Neuroprotection of *n*-Butanol Extract from Roots of *Potentilla anserina* on Hypoxic Injury in Primary Hippocampal Neurons

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Abstract: **Objective** To investigate the protective effect of *n*-butanol extract from the roots of *Potentilla anserina* (NP) on hypoxic hippocampal neurons in neonatal rats. **Methods** Primary cultured hippocampal neurons were pretreated with different concentration of NP (0.25, 0.0625, and 0.0156 mg/mL) before incubation in a low oxygen (0.1%) environment for 4 h. Cell viability was evaluated by Trypan blue staining assay. Lactate dehydrogenase (LDH) released by neurons into the medium was measured. The activity of superoxide dismutase (SOD) in cell cytosol was determined using nitroblue tetrazolium. Morphological changes and mitochondrial function were observed by transmission electron microscopy. **Results** Hypoxic injury could decrease the cells viability of neuron, enhance LDH release ($P < 0.05$), decrease SOD activity, and increase mitochondrial injury. Pretreatment with NP significantly increased cell viability, decreased LDH release ($P < 0.05$), promoted SOD activity ($P < 0.05$), and remarkably improved cellular ultra-microstructure compared with the model group. **Conclusion** NP could protect the primary hippocampal neurons from hypoxic injury by attenuating mitochondrial cell death.

Key words: hypoxia; mitochondria; neuron; *Potentilla anserina*; ultra-microstructure
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Introduction

Hypoxia is a common cause of illness that could lead to serious consequences in different organs. Brain tissues have a high metabolic rate and the brain has the largest oxygen consumption, so the impact of hypoxia on brain tissue is the most serious. Hypoxic brain injury (HBI) is an intensely studied topic in neurological studies; However, its mechanism has not yet been well understood. HBI is a result of multiple factors, such as mitochondrial injury, action of oxygen free radicals, apoptosis, and so on.

Valid prevention and cure for HBI have not yet been found. In China, to integrate traditional Chinese and Western medicine has provided some valid outcomes and showed a broad perspective of Chinese medicine on treatment of cerebral anoxia. The roots of *Potentilla anserina* L., commonly called monorchid herminium herb, belong to the Rosaceae family and contain polysaccharides, amylum, fatty acids, essential amino acids, vitamins, and triterpenes (Hong, Cai, and Xiao, 2006). It possesses a high medicinal and nutritional value (Wang *et al*, 1998). They have been used as a crude drug and Chinese herbal medicine in Tibet. Recent studies have shown that the roots of *P. anserina* could strengthen the immunity (Chen *et al*, 2005) and exhibit anti-oxidative activities and anti-hypoxic properties (Chen and Wang, 2004; Hui and Shang, 2003; Li *et al*, 2012). These provide a possibility for the prevention and cure of cerebral anoxia.

A previous study showed that the root of *P. anserina*

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had antihypoxic and anti-oxidant properties (Li *et al*, 2005); In particular, *n*-butanol extract from the roots of *P. anserina* (NP) could remarkably protect the myocardium from acute ischemic/hypoxic injury (Li *et al*, 2009). Our recent study showed a remarkably protective effect of NP on acute hypobaric hypoxic injury in mice and the pretreatment with NP could significantly attenuate the injury of brain induced by acute hypobaric hypoxia *in vivo* (Wang *et al*, 2011). In the present study, we evaluated the effect of NP on hypoxic injury induced by low oxygen density in the primary hippocampal neurons.

Materials and methods

Reagents

The roots of *Potentilla anserina* L. were collected from Yushu (Qinghai, China) in 2003 and transported to Tianjin after dried in air. The roots were identified by Prof. SUN Qi-shi of Shenyang Pharmaceutical University. A voucher specimen (2003-09-7) was deposited in Logistic University of Chinese People's Armed Police Forces (Tianjin, China). The *n*-butanol extract from the roots of *P. anserina* (NP) was obtained by the method in our previous study (Li *et al*, 2009). NP (151 g) could be obtained from 20 kg roots of *P. anserina* with the yield rate of almost 0.76%. The content of triterpenes in NP was measured by spectrophotometry and was not lower than 0.9%. The NP was dissolved in 0.1% dimethyl sulfoxide (DMSO) diluted with D-Hank's medium before administration.

Tanshinone II_A (Tan II_A, Huike Botanical Development Co., Shaanxi, China), with purity of more than 98%, was dissolved in 0.1% DMSO and made up to prepare concentration of 20 mg/mL in D-Hank's medium (USA). DMEM-F12 medium and N_2 were obtained from GIBCO (USA). Lactose dehydrogenase (LDH) and superoxide dismutase (SOD) assay kits were purchased from Nanking Jianchen Biotechnology Corporation (China).

Primary culture of hippocampal neuron

Primary hippocampal neurons were prepared from the hippocampus of SD neonatal rats (24 h). The neurons were suspended in a culture medium contained DEME-F12, fetal bovine serum (FBS), mycillin, and glucose (4×10^5 / mL), and then plated onto poly-*D*-lysine-coated 60 mm dishes. The medium was changed after 48 h by replacing the FBS with N_2, and half of the medium was replaced every 3 d. The cells were cultured in an incubator at 37 ℃ with 5% CO_2. After 7 d of culture, the observation under a phase-contrast microscope demonstrated that the cells were predominantly neuronal cells (> 96%). All experiments were performed after the cells were cultured for 7 d.

Experimental design

Culture dishes were randomly divided into control, hypoxic injury model, and high-, mid-, and low-dose NP (0.25, 0.0625, and 0.0156 mg/mL, respectively) groups. After 7 d of culture, control dishes were kept in normoxia. D-Hank's media with or without different concentration of NP were used for the hypoxic injury model group and the intervention groups. Tan II_A as a positive control was pre-incubated before hypoxia at the concentration of 0.2 mg/mL. After being incubated in normoxia for 24 h, the model and intervention groups were then exposed to a 95% N_2 and 5% CO_2 air mixture for 4 h.

Determination of cell death by Trypan blue staining assay

The cell death was determined by Trypan blue staining assay as described (Chiu *et al*, 2005; 2009). In brief, cells were treated with control or different doses of NP in medium for 24 h and then exposed to a 95% N_2 and 5% CO_2 air mixture for 4 h. Cells exposed to 0.2% Trypan blue were counted in a hemocytometer and cells stained with Trypan blue were numbered. The death rate was calculated according to the percentage of dead cells over vehicle-treated cells. The assay was done in triplicate, and data were expressed as $\bar{x} \pm s$ ($n = 12$).

LDH and SOD assays

LDH release, which is an indicator of cellular injury, was measured using an LDH kit (Ma *et al*, 2005). The medium (200 μL) was mixed with the reagent, and the absorbance (*A*) at 440 nm was measured. The LDH release was calculated according to the formula in the kits.

SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to the superoxide anion generated by the combination of xanthine with xanthine oxidase. One unit (U) of SOD activity was defined as the quantity of enzyme capable of decreasing NBT reduction by 50%. The results were expressed as U/mg protein in the homogenate of supernatants of the neurons.

Electron microscopy

The cells were washed twice with PBS, scraped, collected, and centrifuged at 1800 r/min for 5 min. The cells were then fixed with 2.5% glutaraldehyde, dewatered, embedded, sliced, and counterstained. The intracellular structures were observed by a transmission electron microscope (TEM).

Data analyses

Statistical analyses were performed using SPSS 10.0 and the results were expressed as $\bar{X} \pm s$. Differences between the means were determined by One-way ANOVA followed by a Student-Newman-Keuls test for multiple comparisons. A value of $P < 0.05$ was considered significantly.

Results

NP significantly increased cell viability in hypoxic hippocampal neurons

Dead cells were stained by Trypan blue. A large number of neurons [(80.66 ± 10.37)%] were stained blue after incubation in low oxygen condition (Fig. 1). However, pretreatment with NP could significantly decrease the number of blue-stained cells. The mortality rates in high-, mid-, and low-dose NP groups were (21.34 ± 3.75)%, (37.36 ± 4.38)%, and (49.29 ± 2.86)% ($P < 0.05$ *vs* model group), respectively. Pretreatment with Tan II_A as a positive control also decreased the mortality to (42.2 ± 4.97)% ($P < 0.05$ *vs* model group).

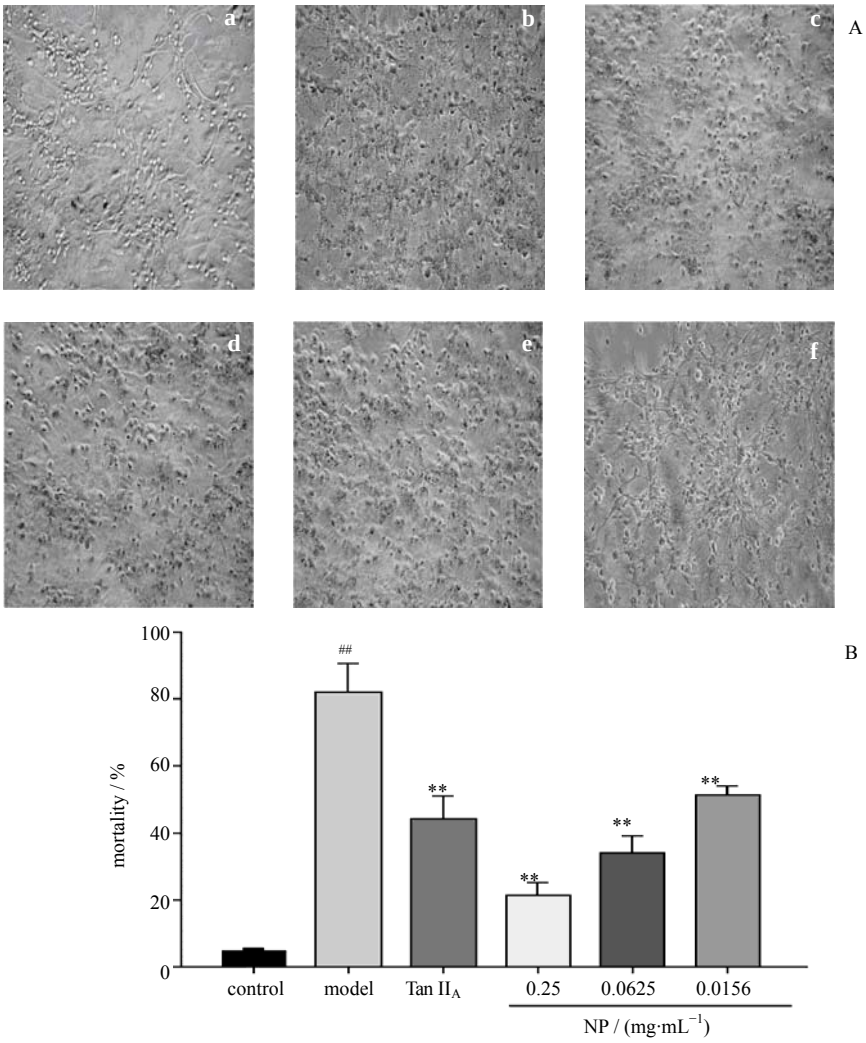

Fig. 1 Effect of NP at high-, mid-, and low-doses on viability of hypoxic hippocampal neurons

A: Trypan blue staining assay showed the dead cells in different groups

a: control group b: model group c: Tan II_A group d: NP 0.25 mg·mL^{-1} group e: NP 0.0625 mg·mL^{-1} group f: NP 0.0156 mg·mL^{-1} group

B: quantification of mortality by Trypan blue staining assay ($\bar{X} \pm s$, $n = 12$)

$^{\#\#}P < 0.01$ *vs* control group $^{**}P < 0.01$ *vs* model group, same as below

NP significantly decreased LDH release from hypoxic hippocampal neurons

Compared with control group, the amounts of LDH in medium increased in model group ($P < 0.01$). When the hypoxic hippocampal neurons were pre-incubated with NP for 24 h, the LDH released by each dosage group remained low ($P < 0.05$ *vs* model group). The increase in LDH leakage was completely recovered by pretreatment with NP, which implied a protective role of NP against neuronal membrane damage induced by hypoxia in primary hippocampal neurons (Fig. 2).

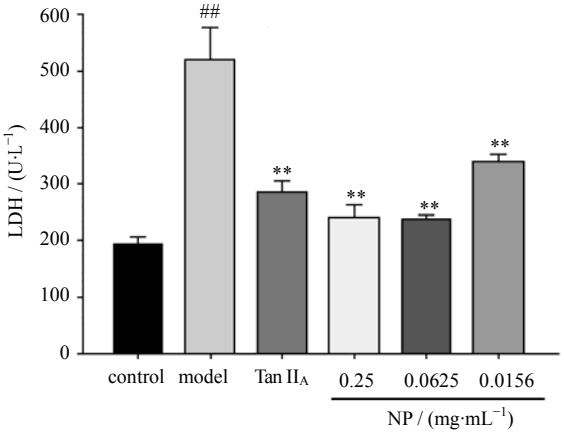

Fig. 2 Effect of NP on LDH release from hypoxic hippocampal neurons ($\bar{X} \pm s$, $n = 12$)

NP significantly promoted SOD activity after hypoxia *in vitro*

The mean SOD level in neurons from control group was (26.914 ± 2.907) U/mg protein (Fig. 3). The SOD level was significantly decreased in model group [(7.962 ± 1.752) U/mg protein, $P < 0.01$ *vs* control group]. The mean SOD levels in neuron from high-, mid-, and low-doses NP groups were (19.388 ± 2.053), (20.782 ± 2.992), and (15.390 ± 1.642) U/mg, respectively. NP in all dose groups could significantly promote SOD activity ($P < 0.01$ *vs* model group).

NP ameliorating injury-induced changes in neuronal morphology

The TEM data showed no obvious autolytic changes in hippocampal neurons in control group. The neurons maintained their normal morphological characteristics, i.e., normal nuclei, normal mitochondria, rough endoplasmic reticulum (RER) with distinct structures, and abundant ribosomes in endochylema (Fig. 4A). After 4 h of hypoxic treatment, the neurons

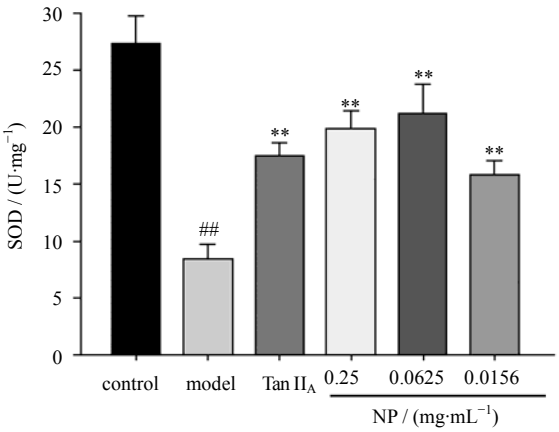

Fig. 3 Effect of NP on SOD levels in neurons ($\bar{X} \pm s$, $n = 12$)

showed obvious autolytic changes. Chromatins were condensed and aggregated, and mitochondrial swelling was observed, which may be the result of increasing membrane permeability. The vacuolization of RER was also observed and a large number of ribosomes were lost (Fig. 4B). In contrast to the neurons subjected to direct hypoxia, the neurons pretreated with NP evidently attenuated the damage caused by direct hypoxia. Cells exposed to NP also showed complete lamellated cristae and membrane constructs in mitochondria and abundant ribosomes in RER and endochylema (Figs. 4D—F). Pretreatment with Tan II_A also markedly lessened the neuronal damage (Fig. 4C).

Discussion

Brain injury models *in vivo* for organ function and treatment studies have been well established (Prieto-Arribas and Moreno-Gutiérrez, 2008; Taoufik and Probert, 2008; Tsukada, Watanabe, and Yamashima, 2001). Many evidences showed that hippocampal neurons were more sensitive to injury than cortical neurons. However, the components of hippocampus are very complex and include multiple kinds of cells, such as astrocytes, oligodendrocytes, and ependymocytes. Primary cultured hippocampal neurons *in vitro* could be used to study the response of a single component to hypoxic injury.

Viable cells could not be stained by Trypan blue. After being treated with hypoxia for 4 h, a large number of dead neurons were stained by Trypan blue. However, pretreatment with NP significantly decreased the number of blue-staining neurons. LDH is an enzyme

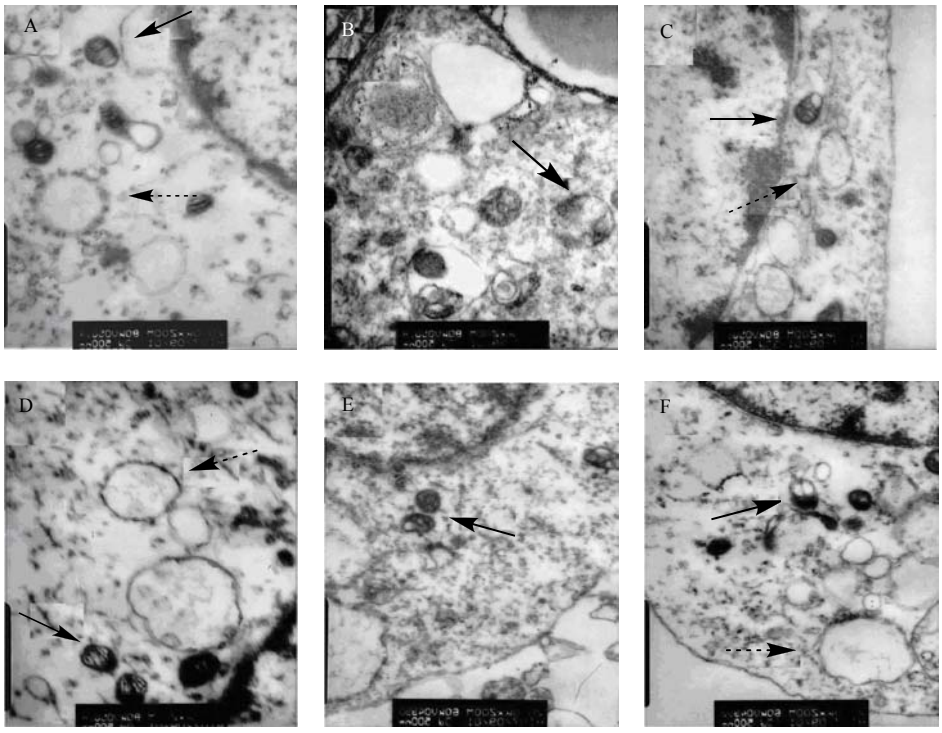

Fig. 4 Characteristic changes of neuronal morphology under TEM

A: control group B: model group C: Tan II_A group D: NP 0.25 mg·mL^{-1} group E: NP 0.0625 mg·mL^{-1} group F: NP 0.0156 mg·mL^{-1} group. Solid arrows indicate mitochondrial and dash arrows indicate RER

found in almost all body tissues. It plays an important role in cellular respiration, wherein glucose from food is converted into usable energy. Acute hypoxia causes cell damage and destroys membrane integrity, thereby causes the release of LDH (Rong, Geng, and Lau, 1996). LDH is an important indicator that reflects the integrity of cell membranes. Pretreatment with NP could attenuate the LDH release and keep the integrity of cell membrane.

Formation of free radicals in tissues under ischemia/hypoxia has been well-established (Schmidley, 1990). Hypoxia induces the release of highly reactive oxygen species (ROS). Oxidative stress is known to induce the extensive lipid peroxidation (LP) of neuronal membranes and to have a key role in hypoxia-induced neuronal injury and cell death. NP possessed anti-oxidative properties and was able to provide tissue protection. A number of studies have shown the anti-oxidative activity of *P. anserina* roots (Chen and Wang, 2004; Li *et al*, 2005). A previous study found that NP also had anti-oxidative activity and protected myocardium and brain tissue from acute ischemic/hypoxic injury (Li *et al*, 2009; Wang *et al*, 2011). All tissues in organism contain some anti-oxidative enzymes to protect themselves from the hazardous effect of an oxidative attack.

Since oxidative stress occurs through excess formation and/or impaired removal of ROS, the anti-oxidant defense system is a crucial component in the maintenance of redox homeostasis. SOD is a major anti-oxidative enzyme that protects cells from the damaging effect of superoxide by accelerating the dismutation reaction of superoxide (Wakatsuki, Okatani, and Shinohara, 2001). In present study, NP at the concentration of 0.25 mg/mL promoted the SOD activity almost 2.5-fold of the model group. These findings are consistent with previous studies.

Fourteen compounds (seven triterpenoids, four isoflavones, two sterols, and one nucleoside) were isolated from NP and were structurally identified. They were ursolic acid (**1**), euscaphic acid (**2**), tormentic acid (**3**), rosamultin (**4**), kaji-ichigoside F1 (**5**), 2α, 3β, 19α-trihydroxyolean-12-en-28oic acid-(28→1)-β-*D*-glucopyranosyl ester (**6**), 2-oxo-3β, 19α-dihydroxyurs-12-en-28-oic acid-(28→1)-β-*D*-glucopyranosyl ester (**7**), β-sitosterol (**8**), β-daucosterol (**9**), adenosine (**10**), daidzin (**11**), puerarin (**12**), 3′-methoxy puerarin (**13**), and daidzein 8-*C*-apiosyl (1→6) glucoside (**14**),

respectively. We conclude that compounds **10** (Sommerschild *et al*, 1999; Zhao *et al*, 2003; Kin *et al*, 2004) and **11**—**14** may partly be responsible for its antihypoxia ability (Miao, Zhao, and Qin, 2005; Cheng *et al*, 2011). Further research will be done in our following works.

In summary, NP has a neuroprotective effect from hypoxic injury on primary culture hippocampal neurons. The possible mechanism is as follows: NP increases the ability of cells to scavenge oxygen free radicals and improves mitochondrial dysfunction, which could prevent cell death.

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