

# Experimental pretreatment with YES-10<sup>®</sup>, a plant extract rich in scutellarin and chlorogenic acid, protects hippocampal neurons from ischemia/reperfusion injury via antioxidant role

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**Abstract.** *Erigeron annuus* (L.) PERS. (EALP) and *Clematis mandshurica* RUPR. (CMR) have been used in traditional remedies due to their medicinal effects. Recently, we reported that pretreatment with 200 mg/kg of YES-10<sup>®</sup> (a combination of extracts from leaves of EALP and CMR) displayed neuroprotective effects against brain ischemia and reperfusion injury. The present study analyzed the major ingredients of YES-10<sup>®</sup> and investigated whether neuroprotection from YES-10<sup>®</sup> was dependent upon antioxidant effects in the cornu ammonis 1 (CA1) field in the gerbil hippocampus, after transient forebrain ischemia for 5 min. YES-10<sup>®</sup> was demonstrated to predominantly contain scutellarin and chlorogenic acid. Pretreatment with YES-10<sup>®</sup> significantly increased protein

levels and the immunoreactivity of copper/zinc-superoxide dismutase (SOD1) and manganese-superoxide dismutase (SOD2) in the pyramidal neurons of the hippocampal CA1 field when these were examined prior to transient ischemia induction. The increased SODs in CA1 pyramidal neurons following YES-10<sup>®</sup> treatment were maintained after ischemic injury. In this case, the CA1 pyramidal neurons were protected from ischemia-reperfusion injury. Oxidative stress was significantly attenuated in the CA1 pyramidal neurons, and this was determined by 4-hydroxy-2-nonenal immunohistochemistry and dihydroethidium histofluorescence staining. Taken together, the results indicated that YES-10<sup>®</sup> significantly attenuated transient ischemia-induced oxidative stress and may be utilized for developing a protective agent against ischemic insults.

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## Introduction

Genus *Erigeron* and *Clematis* belong to the Asteraceae (Compositae) and Ranunculaceae family respectively, and have been used in traditional remedies (1,2) because some species possess anti-apoptotic activity and anti-inflammatory properties (3,4). Many studies have reported the synergistic effects of a mixture of extracts from two or more natural resources in neuroprotection against ischemic brain insults (5-7). In this regard, we recently published a paper that showed that pretreatment with YES-10<sup>®</sup>, a combination of extracts from *Erigeron annuus* (L.) PERS. (EALP) and *Clematis mandshurica* RUPR. (CMR), protected cornu ammonis 1 (CA1) pyramidal neurons and attenuated gliosis (astrogliosis and microgliosis) in the hippocampus of a gerbil model of 5-min transient forebrain ischemia (5).

The hippocampus consists of several subfields. Among them, the CA1 field is vulnerable to 5-min transient ischemia (8). Namely, pyramidal cells in the CA1 field, which are called CA1 pyramidal cells or neurons, die several days (4-5 days) after 5-min transient ischemia. Hence, the death phenomenon is called delayed neuronal death (DND) (8,9). Until now, the mechanisms of DND following 5-min transient ischemia have included glutamate-induced excitotoxicity, oxidative stress due to the immoderate production of reactive oxygen species (ROS) and inflammatory response by pro-inflammatory cytokines (10-13). Among the mechanisms, oxidative stress is one of the major mechanisms of DND (10,14). The overproduction of ROS decreases or stops the functions of molecules such as DNA, lipids and proteins and eventually leads to cell death (15,16). In this regard, many studies on the mechanisms of neuroprotection against ischemic insults have focused on enhancing endogenous antioxidant enzymes, including SOD1 and SOD2 to scavenge overproduced ROS (10,16-19).

The neuroprotective mechanisms of YES-10<sup>®</sup> against brain ischemic injury have been poorly investigated, although we reported neuroprotective effects in a gerbil model of 5-min transient forebrain ischemia (5). In addition, it has been demonstrated that plants belonging to the genus *Clematis*, specifically *Clematis chinensis*, contains scutellarin, a flavonoid glycoside compound (20,21). The scutellarin is also commonly found in the genus *Erigeron* and, in particular, *Erigeron breviscapus* (2,22,23). In addition, *Erigeron annuus* contains chlorogenic acid, a polyphenolic compound, which consists of ester bonds with caffeic acid and quinic acid (24-26). Interestingly, it has been reported that scutellarin and/or chlorogenic acid display neuroprotective effects against brain ischemic insults in rodents (27,28). Therefore, the objective of this research was to analyze scutellarin and chlorogenic acid in YES-10<sup>®</sup> and examine the antioxidant efficacy of YES-10<sup>®</sup> in neuroprotection against ischemic injury induced by 5-min transient forebrain ischemia in gerbils, which have been used as a model of transient ischemia (TI) in the forebrain (19,29).

## Materials and methods

**Experimental animals.** Male gerbils at 6.5 months old (body weight, 77-82 g) obtained from the Experimental Animal Center at Kangwon National University (Chuncheon, Republic of Korea) were used in this study. The animals were housed in conventional environment with suitable room temperature (23±0.5°C) and humidity (approximately 60%). Their room was controlled under constant dark and light cycle every 12 h. The gerbils were provided accessible water and feed. The protocol of this research was approved at Feb. 18, 2020 by the Institutional Animal Care and Use Committee (AICUC) at Kangwon National University (approval no. KW-200113-1). The animal caring and handling complies with the guidelines with the current international laws and policies from the NIH Guide for the Care and Use of Laboratory Animals (The National Academies Press, 8th Ed, 2011).

**Preparation of YES-10<sup>®</sup>.** YES-10<sup>®</sup> was prepared as previously described in our published paper (5,30). Briefly, CMR and EALP were cultivated in Metro-Mix potting soil with a slow releasing fertilizer (Osmocote Plus). They had been grown for

6 weeks, and their leaves were harvested. The collected leaves were washed, dried at 50°C and ground into powder (<1 mm). In turn, 150 g of CMR was extracted with 7-fold volume of 50% EtOH for 60 min and refluxed 2 times (2 h/reflux). The resulting suspension (extract) was filtered, concentrated to be powder by using a rotary evaporator and stored at 4°C. EALP was extracted with 50% EtOH, filtered and dried to be powder by using the same procedure for CMR extract. These extracts were mixed 1:1 ratio to be YES-10<sup>®</sup> which was provided from Famenity Co., Ltd.

**Qualitative analysis of YES-10<sup>®</sup>.** The test sample (YES-10<sup>®</sup>) and standard samples (chlorogenic acid and scutellarin) were precisely weighed and dissolved into 50% methanol. And, thereafter, the test sample (0.5 µl) and standard samples (0.5 µl respectively) were subjected to HPLC (Agilent 1260 infinity II; Agilent Technologies, Inc.) using a Supelco Discovery C18 column (diameter, 4.6 mm; length, 250 mm) filled with octadecylsilyl silica gel (diameter, 5 µm) at 1.0 ml/min of flow rate. Optimum HPLC separation was achieved at 30°C. UV wave length was 335 nm. The mobile phases were set as A (aqueous phosphoric acid) and B (methanol) under following conditions: 0-5 min (5% B), 5-27 min (5-30% B), 27-47 min (30-55% B), 47-47.1 min (55-100% B), 47.1-52 min (100% B), 52-52.1 min (100-5%) and 52.1-55 min (5% B).

**Experimental groups and treatment with YES-10<sup>®</sup>.** Forty two gerbils were assigned to four groups: 1) vehicle/sham group (*n*=14) treated with vehicle (0.85% saline) and sham operation; 2) vehicle/TI group (*n*=28) treated with vehicle (0.85% saline) and TI; 3) YES/sham group (*n*=14) treated with 200 mg/kg YES-10<sup>®</sup> and sham operation; 4) YES/TI group (*n*=28) treated with 200 mg/kg YES-10<sup>®</sup> and TI.

The administration of the vehicle or YES-10<sup>®</sup> was orally given with a feeding needle once/day for 7 days because extracts from natural products have orally been ingested in traditional medicine. The dose of YES-10<sup>®</sup> (200 mg/kg) was chosen on the basis of our published paper, in which pretreatment with 200 mg/kg of YES-10<sup>®</sup> strongly protected the pyramidal neurons located in the hippocampal CA1 field after TI (5). We applied 7 days for YES-10<sup>®</sup> administration according to previous studies with plant extracts, and since data regarding to ADME properties of YES-10<sup>®</sup> had been rarely reported (18,31).

**TI induction.** Surgical procedure for TI was performed as previously described (29,32). In short, the gerbils were anesthetized with 2.5% isoflurane (Hana Pharmaceutical Co., Ltd.) in mixture of oxygen and nitrous oxide (33%:67%). Under anesthesia, a midline incision was made on ventral surface of the neck to find right and left common carotid arteries. Both arteries were exposed and occluded for 5 min by using non-traumatic aneurysm clips. Five minutes after TI, the clips were removed for blood circulation. Complete stop and circulation of blood flow was confirmed in the central arteries located at retinae with ophthalmoscope (HEINE K180<sup>®</sup>) (Heine Optotechnik, Herrsching, Germany). Body temperature was regulated at normal temperature (37.5±0.5°C) with a rectal temperature probe (TR-100; Fine Science Tools) by using thermometric blanket during and after TI surgery.

Sham operated gerbils were subjected to the same TI surgery process without occlusion of both common carotid arteries.

**Expression levels of antioxidant enzymes.** To investigate changes in antioxidant enzymes following treatment with YES-10<sup>®</sup>, Western blot analysis was carried out according to previous study (33,34). Five gerbils per group were deeply anesthetized by intraperitoneal injection of 90 mg/kg pentobarbital sodium (JW Pharm Co Ltd.) (32) and their hippocampal tissues were harvested and homogenized. We used a buffer for homogenization as 50 mM phosphate-buffered saline (PBS, pH 7.4) solution containing 0.1 mM EGTA (pH 8.0), 0.2% Nonidet P-40, 10 mM EDTA (pH 8.0), sodium pyrophosphate, 100 mM  $\beta$ -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM PMSF and 1 mM DTT. After homogenization, the tissue were centrifuged and the supernatants were taken to determine protein levels via using a Micro BCA assay kit with bovine serum albumin as the standard (Pierce Chemical). The aliquots including total protein (20  $\mu$ g) were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The samples were separated by SDS-PAGE (10%) and subsequently the gels were transferred to nitrocellulose membranes (Pall Co.) at 350 mA and 4°C for 90 min. The membranes were incubated with 5% defatted milk at room temperature for 60 min to block non-specific staining. The membrane, thereafter, immunoreacted with each primary antibody at 4°C during overnight: Rabbit anti-Cu, Mn-superoxide dismutase (SOD1; 1:2,000; Abcam), rabbit anti-Mn-superoxide dismutase (SOD2; 1:2,000; Abcam) and rabbit anti- $\beta$ -actin (1:2,000; Sigma-Aldrich; Merck KGaA). The membrane, subsequently, incubated with each peroxidase-conjugated secondary antibody at room temperature for 60 min: Donkey anti-rabbit IgG (1:5,000, Santa Cruz Biotechnology, Inc.). Using a luminol-based chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.) was used for enhancement of visualization.

Each immune blot was analyzed as described previously (35). In brief, the bands were scanned and in order to quantify the bands, densitometric analysis was performed via using the Scion Image software (Scion Crop.). Each level of the target protein was normalized via the corresponding level of  $\beta$ -actin.

**Tissue preparation of histopathology.** In order to examine histopathological and immunohistochemical changes in the gerbil hippocampus following TI, brain sections containing the hippocampus were prepared according to published method (36). Briefly, the gerbils were anesthetized with intraperitoneal injection of pentobarbital sodium (90 mg/kg) (JW Pharm Co Ltd., Republic of Korea) (32) at 2 and 5 days after TI. Under deep anesthesia, the gerbils were transcardially perfused with 0.1 M PBS (pH 7.4) to wash their brains and followed by solution of 4% paraformaldehyde (in 0.1 M PB, pH 7.4) to fix the brains. The fixed brains were removed and post-fixed with the same fixative for 4 h. These brains were cryoprotected with solution of 30% sucrose (in 0.1 M PB, pH 7.4) for 10 h. Finally, serial 30- $\mu$ m coronal sections were made in cryostat (Leisa).

**Immunohistochemistry.** To study antioxidant and neuroprotective effects of YES-10<sup>®</sup> against TI, immunohistochemistry was done according to our previously published method (37). The sections were treated with 0.3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) solution (in 0.1 M PBS, pH 7.4) and immersed in 10% normal goat, horse or chicken serum (in 0.05 M PBS, pH 7.4) for 40 min at room temperature. They were then reacted in solution of each primary antibody for 24 h at 4°C. Primary antibodies were rabbit anti-SOD1 (1:1,500; EMD Millipore) and sheep anti-SOD2 (1:1,500; EMD Millipore) for examining antioxidant enzymes, mouse anti-neuronal nuclei-specific protein (NeuN; a marker for neurons; 1:1,000; Chemicon) for detecting neurons and rabbit anti-4-hydroxy-2-nonenal (4-HNE; 1:1,000; Alexis Biochemicals) for investigating oxidative stress. Subsequently, these sections were exposed to solution of biotinylated goat anti-rabbit IgG (1:250; Vector), chicken anti-sheep IgG (1:250; Vector) or horse anti-mouse IgG (1:250; Vector) as secondary antibody. Finally, these tissues were reacted with solution of avidin-biotin complex (1:300; Vector) and visualized by reacting them with solution of 3, 3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich; Merck KGaA) (in 0.1 M PBS, pH 7.4).

Quantitative analyses of SOD1, SOD2 and 4-HNE immunoreactivity were done according to our published protocol (19). In brief, digital image of each immunoreactive structure was captures in the regions of interest with light microscope (BX53) (Olympus Corporation). Each image was calibrated into an array of 512x512 pixels, and each immunoreactivity was measured by 0-255 gray scale system. After the background density was subtracted, change in each immunoreactivity was calibrated ratio of relative immunoreactivity (RI) by using Adobe Photoshop (version 8.0) and analyzed by using NIH image 1.59 software. The ratio of RI was presented as percent compared with the vehicle/sham group (100%).

Counts of NeuN immunoreactive neurons were done according to our published method (19). Briefly, NeuN immunoreactive neurons were examined with light microscope (BX53) (Olympus Corporation). Digital images of NeuN immunoreactive neurons were captured in the regions of interest (a 250x250  $\mu$ m square), and background density was subtracted as described above. Finally, Cell numbers were analyzed by using an image analyzing software (Optimas 6.5; CyberMetrics).

**Fluoro-Jade B (FJB) histofluorescence staining.** To examine neuronal loss (death) following TI, F-JB (a fluorescent marker for neuronal degeneration) histofluorescence staining was performed as previously described (38). Briefly, the prepared sections were mounted onto microscopy slides which were coated with gelatin. The tissues on the slides were immersed in solution of 0.06% potassium permanganate and incubated in solution of 0.0004% F-JB (Histochem). These tissues were washed with distilled water and placed on slide warmer (approximately 50°C) to be reacted. These stained tissues were dehydrated and mounted by cover glasses with dibutylphthalate polystyrene xylene (D.P.X.; Sigma-Aldrich; Merck KGaA).

As described previously (19), F-JB positive cells were counted. In short, F-JB positive cells were examined with epifluorescent microscope (Carl Zeiss) equipped with a digital camera (DP72) (Olympus Corporation). Digital images of

F-JB positive cells were captured in the regions of interest (250x250  $\mu\text{m}^2$ ), and the back ground density was subtracted as described above (immunohistochemistry section). Finally, F-JB positive cells were counted and analyzed by using image analyzing software (Optimas 6.5; CyberMetrics).

**Dihydroethidium (DHE) fluorescence staining.** In this study, oxidative stress was observed by DHE (Sigma-Aldrich; Merck KGaA) fluorescence staining, which is a method to examine *in situ* production of superoxide anion. As we previously described (36), briefly, the sections were incubated in Krebs-HEPES buffer containing 130 Mm NaCl, 5.6 Mm KCL, 2 Mm CaCl<sub>2</sub>, 0.24 mM MgCl<sub>2</sub>, 8.3 mM HEPES, 11 mM glucose (pH 7.4) for 30 min at 37°C. Ten  $\mu\text{M}$  DHE solution was topically applied on the sections for 2 h at 37°C. At this stage, DHE was oxidized at reaction with superoxide to ethidium, which could bind nuclear DNA and fluoresced red.

Fluorescent intensity of DHE was analyzed as previously described (38). Shortly, the image of DHE histofluorescence was captured in regions of interest with epifluorescence microscope like the method for F-JB staining section). DHE fluorescent intensity was analyzed by using Image-pro Plus 6.0 software. Ratio of DHE fluorescent intensity was calibrated as percentage compared with the vehicle/sham group (100%).

**Statistical analysis.** Data shown in this study represent the means  $\pm$  standard error of the mean (SEM) among the groups were statistically analyzed by two-way analysis of variance (ANOVA) with a post hoc Bonferroni's multiple comparison test was done to determine differences among groups. They were statistically analyzed using SPSS 18.0 (SPSS, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Major ingredients of YES-10<sup>®</sup>.** The result from the HPLC, the retention time of the standard samples were respectively 22.912 min (chlorogenic acid; Fig. 1A) and 37.770 min (scutellarin; Fig. 1B). The major ingredients of the YES-10<sup>®</sup> were revealed as chlorogenic acid (retention time, 22.823 min) and scutellarin (retention time, 22.823 min) (Fig. 1C).

**Expression levels of SODs.** Western blot analysis for SOD1 and SOD2 was carried out to examine alterations in expression levels of endogenous antioxidant enzymes in the hippocampal CA1 field of the sham and TI groups following YES-10<sup>®</sup> pretreatment.

Expression level of SOD1 of the vehicle/sham group was fundamentally observed (Fig. 2A). In the vehicle/TI group, expression levels of SOD1 and SOD2 were decreased at 2 days after TI and significantly reduced at 5 days after TI compared to that in the vehicle/sham group (Fig. 2A and Ba). However, in the YES/sham group expression level of SOD1 was significantly increased compared to that in the vehicle/sham group. In the YES/TI group, expression level of SOD1 was not significantly changed compared with that in the YES/sham group (Fig. 2A and Ba).

A basic expression level of SOD2 was detected in the vehicle/sham group (Fig. 2A). In the vehicle/TI group,

expression level of SOD2 was significantly decreased at 2 days after TI and more significantly reduced at 5 days after TI compared to that in the vehicle/sham group (Fig. 2A and Bb). On the other hand, in the YES/sham group, expression level of SOD2 was significantly increased compared to that in the vehicle/sham group (Fig. 2A and Bb). In the YES/TI group, expression level of SOD2 was not markedly altered compared with that in the YES/sham group (Fig. 2A and Bb).

**Increases of SODs immunoreactivities by YES-10<sup>®</sup>.** Immunohistochemistry for SOD1 and SOD2 was conducted in order to investigate changes in endogenous antioxidant enzymes in the hippocampal CA1 field of the sham and TI groups following YES-10<sup>®</sup> pretreatment.

SOD1 immunoreactivity in the CA1 field of the hippocampus of the vehicle/sham group was found in pyramidal cells of the stratum pyramidale, which are called CA1 pyramidal cells (or neurons), and in non-pyramidal cells which are distributed in strata oriens and radiatum (Fig. 3Aa). In the vehicle/TI group, SOD1 immunoreactivity was significantly reduced in the CA1 pyramidal cells (approximately 60% of the vehicle/sham group) at 2 days after TI and more significantly decreased (approximately 33% of the vehicle/sham group) at 5 days after TI compared to that in the vehicle/sham group (Fig. 3Ab, Ac and B).

In the YES/sham group, SOD1 immunoreactivity was apparently increased in the CA1 pyramidal cells (approximately 109% of the vehicle/sham group) when compared to that in the vehicle/sham group (Fig. 3Ad and B). In the YES/TI group, SOD1 immunoreactivity in the CA1 field was not altered at 2 days post-TI compared with that in the YES/sham group (Fig. 3Ae and B), and SOD1 immunoreactivity at 5 days post-TI was maintained (Fig. 3Af and B).

SOD2 immunoreactivity was detected in the CA1 pyramidal cells of the vehicle/sham group (Fig. 4Aa). In the vehicle/TI group, SOD2 immunoreactivity in the CA1 pyramidal neurons was significantly decreased (approximately 59% of the vehicle/sham group) at 2 days after TI and more significantly reduced (approximately 9% of the vehicle/sham group) at 5 days after TI compared to that in the vehicle/sham group (Fig. 4Ab, Ac and B).

SOD2 immunoreactivity in the CA1 pyramidal cells of the YES/sham group was significantly high (approximately 125% of the vehicle/sham group) compared to that in the vehicle/sham group (Fig. 4Ad and B). In the YES/TI group, SOD2 immunoreactivity in the CA1 pyramidal neurons were slightly decreased after TI compared to that in the YES/sham group (Fig. 4Ae, Af and B).

**Neuroprotective effects by YES-10<sup>®</sup>.** To investigate neuroprotective effects of YES-10<sup>®</sup> in the ischemic hippocampus, immunohistochemistry for NeuN and FJB histofluorescence were performed.

NeuN immunoreactive (NeuN<sup>+</sup>) neurons in the vehicle/sham group were found in all layers in all subfields (CA1-3 fields) of the hippocampus (Fig. 5Aa). In this group, NeuN immunoreactivity was well shown in cells located the stratum pyramidale, which are called pyramidal cells or neurons (Fig. 5Ab), but most of pyramidal cells in the CA1 field, not in the CA2/3 field of the vehicle/TI group were not

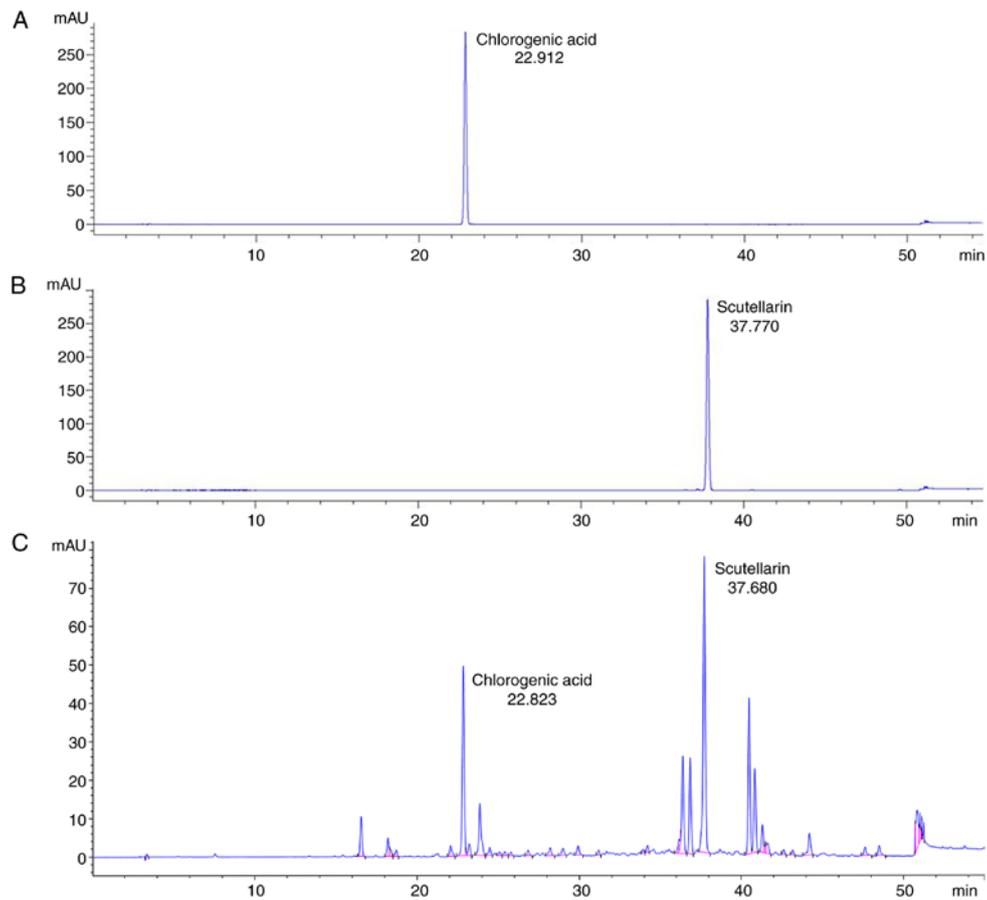


Figure 1. Qualitative analysis of YES-10<sup>®</sup> via High Performance Liquid Chromatography. Representative HPLC chromatograms of (A) standard chlorogenic acid (B) scutellarin and (C) YES-10<sup>®</sup>. The retention times of the standard chlorogenic acid and scutellarin were 22.912 and 37.770 min, respectively. The retention times of the YES-10<sup>®</sup> were 22.823 min (chlorogenic acid) and 37.680 min (scutellarin), respectively.

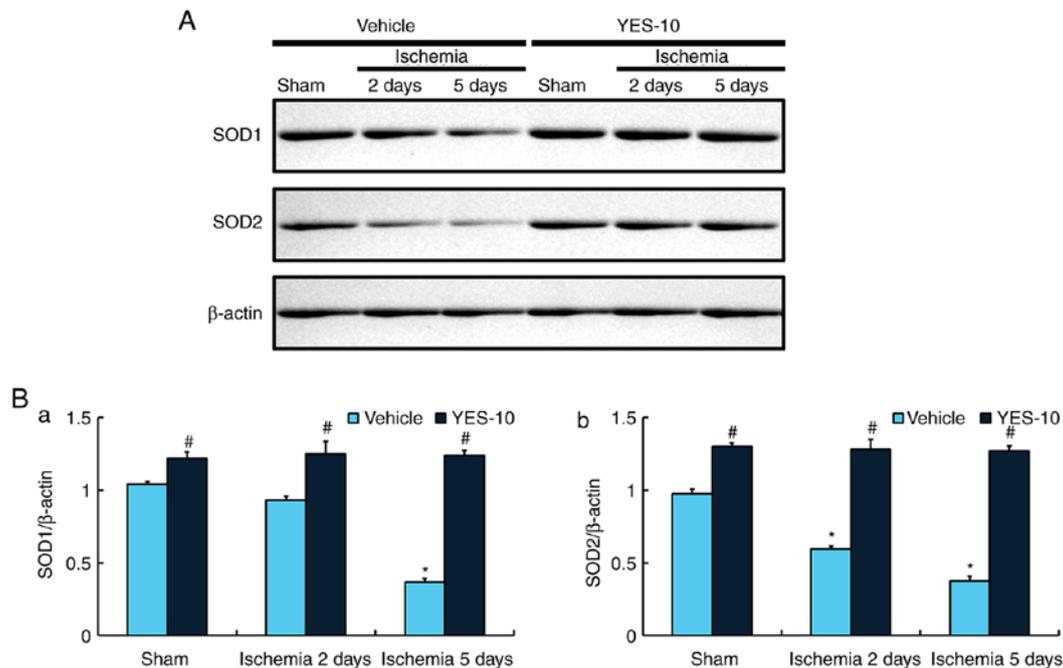


Figure 2. Western blot analysis of changes in antioxidant enzymes in the hippocampus following TI. (A) Representative western blot images of SOD1 and SOD2 in the hippocampal CA1 field of the vehicle/sham, vehicle/TI, YES/sham and YES/TI groups (A), and densitometric analyses of the bands of (Ba) SOD1 and (Bb) SOD2. In the vehicle/TI group, levels of SOD1 and SOD2 are reduced at 2 days post-TI and more significantly decreased at 5 days post-TI. However, levels of SOD1 and SOD2 are elevated in the YES/sham group. In the YES/TI group, the increased levels of SOD1 and SOD2 are maintained at 2 and 5 days post-TI (n=7 at each point in time). \*P<0.05 vs. vehicle/sham group, #P<0.05 vs. corresponding vehicle/sham or TI group. Data are presented as the mean ± SEM. CA1, cornu ammonis 1; SOD, superoxide dismutase; TI, transient ischemia.

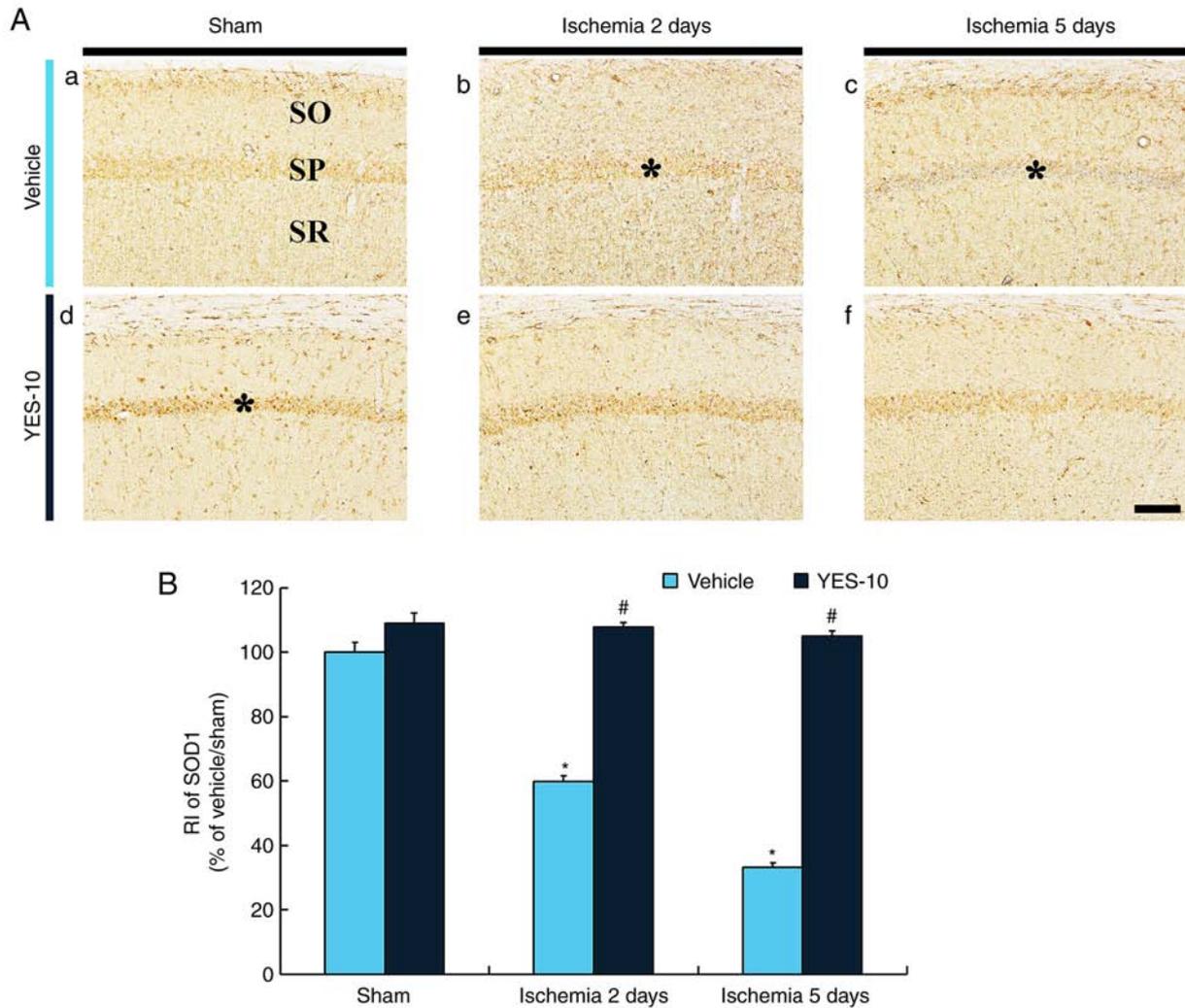


Figure 3. Immunohistochemistry for SOD1 in the hippocampal CA1 field following TI. (Aa) SOD1 immunohistochemistry in the hippocampal CA1 field of the vehicle/sham, (Ab and Ac) vehicle/TI, (Ad) YES/sham and (Ae and Af) YES/TI groups. In the vehicle/TI group, SOD1 immunoreactivity is gradually reduced in the SP (asterisks), and SOD1 immunoreactivity in the SP (asterisk) at 5 days post-TI is hardly shown. SOD1 immunoreactivity in the SP (asterisk) of the YES/sham group is significantly increased compared with that in the vehicle/sham group. SOD1 immunoreactivity in the SP of the YES/TI group is significantly higher than that in the vehicle/TI group. Scale bar, 40  $\mu$ m. (B) RI as % of SOD1 immunoreactivity in the SP (n=7 at each point in time). \*P<0.05 vs. vehicle/sham group, #P<0.05 vs. corresponding vehicle/sham or TI group. Data are presented as the mean  $\pm$  SEM. SOD, superoxide dismutase; SP, stratum pyramidale; SO, stratum oriens; SR, stratum radiatum; TI, transient ischemia; RI, immunoreactivity; CA1, cornu ammonis 1.

stained with NeuN (Fig. 5Ba and Bb). This finding means CA1 pyramidal neurons were damaged or died. In the YES/sham group, NeuN<sup>+</sup> neurons in the hippocampus were not different from those in the vehicle/sham group (Fig. 5Bd and Be). Also, NeuN-immunoreactive CA1 pyramidal neurons of the YES/TI group were similar to those in the vehicle/sham groups (Fig. 5Bd, Be and C). This result means that YES-10<sup>®</sup> pretreatment protected CA1 pyramidal cells from TI injury.

FJB positive (FJB<sup>+</sup>) cells mean that they are dead. In the vehicle/sham group, FJB<sup>+</sup> cells were not found in any layers of the CA1 field (Fig. 5Ac). However, numerous FJB<sup>+</sup> cells were shown in the stratum pyramidale of the CA1 field of the vehicle/TI group at 5 days after TI (Fig. 5Bc and D). This result means that most of CA1 pyramidal cells were dead at 5 days after 5-min TI. In the YES/sham group, FJB<sup>+</sup> cells were not found in the CA1 field (Fig. 5Af). In the YES/TI group, only a few FJB<sup>+</sup> CA1 pyramidal neurons were shown at 5 days after TI (Fig. 5Bf and D). This finding means that most of CA1

pyramidal cells were protected from TI injury by YES-10<sup>®</sup> pretreatment.

*Decrease of oxidative stress by YES-10<sup>®</sup>.* Immunohistochemistry for 4-HNE (an end-product by lipid peroxidation) and DHE (a marker for production of *in situ* superoxide anion) were carried out to investigate alterations in oxidative stress in the ischemic CA1 field following YES-10<sup>®</sup> pretreatment.

4-HNE immunoreactivity was shown in CA1 pyramidal neurons in the vehicle/sham group (Fig. 6Aa). In the vehicle/TI group, 4-HNE immunoreactivity in the CA1 pyramidal neurons at 2 days after TI was significantly increased (approximately 121%) compared to that in the vehicle/sham group (Fig. 6Ab and B), and 4-HNE immunoreactivity at 5 days after TI was very weak due to damage of the CA1 pyramidal cells (Fig. 6Ac and B). In the YES/sham group, 4-HNE immunoreactivity in the CA1 pyramidal cells was not different from that in the vehicle/sham group (Fig. 6Ad and B). In the YES/TI

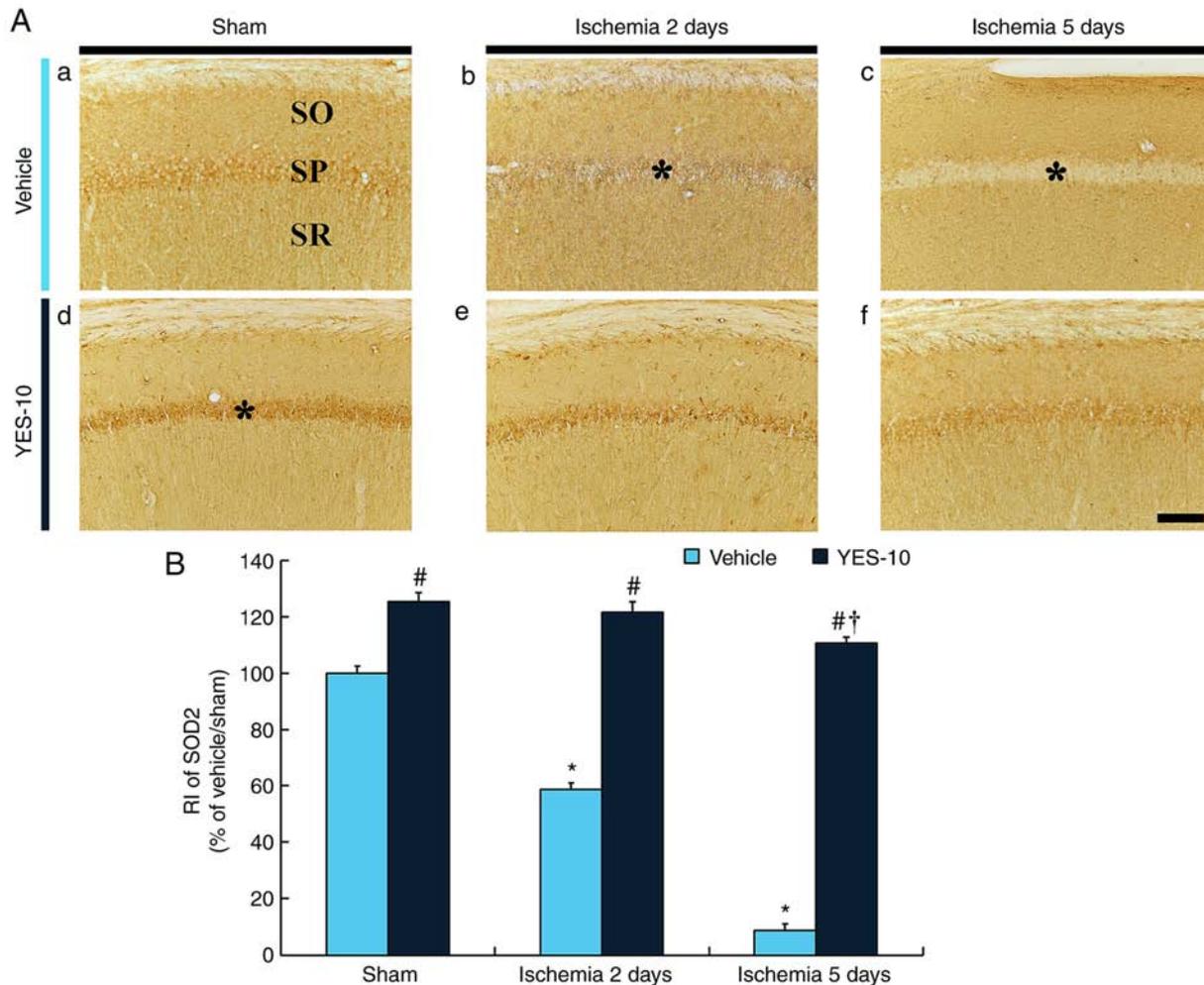


Figure 4. Immunohistochemistry for SOD2 in the hippocampal CA1 field following TI. (Aa) SOD2 immunohistochemistry in the hippocampal CA1 field of the vehicle/sham, (Ab and Ac) vehicle/TI, (Ad) YES/sham and (Ae and Af) YES/TI groups. SOD2 immunoreactivity in the SP (asterisks) of the vehicle/TI group is markedly decreased at 2 days after TI and hardly detected (astrisk) at 5 days after TI. In the YES/sham group, SOD2 immunoreactivity in the SP (asterisk) is significantly higher compared with that in the vehicle/sham group. SOD2 immunoreactivity in the SP of the YES/TI group is significantly high compared to that in the vehicle/TI group. Scale bar, 40  $\mu$ m. (B) RI as % of SOD2 immunoreactivity in the SP (n=7 at each point in time). <sup>\*</sup>P<0.05 vs. vehicle/sham group; <sup>#</sup>P<0.05 vs. corresponding vehicle/sham or TI group; <sup>†</sup>P<0.05 vs. YES/sham group. The bars indicate the mean  $\pm$  SEM. SOD, superoxide dismutase; SP, stratum pyramidale; SO, stratum oriens; SR, stratum radiatum; TI, transient ischemia; CA1, cornu ammonis 1.

group, 4-HNE immunoreactivity in the CA1 pyramidal cells was not altered at 2 days post-TI (Fig. 6Ae and B), and the immunoreactivity at 5 days post-TI was slightly reduced (approximately 83% of the YES/sham group) compared to that in the YES/sham group (Fig. 6Af and B). This finding means that lipid peroxidation in ischemic cells following TI is attenuated by YES-10<sup>®</sup> pretreatment.

Weak DHE fluorescence of the vehicle/sham group was easily detected in CA1 pyramidal cells (Fig. 7Aa). However, in the vehicle/TI group, DHE fluorescence in the CA1 pyramidal cells was significantly enhanced (approximately 168%) at 2 days after TI compared with the vehicle/sham group (Fig. 7Ab and B), and, at 5 day after TI, DHE fluorescence in the CA1 pyramidal cells was decreased, but the fluorescence was higher (approximately 143%) than that in the vehicle/sham group (Fig. 7Ac and B). In this group, particularly, non-pyramidal cells distributed in the other layers (strata oriens and radiatum) expressed strong DHE fluorescence (Fig. 7Ab and Ac). In the YES/sham group, DHE fluorescence in the CA1 pyramidal cells was similar to that

in the vehicle/sham group (Fig. 7Ad and B). In the YES/TI group, DHE fluorescence in the CA1 pyramidal cells was significantly low (approximately 71% at 2 days and 73% at 5 days after TI) compared with that in the corresponding time point of vehicle/TI group (Fig. 7Ae, Af and B). This finding means that the production of superoxide anion in ischemic cells following TI is reduced by YES-10<sup>®</sup> pretreatment.

## Discussion

Many studies have reported that natural products display protective effects against brain ischemic injury. For example, *Chrysanthemum indicum* Linné belonging to the Asteraceae family exerts neuroprotective effects in the hippocampal CA1 field after TI in gerbils (13,19). In addition, some studies have shown the synergistic effects of two extracts from natural products in animal models of ischemic insults. For instance, a combination of *Ligusticum chuanxiong* (from the Apiaceae family) and *Radix Paeoniae*, (from the Paeoniaceae family) ameliorated ischemic brain damage following transient focal

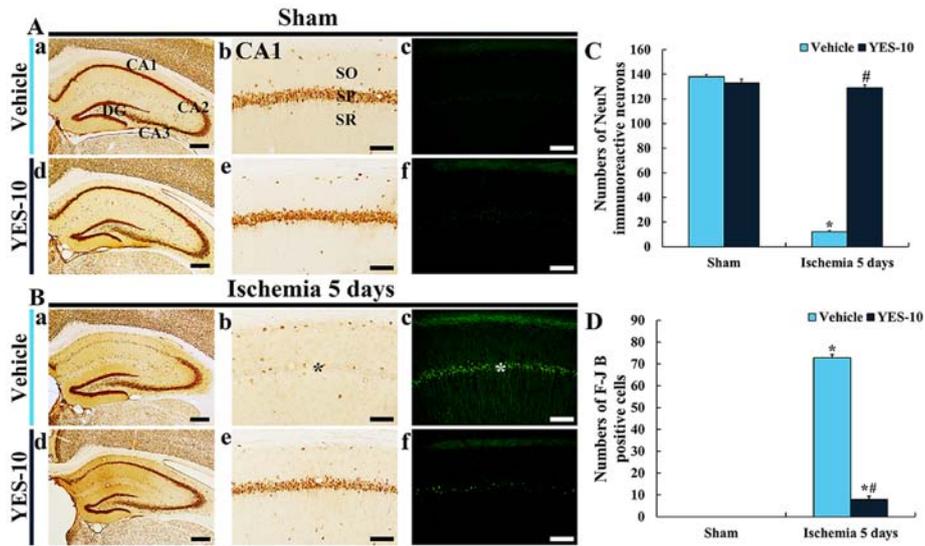


Figure 5. Immunohistochemistry for NeuN and histofluorescence for F-JB in the hippocampus and hippocampal CA1 field following TI. (Aa, Ad, Ba and Bd) NeuN immunohistochemistry in the hippocampus and (Ab, Ae, Bb and Be) CA1 field. (Ac, Af, Bc and Bf) F-JB histofluorescence staining in the CA1 field of the (Aa-Ac) vehicle/sham, (Ad-Af) YES/sham, (Ba-Bc) vehicle/TI and (Bd-Bf) YES/TI groups at 5 days after TI. In all of the sham groups, numerous NeuN+ and no FJB+ cells are present in the SP. In the vehicle/TI group, a few NeuN+ and many FJB+ cells were found in the SP (asterisks). In the YES/TI group, the distribution pattern of NeuN+ and FJB+ cells in the SP is similar to that in the vehicle/sham. Scale bar, 400  $\mu$ m (Aa, Ad, Ba and Bd) and 40  $\mu$ m (Ab, Ac, Ae, Af, Bb, Bc, Be and Bf). (C) Mean numbers of NeuN+ and (D) FJB+ cells in the CA1 SP (n=7 in each group). \*P<0.05 vs. vehicle/TI group, #P<0.05 vs. vehicle/sham group. The bars indicate the mean  $\pm$  SEM. DG, dentate gyrus; CA1, cornu ammonis 1; SP, stratum pyramidale; SO, stratum oriens; SR, stratum radiatum; TI, transient ischemia; NeuN, neuronal nuclei-specific protein; F-JB, fluoro-Jade B.

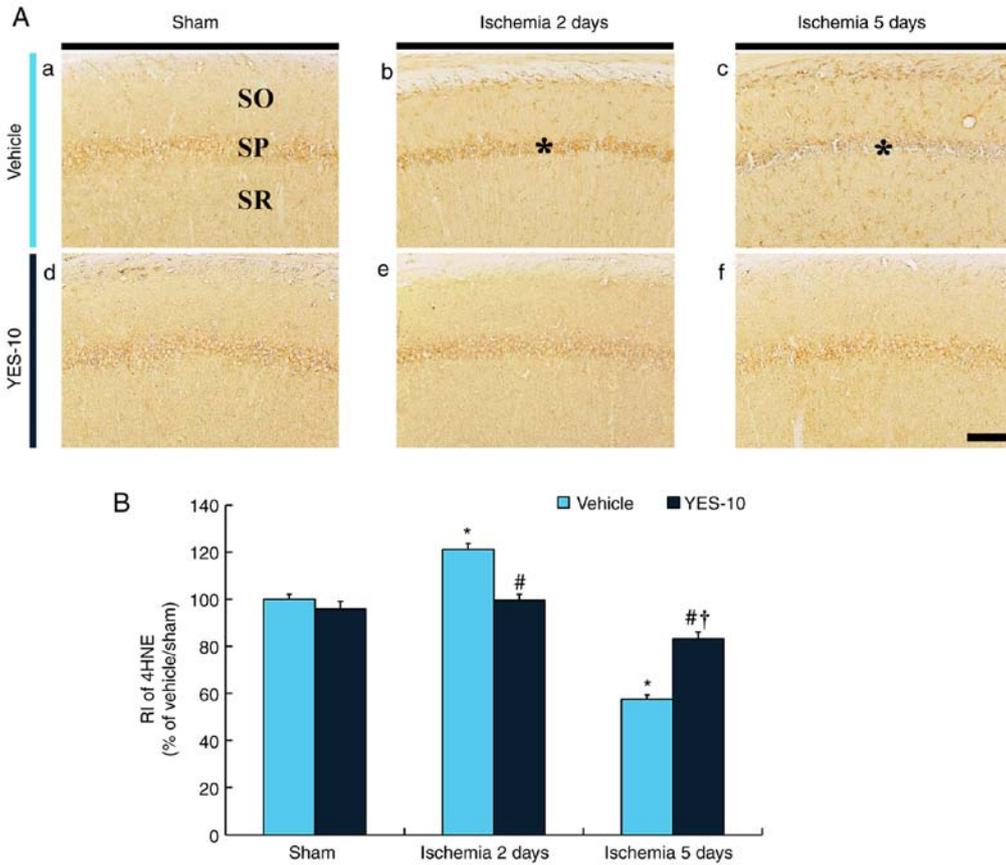


Figure 6. Immunohistochemistry for 4-HNE in the hippocampal CA1 field following TI. (Aa) Immunohistochemistry for 4-HNE in the CA1 field of the vehicle/sham, (Ab and Ac) vehicle/TI, (Ad) YES/sham and (Ae and Af) YES/TI groups. 4-HNE immunoreactivity in the vehicle/sham group is shown in the SP. In the vehicle/TI group, 4-HNE immunoreactivity in the SP (asterisk) is significantly increased at 2 days post-TI, and, at 5 days after TI, 4-HNE immunoreactivity in the SP (asterisk) is more reduced. In the YES/sham group, 4-HNE immunoreactivity in the SP is similar to that of the vehicle/sham group. Also, 4-HNE immunoreactivity in the SP of the YES/TI group is not significantly altered after TI. Scale bar, 40  $\mu$ m. (B) RI as % of 4-HNE in the SP (n=7 at each point in time). \*P<0.05 vs. vehicle/sham group, #P<0.05 vs. corresponding vehicle/sham or TI group, †P<0.05 vs. YES/sham group. The bars indicate the means  $\pm$  SEM. CA1, cornu ammonis 1; TI, transient ischemia; RI, immunoreactivity.

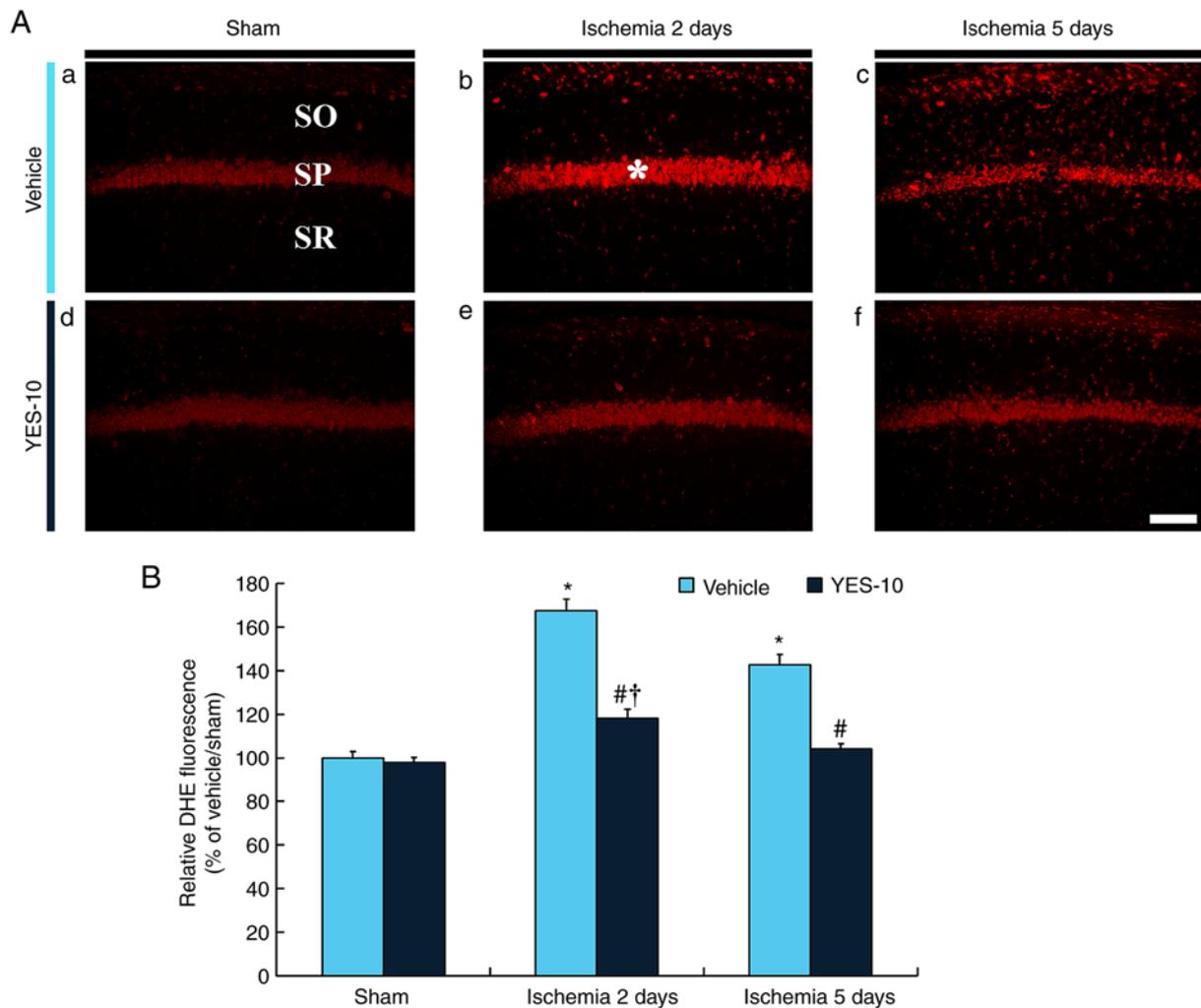


Figure 7. Histofluorescence for DHE in the hippocampal CA1 field following TI. (Aa) DHE histofluorescence staining in the CA1 field of the vehicle/sham, (Ab and Ac) vehicle/TI, (Ad) YES/sham and (Ae and Af) YES/TI groups. In the vehicle/sham group, DHE fluorescence is shown in the SP, but DHE fluorescence is significantly increased (asterisk) at 2 days after TI and decreased at 5 days after TI. In the YES/TI group, DHE fluorescence in the SP is similar to that in the vehicle/sham group. DHE fluorescence of the YES/TI group is slightly increased after TI. Scale bar, 40  $\mu$ m. (B) RI as % of DHE fluorescence in the SP (n=7 at each point in time). \*P<0.05 vs. vehicle/sham group, †P<0.05 vs. corresponding vehicle/TI group, #P<0.05 vs. YES/sham group. The bars indicate the mean  $\pm$  SEM. CA1, cornu ammonis 1; SP, stratum pyramidale; SO, stratum oriens; SR, stratum radiatum; TI, transient ischemia; DHE, dihydroethidium; F-JB, fluoro-Jade B.

brain ischemia induced by middle cerebral artery occlusion in rats (6,7). Additionally, we recently published a paper reporting that pretreatment with 200 mg/kg YES-10<sup>®</sup> strongly protected hippocampal CA1 pyramidal neurons and alleviated reactive gliosis in a gerbil model of TI (5). Gerbils have a unique cerebrovascular system. Namely, they lack posterior communicating arteries which compose Willis' circle (cerebral arterial circle) to supply blood to the brain (39). Therefore, the ligation of only both common carotid arteries is bale to evoke TI in the forebrain, not the hindbrain which supports vital processes (40,41). In this regard, the gerbils with TI show significant reproducibility of TI induction (42). In addition, this model has the advantage of long survival after TI due to intact hindbrain (43,44).

Our current analysis of YES-10<sup>®</sup> identified scutellarin and chlorogenic acid as the major ingredients of YES-10<sup>®</sup>. It has been demonstrated that these compounds confer neuroprotective effects against ischemic insults. In detail, a precedent study reported that pretreatment with scutellarin ameliorated infarct

lesions following focal cerebral ischemia induced by occlusion of the middle cerebral artery (MCA) in rats (27,45). In particular, Wang *et al* (27) demonstrated that the neuroprotective effects resulted from the down regulation of angiotensin-converting enzyme and angiotensin II type 1 receptor and, ultimately, inhibited the production of pro-inflammatory cytokines including tumor necrosis factor  $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 (27). Additionally, Liu *et al* (28) reported the precautionary administration of chlorogenic acid reduced the infarcts induced by transient global cerebral ischemia from two repeated occlusions of the bilateral common carotid artery for 10 min in ten-minute intervals in rats (28), suggesting that the neuroprotective effect of chlorogenic acid treatment to attenuate oxidative stress, which is regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway contributed to the neuroprotective effects of chlorogenic acid. We previously demonstrated neuroprotective effect of chlorogenic acid in a gerbil model of 5-min transient forebrain ischemia (35). In that study, the neuroprotective effect was obtained by treatment of chlorogenic acid as a single

compound (35). In our current study, contrastively, we investigated the neuroprotective effect of YES-10<sup>®</sup> as a mixture of two extracts derived from natural resources, which contained scutellarin and chlorogenic acid as major compounds. Additionally, we found that the synergistic effect in the neuroprotection might be done via excellent antioxidant efficacy.

In this study, we studied whether pretreatment with YES-10<sup>®</sup> exerted antioxidant effects and whether the antioxidant effects protected hippocampal CA1 pyramidal neurons from ischemic injury induced by 5-min TI in gerbils. For this, we performed Western blot analysis and immunohistochemical analysis for SODs and found that these endogenous antioxidant enzymes were markedly increased in the CA1 pyramidal neurons of the YES/sham group and the increases were maintained in the CA1 pyramidal neurons after TI. A number of investigations have been concluded to develop that exert neuroprotective effects against brain ischemia by attenuating oxidative stress. The antioxidant efficacy and neuroprotective effects against cerebral ischemia have been investigated in single compounds (17,46,47), and also natural resource-derived extracts (19,38,48). These materials have a common characteristic that enhances endogenous antioxidant enzymes including SOD1, SOD2, catalase and glutathione peroxidase. It is well known that antioxidant enzymes are defensive factors against oxidative stress (10,49). Therefore, the enhancement of antioxidant enzymes is a driving force in the attenuation of oxidative stress, which eventually can protect neurons or tissues from brain ischemic insults.

Based on the above-mentioned findings, we examined neuroprotection of YES-10<sup>®</sup> in the hippocampal CA1 field in gerbils following TI. In this study, we found death (loss) of the CA1 pyramidal neurons 5 days after 5-min TI. It is well known that, in gerbils, the CA1 pyramidal neurons die 4-5 days after 5-min TI. Thus, this phenomenon is called delayed neuronal death (8,9). When we treated YES-10<sup>®</sup>, neuroprotection was shown in the hippocampal CA1 field of the YES/TI group. However, the neuroprotective effect of YES-10<sup>®</sup> after TI was not examined in other areas (i.e., cerebral cortex and striatum) of the brain induced by 5-min TI. Therefore, further studies on the neuroprotective effect in important areas of the brain should be accomplished in the future.

Finally, we examined whether YES-10<sup>®</sup> pretreatment decreased the production of ROS in the CA1 pyramidal neurons after TI by immunohistochemical analysis of 4-HNE and histofluorescence for DHE. Various approaches to evaluate ROS production have been established and widely used. Among them, two methodologies are commonly used: 1) detecting 4-HNE, an end-product of excessive ROS-induced lipid peroxidation chain reaction (38,50,51) and 2) quantifying *in situ* superoxide anion by the DHE fluorescence assay (36,52). In the YES/TI group, the production of ROS in the CA1 pyramidal neurons was significantly reduced. This finding indicates that the increase in antioxidant enzymes by YES-10<sup>®</sup> ameliorated ROS overproduction, which contributed to neuroprotection against TI injury. Adequate ROS is needed to maintain homeostasis by participating in cellular metabolism (53,54). However, the excessive production of ROS can lead to oxidative stress, which can be the main cause of neuronal damage or death following ischemic insults (10,15). Namely, imbalanced production and neutralization of

antioxidant enzymes following ischemic insults increases ROS production and augments cellular vulnerability through lipid peroxidation, protein oxidation and DNA damage (55). Among cells in the central nervous system, neurons and astrocytes are more susceptible to ROS toxicity than other CNS cells (i.e., microglia), as they have high oxidative metabolism and low antioxidant enzymes as well as high membrane fatty acid content (15).

In conclusion, our current results showed that pretreatment with YES-10<sup>®</sup> increased SODs in the CA1 pyramidal neurons and protected the CA1 pyramidal neurons from TI injury. The TI-induced over-production of ROS in the CA1 pyramidal neurons was significantly reduced after TI. Therefore, we suggest that the strong antioxidant efficacy of YES-10<sup>®</sup> makes it a candidate for developing a therapeutic agent to protect the brains from ischemic damage.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

YP, TL, BK, CP and DK performed the experiments and measurements. YN, JA, JL, JP, JK and YK analyzed and interpreted data. YP and MW confirm the authenticity of all the raw data. IK, JL, SK and MW made substantial contributions to conception and design, and were involved in drafting, revising the manuscript and interpreting all data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The protocol of this research was approved on February 18, 2020 by the Institutional Animal Care and Use Committee (AICUC) at Kangwon National University (approval no. KW-200113-1).

### Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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