Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2019, **17**(6): 0424–0434
Chinese Journal of Natural Medicines

Pretreatment of *Populus tomentiglandulosa* protects hippocampal CA1 pyramidal neurons from ischemia-reperfusion injury in gerbils *via* increasing SODs expressions and maintaining BDNF and IGF-I expressions

Tae-Kyeong Lee[1Δ], Joon Ha Park[2Δ], Ji Hyeon Ahn[2], Hyunjung Kim[1], Minah Song[1], Jae-Chul Lee[1], Jong Dai Kim[3], Yong Hwan Jeon[4], Jung Hoon Choi[5], Choong Hyun Lee[6], In Koo Hwang[7], YAN Bing-Chun[8], Moo-Ho Won[1*], Il Jun Kang[9*]

[1] *Department of Neurobiology, School of Medicine, Kangwon National University, Chuncheon 24341, Republic of Korea;*
[2] *Department of Biomedical Science, Research Institute of Bioscience and Biotechnology, Hallym University, Chuncheon 24252, Republic of Korea;*
[3] *Division of Food Biotechnology, School of Biotechnology, Kangwon National University, Chuncheon 24341, Republic of Korea;*
[4] *Department of Radiology, School of Medicine, Kangwon National University, Chuncheon 24289, Republic of Korea;*
[5] *Department of Anatomy, College of Veterinary Medicine, Kangwon National University, Chuncheon 24341, Republic of Korea;*
[6] *Department of Pharmacy, College of Pharmacy, Dankook University, Cheonan 31116, Republic of Korea;*
[7] *Department of Anatomy and Cell Biology, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 08826, Republic of Korea;*
[8] *Jiangsu Key Laboratory of Integrated Traditional Chinese, Western Medicine for Prevention and Treatment of Senile Diseases, Yangzhou 225001, China;*
[9] *Department of Food Science and Nutrition, Hallym University, Chuncheon 24252, Republic of Korea*

Available online 20 June, 2019

[ABSTRACT] To examine the effects of *Populus tomentiglandulosa* (PT) extract on the expressions of antioxidant enzymes and neurotrophic factors in the cornu ammonis 1 (CA1) region of the hippocampus at 5 min after inducing transient global cerebral ischemia (TGCI) in gerbils, TGCI was induced by occlusion of common carotid arteries for 5 min. Before ischemic surgery, 200 mg·kg^{-1} PT extract was orally administrated once daily for 7 d. We performed neuronal nuclear antigen immunohistochemistry and Fluoro-Jade B staining. Furthermore, we determined *in situ* production of superoxide anion radical, expression levels of SOD1 and SOD2 as antioxidant enzymes and brain-derived neurotrophic factor (BDNF) and insulin-like growth factor I (IGF-I) as neurotrophic factors. Pretreatment with 200 mg·kg^{-1} PT extract prevented neuronal death (loss). Furthermore, pretreatment with 200 mg·kg^{-1} PT extract significantly inhibited the production of superoxide anion radical, increased expressions of SODs and maintained expressions of BDNF and IGF-I. Such increased expressions of SODs were maintained in the neurons after IRI. In summary, pretreated PT extract can significantly increase levels of SODs and protect the neurons against TGCI, suggesting that PT can be a useful natural agent to protect against TGCI.

[KEY WORDS] Transient global cerebral ischemia; *Populus tomentiglandulosa*; Neuroprotective effects; Antioxidant enzymes

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2019)06-0424-11

Introduction

Temporary hindrance of blood circulation in the whole brain causes ischemia-reperfusion injury (IRI), which induces neuronal damage or death in specific regions of the brain including the hippocampus [1]. Cornu ammonis 1 (CA1) in the hippocampus is well known as one of vulnerable regions to ischemia-reperfusion [2]. In this region, death (loss) of pyramidal neurons occurs at several days after ischemia-reperfusion injury. Such neuronal death is called "delayed neuronal death" [3-5].

[Received on] 11-Feb.-2019
[*Corresponding authors] E-mail: mhwon@ kangwon.ac.kr (Moo-Ho Won); E-mail: ijkang@hallym.ac.kr (Il Jun Kang)
[Δ]These authors contributed equally to this work.
These authors have no conflict of interest to declare.

Various mechanisms of neuronal death following IRI have been reported including glutamate-induced excitotoxicity, oxidative stress by excessive production of reactive oxygen species (ROS) and glial activation-mediated inflammatory reaction [6-9]. Superoxide anion production has been measured by dihydroethidium (DHE) fluorescence [10-11]. Cu, Zn-superoxide dismutase (SOD1) and Mn-superoxide dismutase (SOD2) are known to play a protective role in the pathogenesis of superoxide radical-mediated brain injury [12]. Elevation of SODs levels can prevent neuronal loss from ischemic insults in the brain [13]. In addition, neurotrophic factors in mammalian brains are critically involved in maintaining neuronal function [14]. Increase of levels of insulin-like growth factor I (IGF-I) and brain-derived neurotrophic factor (BDNF) can improve cognitive function and neurogenesis [15] and protects neurons from ischemic injury [16].

To prevent neuronal death following IRI, many researchers have tried to find medical plants that protect neurons from ischemia-reperfusion IRI using animal models of transient brain ischemia because medical plants possess diverse biological properties including antioxidant and anti-inflammatory effects [13, 17-18]. Mechanisms underlying such effects of the medical plants have also been determined [13, 17-18].

The genus *Populus* belongs to family Salicaceae that includes diverse species. Some species have shown various medical actions. For example, *Populus balsamifera* L. possesses antibacterial activity [19]. *Populus nigra* and *Populus davidiana* has pharmacological potential due to antioxidant, anti-inflammatory and hepatoprotective properties [20-21]. However, to the best of our knowledge, few studies have investigated pharmacological potentials of *Populus tomentiglandulosa* (PT), one species belonging to genus *Populus*. Therefore, the objective of this study was to examine protective effects of *Populus tomentiglandulosa* against IRI and its protective mechanisms using gerbil as a model for transient global cerebral ischemia(TGCI) [22].

Materials and Methods

Experimental animals

Six months old male Mongolian gerbils (body weight 70–80 g) were obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, South Korea. The gerbils were housed in an optimum status under suitable temperature (23 °C) and humidity (60%). 12 hours of light and dark cycle was controlled, and water and feed were freely accessed. Experimental protocol was approved (Approval No. KW-160802-1) by the Institutional Animal Care and Use Committee (IACUC) at Kangwon National University and adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011).

Preparation of Populus tomentiglandulosa (PT) extract

Populus tomentiglandulosa (voucher: L110775; specimen No.: KWNA200004212082, Kangwon National University) was collected from Kangwon Province (South Korea), in October 2015 by Professor Jong Dai Kim. In order to prepare ethanol PT, *Populus tomentiglandulosa* was washed with distilled water, air-dried at 60 °C and ground into fine powder by a grinder (IKA M20, IKA, Staufen, Germany), and the powder was refluxed with 10 vol (*V*/*W*) of 70% ethanol at 70 °C for 24 h. The extraction procedure was repeated 3 times. The extract was filtered *via* Whatman No. 1 filter paper (Whatman Ltd., Maidstone, Kent, UK), concentrated with a vacuum evaporator, and completely dried with a freeze-drier. The extraction yield was 14.7%.

Administration with PT

We had conducted a pilot experiment about dose of the PT extract. We applied 50, 100 and 200 mg·kg^{-1}, respectively, to the experimental animals and induced cerebral ischemia. Among the 3 doses, we found that pyramidal neurons in the hippocampal CA1 region were protected from cerebral ischemia when we treated 200 mg·kg^{-1} PT extract. In this reason, we chose that dose.

Animals were divided into 6 groups (n = 14 in each group): 1) vehicle (sterilized normal saline; 0.9% *W*/*V* NaCl)-treated sham-operated group (vehicle-sham group), 2) vehicle-treated ischemia-operated group (vehicle-ischemia group), 3) 100 and 200 mg·kg^{-1} PT-treated sham-operated groups (PT-sham groups), and 4) 100 and 200 mg·kg^{-1} PT-treated ischemia-operated groups (PT-ischemia groups). PT was dissolved in saline. PT or saline was orally administrated once a day for 1 week before ischemic surgery.

Induction of ischemia-reperfusion injury

IRI was induced by 5 min of TGCI as described previously [23]. In short, the animals treated with PT were anesthetized with a mixture of 2.5% isoflurane (Baxtor, Deerfeld, IL) in 33% oxygen and 67% nitrous oxide. Both common carotid arteries were occluded for 5 min using non-traumatic aneurysm clips. Using an ophthalmoscope, the complete occlusion of common carotid arteries was confirmed by obstruction of blood flow in the central retinal artery. The body temperature under normothermic (37 ± 0.5 °C) condition was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, USA) and maintained using a thermometric blanket from this surgery until the animals recovered completely from anesthesia. Sham-operated animals received the operation, but the common carotid arteries were not occluded.

Western blot analyses

To examine effects of PT on SOD1, SOD2, BDNF and IGF-I levels in the hippocampus, the animals (n = 7 in each group) were used for Western blotting analysis at sham, 2 and 5 days after the ischemic surgery. For tissue preparation, the animals were anesthetized with of pentobarbital sodium (60 mg·kg^{-1}, intraperitoneal injection) [24-25]. Their brains were removed and transversely cut into 400-μm thickness on a vibratome (Leica), and the hippocampi were then dissected with a surgical blade. According to our published method [16],

in short, the tissues were homogenized in 50 mmol·L^{-1} PBS (pH 7.4) containing EGTA (pH 8.0), 0.2% NP-40, 10 mmol·L^{-1} EDTA (pH 8.0), 15 mmol·L^{-1} sodium pyrophosphate, 100 mmol·L^{-1} β-glycerophosphate, 50 mmol·L^{-1} NaF, 150 mmol·L^{-1} NaCl, 2 mmol·L^{-1} sodium orthvanadate, 1 mmol·L^{-1} PMSF and 1 mmol·L^{-1} DTT. After centrifugation, each protein level in the supernatant was determined using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, USA). Aliquots containing 20 μg of total protein were boiled at 95 °C in loading buffer containing 150 mmol·L^{-1} Tris (pH 6.8), 3 mmol·L^{-1} DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol for 5 min. The aliquots were then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membrane (Pall Crop, East Hills, NY, USA). In order to reduce background staining, the membranes were incubated with 5% skimmed milk in PBS containing 0.1% Tween 20 for 45 min at 25°C Sheep anti-SOD1 (1 : 1000, Calbiochem, CA, USA), sheep anti-SOD2 (1 : 1000, Calbiochem, CA, USA), rabbit anti-BDNF (1 : 500, Abcam), rabbit anti-IGF-I (1 : 1000, Santa Cruz, CA, USA) and mouse anti-β-actin (1 : 5000, Abcam, Cambridge, UK) were used as primary antibodies. Peroxidase conjugated donkey anti-sheep IgG (1 : 1000; Cat. No. HAF016, R&D Systems), goat anti-rabbit IgG (1 : 3000; cat. no. 65-6120, Thermo Fisher Scientific Inc.) and goat anti-mouse IgG (1 : 2 000; Cat. No. 62-6520, Thermo Fisher Scientific Inc.) were used as secondary antibodies and enhanced luminol-based chemiluminescence kit (Pierce; Thermo Fisher Scientific Inc.) was used for visualization.

Tissue processing for histology

The animals (n = 7 at each point in time in each group) were sacrificed at sham, 2 and 5 days after ischemia-reperfusion). Briefly, for the tissue preparation, as described previously [24-25], the animals were anesthetized with pentobarbital sodium (60 mg·kg^{-1}, intraperitoneal injection) and perfused transcardially with 4% paraformaldehyde (in 0.1 mol·L^{-1} PB, pH 7.4). Their brains were removed, cryoprotected by infiltration with 30% sucrose (in 0.1 mol·L^{-1} PB, pH 7.4), and serially sectioned into 30-μm coronal sections in a cryostat (Leica, Germany).

DHE fluorescence staining

To investigate *in situ* production of superoxide anion in the hippocampal CA1, oxidative fluorescent DHE (Sigma-Aldrich, St. Louis, USA) was used. In brief, according to our published method [11], the sections were equilibrated under identical conditions for 30 min at 37 °C in Krebs-HEPES buffer (130 mmol·L^{-1} NaCl, 5.6 mmol·L^{-1} KCl, 2 mmol·L^{-1} $CaCl_2$, 0.24 mmol·L^{-1} $MgCl_2$, 8.3 mmol·L^{-1} HEPES, 11 mmol·L^{-1} glucose, pH 7.4). Fresh buffer containing 10 μmol·L^{-1} DHE was topically applied on the sections for 2 h at 37 °C. DHE was oxidized on the reaction with superoxide to ethidium, which could bind DNA in nuclei and fluoresced red.

Immunohistochemistry

To examine neuroprotective effect of PT against IRI, immunohistochemistry was performed according our published protocol [13]. In brief, the sections were incubated with diluted mouse anti-neuronal nuclei antigen (NeuN, a marker of neurons) (1 : 1000, Chemicon, Temecula, CA, USA). Thereafter, the tissues were exposed to biotinylated goat anti-mouse IgG and avidin-biotin complex subsequently (1 : 200, Vector Laboratories, Burlingame, CA, USA). Finally, they were visualized by staining with 3, 3'-diaminobenzidine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

To examine effects of PT on changes in antioxidant enzymes in the CA1 before and after ischemia-reperfusion, for SODs immunohistochemistry, sheep anti-SOD1 (1 : 1500, EMD Millipore, Billerica, MA, USA) and sheep anti-SOD2 (1 : 1500, EMD Millipore, Billerica, MA, USA) was used as primary antibody according to the above-mentioned method. In addition, to elucidate change in neurotrophic factors, we used rabbit anti-BDNF (1 : 200, Abcam, UK) rabbit anti-IGF-I (1 : 200, Santa Cruz, CA, USA) as primary antibodies according to the above-mentioned method.

To establish specificity of each immunostaining, a negative control test was done with pre-immune serum instead of each primary antibody. The negative control was done by absence of immunoreactivity in all structures observed.

Fluoro-Jade B (F-J B) histofluorescence staining

F-J B (a high affinity fluorescent marker for neuronal degradation) histofluorescence staining was performed to examine the neuroprotective effect of PT against ischemic damage. As described previously [26], in brief, the sections were immersed in a solution of 1% sodium hydroxide, and transferred to solution of 0.06% potassium permanganate for 20 min and subsequently incubated with a 0.000 4% F-J B (Histochem, Jefferson, AR) staining solution for 45 min. After washing them with distilled water, they were placed on a slide warmer (approximately 50 °C). Finally, the sections were examined using an epifluorescent microscope (Carl Zeiss, Germany) with blue (450–490 nm) excitation light and a barrier filter.

Data analysis

Western blotting analyses, as previously described [16], the bands were scanned, and densitometric analysis for the quantification of the bands was done using Scion Image software (Scion Crop., Frederick, MD), which was used to count ROD. A ratio of the ROD was calibrated as %, with the vehicle-sham group designated as 100%.

To analyze the production of superoxide anion, the sections were examined with epifluorescent microscope (Olympus, Japan) using an excitation wavelength of 520 to 540 nm. The fluorescence intensity was analyzed from 7 sections in each animal. Ethidium fluorescence was quantified from the images using Image-pro Plus 6.0 software. A ratio of the DHE fluorescence intensity was calibrated as % and the vehicle-sham group was considered as 100%.

Numbers of NeuN immunoreactive neurons and F-J B positive cells were analyzed according to our published method [26]. In brief, digital images of the positive cells in the CA1 were captured with a light microscope (BX53, Olympus,

Germany) which was added a digital camera (DP72, Olympus) connected with a PC monitor. The positive cells were counted in a 250 μm × 250 μm square applied approximately at the center of the CA1 using an image analyzing system (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ).

To analyze each SOD1, SOD2, BDNF and IGF-I immunoreactivity, 5 sections per animal were selected and analyzed according to our published method [27]. In brief, digital images of all immunoreactive structures stained by each antibody were taken from the CA1 through a light microscope (BX53, Olympus, Germany) equipped with digital camera (DP72, Olympus) connected to a PC monitor. The images were calibrated into an array of 512 × 512 pixels corresponding to a tissue area of 140 μm × 140 μm (40× primary magnification). Each immunoreactivity was measured by a 0–255 gray scale system, and the background density was subtracted. A ratio of the relative immunoreactivity (RI) for each antibody was calibrated as % using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software. A ratio of the RI was calibrated as %, with the vehicle-sham group designated as 100%.

Statistical analysis

Data were presented as the mean ± standard error of mean (SEM). The data were measured by two-way analysis of variance (ANOVA) with a post hoc Bonferroni's multiple comparison test, in order to express differences among experimental groups. $P < 0.05$ was considered as indicating statistically significant.

Results

Neuroprotection

Neurons of the pyramidal cell layer, which are named "pyramidal neurons", were well stained with NeuN (Figs. 1Aa and 1Ab). In the vehicle-ischemia group, NeuN-immunoreactive pyramidal neurons were significantly decreased in the CA1 at 5 days after ischemia-reperfusion (Figs. 1Ba and 1Bb). In all the PT-sham groups, distribution pattern and numbers of NeuN-immunoreactive CA1 pyramidal neurons were similar to those in the vehicle-sham group (Figs. 1Ad, 1Ae, 1Ag, 1Ah and 1C). In the PT-ischemia groups, NeuN-immunoreactive CA1 pyramidal neurons were rarely found in the 100 mg·kg^{-1} PT-ischemia group (Figs. 1Bd and 1Be); however, in the 200 mg·kg^{-1} PT-ischemia group, many NeuN-immunoreactive CA1 pyramidal neurons were observed (about 89% of the sham-group) 5 days after ischemia-reperfusion (Figs. 1Bg, 1Bh and 1C).

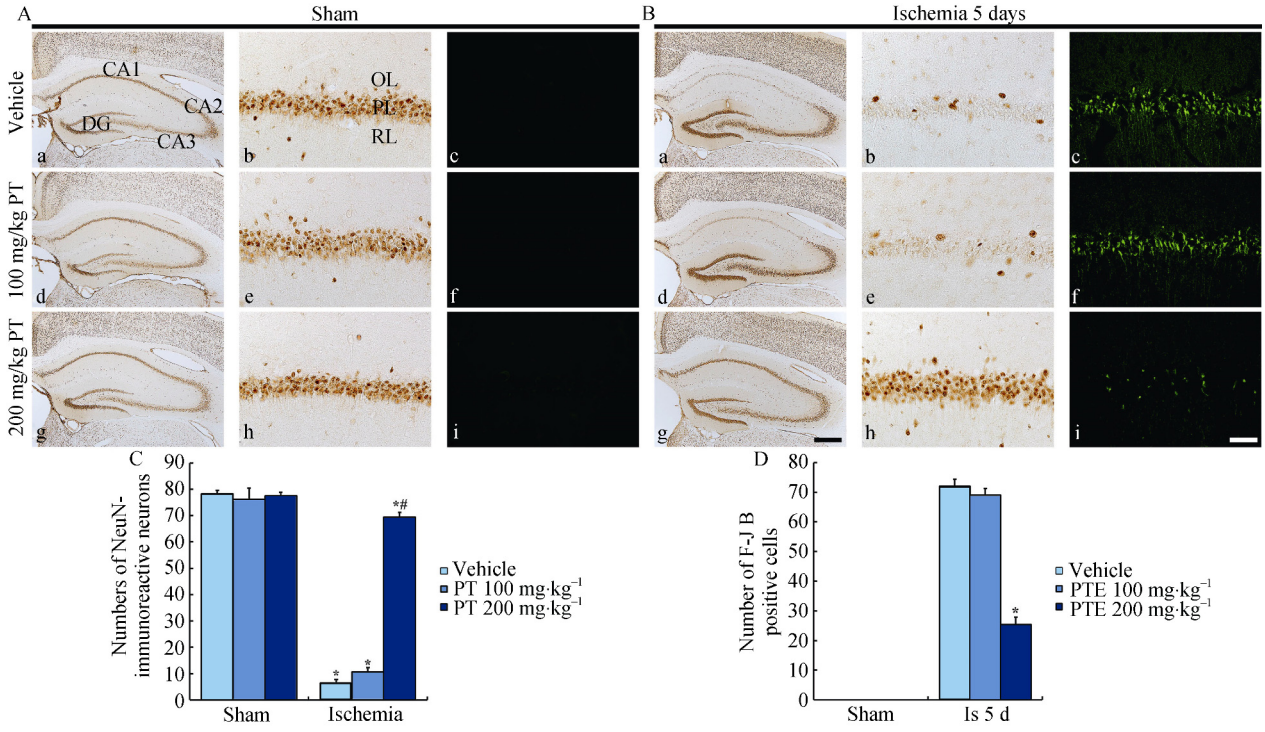


Fig. 1 NeuN immunohistochemistry and F-J B histofluorescence staining in the CA1 of the vehicle-sham (Aa–Ac), vehicle-ischemia (Ba–Bc), 100 mg·kg^{-1} PT-sham (Ad–Af), 100 mg·kg^{-1} PT-ischemia (Bd–Bf), 200 mg·kg^{-1} PT-sham (Ag–Ai) and 200 mg·kg^{-1} PT-ischemia (Bg–Bi) groups 5 d after ischemia-reperfusion. In the vehicle-ischemia and 100 mg·kg^{-1} PT-ischemia groups, a few NeuN-immunoreactive neurons and abundant F-J B positive cells are detected in the pyramidal layer (PL) (asterisks). However, in the 200 mg·kg^{-1} PT-ischemia group, many NeuN-immunoreactive and few F-J B positive cells are shown in the PL. OL: oriens layer; RL: radiatum layer. Scale bar = 400 μm (Aa, Ad, Ag, Ba, Bd and Bg) and 40 μm (Ab, Ac, Ae, Af, Ah, Ai, Bb, Bc, Be, Bf, Bh and Bi). S and T: Numbers of NeuN-immunoreactive neurons (C) and F-J B positive cells (D) in the CA1 (n = 7 in each group, $^{*}P < 0.05$ *vs* vehicle-ischemia group, $^{\#}P < 0.05$ *vs* 100 mg·kg^{-1} PT-ischemia group). The bars indicate the means ± SEM.

F-J B positive cells were not found in the hippocampus of the vehicle-sham group (Fig. 1Ac). However, in the vehicle-ischemia group, F-J B positive cells were shown the pyramidal layer of the CA1 at 5 days after ischemia-reperfusion (Fig. 1Bc). In all the PT-sham groups, F-J B positive cells were not detected (Figs. 1Af and 1Ai). In the 100 mg·kg^{-1} PT-ischemia group, many F-J B positive cells were found in the pyramidal layer like those in the vehicle-ischemia group (Figs. 1Bf and 1D). On the other hand, in the 200 mg·kg^{-1} PT-ischemia group, F-J B positive cells were significantly decreased (about 35% of vehicle-ischemia group) compared to the vehicle-ischemia group (Fig. 1Bi and 1D).

Levels of SOD1, SOD2, BDNF and IGF-I

We found that pretreated PT (200 mg·kg^{-1}) affected levels of SOD1, SOD2, BDNF and IGF-I proteins in the CA1 before and after IRI (Fig. 2).

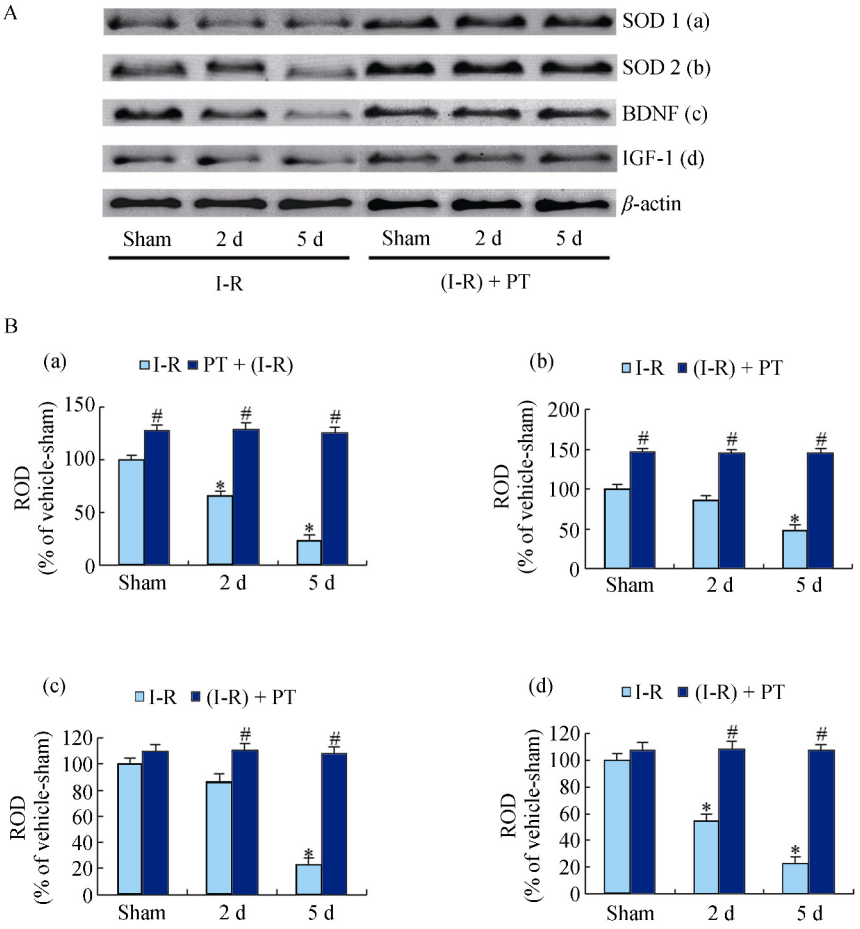


Fig. 2 Western blotting analysis for SOD1 (a), SOD2 (b), BDNF (c) and IGF-I (d) in the CA1 of the vehicle-sham, vehicle-ischemia 2 and 5 d after ischemia reperfusion, PT-sham and PT-ischemia 2 and 5 d after ischemia-reperfusion. In all vehicle-ischemia groups, protein levels of SOD1, SOD2, BDNF and IGF-I are gradually decreased respectively, compare to those in their vehicle-sham groups. However, in the PT-sham groups, protein levels of SOD1 and SOD2 are higher than those in their vehicle-sham groups. And their protein levels are maintained with time after ischemia-reperfusion. On the other hand, in the PT-sham groups, protein levels of BDNF and IGF-I are similar to those in their vehicle-sham groups. And their protein levels are sustained with time after ischemia-reperfusion. Relative optical density (ROD) as the mean percentage values of immunoblot band is represented. I-R: ischemia-reperfusion. (n = 7 in each time point of each group, $^{*}P < 0.05$ *vs* vehicle-sham group, $^{\#}P < 0.05$ *vs* vehicle-sham or vehicle-ischemia groups). The bars indicate the means ± SEM.

SOD1 level in the vehicle-ischemia group was signify-cantly decreased (about 66% of the vehicle-sham group) 2 days after ischemia-reperfusion and very low (about 23% of the vehicle-sham group) 5 days after ischemia-reperfusion. In the PT-sham group, SOD1 level was about 126% of the vehicle-sham group, and the level was not significantly altered until 5 days after ischemia-reperfusion (Figs. 2Aa and 2Ba).

SOD2 level in the vehicle-ischemia group was about 87% of the vehicle-sham group 2 days after ischemia-reperfusion and more decreased (about 49% of the vehicle-sham group) 5 days after ischemia-reperfusion. SOD2 level in the PT-sham group was significantly higher (about 147% of the vehicle-sham group) compared to that in the vehicle-sham group, and the level was maintained until 5 days after ischemia-reperfusion (Figs. 2Ab and 2Bb).

BDNF level in the vehicle-ischemia group was decreased

to about 87% of the vehicle-sham group 2 days after ischemia-reperfusion and very low (about 23% of the vehicle-sham group) 5 days after ischemia-reperfusion. In the PT-sham group, BDNF level was slightly increased (about 110% of the vehicle-sham group) compared to that in the vehicle-sham group, and the level was not changed until 5 days after ischemia-reperfusion (Figs. 2Ac and 2Bc).

IGF-I level in the vehicle-ischemia group was significantly decreased (about 55% of the vehicle-sham group) 2 days after ischemia-reperfusion and very weak (about 23% of the vehicle-sham group) 5 days after ischemia-reperfusion. In the PT-sham group, IGF-I level was similar (about 108% of the vehicle-sham group) to that in the vehicle-sham group, and the level was not altered until 5 days after ischemia-reperfusion (Figs. 2Ad and 2Bd).

DHE fluorescence

In all the sham groups, DHE fluorescence was detected very weakly in the pyramidal layer of the CA1 (Figs. 3Aa and 3Ad), showing that no significant differences were observed between the groups (Fig. 3B).

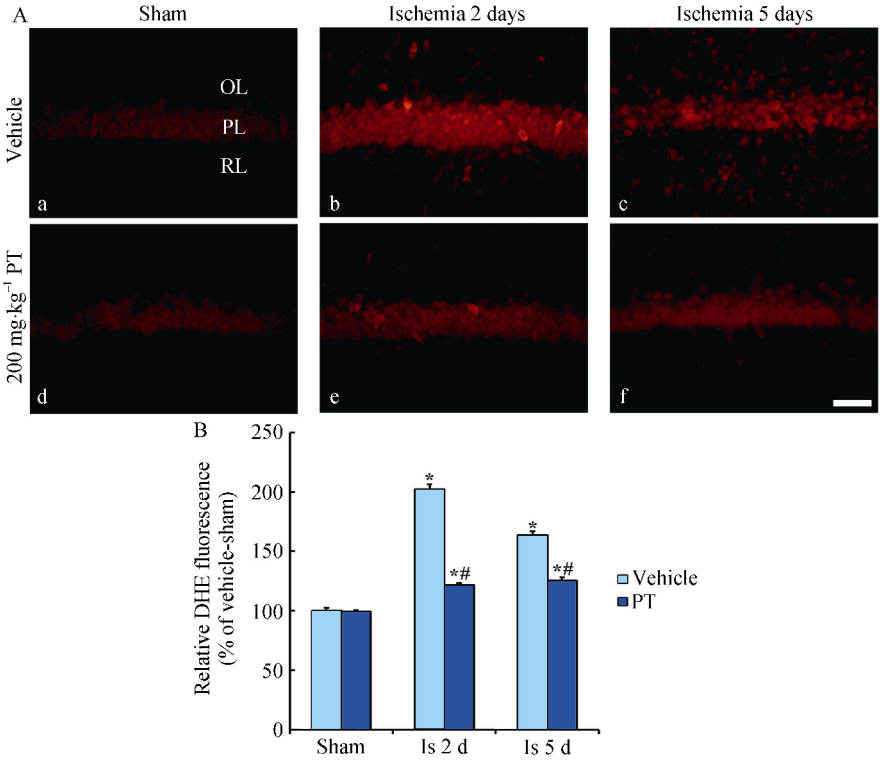


Fig. 3 DHE fluorescence staining in the CA1 of the vehicle-sham (Aa), vehicle-ischemia (Ab and Ac), 200 mg·kg^{-1} PT-sham (Ad) and 200 mg·kg^{-1} PT-ischemia groups 2 d (Ae) and 5 d (Af) after ischemia-reperfusion. ROS prodction is weakly observed in all the sham-groups. In the vehicle-ischemia group, superoxide anion radical level is significantly increased in the pyramidal cell layer (PL). However, in the 200 mg·kg^{-1} PT-ischemia group, ROS production is significantly lower than that in the vehicle-ischemia group. OL: oriens layer; RL: radiatum layer. Scale bar = 40 μm. B: Relative DHE flurorescence as % of DHE fluorescence in CA1 pyramidal neurons (n = 7 in each time point of each group, $^{*}P < 0.05$ *vs* vehicle-sham group, $^{\#}P < 0.05$ *vs* corresponding time point of vehicle-ischemia group). The bars indicate the means ± SEM.

In the vehicle-ischemia group, DHE fluorescence in the pyramidal layer was significantly increased at 2 and 5 days after ischemia-reperfusion (about 202% and 163%, respectively, of the vehicle-sham group) compared to that in the vehicle-sham group (Figs. 3Aa–3Ac and 3Ae).

In the 200 mg·kg^{-1} PT-ischemia group, DHE fluorescence was significantly low (about respectively 60% and 77% at 2 and 5 days, respectively, after ischemia-reperfusion compared to corresponding vehicle-ischemia group (Figs. 3Ab, 3Ac, 3Ae, 3Af and 3B).

Immunoreactivity of SOD1

SOD1 immunoreactivity was easily detected in CA1 pyramidal neurons of the CA1 of the vehicle-sham group (Fig. 4Aa). In the vehicle-ischemia group, SOD1 immunoreactivity in CA1 pyramidal neurons was significantly decreased (about 65% of the vehicle-sham group) 2 days after ischemia-reperfusion and hardly observed (about 26% of the vehicle-sham group) 5 days after ischemia-reperfusion (Figs. 4Ab, 4Ac and 4B).

In the PT-sham group, SOD1 immunoreactivity in CA1 pyramidal neurons was significantly higher (about 122% of the vehicle-sham group) than that in the vehicle-sham group (Figs. 4Ad and 4B). In addition, in the PT-ischemia group, SOD1 immunoreactivity in the CA1 pyramidal neurons was maintained until 5 days after ischemia-reperfusion (Figs. 4Ae, 4Af and 4B).

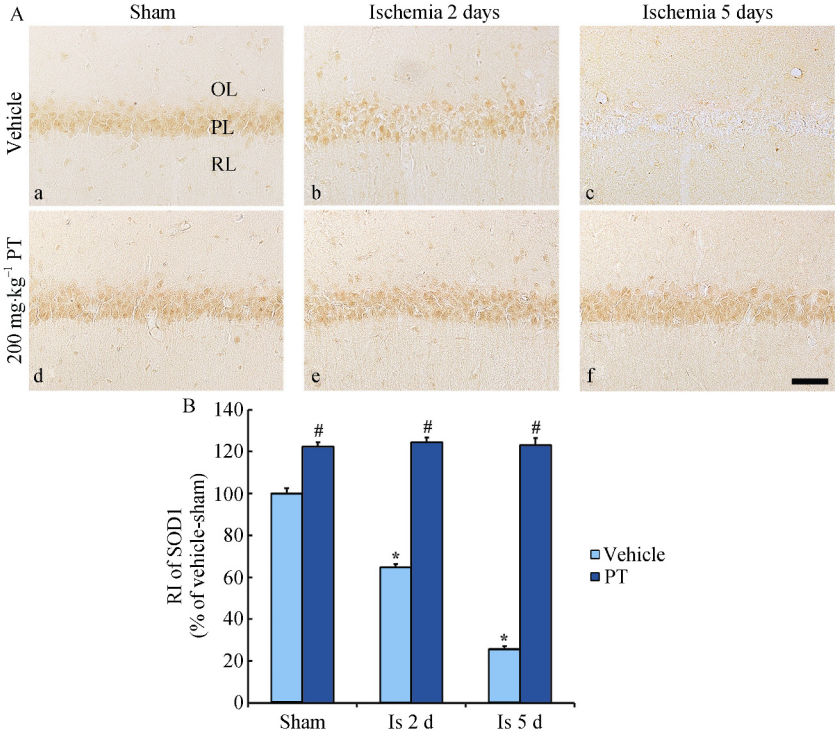


Fig. 4 Immunohistochemical staining for SOD1 in the CA1 of the vehicle-sham (Aa), vehicle-ischemia (Ab and Ac), 200 mg·kg^{-1} PT-sham (Ad) and -ischemia groups 2 d (Ae) and 5 d (Af) after ischemia-reperfusion. SOD1 immunoreactivity is detected in the pyramidal cell layer (PL) in the vehicle-sham group. In the vehicle-ischemia group, SOD1 immunoreactivity is hardly shown in the PL 5 d after ischemia-reperfusion. In the 200 mg·kg^{-1} PT-sham and -ischemia groups, SOD1 immunoreactivity in the PL is significantly increased compared to that in the vehicle-sham group and maitained until 5 d after ischemia-reperfusion. OL: oriens layer; RL: radiatum layer. Scale bar = 40 μm. B: Reactive immunoreactivity as % of SOD1 immunoreactivity in CA1 pyramidal neurons (n = 7 in each time point of each group, $^{*}P < 0.05$ *vs* vehicle-sham group, $^{\#}P < 0.05$ *vs* vehicle-sham and vehicle-ischemia groups). The bars indicate the means ± SEM.

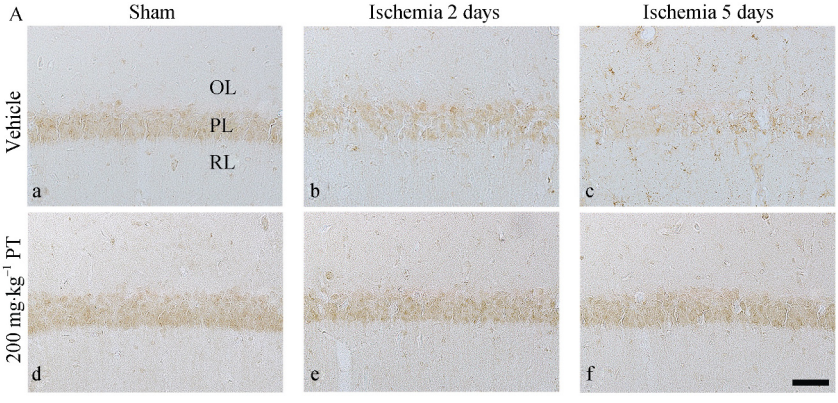
Immunoreactivity of SOD2

In the vehicle-sham group, SOD2 immunoreactivity was also found in CA1 pyramidal neurons (Fig. 5Aa). In the vehicle-ischemia group, SOD2 immunoreactivity in CA1 pyramidal neurons was decreased to about 85% of the vehicle-sham group 2 days after ischemia-reperfusion and more significantly decreased (about 57% of the vehicle-sham group) 5 days after ischemia-reperfusion (Figs. 5Ab, 5Ac and 5B).

SOD2 immunoreactivity in CA1 pyramidal neurons of the PT-sham group was significantly high (about 144% of the vehicle-sham group) compared to that in the vehicle-sham group (Figs. 5Ad and 5B). In the PT-ischemia group, SOD2 immunoreactivity in CA1 pyramidal neurons was not altered 2 and 5 days after ischemia-reperfusion (Figs. 5Ae, 5Af and 5B).

Immunoreactivity of BDNF

BDNF immunoreactivity in the vehicle-sham group was easily observed in CA1 pyramidal neurons (Fig. 6Aa). In the vehicle-ischemia group, BDNF immunoreactivity in CA1 pyramidal neurons was weak (about 86% of the vehicle-sham group) 2 days after ischemia-reperfusion and significantly decreased to about 21% of the vehicle-sham group 5 days after ischemia-reperfusion (Figs. 6Ab, 6Ac and 6B).



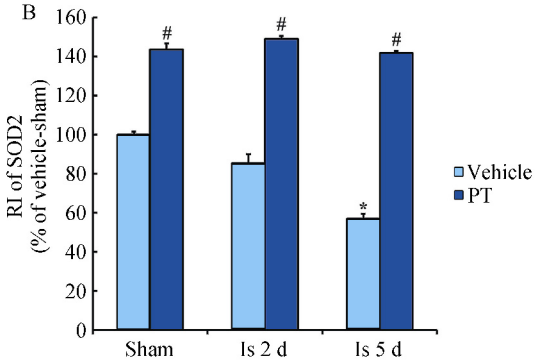


Fig. 5 Immunohistochemistry for SOD2 in the CA1 of the vehicle-sham (Aa), vehicle-ischemia (Ab and Ac), 200 mg·kg^{-1} PT-sham (Ad) and -ischemia groups 2 d (Ae) and 5 d (Af) after ischemia-reperfusion. In the vehicle-sham group, SOD2 immunoreactivity is clearly observed in the pyramidal layer (PL). In contrast, in the vehicle-ischemia groups, SOD2 immunoreactivity is gradually decreased in the PL with time after ischemia-reperfusion. In the 200 mg·kg^{-1} PT-sham group, SOD2 immunoreactivity in the PL is significantly higher than that in the vehicle-sham group. And SOD2 immunoreactivity in the 200 mg·kg^{-1} PT-ischemia group is maintained with time after ischemia-reperfusion. OL: oriens layer; RL: radiatum layer. Scale bar = 40 μm. B: Reactive immunoreactivity as % of SOD2 immunoreactivity in CA1 pyramidal neurons (n = 7 in each time point of each group, $^{*}P < 0.05$ *vs* vehicle-sham group, $^{\#}P < 0.05$ *vs* vehicle-sham and vehicle-ischemia groups). The bars indicate the means ± SEM.

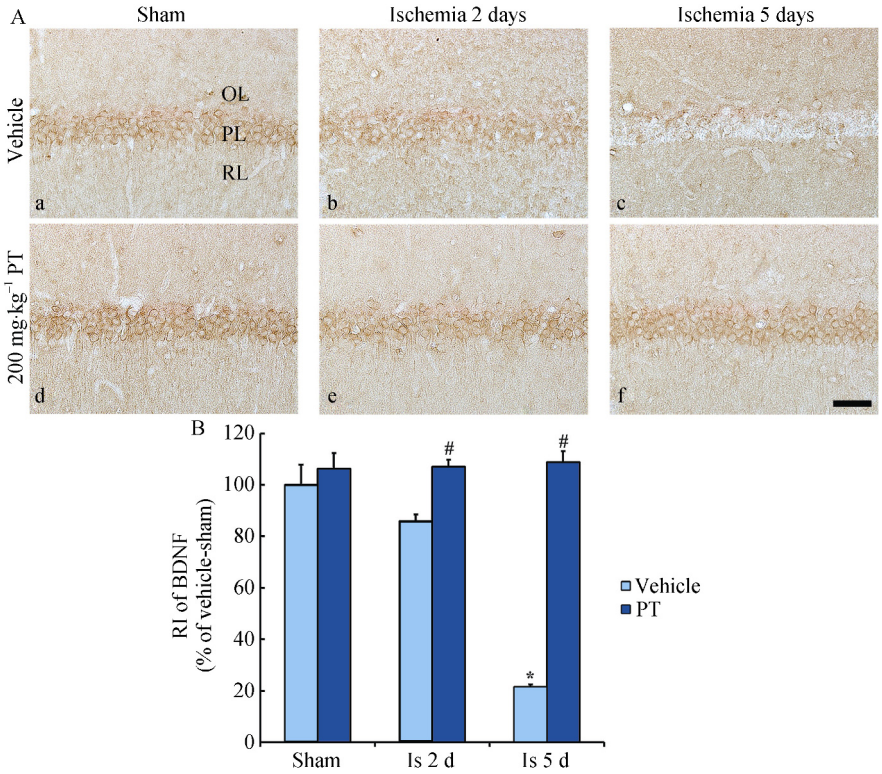


Fig. 6 Immunohistochemistry for BDNF in the CA1 of the vehicle-sham (Aa), vehicle-ischemia (Ab and Ac), 200 mg·kg^{-1} PT-sham (Ad) and -ischemia groups 2 d (Ae) and 5 d (Af) after ischemia-reperfusion. BDNF immunoreactivity is well detected in the pyramidal layer (PL) in the vehicle-sham group. However, in the vehicle-ischemia group, BDNF immunoreactivity decreases in the PL with time after ischemia-reperfusion. Especially, BDNF immunoreactivity in the vehicle-ischemia group 5 d after ishcmia-reperfusion is significantly weaker than that in the vehicle-sham group. In contrast, in the 200 mg·kg^{-1} PT-sham group, BDNF immuoreactivity is slightly higher than that in the vehicle-sham group. Furthermore, BDNF immunoreactivity in the 200 mg·kg^{-1} PT-ischemia group is sustained with time after ischemia reperfusion. OL: oriens layer; RL: radiatum layer. Scale bar = 40 μm. B: Reactive immunoreactivity as % of SOD1 immunoreactivity in CA1 pyramidal neurons (n = 7 in each time point of each group, $^{*}P < 0.05$ *vs* vehicle-sham group, $^{\#}P < 0.05$ *vs* vehicle-ischemia groups). The bars indicate the means ± SEM.

In the PT-sham group, BDNF immunoreactivity in CA1 pyramidal neurons was slightly increased (about 106% of the vehicle-sham group) compared to that in the vehicle-sham group (Figs. 6Ad and 6Ag). In the PT-ischemia group, BDNF

immunoreactivity was about 107% and 109% of the vehicle-sham group, respectively, at 2 and 5 days after ischemia-reperfusion (Figs. 6Ae, 6Af and 6B).

Immunoreactivity of IGF-I

IGF-I immunoreactivity in the vehicle-sham group was detected in CA1 pyramidal neurons (Fig. 7Aa). In the vehicle-ischemia group, IGF-I immunoreactivity in CA1 pyramidal neurons was significantly decreased (about 50% of the vehicle-sham group) 2 days after ischemia-reperfusion and very weak (about 21% of the vehicle-sham group) 5 days after ischemia-reperfusion (Figs. 7Ab, 7Ac and 7B).

In the PT-sham group, IGF-I immunoreactivity in CA1 pyramidal neurons was similar (about 102% of the vehicle-sham group) to that in the vehicle-sham group (Figs. 7Ad and 7Ag). In the PT-ischemia group, IGF-I immunoreactivity in CA1 pyramidal neurons was about 103% and 101% of the vehicle-sham group, respectively, 2 and 5 days after ischemia-reperfusion (Figs. 7Ae, 7Af and 7Ag).

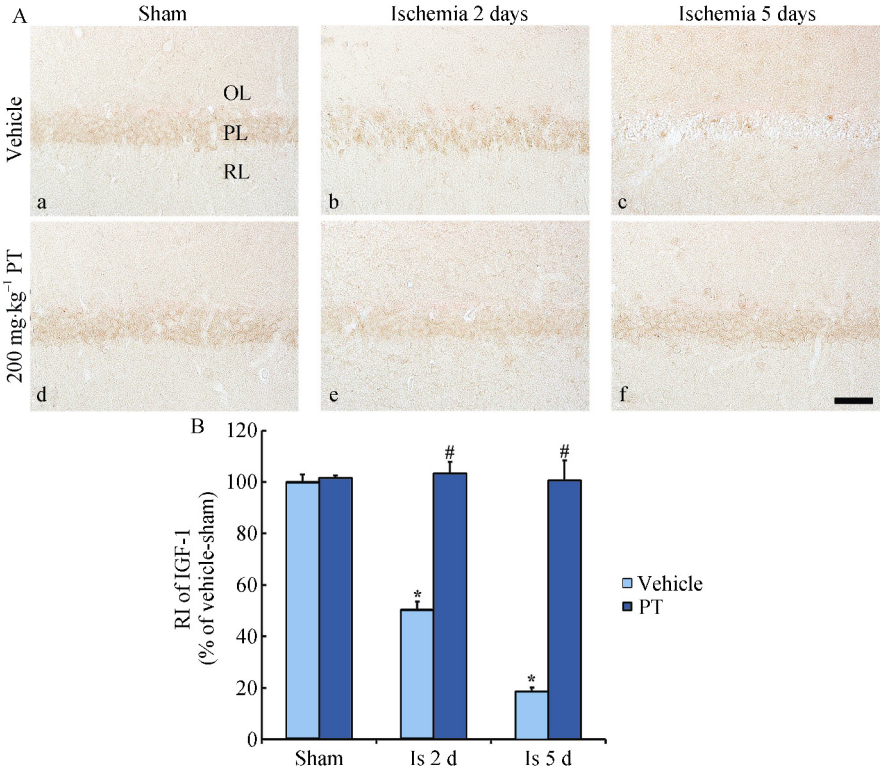


Fig. 7 Immunohistochemistry for IGF-I in the CA1 of the vehicle-sham (Aa), vehicle-ischemia (Ab and Ac), 200 mg·kg^{-1} PT-sham (Ad) and -ischemia groups 2 d (Ae) and 5 d (Af) after ischemia-reperfusion. In the vehicle-sham group, IGF-I immunoreactivity is cearly observed in the pyramidal layer (PL). However, in the vehicle-ischemia group, BDNF immunoreactivity is gradually decreased in the PL with time after ischemia-reperfusion. In contrast, IGF-I immuoreactivity in the 200 mg·kg^{-1} PT-sham group is similar to that in the vehicle-sham group. BDNF immunoreactivity in the 200 mg·kg^{-1} PT-ischemia group is maintained with time after ischemia-reperfusion. OL: oriens layer; RL: radiatum layer. Scale bar = 40 μm. B: Reactive immunoreactivity as % of SOD1 immunoreactivity in CA1 pyramidal neurons (n = 7 in each time point of each group, $^{*}P < 0.05$ *vs* vehicle-sham group, $^{\#}P < 0.05$ *vs* vehicle-ischemia groups). The bars indicate the means ± SEM.

Discussion

Many botanical extracts display pharmacological potential against cerebral ischemic insults [8-9, 13, 28-30]. However, studies about precautionary effects of PT on TGCI are lacking. In our previous study, we found that pre-treatment of PT extract attenuated neuronal loss and glial activation in the hippocampal CA1 region after TGCI [31]; however, mechanisms of PT-mediated neuroprotection were not covered. Therefore, in this study, we examined effects of PT on expression levels of SODs, BDNF and IGF-I before and after TGCI in gerbils via western blotting and immunohistochemistry.

It is well known that cerebral ischemia can induce oxidative stress by overproduction of ROS, which can lead to accumulation of ROS and cause malfunctions of cellular macromolecules including DNA, proteins, and lipid, and, finally, cells lose their own function [32-33]. DHE has been used for detection of superoxide anion radical with fluorescent end-product and it has been well established measurement of superoxide anion radical production [10]. In the present study, DHE fluorescence was significantly decreased by pretreatment with 200 mg·kg^{-1} PT after ischemic insult. SODs are endogenous antioxidant enzymes that act as ROS scavengers. Many precedent studies have reported the relationship between

SODs and neuroprotective effects in experimental animal or cell models of cerebral ischemic insults [16, 34-35]. In the present study, we discovered that pretreatment with 200 mg·kg^{-1} PT, not 100 mg·kg^{-1} PT, significantly increased expression levels of SODs in CA1 pyramidal neurons. Such increases were maintained in neurons after ischemia. Our findings along with above-mentioned papers and indicate that pretreatment with PT has neuroprotective effect against brain ischemic diseases.

We examined the neuroprotection of PT in hippocampal CA1 at 5 days after TGCI using NeuN immunohistochemistry and F-J B histofluorescence staining commonly used for histopathological measurement of neuronal death/loss in the central nerve system. We found that pretreatment with 200 mg·kg^{-1} PT significantly protected CA1 pyramidal neurons from transient global cerebral ischema.

Many studies have shown that neurotrophic factors including BDNF and IGF-I play important roles in enhancing neuronal differentiation and survival in the hippocampus [36-38]. Some studies have shown that the elevation or maintenance of levels of neurotrophic factors can attenuate neuronal loss following cerebral ischemic insults [16, 24]. Furthermore, some precedent studies have described that exogenous BDNF and IGF-I prevent hippocampal neuronal death from IRI [39-41]. Based on these studies, we examined effects of pretreatment with 200 mg·kg^{-1} PT on expression levels of BDNF and IGF-I in the CA1 before and after ischemia-reperfusion. Our results revealed that pretreatment with PT did not increase expression levels of BDNF and IGF-I in CA1 pyramidal neurons before ischemia. However, the treatment of 200 mg·kg^{-1} PT maintained their expression levels after ischemia-reperfusion. This finding indicates that the pretreatment of 200 mg·kg^{-1} PT does not affect BDNF or IGF-I expressions in neurons before TGCI, but it could maintain their expressions in neurons after ischemic insults. This suggests that maintained BDNF or IGF-I expressions in ischemic CA1 pyramidal neurons might play important roles in the survival of the CA1 pyramidal neurons after ischemic insults.

In conclusion, pretreatment with 200 mg·kg^{-1} PT significantly enhanced expression levels of SODs before and after ischemia-reperfusion, and the treatment maintained expressions of BDNF and IGF-I after ischemia-reperfusion. These expressions might be involved in the protection of CA1 pyramidal neurons from IRI. Based on this study, we suggest that *Populus tomentiglandulosa* could be a useful resource to protect against cerebral ischemic insults due to its strong antioxidant effects.

Acknowledgement

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Bio-Synergy Research Project (NRF-2018M3A9C4076478) of the Ministry of Science and ICT through the NRF and by the Bio & Medical Technology Development Program of the NRF funded by the Korean government, MSIP (NRF-2015M3A9B6066835) and by Basic Science Research Program through the NRF funded by the MSIP (NRF-2017R1A2B4008403).

References

[1] Park JH, Kim YH, Ahn JH, *et al*. Atomoxetine protects against NMDA receptor-mediated hippocampal neuronal death following transient global cerebral ischemia [J]. *Curr Neurovasc Res*, 2017, **14**(2): 158-168.

[2] Schmidt-Kastner R, Freund TF. Selective vulnerability of the hippocampus in brain ischemia [J]. *Neuroscience*, 1991, **40**(3): 599-636.

[3] Crain BJ, Westerkam WD, Harrison AH, *et al*. Selective neuronal death after transient forebrain ischemia in the Mongolian gerbil: a silver impregnation study [J]. *Neuroscience*, 1988, **27**(2): 387-402.

[4] Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia [J]. *Brain Res*, 1982, **239**(1): 57-69.

[5] Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia [J]. *Ann Neurol*, 1982, **11**(5): 491-498.

[6] Lewen A, Matz P, Chan PH. Free radical pathways in CNS injury [J]. *J Neurotrauma*, 2000, **17**(10): 871-890.

[7] Lee JC, Won MH. Neuroprotection of antioxidant enzymes against transient global cerebral ischemia in gerbils [J]. *Anat Cell Biol*, 2014, **47**(3): 149-156.

[8] Mdzinarishvili A, Sumbria R, Lang D, *et al*. *Ginkgo* extract EGb761 confers neuroprotection by reduction of glutamate release in ischemic brain [J]. *J Pharm Pharm Sci*, 2012, **15**(1): 94-102.

[9] Yoo KY, Kim IH, Cho JH, *et al*. Neuroprotection of *Chrysanthemum indicum* Linne against cerebral ischemia/reperfusion injury by anti-inflammatory effect in gerbils [J]. *Neural Regen Res*, 2016, **11**(2): 270-277.

[10] Chen J, Rogers SC, Kavdia M. Analysis of kinetics of dihydroethidium fluorescence with superoxide using xanthine oxidase and hypoxanthine assay [J]. *Ann Biomed Eng*, 2013, **41**(2): 327-337.

[11] Lee JC, Park JH, Kim IH, *et al*. Neuroprotection of ischemic preconditioning is mediated by thioredoxin 2 in the hippocampal CA1 region following a subsequent transient cerebral ischemia [J]. *Brain Pathol*, 2017, **27**(3): 276-291.

[12] Kawase M, Murakami K, Fujimura M, *et al*. Exacerbation of delayed cell injury after transient global ischemia in mutant mice with CuZn superoxide dismutase deficiency [J]. *Stroke*, 1999, **30**(9): 1962-1968.

[13] Kim IH, Lee TK, Cho JH, *et al*. Pretreatment with *Chrysanthemum indicum* Linne extract protects pyramidal neurons from transient cerebral ischemia via increasing antioxidants in the gerbil hippocampal CA1 region [J]. *Mol Med Rep*, 2017, **16**(1): 133-142.

[14] Hefti F. Pharmacology of neurotrophic factors [J]. *Annu Rev Pharmacol Toxicol*, 1997, **37**: 239-267.

[15] Chen BH, Ahn JH, Park JH, *et al*. Rufinamide, an antiepileptic drug, improves cognition and increases neurogenesis in the aged gerbil hippocampal dentate gyrus *via* increasing expressions of IGF-1, IGF-1R and p-CREB [J]. *Chem Biol Interact*, 2018, **286**: 71-77.

[16] Park JH, Park OK, Yan B, *et al*. Neuroprotection *via* maintenance or increase of antioxidants and neurotrophic factors in ischemic gerbil hippocampus treated with tanshinone I [J]. *Chin Med J (Engl)*, 2014, **127**(19): 3396-3405.

[17] Venkatesan T, Choi YW, Lee J, *et al*. Pinus densiflora needle supercritical fluid extract suppresses the expression of pro-inflammatory mediators iNOS, IL-6 and IL-1beta, and activation of inflammatory STAT1 and STAT3 signaling proteins in bacterial lipopolysaccharide-challenged murine macrophages [J]. *Daru*, 2017, **25**(1): 18.

[18] Cheng W, Li J, You T, *et al*. Anti-inflammatory and immunomodulatory activities of the extracts from the inflorescence of *Chrysanthemum indicum* Linne [J]. *J Ethnopharmacol*, 2005, **101**(1-3): 334-337.

[19] Simard F, Legault J, Lavoie S, *et al*. Balsacones D-I, dihydrocinnamoyl flavans from *Populus balsamifera* buds [J]. *Phytochemistry*, 2014, **100**: 141-149.

[20] Debbache-Benaida N, Atmani-Kilani D, Schini-Keirth VB, *et al*. Pharmacological potential of *Populus nigra* extract as antioxidant, anti-inflammatory, cardiovascular and hepatoprotective agent [J]. *Asian Pac J Trop Biomed*, 2013, **3**(9): 697-704.

[21] Zhang X, Thuong PT, Min BS, *et al*. Phenolic glycosides with antioxidant activity from the stem bark of *Populus davidiana* [J]. *J Nat Prod*, 2006, **69**(9): 1370-1373.

[22] Dekanski D, Selakovic V, Piperski V, *et al*. Protective effect of olive leaf extract on hippocampal injury induced by transient global cerebral ischemia and reperfusion in Mongolian gerbils [J]. *Phytomedicine*, 2011, **18**(13): 1137-1143.

[23] Lee JC, Park JH, Ahn JH, *et al*. New GABAergic neurogenesis in the hippocampal CA1 region of a gerbil model of long-term survival after transient cerebral ischemic injury [J]. *Brain Pathol*, 2016, **26**(5): 581-592.

[24] Park JH, Lee TK, Yan BC, *et al*. Pretreated *Glehnia littoralis* extract prevents neuronal death following transient global cerebral ischemia through Increases of superoxide dismutase 1 and brain-derived neurotrophic factor expressions in the gerbil hippocampal cornu ammonis 1 area [J]. *Chin Med J (Engl)*, 2017, **130**(15): 1796-1803.

[25] Carpenter JW. Exotic animal formulary (4 ed) [J]. *J Exotic Pet Medicine*, 2013, **22**: 308-309.

[26] Park JH, Shin BN, Chen BH, *et al*. Neuroprotection and reduced gliosis by atomoxetine pretreatment in a gerbil model of transient cerebral ischemia [J]. *J Neurol Sci*, 2015, **359**(1-2): 373-380.

[27] Lee TK, Park JH, Ahn JH, *et al*. Pretreated duloxetine protects hippocampal CA1 pyramidal neurons from ischemia-reperfusion injury through decreases of glial activation and oxidative stress [J]. *J Neurol Sci*, 2016, **370**: 229-236.

[28] Safwen K, Selima S, Mohamed E, *et al*. Protective effect of grape seed and skin extract on cerebral ischemia in rat: implication of transition metals [J]. *Int J Stroke*, 2015, **10**(3): 415-424.

[29] Saleem S, Zhuang H, Biswal S, *et al*. *Ginkgo biloba* extract neuroprotective action is dependent on heme oxygenase 1 in ischemic reperfusion brain injury [J]. *Stroke*, 2008, **39**(12): 3389-3396.

[30] Yoo KY, Hwang IK, Kim JD, *et al*. Antiinflammatory effect of the ethanol extract of *Berberis koreana* in a gerbil model of cerebral ischemia/reperfusion [J]. *Phytother Res*, 2008, **22**(11): 1527-1532.

[31] Park JH, Lee TK, Ahn JH, *et al*. Pre-treated *Populus tomentiglandulosa* extract inhibits neuronal loss and alleviates gliosis in the gerbil hippocampal CA1 area induced by transient global cerebral ischemia [J]. *Anat Cell Biol*, 2017, **50**(4): 284-292.

[32] Lee JC, Kim IH, Park JH, *et al*. Ischemic preconditioning protects hippocampal pyramidal neurons from transient ischemic injury *via* the attenuation of oxidative damage through upregulating heme oxygenase-1 [J]. *Free Radic Biol Med*, 2015, **79**: 78-90.

[33] Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain [J]. *J Cereb Blood Flow Metab*, 2001, **21**(1): 2-14.

[34] Sato R, Inanami O, Syuto B, *et al*. The plasma superoxide scavenging activity in canine cancer and hepatic disease [J]. *J Vet Med Sci*, 2003, **65**(4): 465-469.

[35] Greenlund LJS, Deckwerth TL, Jr Johnson EM. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death [J]. *Neuron*, 1995, **14**(2): 303-315.

[36] Wine RN, McPherson CA, Harry GJ. IGF-1 and pAKT signaling promote hippocampal CA1 neuronal survival following injury to dentate granule cells [J]. *Neurotox Res*, 2009, **16**(3): 280-292.

[37] Lindholm D, Carroll P, Tzimagiorgis G, *et al*. Autocrine-paracrine regulation of hippocampal neuron survival by IGF-1 and the neurotrophins BDNF, NT-3 and NT-4 [J]. *Eur J Neurosci*, 1996, **8**(7): 1452-1460.

[38] Nagahara AH, Tuszynski MH. Potential therapeutic uses of BDNF in neurological and psychiatric disorders [J]. *Nat Rev Drug Discov*, 2011, **10**(3): 209-219.

[39] Tagami M, Ikeda K, Nara Y, *et al*. Insulin-like growth factor-1 attenuates apoptosis in hippocampal neurons caused by cerebral ischemia and reperfusion in stroke-prone spontaneously hypertensive rats [J]. *Lab Invest*, 1997, **76**(5): 613-617.

[40] Traub ML, De Butte-Smith M, Zukin RS, *et al*. Oestradiol and insulin-like growth factor-1 reduce cell loss after global ischaemia in middle-aged female rats [J]. *J Neuroendocrinol*, 2009, **21**(12): 1038-1044.

[41] Kiprianova I, Freiman TM, Desiderato S, *et al*. Brain-derived neurotrophic factor prevents neuronal death and glial activation after global ischemia in the rat [J]. *J Neurosci Res*, 1999, **56**(1): 21-27.

Cite this article as: Tae-Kyeong Lee, Joon Ha Park, Ji Hyeon Ahn, Hyunjung Kim, Minah Song, Jae-Chul Lee, Jong Dai Kim, Yong Hwan Jeon, Jung Hoon Choi, Choong Hyun Lee, In Koo Hwang, YAN Bing-Chun, Moo-Ho Won, Il Jun Kang. Pretreatment of *Populus tomentiglandulosa* protects hippocampal CA1 pyramidal neurons from ischemia-reperfusion injury in gerbils *via* increasing SODs expressions and maintaining BDNF and IGF-I expressions [J]. *Chin J Nat Med*, 2019, **17**(6): 424-434.