Effects of total saponins of Panax notoginseng on immature neuroblasts in the adult olfactory bulb following global cerebral ischemia/reperfusion

Xu He1,2,*, Feng-jun Deng1,9, Jin-wen Ge1, Xiao-xin Yan1, Ai-hua Pan1,4, Zhi-yuan Li1,4

1 Department of Anatomy and Neurobiology, School of Basic Medical Sciences, Central South University, Changsha, Hunan Province, China
2 Department of Anatomy, Yiyang Medical College, Yiyang, Hunan Province, China
3 Department of Pharmacy, Yiyang Medical College, Yiyang, Hunan Province, China
4 Department of Integrated Traditional and Western Medicine, Hunan University of Traditional Chinese Medicine, Changsha, Hunan Province, China

Abstract

The main active components extracted from Panax notoginseng are total saponins. They have been shown to inhibit platelet aggregation, increase cerebral blood flow, improve neurological behavior, decrease infarct volume and promote proliferation and differentiation of neural stem cells in the hippocampus and lateral ventricles. However, there is a lack of studies on whether total saponins of Panax notoginseng have potential benefits on immature neuroblasts in the olfactory bulb following ischemia and reperfusion. This study established a rat model of global cerebral ischemia and reperfusion using four-vessel occlusion. Rats were administered total saponins of Panax notoginseng at 75 mg/kg intraperitoneally 30 minutes after ischemia then once a day, for either 7 or 14 days. Total saponins of Panax notoginseng enhanced the number of doublecortin (DCX)+ neural progenitor cells and increased co-localization of DCX with neuronal nuclei and phosphorylated cyclic adenosine monophosphate response element-binding/DCX+ neural progenitor cells in the olfactory bulb at 7 and 14 days post ischemia. These findings indicate that following global brain ischemia/reperfusion, total saponins of Panax notoginseng promote differentiation of DCX+ cells expressing immature neuroblasts in the olfactory bulb and the underlying mechanism is related to the activation of the signaling pathway of cyclic adenosine monophosphate response element binding protein.

Key Words: nerve regeneration; total saponins of Panax notoginseng; cerebral ischemia/reperfusion; immature neurons; neurogenesis; doublecortin; olfactory bulb; neural regeneration

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Introduction

The subventricular zone of the lateral ventricle is a neurogenic niche because it can maintain neurogenesis throughout life (Luskin, 1993; Alvarez-Buylla and Garcia-Verdugo, 2002; Ponti et al., 2013). Precursors from the subventricular zone migrate toward the olfactory bulb following the rostral migratory stream pathway where they are functionally integrated into circularity networks (Winner et al., 2002; Ma et al., 2009; Nissant et al., 2009; Macklis, 2012). Doublecortin (DCX) is an endogenous microtubule-associated protein with an immature phenotype and is a good marker of neuroblasts (Francis et al., 1999; Brown et al., 2003; Couillard-Despres et al., 2005; Saalting et al., 2012). Transient cerebral ischemia or brain trauma can stimulate endogenous neurogenesis in certain areas of the brain including the olfactory bulb, and this contributes to neuronal recovery to varying degrees (Zhang et al., 2001; Iwai et al., 2003; Choi et al., 2010; Pan et al., 2013). However, the capacity of endogenous self-repair is limited, so it is imperative to find either candidate drugs or other strategies to induce and amplify endogenous adult precursors following global cerebral ischemia.

Panax notoginseng is a traditional Chinese medicine widely used in China to remove blood stasis. Total saponins of Panax notoginseng (TSPN) are the bioactive constituents of Panax notoginseng (Ng, 2006), and are responsible for inhibiting apoptosis and caspase activation (Li et al., 2009), enhancing endogenous brain-derived neurotrophic factor expression, reducing cytokines and blood-brain barrier permeability, and stimulating neural stem cell proliferation and differentiation in the hippocampus (Si et al., 2011). Although the olfactory bulb is an area that displays neurogenesis and is an important part of the rostral migratory stream, there are no reports on...
whether TSPN could enhance neurogenesis in the olfactory bulb following global cerebral ischemia/reperfusion (I/R). This study evaluated the effects of TSPN on immature neuroblasts in the olfactory bulb of a rat model of transient global cerebral I/R induced by four-vessel occlusion.

Materials and Methods
Establishment of transient global cerebral I/R models
A total of 80 specific-pathogen-free male Sprague-Dawley rats weighing 250–290 g and aged 8 weeks were obtained from the Animal Center of Xiangya School of Medicine, Central South University, China (license No. SCXK (Xiang) 2009-0012). All animal procedures conformed to the National Institutes of Health Guide for the care and use of laboratory animals, and all procedures were approved by the Ethics Committee of Central South University, China. All efforts were made to minimize animal discomfort and reduce the number of animals used.

Four-vessel occlusion was used to induce cerebral ischemia (Pulssineli and Brierley, 1979). Briefly, all rats were anesthetized with 1% sodium pentobarbital diluted in distilled water (50 mg/kg, intraperitoneally) in a sterile room. The rats were positioned in Kopf stereotactic apparatus (David Kopf Instruments, Tujunga, CA, USA) and the vertebral arteries were irreversibly occluded by electrocoagulation. A non-absorbable suture was looped around the carotid arteries. On the following day, rats were re-anesthetized and carotid arteries were disconnected carefully to avoid damage to the vagus nerve, and then occluded with micro-arterial clamps for 30 minutes. Rats lost their righting reflex during I/R and this was achieved by de-clamping the arteries. During the surgery, rectal temperature was monitored and maintained at 37 ± 0.5°C with a rectal probe and a heat lamp was used to ensure the body temperature at 37°C. Eight rats died, so the remaining 72 animals were equally and randomly divided into the TSPN group and vehicle group.

TSPN administration
The rats in the TSPN group were administered TSPN (reagent No. M004538, high-performance liquid chromatography ≥ 98%, Chengdu Maikax Chemical Co., Ltd., Chengdu, China) intraperitoneally 30 minutes after brain ischemia. The dose of TSPN was 75 mg/kg suspended in 0.9% sodium salt 10 g/L, once per day for 1, 7 and 14 days after reperfusion. Rats in the vehicle group were treated with an equal volume of sodium salt, one injection per day until the rats were sacrificed at either 1, 7 or 14 days post ischemia.

Tissue preparation
Rats in both groups were intraperitoneally anesthetized with a high dose of sodium pentobarbital (400 mg/kg) and then perfused transcardially with saline followed by 4% paraformaldehyde. The brains were post-fixed at 4°C overnight, and were treated in gradual concentrations of sucrose (15% and 30%) until they sank. Coronal sections were prepared across the olfactory bulb (Paxinos and Watson, 2005) in a cryostat (Thermo Shandon Limited, UK). Twelve sets of 30-μm sections were cut and collected for western blot assay. For double immunofluorescence, 12 sets of 8-μm sections were also collected by thaw-mounting on positively charged microslides.

Immunohistochemistry and immunofluorescent labeling
Immunohistochemistry was carried out using the avidin-biotin complex method. Sections were treated with 3% H2O2 for 30 minutes at 24 ± 1°C, and pre-incubated for 1 hour, followed by incubation with goat anti-DCX (1:2,000; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4°C. Sections were further reacted with rabbit anti-goat IgGs (Boster, Wuhan, Hubei Province, China) at 1:400 for 2 hours at 24 ± 1°C and subsequently with avidin-biotin complex reagents for an additional 2 hours. Immunoreactivity was visualized using 0.003% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine.

Immunofluorescent labeling was carried out as follows: sections were immersed in PBS containing 5% donkey serum and 0.1% Triton X-100 for 1 hour, treated with goat anti-DCX (1:2,000), mouse anti-neuronal nuclei (NeuN) (1:4,000; Merck Millipore, Darmstadt, Germany), rabbit anti-phosphorylated cyclic adenosine monophosphate response element binding protein (p-CREB) (1:2,000; Cell Signaling, Boston, MA, USA) overnight at 4°C, followed by a 2-hour reaction with Alexa-Fluor 488 and Alexa-Fluor 594-donkey anti-goat, anti-mouse, anti-rabbit IgGs (1:200, Invitrogen, Carlsbad, CA, USA). Sections were counterstained with bisbenzimide (1:50,000; Hoechst 33342).

Immunostained sections were examined and imaged on a microscope equipped with a digital camera and imaging system (BX40, Olympus, Tokyo, Japan). The sections of the olfactory bulb used for comparison were on the same horizontal plane. Because the DCX+ cells in the olfactory bulb were in a cluster, the mean optical density of images was captured using an identical photographic setting and compared using NIH Image J (NIH, MD, USA). Images at 40× magnification were taken for morphological analyses of positive cells. Co-localization of cells in the olfactory bulb was calculated in a set of sections. In each section, five fields were captured with a 20× objective lens. The total DCX+ cells and co-localized cells were added together for each section, brain, and group. The formula for co-localization rate was: co-localized DCX+ cells/total DCX+ cells × 100%.

Western blot assay
The olfactory bulb was snap-frozen in liquid nitrogen. Frozen tissues were homogenized in a cocktail buffer, including phosphatase inhibitors and protease inhibitors (Roche Applied Science, Mannheim, Germany). The samples were then centrifuged at 1,200 × g for 15 minutes at 4°C. Bicinchoninic acid assay was used to measure the protein concentrations from supernatants. A total of 20 μg protein was loaded in each lane and separated by 12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the blotted proteins were transferred to nitrocellulose membrane. The membrane was blocked using 10% nonfat milk for 2 hours at 24 ± 1°C, then incubated overnight with goat anti-DCX.
(1:1,000, Santa Cruz Biotechnology) and mouse anti-GAPDH (1:10,000; Boster). The membrane was washed and incubated with horseradish peroxidase-conjugate rabbit anti-goat or anti-mouse (1:5,000; Merck Millipore, Merck KGaA, Germany) for 2 hours at 24 ± 1°C, followed by exposure to photographic films for 1 to 5 minutes. Western blot band intensity (integrated optical density) was measured using NIH Image J. GAPDH was used as an internal reference.

**Statistical analysis**

All data are expressed as the mean ± SD, and were analyzed using Prism GraphPad5.0 (GraphPad Software Inc., La Jolla, CA, USA). Comparisons were conducted using one-way analysis of variance followed by Bonferroni post hoc tests. A value of $P \leq 0.05$ was considered statistically significant.

**Results**

TSPN up-regulated the number of DCX$^+$ cells in the olfactory bulb following global cerebral I/R

DCX has been used as a marker for immature neurons, and DCX$^+$ cells populate in the piriform cortex and olfactory bulb of rabbits, cats, rats, guinea pigs and non-human primates...
Figure 3 Double immunofluorescence staining for DCX and NeuN (A) and quantitative data (B) in the olfactory bulb of adult rats at 14 days post ischemia. E' and F' are enlarged images of boxes in E and F, respectively. Scale bar: 100 μm in E and F; scale bar: 50 μm in E' and F. All data are expressed as the mean ± SD (six rats per group), and comparisons were conducted using one-way analysis of variance followed by Bonferroni post hoc tests. **P < 0.01, ***P < 0.001, vs. vehicle group. ns: P > 0.05, vs. vehicle group. TSPN: Total saponins of Panax notoginseng; GCL: granule cell layer; DCX: doublecortin; NeuN: neuronal nuclei.

Figure 4 Double immunofluorescence staining for DCX and p-CREB (A) and quantitative data (B) in the olfactory bulb of adult rats at 14 days post ischemia. C' and D' are enlarged images of boxes in C and D, respectively. Scale bar: 100 μm in C and D; scale bar: 50 μm in C' and D'. All data are expressed as the mean ± SD (six rats per group), and comparisons were conducted using one-way analysis of variance followed by Bonferroni post hoc tests. **P < 0.01, ***P < 0.001, vs. vehicle group. ns: P > 0.05, vs. vehicle group. TSPN: Total saponins of Panax notoginseng; p-CREB: phosphorylated cyclic adenosine monophosphate response element binding protein; GCL: granule cell layer; DCX: doublecortin.
(Xiong et al., 2008; Cai et al., 2009; Zhang et al., 2009; Klemm et al., 2011; He et al., 2014; Zeng et al., 2014). In the present study, global cerebral I/R affected DCX labeling, with varied shapes and sizes of cells detectable mainly around the granule cell layer, internal plexiform layer, and mitral cell layer. Dendrite-like processes chain in a cluster in the granule cell layer (Figure 1A3, B3). There was no significant difference in the mean optical density of DCX in the granule cell layer in vehicle or TSPN groups 1 day post ischemia (P > 0.05). In comparison with the vehicle group, TSPN significantly increased the mean optical density of DCX+ cells in the granule cell layer at 7 and 14 days post ischemia (day 7: P < 0.05; day 14: P < 0.001; Figure 1C). Furthermore, the mean optical density of DCX+ cells was significantly higher in the TSPN group than in the vehicle group in the internal plexiform layer and mitral cell layer (P < 0.0001, F = 16.8, degrees of freedom (df) = 5, 24; P < 0.0001, F = 10.7, df = 5, 24). However, there was no significant difference in the internal plexiform layer and mitral cell layer between the TSPN and vehicle groups 1 day post ischemia (P > 0.05). At 7 and 14 days after ischemia, the mean optical density of DCX+ cells in the internal plexiform layer was significantly higher in the TSPN group than in the vehicle group (day 7: P < 0.05; day 14: P < 0.01). The mean optical density of DCX+ cells in the mitral cell layer was significantly higher in the TSPN group than in the vehicle group at 14 days post ischemia (P < 0.05; Figure 1D and E).

TSPN elevated DCX protein levels in the olfactory bulb following global cerebral I/R

DCX protein levels in the olfactory bulb formation were determined by western blot assay. Immunoblotted DCX signal increased in the TSPN group compared with the vehicle group at 7 and 14 days post ischemia (Figure 2A). The mean optical density of DCX protein level at 7 and 14 days post ischemia was significantly higher in the TSPN group than in the vehicle group (day 7: P < 0.001; day 14: P < 0.001), but not at 1 day post ischemia (P > 0.05; Figure 2B).

TSPN enhanced co-localization of DCX with NeuN in the olfactory bulb following global cerebral I/R

There were a higher number of DCX+ cells in the granule cell layer than in any other layer between the vehicle and TSPN groups (Figure 1). We therefore focused on the granule cell layer. DCX+ cells expressing NeuN were detected in the olfactory bulb at all time points post ischemia (Figure 3A), and the ratios of DCX+ cells co-expressing NeuN to the total DCX-labeled cells were higher in the TSPN group than in the vehicle group at both 7 and 14 days post ischemia (day 7: P < 0.01; day 14: P < 0.001), but not at 1 day (P > 0.05; Figure 3B).

TSPN induced immature neuroblasts in the olfactory bulb through phosphorylation of cyclic adenosine monophosphate response element binding protein (CREB) following global cerebral I/R

To further understand the underlying mechanism by which TSPN regulated immature neurons in the olfactory bulb, we tested the activation of p-CREB in the olfactory bulb following global cerebral ischemia using immunohistochemistry. A number of p-CREB/DCX double-labeled cells were found in both groups at various time points (Figure 4A). Moreover, the percentage of p-CREB/DCX double-labeled cells significantly increased in the TSPN group compared with the vehicle group at 7 days (P < 0.01) and at 14 days (P < 0.001), but not at 1 day post ischemia (P > 0.05; Figure 4B).

Discussion

We examined TSPN effects on immature neuroblasts in an animal model of global cerebral I/R. TSPN evidently enhanced DCX-expressing immature neurons in the olfactory bulb. Moreover, TSPN noticeably increased DCX/NeuN co-expression and p-CREB/DCX+ cells, which suggests that TSPN can promote neurochemical differentiation of immature neural cells in the olfactory bulb following global ischemia by up-regulating the p-CREB signaling pathway.

Neurogenesis is a process whereby precursor cells divide into immature neurons and then differentiate into neurons with a mature phenotype (Lazarini et al., 2014). Immature neurons expressing DCX are of great significance to brain plasticity and development (Nacher et al., 2001; Bloch et al., 2011) and are involved in cytoskeletal changes (Kutsuna et al., 2014) and structural plasticity, and are thought to occur at synaptic and neuritic levels under physiological conditions (Luo, 2002; He et al., 2014). Neurogenesis can be accelerated after brain ischemia in the adult rat, monkey, human cerebrum including the olfactory bulb (Jin et al., 2001b; Tonchev et al., 2003; Gould, 2007; Kutsuna et al., 2014). In our study, the increased numerical density of immature DCX-expressing neurons indicates an increased population of newborn immature neurons and dendritic processes. To further confirm the immunohistochemistry result, western blots were carried out to detect DCX protein levels. Densitometry was consistent with the immunofluorescence, suggesting that TSPN increased the population of immature neurons in the olfactory bulb following global cerebral I/R.

It is well known that when newly generated precursor cells in the adult brain begin developing and maturing biochemically and morphologically (Brown et al., 2003), newly generated neurons in the olfactory bulb are crucial for olfactory function (Gheusi et al., 2000; Lledo and Saghatelayan, 2005; Kermen et al., 2010). NeuN is a mature neuronal marker (Mullen et al., 1992; Sarnat et al., 1998) and is both apparent and increased during hippocampal neurogenesis. Therefore, the extent of DCX/NeuN co-expression may implicate the neurochemical maturity of immature olfactory DCX-expressing neurons. Results from this study showed a significant increase in NeuN co-expression among the DCX+ neurons in the olfactory bulb of global cerebral ischemia rats following intraperitoneally injecting TSPN at 7 and 14 days. These findings suggest that TSPN could enhance and drive the neurochemical differentiation of immature neurons in the olfactory bulb toward the phenotype of maturation.

CREB is an important nuclear transcription factor, and
experiments in vivo and vitro demonstrated that CREB signaling pathways play a vital role in the neurogenesis and survival of immature neuroblasts in the olfactory bulb (Giachino et al., 2005), subventricular zone (Gampe et al., 2011; Herold et al., 2011) and hippocampus (Jagasia et al., 2009; Kim et al., 2010). Activated CREB is involved in many biological functions such as enhancement of neuronal regeneration, synaptic formation and spatial learning (Walton et al., 1996; O’Connell et al., 2000; Rajan et al., 2015). In transient ischemic adult rodents, high levels of p-CREB existed (Walton et al., 1996; Tanaka et al., 2000; Jin et al., 2001a) in immature DCX-expressing neurons in the dentate gyrus (Nakagawa et al., 2002). Cell counting showed that there were a large number of p-CREB+DCX+ neural progenitor cells in the olfactory bulb of the two groups at different time points, which suggested engagement of the CREB signaling pathway in adult neurogenesis and recruitment of immature DCX-expressing neurons for brain repair following global cerebral ischemia. Furthermore, TSPN apparently increased the number of the DCX+ cells expressing p-CREB in the olfactory bulb of rats surviving 7 and 14 days, implying that TSPN could modulate the newborn immature neuroblasts by up-regulating the p-CREB signaling pathway.

In summary, TSPN could enhance regeneration and maturation of DCX+ cells expressing immature neuroblasts in the olfactory bulb of adult rats through activating the CREB signaling pathway following global cerebral I/R.

Author contributions: ZYL and AHP designed the study. XH, FJD, and JWG implemented the experiments. XH wrote and revised the paper. XXY analyzed the data and supervised the whole research. All authors approved the final version of the paper.

Conflicts of interest: None declared.

References


