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

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Abstract

Background: *Glehnia littoralis*, as a traditional herbal medicine to heal various health ailments in East Asia, displays various therapeutic properties including antioxidant effects. However, neuroprotective effects of *G. littoralis* against cerebral ischemic insults have not yet been addressed. Therefore, in this study, we first examined its neuroprotective effects in the hippocampus using a gerbil model of transient global cerebral ischemia (TGCI).
Methods: Gerbils were subjected to TGCI for 5 min. *G. littoralis* extract (GLE; 100 and 200 mg/kg) was administrated orally once daily for 7 days before ischemic surgery. Neuroprotection was examined by neuronal nuclear antigen immunohistochemistry and Fluoro-Jade B histofluorescence staining. Gliosis was observed by immunohistochemistry for glial fibrillary acidic protein and ionized calcium-binding adapter molecule 1. For neuroprotective mechanisms, immunohistochemistry for superoxide dismutase (SOD) 1 and brain-derived neurotrophic factor (BDNF) was done.
Results: Pretreatment with 200 mg/kg of GLE protected pyramidal neurons in the cornu ammonis 1 (CA1) area from ischemic insult area ($F = 29.770$, $P < 0.05$) and significantly inhibited activations of astrocytes ($F = 22.959$, $P < 0.05$) and microglia ($F = 44.135$, $P < 0.05$) in the ischemic CA1 area. In addition, pretreatment with GLE significantly increased expressions of SOD1 ($F = 28.561$, $P < 0.05$) and BDNF ($F = 55.298$, $P < 0.05$) in CA1 pyramidal neurons of the sham- and ischemia-operated groups.
Conclusions: Our findings indicate that pretreatment with GLE can protect neurons from ischemic insults, and we suggest that its neuroprotective mechanism may be closely associated with increases of SOD1 and BDNF expressions as well as attenuation of glial activation.

Key words: Antioxidant; Glial Activation; Neurotrophic Factor; Neuroprotection; Pyramidal Neurons

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INTRODUCTION

Transient global cerebral ischemia (TGCI) is caused by a temporary deficiency of cerebral blood supply and leads to selective neuronal death in specific brain areas. Especially, neuronal death occurs in the hippocampus and results in learning and memory deficits.[1,2] In humans and experimental animals, the cornu ammonis 1 (CA1) area in the hippocampus is known as one of the most vulnerable regions to TGCI,[3,4] in which pyramidal neurons in the pyramidal layer die from several days after TGCI.[5,6]

Until now, many researchers have attempted to find neuroprotective actions of medical plants and their components and studied their mechanisms of ischemia-induced neuronal death, such as glutamate-induced excitotoxicity, oxidative stress by excessive production of reactive oxygen species (ROS), and glial activation-mediated inflammatory response.[7-11]

Glehnia littoralis, a species of plants in the *Apiaceae* family, is a traditional herb in East Asian countries and has been widely used as diaphoretic, antipyretic, and analgesic agents in the traditional medicine.[12] In addition, recent *in vitro* and *in vivo* studies have demonstrated that *G. littoralis* possesses various therapeutic properties such as anticancer,[13] antioxidant,[14] and anti-inflammatory effects.[15] However, to the best of our knowledge, there have been few reports regarding protective effects of *G. littoralis* against cerebral ischemic insults. Therefore, in this study, we examined neuroprotective effects of *G. littoralis* extract (GLE) and its related mechanisms using a gerbil model of 5 min of TGCI, which has been widely used for the evaluation of neuroprotection against TGCI.[16,17]

METHODS

Ethical approval

Experimental protocol was approved (Approval No. KW_160802_1) by the Institutional Animal Care and Use Committee at Kangwon National University (Chuncheon, Korea).

Preparation of *Glehnia littoralis* extract

G. littoralis was collected by Professor Jong Dai Kim in Kangwon Province (Korea) in October 2014. For the preparation of ethanol GLE, roots and rhizomes of *G. littoralis* were washed with distilled water, air-dried at 60°C, and ground into fine powder by a grinder (IKA M20, IKA, Staufen, Germany). The *G. littoralis* powder was refluxed with 10 vol (v/w) of 70% ethanol at 70°C for 24 h, and the extraction procedure was repeated three times. The extract was filtered through Whatman No. 1 filter paper (Whatman Ltd., Maidstone, Kent, UK), concentrated with a vacuum evaporator, and it was completely dried with a freeze drier. The extraction yield was 8.68%. A voucher specimen (No. KIOM0077036) was deposited at the herbarium of the Center of Herbal Resources Research (Korea Institute of Oriental Medicine, Daejeon, Korea).

Administration with *Glehnia littoralis* extract

We used male Mongolian gerbils (6 months of age and 70–80 g of body weight). Animals were divided into six groups (*n* = 7 in each group): (1) vehicle-sham group, which was treated with vehicle (sterile normal saline; 0.9% w/v NaCl) and given sham operation, (2) vehicle-ischemia group, which was treated with vehicle and given TGCI, (3 and 4) GLE-sham group, which was treated with 100 and 200 mg/kg GLE, respectively, and given sham operation, and (5 and 6) GLE-ischemia group, which was treated with 100 and 200 mg/kg GLE, respectively, and given TGCI. GLE was dissolved in saline, and GLE or saline was orally administered once a day for 7 days before ischemic surgery. We adapted the oral administration of GLE for 1 week because extracts from plants had been taken orally in the traditional medicine and rarely data regarding the absorption and metabolism of GLE had been reported.

Induction of transient global cerebral ischemia

TGCI was induced according to our method.[1] In short, gerbils were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide and bilateral common carotid arteries were occluded for 5 min. Restoration of the blood flow (reperfusion) was directly observed under the ophthalmoscope. Body (rectal) temperature was maintained under normothermic (37.0 ± 0.5°C) condition before, during, and after TGCI. Sham groups were subjected to the same operation, except that the common carotid arteries were not occluded. The gerbils were weighed 1 day before TGCI and 5 days after TGCI. No significant differences were observed in the body weight of the gerbils of the experimental group (data not shown).

Histochemistry and immunohistochemistry

Gerbils (*n* = 7 at each point in time in each group) were sacrificed 2 and 5 days after TGCI. As we previously described,[6] in brief, the animals were perfused transcardially with 4% paraformaldehyde, and their brains were cryoprotected by infiltration with 30% sucrose. Frozen brains were serially sectioned into 30-μm coronal sections in a cryostat (Leica, Germany).

To investigate neuronal damage/death in the hippocampal CA1 area 5 days after TGCI, neuronal nuclear antigen (NeuN, a marker for neurons) immunohistochemistry and Fluoro-Jade B (F-J B, a high-affinity fluorescent marker for neuronal degeneration) histofluorescence staining were performed according to our method.[6] In short, for NeuN immunohistochemistry, the sections were incubated with diluted mouse anti-NeuN (1:1000, Chemicon, Temecula, CA) and subsequently exposed to biotinylated goat anti-mouse IgG and streptavidin peroxidase complex (1:200, Vector, Burlingame, CA, USA). Moreover, they were visualized by staining with 3,3'-diaminobenzidine (Sigma). For F-J B histofluorescence staining, the sections were first 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 (Histochem, Jefferson, AR, USA) staining solution. The sections were examined using an epifluorescent microscope (Carl Zeiss, Germany), with blue (450–490 nm) excitation light and a barrier filter.

To examine changes in glial cells in the CA1 following TGCI, immunohistochemistry for astrocytes and microglia was performed using mouse anti-glial fibrillary acidic protein (GFAP, 1:800, Chemicon) and rabbit anti-ionized calcium binding adapter molecule 1 (Iba-1, 1:800, Wako), respectively, according to the above-mentioned method. In addition, sheep-anti-Cu, Zn-superoxide dismutase (SOD1, 1:1000; Calbiochem, USA), and rabbit anti-brain-derived neurotrophic factor (BDNF, 1:200, Abcam, UK) were used as primary antibodies to investigate possible mechanisms of neuroprotection by GLE.

Data analysis

Data analysis was done as previously described.[6,18] In short, digital images from five sections per animal were captured with a light microscope (BX53, Olympus, Germany) equipped with a digital camera (DP72, Olympus) connected to a PC monitor. For quantitative analyses of NeuN-immunoreactive (NeuN+) and F-J B-positive (F-J B+) cells, we counted cells in a 250 μm × 250 μm including the stratum pyramidale at the center of the CA1 using an image analyzing system (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ, USA). Cell counts were obtained by averaging the counts. In addition, for quantitative analyses of GFAP, Iba-1, SOD1, and BDNF immunoreactivities, their 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 a ratio of the relative optical density (ROD) for GFAP- and Iba-1-immunoreactive structures and relative immunoreactivity (RI) for SOD1 and BDNF was calibrated as % using Adobe Photoshop version 8.0 and then analyzed using ImageJ software version 1.49 (National Institutes of Health, Bethesda, MD, USA). A ratio of the ROD or RI was calibrated as %, with the vehicle-sham group designated as 100%.

Statistical analysis

The data shown here represent the means ± standard error. Differences of the means among the groups were statistically analyzed by two-way analysis of variance with a *post hoc* Bonferroni's multiple comparison test to elucidate the neuroprotective effects of GLE. Statistical significance was considered at $P < 0.05$.

RESULTS

Neuroprotection

The neuroprotective effect of GLE against TGCI in the hippocampus was examined 5 days after TGCI using NeuN immunohistochemistry and F-J B histofluorescence staining. In the vehicle-sham and GLE (100 and 200 mg/kg)-sham groups, NeuN-immunoreactive neurons were distributed in the striatum pyramidale, and no F-J B-positive cells were detected [Figures 1A-1F and 2a-2c]; no significant difference was found in the number of NeuN-immunoreactive neurons between these groups [Table 1].

In the vehicle-ischemia group, numbers of NeuN-immunoreactive neurons were significantly decreased in the stratum pyramidale of the CA1 alone, and many F-J B-positive cells, which show green fluorescence and are dead cells, were detected in the striatum pyramidale of the CA1 [Figures 1a, 1b, 2d and Table 1].

In the 100 mg/kg GLE-ischemia group, numbers of NeuN-immunoreactive neurons and F-J B-positive cells were similar to those in the vehicle-ischemia group [Figures 1c, 1d, 2e and Table 1]. However, abundant NeuN-immunoreactive neurons and a small number of F-J B-positive cells were observed in the 200 mg/kg GLE-ischemia group [Figures 1e, 1f, 2f and Table 1].

Based on these findings, we examined neuroprotective mechanisms of GLE in the 200 mg/kg GLE-ischemia group in the CA1.

Glial activation
Astrocytosis
In the vehicle-sham group, GFAP-immunoreactive astrocytes were observed in all layers of the CA1, and they showed small cell bodies and fine cytoplasmic processes as a resting form [Figure 3a].

In the vehicle-ischemia group, GFAP-immunoreactive astrocytes were altered in the CA1 with time after TGCI, namely, the cytoplasm of astrocytes was hypertrophied with thick processes, indicating an active state [Figure 3c and 3e]. In addition, the ROD of GFAP-immunoreactive structures began to be increased in the CA1 with time, and the ROD 5 days after TGCI was significantly increased (about 193% of the vehicle-sham group) [Figure 3m].

In the 200 mg/kg GLE-sham group, the morphology and ROD of GFAP-immunoreactive astrocytes were similar to those in the vehicle-sham group [Figure 3b and 3m].

In the 200 mg/kg GLE-ischemia group, GFAP-immunoreactive astrocytes were less hypertrophied and their ROD was decreased compared with those in the vehicle-ischemia group [Figures 3d and 3f]; especially, a significant decrease (about 138% of the vehicle-sham group) in the ROD was observed 5 days after TGCI [Figure 3m].

Microgliosis
In the vehicle-sham group, Iba-1-immunoreactive microglia were scattered throughout the CA1 and showed a typical ramified form with small cytoplasm [Figure 3g].

In the vehicle-ischemia group, microglia were hypertrophied with highly branched processes, indicating an active state, 2 days after TGCI [Figure 3i]. Five days after TGCI, microglia were more activated, and many microglia were aggregated near or in the stratum pyramidale [Figure 3k]; at this time, the ROD was significantly increased (about 235% of the vehicle-sham group) [Figure 3n].
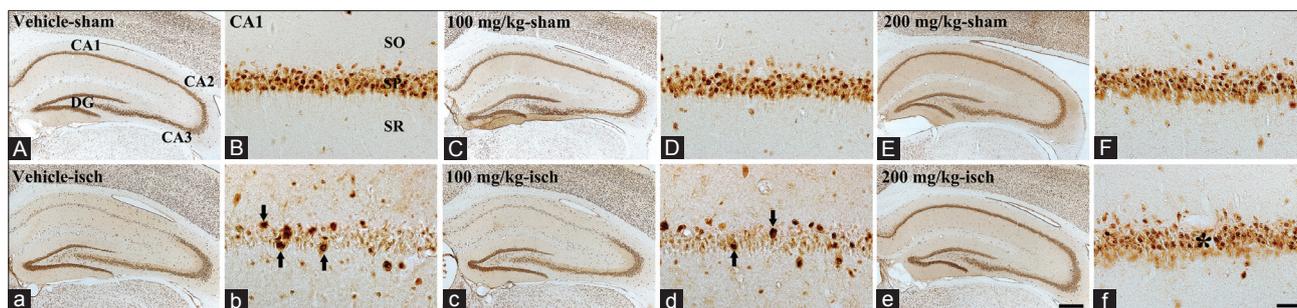
Figure 1: NeuN immunohistochemistry in the cornu ammonis 1 five days after transient global cerebral ischemia. In all sham groups (A-F), abundant NeuN-immunoreactive neurons are shown in the SP. A few NeuN-immunoreactive neurons (arrows) are observed in the SP of the vehicle-ischemia (a and b) and 100 mg/kg GLE-ischemia (c and d) groups. In the 200 mg/kg GLE-ischemia group (e and f), many NeuN-immunoreactive neurons (asterisk) are found. SO: Stratum oriens; SR: Stratum radiatum. NeuN: Neuronal nuclear antigen; SP: Stratum pyramidale; GLE: *Glehnia littoralis* extract. Scale bar = 400 μm (A, C, E, a, c, e) and 40 μm (B, D, F, b, d, f).
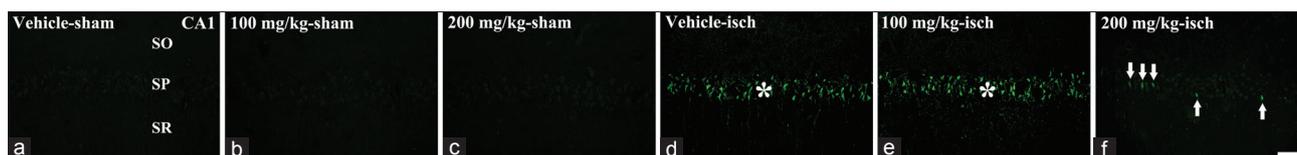

Figure 2: F-J B histofluorescence staining in the cornu ammonis 1 five days after transient global cerebral ischemia. In all sham groups (a-c), no F-J B-positive cells are detected. Many F-J B-positive cells (asterisks, green) are observed in the stratum pyramidale of the vehicle-ischemia (d) and 100 mg/kg GLE-ischemia (e) groups. However, in the 200 mg/kg GLE-ischemia-group (f), a few F-J B-positive cells (arrows) are detected. SO: Stratum oriens; SR: Stratum radiatum. F-J: Fluoro-Jade; GLE: *Glehnia littoralis* extract. Scale bar = 40 μm.

Table 1: The mean number of NeuN-immunoreactive neurons and F-J B-positive cells in the CA1 of the vehicle- and GLE-ischemia groups (*n* = 7)

Groups	NeuN-immunoreactive neurons			F-J B-positive cells		
	Vehicle	GLE		Vehicle	GLE	
		100 mg/kg	200 mg/kg		100 mg/kg	200 mg/kg
Sham	83.00 ± 4.41	79.00 ± 8.38	81.00 ± 6.17	0	0	0
Ischemia	10.00 ± 2.72*	9.00 ± 9.14*	75.00 ± 6.79†	63.00 ± 8.46	66.00 ± 1.85	5.00 ± 2.24†

The number of NeuN-immunoreactive neurons and F-J B-positive cells was counted in a 250×250 μm square of the stratum pyramidale of the CA1 5 days after TGCI ($F = 29.770$, *P<0.05 vs. vehicle_sham group, $F = 80.348$, †P<0.05 vs. vehicle_ischemia group). Data were analyzed by two-way analysis of variance with a post hoc Bonferroni's multiple comparison test.

In the 200 mg/kg GLE-sham group, the morphology and distribution of microglia and their ROD were similar to those in the vehicle-sham group [Figures 3n and 3h].

In the 200 mg/kg GLE-ischemia group, a few Iba-1-immunoreactive microglia were activated and the ROD was low compared with that in the vehicle-ischemia group [Figure 3j, 3l, and 3n]; 5 days after TGCI, the ROD was significantly decreased (about 139% of the vehicle-sham group) [Figure 3n].

Superoxide dismutase 1 and brain-derived neurotrophic factor immunoreactivities

Superoxide dismutase 1 immunoreactivity

In the vehicle-sham group, SOD1 immunoreactivity was easily shown in neurons of the stratum pyramidale of the CA1 [Figure 4a].

In the vehicle-ischemia group, SOD1 immunoreactivity in neurons of the stratum pyramidale decreased with time after TGCI, showing about 79% of the vehicle-sham group 2 days after TGCI and about 43% of the vehicle-sham group 5 days after TGCI [Figure 4c, 4e, and 4m].

In the GLE-sham group, SOD1 immunoreactivity in neurons of the stratum pyramidale was significantly higher (about 134% of the vehicle-sham group) than that in the vehicle-sham group [Figure 4b and 4m].

In the GLE-ischemia group, SOD1 immunoreactivity in neurons of the stratum pyramidale was continuously maintained after TGCI [Figure 4d, 4f, and 4m].

Brain-derived neurotrophic factor immunoreactivity

In the vehicle-sham group, BDNF immunoreactivity was observed in neurons of the stratum pyramidale of the CA1 [Figure 4g].

In the vehicle-ischemia group, BDNF immunoreactivity in neurons of the stratum pyramidale was significantly decreased (about 58% of the vehicle-sham group) 2 days after TGCI and was very low (about 32% of the vehicle-sham group) 5 days after TGCI [Figure 4i, 4k, and 4n].

In the GLE-sham group, BDNF immunoreactivity in neurons of the stratum pyramidale was significantly increased (about 148% of the vehicle-sham group) [Figures 3n and 4h].

In the GLE-ischemia group, BDNF immunoreactivity in neurons of the stratum pyramidale was consistently maintained after TGCI [Figure 4j, 4l, and 4n].

DISCUSSION

Plants belonging to the family *Apiaceae* have been extensively used for food and medicinal purpose. Although it has been reported that some plant extracts of this family including Angelica and Water dropwort show strong neuroprotective properties in experimental animal models of cerebral ischemia,[19-21] there were few studies regarding neuroprotective effects of GLE against brain ischemic insults. Therefore, in this study, we examined neuroprotective effects of GLE in a gerbil model of TGCI using NeuN immunohistochemistry and F-J B histofluorescence staining, which are widely used for the histological evaluation of neuronal damage/death in the central
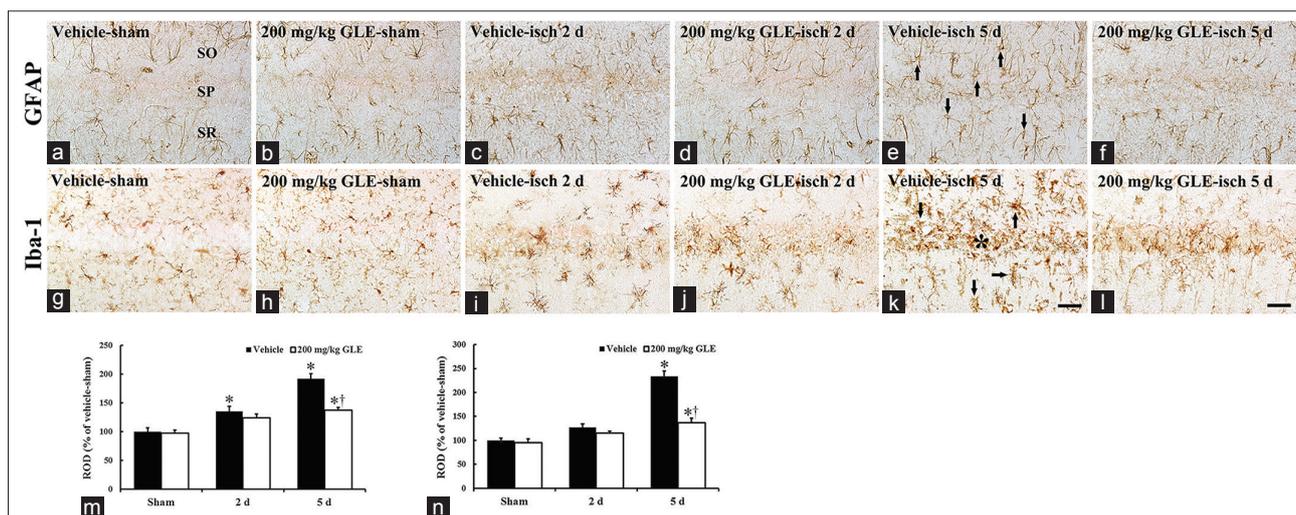

Figure 3: Immunohistochemistry for GFAP (a-f) and Iba-1 (g-l) in the cornu ammonis 1 two and five days after TGCI. In the vehicle-ischemia group, GFAP- (c and e) and Iba-1- (i and k) immunoreactive glia (arrows) are activated; especially, many activated microglia are aggregated in the stratum pyramidale (asterisk) 5 days after TGCI. In the 200 mg/kg GLE-ischemia groups (d, f, j, and l), activations of glia are significantly reduced. SO: Stratum oriens; SR: Stratum radiatum. Scale bar = 40 μm. (m and n) Relative optical density as % of GFAP- and Iba-1-immunoreactive structures ($n = 7$ at each point in time, $*P < 0.05$ vs. vehicle-sham group, $†P < 0.05$ vs. vehicle-ischemia group). Bars indicate the means ± standard error. GFAP: Glial fibrillary acidic protein; Iba-1: Ionized calcium binding adapter molecule 1; TGCI: Transient global cerebral ischemia; GLE: *Glehnia littoralis* extract.
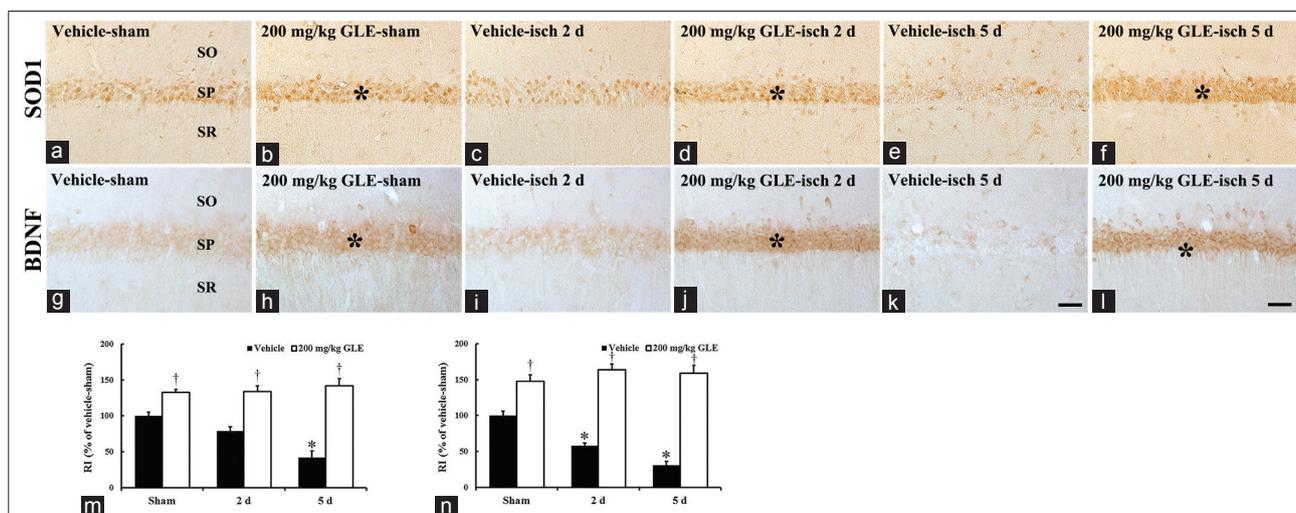

Figure 4: Immunohistochemistry for SOD1 (a-f) and BDNF (g-l) in the cornu ammonis 1 two and five days after TGCI. In the vehicle-ischemia groups, SOD1 (c and e) and BDNF (i and k) immunoreactivity is gradually decreased in the stratum pyramidale after TGCI. In the 200 mg/kg GLE-sham- (b and h) and 200 mg/kg GLE-ischemia (d, f, j and l) groups, increased SOD1 and BDNF immunoreactivities (asterisks) are maintained. SO: Stratum oriens; SR: Stratum radiatum. Scale bar = 40 μm. (m and n) Relative immunoreactivity as % of SOD1 and BDNF immunoreactivities in the pyramidal neurons ($n = 7$ at each point in time, $*P < 0.05$ versus vehicle-sham group, $†P < 0.05$ versus vehicle-sham or vehicle-ischemia group). Bars indicate the means ± standard error. SOD: Superoxide dismutase; BDNF: Brain-derived neurotrophic factor; TGCI: Transient global cerebral ischemia; GLE: *Glehnia littoralis* extract.

nervous system (CNS),[22,23] and we found that pretreatment with 200 mg/kg of GLE effectively protected pyramidal neurons in the CA1 from ischemic damage following TGCI. Recently, some studies have shown that major constituents isolated from *G. littoralis* such as quercetin, isoquercetin, and rutin protect neurons in *in vitro* and *in vivo* models of cerebral ischemia.[24-26] However, to the best of our knowledge, our present study is the first that shows that GLE exhibited neuroprotective properties against TGCI.

It has been reported that TGCI leads to the proliferation and activation of glial cells including astrocytes and microglia in the ischemic CA1[6,27] and that activated astrocytes and microglia take part in ischemia-induced inflammatory response through the synthesis and release of diverse neurotoxic substances including pro-inflammatory cytokines and nitric oxide, which contribute to the development of ischemic neuronal damage/death.[28-30] From these reports, it has been thought that the inhibition of astrocytes and microglia activation induced by ischemic insults contributes to neuroprotection against ischemic insults. [6,31,32] In this regard, we show, in the present study, that pretreatment with 200 mg/kg of GLE significantly inhibited the activation of astrocytes and microglia in the ischemic CA1. Although there were no studies regarding effects of GLE on the TGCI-induced activation of glial cells, Yoon *et al.*,[33] reported that GLE inhibited the production of inflammatory mediators (nitric oxide and prostaglandin E_2) and pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1β) in lipopolysaccharide-activated macrophages. Therefore, it is likely that the neuroprotective effect of GLE against TGCI might involve the inhibition of ischemia-induced astrocyte and microglia activation.

Neurons in the brain are highly vulnerable to oxidative stress which is induced by the overgeneration of ROS in various neurological disorders including cerebral ischemic insults.[34] It has been well known that oxidative stress in neurons is a crucial underlying factor of neuronal death following cerebral ischemic injury and that the excessive formation of ROS is prevented by endogenous antioxidant enzymes.[35,36]

Among endogenous antioxidant enzymes, SOD1 is one of the most important antioxidant enzymes against oxidative stress, and the importance of SOD1 in cerebral ischemia has been emphasized by extensive experiments, for example, SOD1 displays neuroprotective action in animal models of cerebral ischemia. Namely, Chan reported that an increased level of SOD1 in transgenic mice protected neurons from transient focal cerebral ischemic injury.[37] In addition, it was reported that the overexpression of SOD1 reduced neuronal death in the CA1, following TGCI using transgenic mice and rats.[38,39] Furthermore, we recently reported that an increase of endogenous SOD1 expression in pyramidal neurons in the CA1 provided neuroprotection against TGCI in gerbils.[18] Based on the these reports, in this study, we examined change in SOD1 immunoreactivity in the ischemic CA1 following pretreatment with GLE to elucidate neuroprotective mechanisms of GLE against TGCI and found that SOD1 immunoreactivity in pyramidal neurons of the CA1 was significantly increased by pretreatment with 200 mg/kg of GLE in the sham and ischemia groups compared with the vehicle-sham group. Therefore, it is likely that the increase and maintenance of SOD1 expression following pretreatment with GLE might alleviate TGCI-induced neuronal damage.

BDNF is a representative neurotrophic factor in the CNS and plays a significant role in neuronal survival after various brain insults including brain ischemia.[40] Exogenous BDNF-mediated neuroprotective effects have been observed in animal models of cerebral ischemia,[41,42] and the inhibition of endogenous BDNF activity aggravates neuronal damage following cerebral ischemia.[43] In addition, some researchers have reported that upregulated BDNF expression following treatment with some herbal medicines attenuates cerebral ischemia-induced neuronal damage.[44-46] Furthermore, it was reported that BDNF signaling mediated the upregulation of antioxidant enzymes and antioxidant processes, which are closely associated with neuroprotection, in neuronal cells.[47] Together these published studies, in the present study, we found that pretreatment with 200 mg/kg of GLE significantly increased BDNF immunoreactivity in pyramidal neurons of the CA1 of the sham and ischemia groups. Therefore, this result suggests that the increased and sustained BDNF expression following pretreatment with GLE in the sham and ischemia groups might contribute to neuroprotective effects of GLE against TGCI.

In conclusion, results of this study clearly show, for the first time, that pretreatment with GLE protected pyramidal neurons of the CA1 from TGCI and that the neuroprotective effects of GLE against TGCI are closely associated with the attenuation of glial activation as well as increases of SOD1 and BDNF expressions. Therefore, we suggest that *G. littoralis* can be used as a potential candidate for the prevention of cerebral ischemic insults.

Financial support and sponsorship

Conflicts of interest

There are no conflicts of interest.

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