Pre-treatment with *Chrysanthemum indicum* Linné extract protects pyramidal neurons from transient cerebral ischemia via increasing antioxidants in the gerbil hippocampal CA1 region

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Abstract. *Chrysanthemum indicum* Linné extract (CIL) is used in herbal medicine in East Asia. In the present study, gerbils were orally pre-treated with CIL, and changes of anti-oxidant enzymes including superoxide dismutase (SOD) 1 and SOD2, catalase (CAT) and glutathione peroxidase (GPX) in the hippocampal CA1 region following 5 min of transient cerebral ischemia were investigated and the neuroprotective effect of CIL in the ischemic CA1 region was examined. SOD1, SOD2, CAT and GPX immunoreactivities were observed in the pyramidal cells of the CA1 region and their immunoreactivities were gradually decreased following ischemia-reperfusion and barely detectable at 5 days post-ischemia. CIL pre-treatment significantly increased immunoreactivities of SOD1, CAT and GPX, but not SOD2, in the CA1 pyramidal cells of the sham-operated animals. In addition, SOD1, SOD2, CAT and GPX immunoreactivities in the CA1 pyramidal cells were significantly higher compared with the ischemia-operated animals. Furthermore, it was identified that pre-treatment with CIL protected the CA1 pyramidal cells in the CA1 region using neuronal nuclei immunohistochemistry and Fluoro-Jade B histofluorescence staining; the protected CA1 pyramidal cells were 67.5% compared with the sham-operated animals. In conclusion, oral CIL pre-treatment increased endogenous antioxidant enzymes in CA1 pyramidal cells in the gerbil hippocampus and protected the cells from transient cerebral ischemic insult. This finding suggested that CIL is promising for the prevention of ischemia-induced neuronal damage.

Introduction

Ischemic stroke occurs due to the temporary or permanent blockage of blood circulation in the brain in several circumstances, including brain ischemia, cardiac arrest and cardiovascular surgery (1-3). Transient cerebral ischemia, which is a major cause of ischemic stroke, leads to selective neuronal damage/death in vulnerable brain areas, including the cerebral cortex, the striatum and the hippocampus (3,4). In particular, the most vulnerable area to transient cerebral ischemia is the CA1 region of the hippocampus, in which pyramidal neurons of the stratum pyramidale of the CA1 region die several days following ischemia-reperfusion injury (5,6).

One of mechanisms regarding neuronal damage/death induced by cerebral ischemia is oxidative stress, which is associated with the excessive production of reactive oxygen species (ROS) (7,8). The accumulation of ROS in ischemic conditions induces DNA damage, lipid peroxidation and...
changes in cellular proteins (8,9). ROS is converted into nontoxic compounds by enzymatic antioxidants including superoxide dismutases (SODs), catalase (CAT) and glutathione peroxidase (GPX); known antioxidant enzymes (8,10). Various antioxidants, including antioxidant enzymes, have been recognized as beneficial in therapies for neurologic diseases (11,12).

Many studies on neuroprotection by plant extracts have been reported using animal models of cerebral ischemic insults (13,14). *Chrysanthemum indicum* Linné (Compositae; CIL) is a traditional herb used for medicines in East Asia. It has been used for the treatment of immune-related disorders, hypertension, infectious diseases and respiratory illness (15,16). The major components of CIL are bornyl acetate (10.00-21.33%), borneol (8.34-18.34%), camphor (7.75-23.52%) and germacrene D (1.08-12.67%). Significant minor components of CIL include α-terpineol (1.28-3.32%), terpinen-4-ol (0.70-1.59%) and caryophyllene oxide (0.13-2.73%). However, 1,8-cineole is present at 30.41% in fresh flower oil and only 0.12-0.61% in the oil of air-dried and processed flowers (17,18). CIL exhibits anti-bacterial, anti-viral, antioxidant, anti-inflammatory and immunomodulatory functions (17,19). To the best of the authors' knowledge, few studies regarding neuroprotective effects of CIL and its antioxidant mechanism in brain ischemic insults have been published; therefore, the present study investigated the neuroprotective effect of CIL and whether endogenous antioxidant enzymes, including SOD1, SOD2, CAT and GPX, were associated with the neuroprotection in the hippocampus of the gerbil, a good animal model of transient cerebral ischemia (20,21).

**Materials and methods**

*Preparation of extract from CIL.* CIL was collected by Professor Jong Dai Kim in Kangwon Province (South Korea), in October 2013 and maintained in a deep freezer (-70°C). The CIL was extracted with 70% ethanol at 70°C for 4 h, and extraction was repeated three times. The extract was filtered through Whatman filter paper (no. 2), concentrated with a vacuum evaporator, and completely dried with a freeze-drier. The extraction yield was 14.5%.

*Groups of experimental animals.* Male Mongolian gerbils (*Meriones unguiculatus*; weight, 65-75 g; age, 6 months) were obtained from the Experimental Animal Center, Kangwon National University, Chuncheon, South Korea. The animals were housed in standard conditions under adequate temperature (23°C) and humidity (60%) control with a 12-h:12-h light:dark cycle, and were provided with free access to food and water. All the experimental protocols were approved (approval no. KW-130424-1) by the Institutional Animal Care and Use Committee at Kangwon 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 edition, 2011).

The experimental animals were divided into four groups (n=7 at each time point in each groups): i) Vehicle-sham-group, which was treated with vehicle and underwent no ischemia; ii) CIL-sham-group, which was treated with CIL and underwent no ischemia; iii) vehicle-ischemia-group, which was treated with vehicle and underwent ischemia surgery; and iv) CIL-ischemia-group, which was treated with CIL and underwent ischemia surgery.

**Administration with CIL.** CIL extract was dissolved in saline and administrated orally using a feeding needle once a day for seven days prior to ischemia surgery, according to previously published procedure (22). The preliminary tests were carried out with 25, 50, 100 and 200 mg/kg CIL. There were no neuroprotective effects in doses of 25, 50 and 100 mg/kg, but protective effects were demonstrated in animals treated with 200 mg/kg. Thus, 200 mg/kg was selected. The last treatment was at 30 min prior to the surgery to maintain the level of CIL in animals prior to and following surgery.

**Induction of transient cerebral ischemia.** As previously described (23), the experimental animals were anesthetized with a mixture of ~2.5% isoflurane (Baxtor Healthcare Corp., Deerfield, IL, USA) in 33% O2 and 67% N2. Bilateral common carotid arteries were isolated and occluded for 5 min using non-traumatic aneurysm clips. Rectal temperature was controlled under normothermic (37±0.5°C) conditions during the surgery with a rectal temperature probe (TR-100; Fine Science Tools, Inc., Foster City, CA, USA).

**Tissue preparation for histology.** As previously described (24), gerbils (n=7 at each time point in each group) were anesthetized with pentobarbital sodium at the designated times and perfused transcardially with 0.1 M phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB; pH 7.4). The brains were removed and postfixed in the same fixative for 6 h, and the brain tissues were sectioned on a cryostat (Leica Microsystems GmbH, Wetzlar, Germany) into 30 µm coronal sections.

**Immunohistochemistry.** Immunohistochemistry was performed according to the previously published procedure (24). In short, the sections were incubated with diluted mouse anti-neuronal nuclei (NeuN; 1:800; cat. no. MAB377), sheep anti-copper, zinc-superoxide dismutase (SOD1; 1:1,000; cat. no. 574597) and sheep anti-copper, zinc-superoxide dismutase (SOD2; 1:1,000; cat. no. 574597) and sheep anti-mangan-superoxide dismutase (SOD2; 1:1,000; cat. no. 574597; all from EMD Millipore, CA, USA) and were visualized with 3,3'-diaminobenzidine complex (1:200, all from Vector Laboratories, Burlingame, CA, USA). Thereafter the tissues were exposed to biotinylated horse anti-mouse IgG (1:250; cat. no. BA-1000) and streptavidin peroxidase (1:1,000; cat. no. BA-6000) and goat anti-mouse (1:250; cat. no. BA-2000), rabbit anti-sheep (1:250; cat. no. ab52477) and sheep anti-glutathione peroxidase (GPX; 1:1,000; cat. no. ab52477; all from Abcam, Cambridge, MA, UK). Thereafter the sections were exposed to biotinylated horseradish peroxidase (1:1,000; cat. no. ab21966; both from Abcam, Cambridge, MA, UK) and streptavidin peroxidase complex (1:200, all from Vector Laboratories, Burlingame, CA, USA) and were visualized with 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

**Fluoro-Jade B histochemistry staining.** Fluoro-Jade B (F-J B) histochemistry staining was conducted according to a published procedure (25) in order to examine neuronal death. In brief, the sections were immersed in a solution containing 1% sodium hydroxide, transferred to a solution of 0.06% potassium permanganate and transferred to a 0.0004% F-J B (Histo-Chem Inc., Jefferson, AR, USA) staining solution.
The stained sections were observed using an epifluorescent microscope (Zeiss AG, Oberkochen, Germany) with a blue (450–490 nm) excitation source and a barrier filter.

Data analysis. Data were analyzed according to published procedure (26). Briefly, to quantitatively analyze immunoreactivities of antioxidant enzymes, the immunoreactivity of SOD1, SOD2, CAT and GPX-immunoreactive structures was evaluated on the basis of optical density (OD), which was obtained following the transformation of the mean gray level using the formula: OD = log (256/mean gray level). A portion of the OD of an image file was calibrated in Adobe Photoshop 8.0 (Adobe Systems, Inc., San Jose, CA, USA) and then analyzed as a percentage, with the sham-operated-group designated as 100%, in ImageJ version 1.59 (National Institutes of Health, Bethesda, MD, USA). For cell counting, NeuN- and F-J B-positive cells were imaged from the stratum pyramidale through an AxioM1 light microscope (Zeiss AG) equipped with a digital camera (Axiocam; Zeiss AG) connected to a PC monitor. The mean number of NeuN- and F-J B-positive cells was counted in a 200x200 µm square applied approximately at the center of the CA1 region. Cell counts were obtained by averaging the total cell numbers from each animal per group and analyzing them as a percentage, with the vehicle-sham-group designated as 100%.

Statistical analysis. The data was presented as mean ± standard error of mean of the means among the groups and were statistically analyzed by analysis of variance with a post hoc Bonferroni’s multiple comparisons test, in order to present differences among experimental groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Antioxidant immunoreactivities

SOD1 immunoreactivity. SOD1 immunoreactivity was easily detected in pyramidal cells of the hippocampal CA1 region of the vehicle-sham-group (Fig. 1A). In the vehicle-ischemia-group, SOD1 immunoreactivity was significantly decreased in the CA1 pyramidal cells 2 days following ischemia-reperfusion and SOD1 immunoreactivity was hardly detected in the CA1 pyramidal cells 5 days following ischemia-reperfusion and SOD1 immunoreactivity was significantly decreased at 2 days post-ischemia (Fig. 1C, E and G). In the CIL-sham-group, SOD1 immunoreactivity in the CA1 pyramidal cells was slightly increased compared with the vehicle-sham-group; however, it was not statistically significant (Fig. 2B and G). In the CIL-ischemia-group, the changing pattern of SOD2 immunoreactivity in the CA1 pyramidal cells was similar to that of the SOD1 immunoreactivity (Fig. 2D, F and G).

CAT immunoreactivity. In the vehicle-sham-group, CAT immunoreactivity was clearly observed in the pyramidal cells of the stratum pyramidale layer of the CA1 region (Fig. 3A). In the vehicle-ischemia-group, CAT immunoreactivity was significantly decreased in the CA1 pyramidal cells at 2 days post-ischemia (Fig. 3C and G). At 5 days post-ischemia, CAT immunoreactivity in the CA1 pyramidal cells was barely observable; however, CAT immunoreactivity was newly expressed in non-pyramidal cells in the stratum oriens and radiatum of the CA1 region (Fig. 3E and G).

In the CIL-sham-group, CAT immunoreactivity in the CA pyramidal cells was significantly increased compared with the vehicle-sham-group (Fig. 3B and G). In the CIL-ischemia-group, although the CAT immunoreactivity in the CA1 pyramidal cells was decreased following ischemia-reperfusion, the CAT immunoreactivity was significantly higher compared with the corresponding vehicle-ischemia-group (Fig. 3D, F and G).

GPX immunoreactivity. GPX immunoreactivity in the vehicle-sham-group was easily detected in the CA1 pyramidal cells (Fig. 4A). GPX immunoreactivity in the CA1 pyramidal cells was markedly decreased at 2 days post-ischemia and barely identified at 5 days post-ischemia (Fig. 4C, E and G).

GPX immunoreactivity in the CA1 pyramidal cells of the CIL-sham-group was significantly higher compared with the vehicle-sham-group (Fig. 4B and G). In the CIL-ischemia-group, GPX immunoreactivity in the CA1 pyramidal cells was decreased following ischemia-reperfusion; however, the GPX immunoreactivity was significantly higher compared with the corresponding vehicle-ischemia-group (Fig. 4D, F and G).

Neuroprotective effects

NeuN-positive neurons. NeuN-positive neurons were identified in the stratum pyramidale of the hippocampus proper (CA1-3 regions) of the vehicle-sham-group (Fig. 5A and B). The distribution of NeuN-positive neurons in the CIL-sham-group was similar to the vehicle-sham-group and the number of NeuN-positive neurons remained unchanged (Fig. 5D, E and M).

In the vehicle-ischemia-group, a small number of NeuN-positive neurons were observed in the stratum pyramidale of the CA1 region, and none in the CA2-3 region, 5 days following ischemia-reperfusion (Fig. 5G and H); the relative number of NeuN-positive neurons was ~9% compared with the vehicle-sham-group (Fig. 5M). However, in the CIL-ischemia-group, numerous NeuN-positive neurons were detected in the stratum pyramidale of the CA1 region 5 days following ischemia-reperfusion (Fig. 5J and K); the protected neurons were ~68% of the vehicle-sham-group (Fig. 5M); this finding was identical to our previously study (22).
**F-J B-positive cells.*** In the vehicle-sham- and CIL-sham-groups, F-J B-positive cells were not observed in the stratum pyramidale of the CA1 region (Fig. 5C, F and N). In the vehicle-ischemia-group, however, numerous F-J B-positive cells were detected in the stratum pyramidale of the CA1 region 5 days following ischemia-reperfusion (Fig. 5I and N). However, in the CIL-ischemia-group, only a few F-J B-positive cells were detected, and the relative number of F-J B-positive cells was ~24% that of the vehicle-ischemia-group (Fig. 5L and N); this finding was identical to a previous study of the authors (22).
Discussion

Just five min of transient cerebral ischemia results in the damage/death of pyramidal neurons in the hippocampus and neuronal death occurs selectively in the hippocampal CA1 region (27,28). It has been reported (6,29) that pyramidal cells of the stratum pyramidale in the hippocampal CA1 region die several days following transient cerebral ischemia. Mongolian gerbils have been generally used as a good experimental animal model of transient cerebral ischemia as the posterior...
communicating arteries in Willis' circle, which connect the vertebrobasilar and carotid arterial system, are lacking in gerbils and transient cerebral ischemia can easily be made by the ligation of bilateral common carotid arteries (24,30,31).

The present study observed the death of pyramidal cells in the hippocampal CA1 region by NeuN immunohistochemistry and F-J B histofluorescence; a noticeable loss of CA1 pyramidal cells was identified in the stratum pyramidale of the CA1 region 5 days following ischemia-reperfusion. This result corresponds to findings of previous studies (23,32,33). In addition, it was identified that the oral pre-treatment of 200 mg/kg CIL to the gerbils protected CA1 pyramidal cells

Figure 3. CAT immunohistochemistry in the hippocampal CA1 region of the (A) vehicle-sham-,(B) CIL-sham-,(C and E) vehicle-ischemia- and (D and F) CIL-ischemia-groups following ischemia-reperfusion. CAT immunoreactivity is markedly decreased in the SP (asterisk) of the vehicle-ischemia-group at 5 days following ischemia-reperfusion; however, in the CIL-sham- and CIL-ischemia-groups, CAT immunoreactivity in the SP is significantly higher compared with the vehicle-sham- and vehicle-ischemia-groups. Scale bar, 100 µm. (G) ROD as % values of CAT immunoreactivity in the SP of each group (‘P<0.05 vs. vehicle-sham-group; ‘P<0.05 vs. corresponding vehicle-ischemia-group; †P<0.05 vs. respective pre-time point group). The bars indicate the means ± standard error of mean. SP, stratum pyramidale; CAT, catalase; CIL, Chrysanthemum indicum Linné extract; ROD, relative optical density; SO, stratum oriens; SR, stratum radiatum.
(~67% of the sham-operated gerbils) from 5 min of transient cerebral ischemia; this finding was identical to a previous study of the authors (22).

Although mechanisms regarding neuronal death by transient cerebral ischemic insult are complex, it has been demonstrated that, among the mechanisms, endogenous antioxidant enzymes are associated with neuroprotection via the efficient scavenging of ROS (2,9,34,35). Excessive ROS production is a cause of neuronal damage/death following ischemia-reperfusion injury and has been implicated in the

Figure 4. GPX immunohistochemistry in the hippocampal CA1 region of the (A) vehicle-sham-, (B) CIL-sham-, (C and E) vehicle-ischemia- and (D and F) CIL-ischemia-groups following ischemia-reperfusion. GPX immunoreactivity is detected well in the SP of the vehicle-sham-group; the immunoreactivity is significantly increased in the CIL-sham-group. In the vehicle-ischemia-group, GPX immunoreactivity is hardly observed in the SP at 5 days post-ischemia; however, in the CIL-ischemia-group, GPX immunoreactivity is significantly higher than that in the vehicle-ischemia-group. Scale bar, 100 µm. (G) ROD as % values of GPX immunoreactivity in the SP of each group (*P<0.05 vs. vehicle-sham-group; †P<0.05 vs. corresponding vehicle-ischemia-group; ‡P<0.05 vs. respective pre-time point group). The bars indicate the means ± standard error of mean. GPX, glutathione peroxidase; CIL, Chrysanthemum indicum Linné extract; ROD, relative optical density; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.
development of numerous neurologic disorders and brain dysfunctions (10,36-38). Accumulated ROS cause the injurious modification of cellular elements including DNA, proteins and lipids; eventually, the accumulated ROS can impair cellular function and result in neuronal damage/death (39,40). In the present study, SOD1, SOD2, CAT and GPX immunoreactivities of the vehicle-ischemia-group were significantly decreased and barely identified in the CA1 pyramidal cells 5 days following ischemia-reperfusion. This result is coincident with the finding of a previous study using gerbils (40).
Kim et al (41) investigated the protective effect of Chrysanthemum indicum ethanol extract against cisplatin-induced nephrotoxicity in vitro. Their findings may be associated with the antioxidative effects of Chrysanthemum indicum ethanol extract since the antioxidative effects of the vehicle-sham-group, and SOD1, SOD2, CAT and GPX immunoactivities in the CA1 pyramidal cells were significantly higher compared with the vehicle-ischemia-group. These results suggested that the administration of CIL increases antioxidant enzymes and it exhibits neuroprotection following transient cerebral ischemia.

ROS are scavenged by SODs, GPX and CAT, and functions of the antioxidant enzymes have been studied by a number of researchers. It has been reported that SOD1 overexpression demonstrated a neuroprotective effect in the hippocampal CA1 region against cerebral ischemic insults in rodents (42,43). Kondo et al (44) reported that SOD1 knockout mice had demonstrated the increase of cell death and edema of the brain following focal cerebral ischemia, and Murakami et al (45) demonstrated that, in SOD2 knockout mice, exacerbated infarct volume was identified in the brain following permanent focal cerebral ischemia, and suggested that SOD2 was an important enzyme in protecting brain from ischemic injury. It has also been reported that the administration of PEP-1-CAT fusion protein demonstrated significant neuroprotection in the hippocampal CA1 region following transient cerebral ischemia (46). Furthermore, it was recently reported that GPX, which is another antioxidant enzyme contributing to H$_2$O$_2$ scavenging, exhibited a stronger neuroprotective antioxidant against oxidative stress than SOD (8).

It has been demonstrated that CIL is associated with the inhibition of inflammatory responses (15,16,19,22,47). Cheng et al (15) reported that a butanol soluble fraction of CIL possessed anti-inflammatory, immunomodulatory and mono-nuclear phagocytic activities by the enhancement of serum IgG and IgM levels in response to sheep red blood cells in cyclophosphamide-induced mice, and Cheon et al (19) demonstrated that CIL suppressed the production of inflammatory mediators and proinflammatory cytokines via the downregulation of nuclear factor-kb and mitogen-activated protein kinases in RAW264.7 macrophages (15,19). Previously, Kim et al (48) reported that CIL protected against 1-methyl-4-phenylpyridinium ions and lipopolysaccharide-induced cytotoxicity in a cellular model of Parkinson's disease. In addition, Yoo et al (22) recently reported that CIL pre-treatment increased anti-inflammatory cytokines in the hippocampus and that the increased anti-inflammatory cytokines were associated with neuroprotection in the gerbil hippocampus induced by transient cerebral ischemia.

In brief, the present study identified that CIL pre-treatment enhanced SOD1, CAT and GPX, although not SOD2, in pyramidal cells in the gerbil hippocampal CA1 region and protected the cells from transient cerebral ischemia. These results indicated that CIL-mediated neuroprotective effect may be associated with increases of antioxidant enzymes in the CA1 pyramidal cells and suggested that CIL may be used for the prevention of ischemic damage in the brain.

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