30 min, 1 h, 2 h, and 4 h, and measuring survival rate of the cell in each concentration and at each time point, and finally the proper concentration of sodium dithionite which caused the most severest and stablest damage being chosen as 1 mmol/L and the hypoxia time period as 4 h.

Cultivation of the cells in all groups

In the experiment, there were three groups designed as normal control, hypoxia model, and NP (which also consists of three concentration groups: 1, 0.25, and 0.0625 g/L) groups. D-Hank's solution replacing culture media was added in normal control group, 1 mmol sodium dithionite solution in model group, and NP of different concentrations dispensed by D-Hank's in NP groups along with 1 mmol/L sodium dithionite solution. Then, these groups were incubated for 4 h in 5% CO₂ incubator at 37°C.

The vitality of cell metabolism by MTT colorimetry

After 4 h of incubation at 37°C, we added 50 μ l MTT (1 g/L) in each group, incubated them in the 5% CO₂ for another 4 h, stopped the culture and discarded the liquid. After adding 150 μ l DMSO into each group and shaking for 1 min to make the crystals dissolve fully, we measured the optical density (OD) value where the detected wave length is 570 nm and the wave length for reference is 630 nm. The higher the OD value is, the stronger cellular metabolic activity is.

LDH-release assay

We got cells of logarithmic growth phase digested with trypsin to make cell suspension and took separately 1 ml of cell suspension to add into a 24 pore plate. After 24 h, we took separately 1 ml serum-free culture media and 1 ml Na₂S₂O₄ solution, whose concentration was 2 mmol/L, to add, respectively into the 24 pore plate, which was then put into a hermetic container. Subsequently, the hermetic container was put into CO₂ incubator for culture, respectively for 2, 4, and 6 h. Then, at the end of the three-time periods, supernatant fluids of cell culture in hypoxia group and control group were collected into different Eppendorf tubes and were preserved for use at 4°C. The way to measure LDH leakage rate outside the cells was as follows: put corresponding reagents into vacant tube, typical tube, measured tube, and control tube, and ensure their mixture; give the four tubes water baths twice at 37°C for 15 min, add to them 0.25 ml of 2,4-dinitro-phenyl-hydrazine and 2.5 ml of NaOH solution (0.4 mol/L) separately, and place them at room temperature for 3 min; measure the absorbance of each tube. Formula for calculation is as follows:

$$\text{LDH vitality in serum (U/L)} = \frac{(\text{absolute value of measured tube} - \text{absolute value of control tube}) / (\text{absolute value of typical tube} - \text{absolute value of vacant tube}) \times \text{standard concentration (2 mmol/L)} \times \text{dilution times of sample} \times 1000}$$

Hematoxylin and eosin (HE) staining

The cells of logarithmic growth phase were exposed to 0.25% trypsinase to cause them to be digested, made them into cell suspension, and further into climbing slice. Then, we covered it with coverslip and fixed it with paraform for 20 min, then washed it twice with phosphate buffered solution to get ready for staining. We immersed it into hematoxylin staining solution for 5 to 10 min, washed it with tap water before we immersed it into dilute hydrochloric acid and alcohol, respectively for color separation which only took several seconds, and finally washed it with tap water. We immersed it into light ammonia water to cause nucleus to become blue, and then after 3 to 5 min, we got it washed with tap water. We immersed it into eosin staining solution for 5 to 10 min to get it stained and then washed it with tap water. We dehydrated it by turns with 70, 80, and 90% alcohol once, then 95% alcohol twice and 100% alcohol three times. Each process of dehydration took 1 min. Then, we treated it with xylene three times which took 1 min each time. Finally, we dripped neutral gum on it and fixed the cell side downward on another slide.

Microsphere suspension chips assay

Cellular proteins in each group were extracted and the cytokine levels were detected in accordance with the instructions on the suspended chips kit and the operating method of Bio-Plex200 suspension array system.

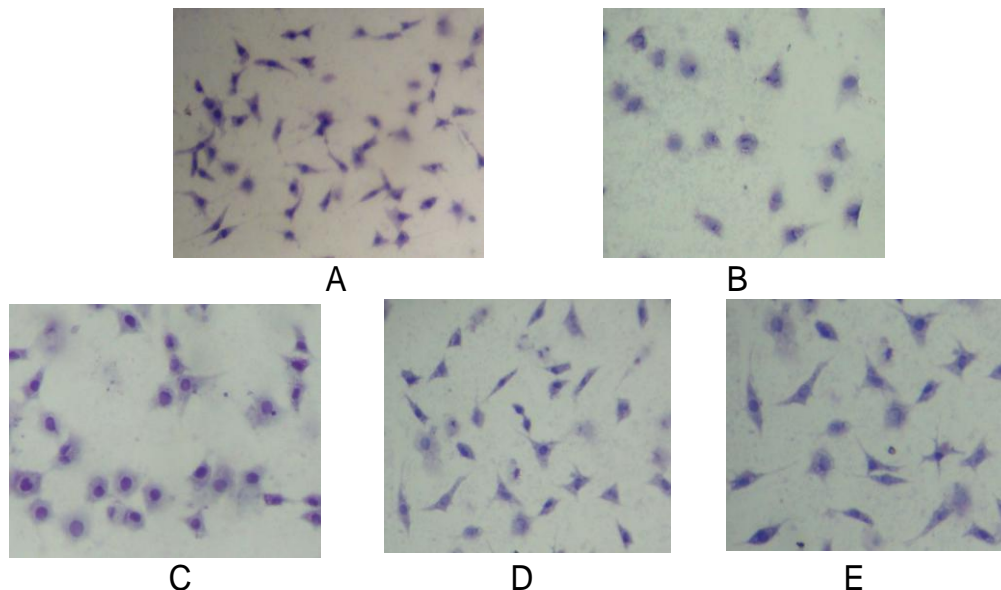


Figure 1. HE staining to observe the effect of different treatments on cell morphous. A: normal control group; B: hypoxia model group; C to E: NP groups (0.0625, 0.25, and 1 g/L) ($\times 40$). In control group, the cytoplasm looked pink, the nucleus blue, and the cell body was oval, with as many as 3 to 4 apophysis sticking out of the majority of cells. In hypoxia group, the cell body looked remarkably swollen with many vacuoles in it, most of the plasmalemmas ruptured, resulting in a lot of debris, and a small number of cells survived. In 0.0625 g/L of NP group, some cells still looked swollen with vacuoles, but compared with that in hypoxia group, the cell morphous recovered a bit; in 1 and 0.25 g/L of NP groups, the cell morphous recovered to the normal condition and some cytoplasm apophysis, thick and thin, long and short, could be observed.

Statistical analysis

Values are presented as means \pm standard deviation (SD). The normality test was completed by the Shapiro-Wilk test. Group means were compared by analysis of variance (ANOVA) using Fisher's post hoc analysis with Excel software (Microsoft). $P < 0.01$ was considered statistically significant.

RESULTS

Succinate dehydrogenase (SDH) concentration inside SH-SY5Y cell and LDH concentration outside the cell

Measurement by MTT assay indicated that the SDH concentration in hypoxia model group was 168.67 ± 21.14 ($P < 0.01$), and when compared with that in normal control group (351.18 ± 39.39), it decreased significantly; it also showed that LDH leakage increased significantly in hypoxia group (599.65 ± 21.62 U/ml), when compared with that in control group (185.29 ± 9.73 U/ml) ($P < 0.01$); and when compared with hypoxia model group, SDH concentration increased significantly in NP groups, while LDH leakage decreased significantly ($P < 0.01$) (Table 1).

Changes of SH-SY5Y cell in all groups with HE staining

In the control group, the cytoplasm looked pink, the

nucleus blue, and the cell body was oval, with as many as 3 to 4 apophysis sticking out of the majority of the cells. In hypoxia group, the cell body looked remarkably swollen with many vacuoles in it, most of the plasmalemmas ruptured, resulting in a lot of debris, and a small number of cells survived. In 0.0625 g/L of NP group, some cells still looked swollen with vacuoles, but compared with that in hypoxia group, the cell morphous recovered a bit. In 1 and 0.25 g/L of NP groups, the cell morphous recovered to the normal condition and some cytoplasm apophysis, thick and thin, long and short, could be observed (Figure 1).

Cytokines expression profiling of hypoxic SH-SY5Y cells treated with NP

Compared to that in the control group, the expression of MCP-1, IL-1 β , IFN- γ , and TNF- α increased significantly, while the expression of VEGF, IL-6, and IL-10 reduced significantly ($P < 0.01$) in hypoxia model group; while in NP groups, the level of MCP-1, IL-1 β , IFN- γ , and TNF- α were down-regulated significantly ($P < 0.01$) and the level of VEGF, IL-6, and IL-10 were up-regulated significantly ($P < 0.01$), as compared to that in the model group (Table 2).

Table 1. The effects of different treatments on LDH in the hypoxia induced damage of SH-SY5Y cells and its metabolism activity SDH ($\bar{x} \pm s$).

Group	n	SDH (pg/ml)	LDH (U/L)
Normal control	6	0.472 \pm 0.062	185.29 \pm 9.73
Hypoxia model	6	0.232 \pm 0.038*	599.65 \pm 21.62*
NP (1g/L)	6	0.348 \pm 0.050 ^Δ	452.68 \pm 35.34 ^Δ
NP (0.25 g/L)	6	0.334 \pm 0.042 ^Δ	394.23 \pm 24.63 ^Δ
NP (0.0625g/L)	6	0.298 \pm 0.02 ^Δ	479.82 \pm 46.61 ^Δ

SDH value by MTT assay can indirectly reflect the number of live cells. In a certain number of cells, the number of cells crystallized by MTT is in direct proportion to the number of live cells, SDH concentration of NP groups lay between normal group and hypoxia group, with its concentration in 1 g/L NP group close to that in normal group. LDH (l-lactate dehydrogenase), as a kind of steady protein, lies in cytoplasm of normal cells and it will be released out of the cell once cell membrane is damaged. Therefore, by testing LDH activity in supernatant of cultivated cells, the damage degree of cells can be identified. LDH concentration in NP groups lay between normal group and hypoxia group, with its concentration in 0.25 g/L of NP group close to that normal group. *P < 0.01 (compared to the normal control group); ^ΔP < 0.01 (compared to the hypoxia model group).

Table 2. The effect of different treatments on the levels of cytokines of hypoxia induced damage on SH-SY5Y($\bar{x} \pm s$, n = 3).

Group	MCP-1 (pg/ml)	IL-1 β (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	IFN- γ (pg/ml)	TNF- α (pg/ml)	VEGF (pg/ml)
Normal control	140.78 \pm 3.85	11.58 \pm 1.35	496.54 \pm 20.35	310.14 \pm 17.2	26.88 \pm 2.69	5.63 \pm 0.22	6138.65 \pm 126.50
Hypoxia Model	169.35 \pm 3.61	26.82 \pm 1.21	196.59 \pm 4.40	144.19 \pm 8.13	77.82 \pm 1.76	18.55 \pm 0.84	3120.5 \pm 97.37
NP (1g/L)	149.36 \pm 3.55	18.93 \pm 1.15	330.18 \pm 4.51	217.66 \pm 8.37	50.36 \pm 2.68	7.29 \pm 0.13	3662.54 \pm 516.92
NP (0.25g/L)	150.63 \pm 6.35 [Ⓢ]	19.63 \pm 2.32 [Ⓢ]	312.78 \pm 5.23 [Ⓢ]	225.96 \pm 7.89 [Ⓢ]	59.63 \pm 3.69 [Ⓢ]	8.96 \pm 0.93 [Ⓢ]	3749.52 \pm 369.25 [Ⓢ]
NP (0.0625g/L)	153.61 \pm 4.09	17.43 \pm 0.85	293.85 \pm 11.36	239.84 \pm 11.92	60.55 \pm 1.85	7.03 \pm 0.19	3730.01 \pm 217.10

Compared to that in the control group, the expression of MCP-1, IL-1 β , IFN- γ , and TNF- α increased significantly, while the expression of VEGF, IL-6, and IL-10 reduced significantly (P < 0.01) in hypoxia model group; while in NP groups, the level of MCP-1, IL-1 β , IFN- γ , and TNF- α were down-regulated significantly (P < 0.01) and the level of VEGF, IL-6, and IL-10 were up-regulated significantly (P < 0.01), compared to that in the model group. P < 0.01(compared to the normal control group); [Ⓢ]P < 0.01(compared to the hypoxia model group).

DISCUSSION

The stimulation of hypoxia in high altitude environment can cause a series of compensatory responses in the body accompanied by a sequence of stress reaction, one of which is hypoxia induced pulmonary and vascular inflammation resulting from cytokine and chemokines rather than infection (Minamino et al., 2001; Mou et al.,

2002). Meanwhile, hypoxia is a basic pathological process shared by many diseases. Neurons are extremely sensitive to hypoxia. SH-SY5Y cell line of human neuroblastoma strain is lowly differentiated tumor cells, which multiply fast and resemble normal nerve cells in physiological and biochemical functions, and are widely used in the researches related to nervous system diseases pathogenesis and their drug action mechanism.

When hypoxia led to acute damage of cells, the integrality of cell membrane would be damaged and cell permeability would increase (Ray et al., 2011; Sethy et al., 2011). As a result, LDH would be released from cytoplasm. The release rate of LDH and MTT assay are commonly used as the indicators of cell survival, growth status, and injuries in that the extra-cellular LDH activity can reflect indirectly the integrity of the cell membrane.

The experiments demonstrated that the exposure to 1 mmol/L sodium dithionite for 4 h can induce cell injury, while 1, 0.25, and 0.0625 g/L NP can inhibit hypoxia-induced LDH release and improve the cell vitality, which demonstrated that NP could relieve neuroglioma cell of hypoxia-induced damage to succinic dehydrogenase activity and maintain respiratory function of damaged cells (Goodman et al., 2011; Shrivastava et al., 2008). The cell hypoxia model established in this study was proved by the release rate of LDH and MTT assay to be effective and easy to manipulate, which is positively meaningful for further studies in mechanism and clinical therapeutics.

Under normal circumstances, the concentration of cytokines is very low in the body and it will increase rapidly soon after the attack of stimulus from outside. Cytokines unite not only with organ cell receptors to produce biological effect, but also with circulatory soluble receptors to initiate excessive release of cytokines. The cytokines overproduction induced injury is closely related to hypoxia process and the synergism among cytokines brings about water fall effect, which will in turn worsen the injury of cells. The results of the *in-vitro* and *in-vivo* experiments show that the monocyte chemoattractant protein-1 (MCP-1) is characterized by chemotactic activity for monocytes and it can activate monocytes and macrophages to produce and release superoxide anions and lysozyme so as to induce the production of cytokines of IL-1 and IL-6 (Weber et al., 2010), and that IL-1 β , as a subtype of IL-1, has a high expression in the inflammatory reaction. It was reported that brain ischemia-hypoxia suffered by new-born rats could lead to the rise of the expression of IL-1 β and mRNA in gliocytes and neurons (Saliba et al., 2001; Dinarello, 2010). IL-1 β produced from the damage can stimulate the production of other cytokines (Gabay et al., 2010; Weber et al., 2010); IFN- γ and TNF- α , as the important immunoregulation factors, participate in the body's inflammation and immune reactions. IFN- γ can induce macrophages to produce nitric-oxide synthase (iNOS), promote synthesis of NO and increase the transcription, translation, and secretion of LPS induced IL-1 (Chang et al., 2010; Us, 2008). It has been recently showed that brain hypoxia and ischemia trigger the increase of TNF- α and mRNA in neurons and the notably higher concentration of TNF- α in neurons than in plasma aggrates brain tissual lesion (Frede et al., 2007; Trayhum et al., 2008). In this study, the expression of MCP-1, IL-1 β , IFN- γ , and TNF- α increased significantly in the model group, while in NP groups ranging from high-concentration to low-concentration, their expressions all decreased significantly, which indicates that NP could alleviate hypoxia induced inflammatory reaction by reducing the levels of inflammatory cytokines. IL-10, being an inhibitor of cellular immune reaction (Ogawa et al., 2008; Cyktor et al., 2011), can inhibit the secretion of IL-1, IL-6, and TNF- α and other cytokines in white blood cells and microglia, the aggregation of white blood cells and the production of chemotactic factors, and it is also

reported to be able to protect nerves (Jiang et al., 2005). IL-6 has a dual effect on brain injury and protection. At acute stage of moderate and severe hypoxic ischemic encephalopathy (HIE), the level of IL-6 increases significantly, while Loddick et al. (1998) and other studies indicated that the injection of recombinant IL-6 into continuing middle cerebral artery (MCA) occlusive brain ventricle of rats can remarkably reduce the ischemic brain damage. It has been reported that VEGF had neuro-protective effects in many types of damage, providing types of nerve cells with direct neuro-trophy and neuroprotectin (Rosenstein et al., 2003). It is generally believed that hypoxia induced high expression of VEGF, and it is mainly realized by hypoxia-inducible factor (HIF). VEGF is the target gene shared by HIF-1 and HIF-2 (Liu et al., 2003), but there is also material indicating that HIF-2 has a stronger role than HIF-1 in the regulation and the transcription and activation of VEGF (Akeno et al., 2002). In this study, the significant decrease of IL-10, IL-6, and VEGF in the model and their significant increase in NP groups showed that NP may alleviate hypoxia induced immune damage by increasing inflammation inhibitive factors and nerve protectins, such as IL-10, IL-6, and VEGF.

The cytokines expression profiling can be deduced as a window into hypoxia SH-SY5Y cells, and n-butyl extract of *P. anserina* L. can significantly protect the cell against the hypoxia induced damage set up *in vitro* by sodium dithionite.

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