— Regular Article —

Neuroprotective Effect of Repeated Treatment with *Hericium erinaceum* in Mice Subjected to Middle Cerebral Artery Occlusion

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The neuroprotective effects of *Hericium erinaceum* (*H. erinaceum*) were studied in mice subjected to middle cerebral artery (MCA) occlusion. Infarct volumes were markedly reduced in mice receiving 14 days of *H. erinaceum* (300 mg/kg) treatment prior to 4-hr MCA occlusion. Moreover, 14-day pre-ischemic *H. erinaceum* treatment significantly increased the levels of nerve growth factor (NGF) in both the cortex and striatum of mice subjected to 4-hr MCA occlusion. However, pre-ischemic *H. erinaceum* treatment had no effect on cerebral blood flow (CBF) in the cortex of mice subjected to MCA occlusion. Treatment with *H. erinaceum* for 1 day prior to MCA occlusion-induced ischemia had no effect on infarct volume or NGF level. These results suggest that 14 days of treatment with *H. erinaceum* prior to MCA occlusion protected against focal cerebral ischemia, by increasing NGF levels. This implies that *H. erinaceum* and its components could be useful for preventing cerebral infarction.

Key words —— *Hericium erinaceum*, middle cerebral artery occlusion, nerve growth factor, cerebral ischemia, neuroprotection

INTRODUCTION

Hericium erinaceum (*H. erinaceum*) is a white fungus found in temperate areas in the northern hemisphere, including China, Japan, and the U.S.A. In Japan, it is called yamabushitake, which is similar to the term used for the decoration of a mountain priest's costume. *H. erinaceum* is a traditional edible mushroom in several Asian countries, and has generally been used as a herbal medicine for the treatment of dyspepsia, gastric ulcers and enervation in China.[1–6)]

H. erinaceum consists of several components, including polysaccharides, proteins, lectins, erinacol and terpenoids, some of whose biological activities have been studied.[1, 7)] Hericenone and erinacine have been isolated from the fruiting body[8)] and mycelia[9)] of *H. erinaceum,* respectively, and hericenone C-H and erinacine A-I have been shown to stimulate nerve growth factor (NGF) synthesis[10–12)] in cultured astrocytes. The potential neuroprotective effects of *H. erinaceum* in neurodegenerative diseases, including dementia and motor dysfunction, have therefore attracted considerable attention, because both hericenone and erinacine are low-molecular weight, relatively lipid soluble compounds that are able to pass the blood-brain barrier.[13)] However, no studies have reported on the cerebroprotective effects of *H. erinaceum in vivo*.

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Previous studies have shown that NGF is involved in cerebral ischemia; a transient increase in NGF in both cortices was detected 6 hr after middle cerebral artery (MCA) occlusion.[14] Selegiline increased NGF gene expression in the cerebral cortex after MCA occlusion.[15] Butylphthalide significantly up-regulated the expression of NGF at the genetic transcription level, and protected against ischemic injury.[16] Dexamethasone may play a role in modulating NGF mRNA expression during the hippocampal neuronal response to brain ischemia.[17] These results indicate that NGF could play an important role in preventing cerebral ischemia.

In the present study, we examined the neuroprotective effect of *H. erinaceum* during cerebral infarction induced by MCA occlusion. Moreover, we investigated the effects of *H. erinaceum* on NGF levels and cerebral blood flow (CBF) in mice subjected to MCA occlusion.

MATERIALS AND METHODS

Animals —— Male ddY mice weighing 25–35 g were purchased from the Kiwa Experimental Animal Laboratory (Wakayama, Japan). Animals were kept under a 12-hr light/dark cycle (lights on at 07 : 00) in an air-conditioned room (temperature 23 ± 2°C, humidity 55 ± 5%) with food (CE-2; CLEA Japan; Tokyo, Japan) and water available *ad libitum*. All experimental procedures regarding animal care and use were performed in accordance with the regulations established by the Experimental Animal Care and Use Committee of Fukuoka University. All experiments conformed to the guidelines on the ethical use of animals of the Japanese Government Notification, and all efforts were made to minimize both the number of animals used for experiments and their suffering.

Preparation of *H. erinaceum* Solution —— Dried mushrooms, the fruiting bodies of *H. erinaceum*, were obtained from Murata Industrial Limited Company (Miyazaki, Japan). The *H. erinaceum* powder was suspended in purified water.

Transient Focal Cerebral Ischemia —— Focal cerebral ischemia was induced according to the method described in our previous study.[18] Mice were anesthetized with 2% halothane and anesthesia was maintained thereafter with 1% halothane (Flosen; Takeda Chemical Industries, Osaka, Japan). After a midline neck incision, the left common and external carotid arteries were isolated and ligated. A nylon monofilament (8–0; Ethilon; Johnson & Johnson, Tokyo, Japan) coated with silicon resin (Xantopren; Heleus Dental Material, Osaka, Japan) was introduced through a small incision into the common carotid artery and advanced to a position 9 mm distal to the carotid bifurcation for occlusion of the MCA. Four hours after the occlusion, the mice were re-anesthetized with halothane and reperfusion was established by withdrawal of the filament. Twenty-four hours after MCA occlusion, the animals were sacrificed by decapitation. The brains were carefully removed and sectioned coronally into four 2-mm slices, using a mouse brain matrix. Slices were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, U.S.A.). TTC is a marker of mitochondrial function and can reliably indicate the ischemic areas for up to 3 days after ischemia.[10] The area of the infarction (mm^2) in each section was measured using a computerized image analysis system (Image J, version 1.37), and the volume was calculated. For acute treatment, *H. erinaceum* was administrated orally, 1 hr before and 2 hr after MCA occlusion. In repeated treatments, *H. erinaceum* and β-1,3-glucan (Curdlan; Wako, Osaka, Japan) suspended in 1% Tween were orally administered once a day for 14 days. 1% Tween was used as dispersant of *H. erinaceum* powder and β-1,3-glucan.

NGF Determination —— NGF levels were measured in the cortex, striatum and hippocampus, 24 hr after the last administration of *H. erinaceum*. Mice were sacrificed and brain regions quickly removed. NGF was measured using an NGF kit (Emax ImmunoAssay System number G7631; Promega, Madison, WI, U.S.A.). Briefly, tissues were homogenized in kit calibration buffer and centrifuged at $15000 \times g$ for 20 min. Ninety-six well immunoplates were coated with 100 µl per well of polyclonal sheep anti-NGF antibody. After overnight incubation at 4°C, the plates were washed with wash buffer and incubated with a blocking buffer for 1 hr at room temperature. After washing, the samples which was homogenized tissues in kit calibration buffer and the NGF standard solution were diluted with sample buffer, distributed into the wells and incubated for 6 hr at room temperature in a shaking container. After washing five times with wash buffer, monoclonal sheep anti-NGF antibody was added to each well. Following overnight incubation at 4°C, the plates were washed five times with wash buffer. The plates were then incubated with anti-rat IgG-horseradish peroxidase con-

jugate for 2.5 hr at room temperature in a shaking container. This was followed by incubation with 3,3′,5,5′-tetramethylbenzidine (TMB) one solution for 10 min, and 100 μl of 1 N hydrochloric acid was then added to each well. The colorimetric product was measured at 450 nm using a microplate reader (Model 550; Bio-Rad, Hercules, CA, U.S.A.). NGF concentrations were determined from the regression line for the NGF standard curve (ranging from 250 pg/ml purified NGF), incubated under similar conditions. According to the manufacturer's instructions, the sensitivity of the assay is about 7.8 pg/ml of NGF, and there is no cross-reactivity with other related neurotrophic factors (BDNF, NT-3, and NT-4). The NGF concentration was measured 24 hr after the last *H. erinaceum* treatment in intact mice or 24 hr after MCA occlusion.

Measurement of CBF —— CBF was monitored by laser Doppler flowmetry (ALF21, Advance Co., Tokyo, Japan) using a laser Doppler flowmeter (ALF2100, Advance Co.) probe (diameter 0.5 mm) inserted into the left cortex (anterior: −0.22 mm; lateral: 2.5 mm from bregma; depth: 2.0 mm from the skull surface) through a guide cannula.[19] CBF was measured during 4-hr MCA occlusion.

Statistical Analysis —— Results are expressed as means ± standard error of the mean (S.E.M.). Dunnett's test after one-way analysis of variance (ANOVA) was used to compare infarct volumes. Tukey-Kramer test after one-way ANOVA was used to compare NGF levels. Student's *t*-test was used to compare CBFs. A p value of < 0.05 was considered to be significant.

RESULTS

Effect of 14-day Repeated *H. erinaceum* Treatment on NGF Levels in Intact Mice

Treatment with *H. erinaceum* 300 mg/kg once daily for 14 days significantly increased NGF levels in the striatum and hippocampus of intact mice (striatum: vehicle, 0.73 ± 0.09 pg/mg, $n = 15$; *H. erinaceum*, 1.04 ± 0.10 pg/mg, $n = 11$, $p < 0.05$; hippocampus: vehicle, 3.31 ± 0.25 pg/mg, $n = 14$; *H. erinaceum*, 4.28 ± 0.30 pg/mg, $n = 12$, $p < 0.05$, Student's *t*-test, Fig. 1). Treatment also tended to increase NGF levels in the cortex, though the difference was not significant (vehicle, 1.35 ± 0.11 pg/mg, $n = 15$; *H. erinaceum*, 1.66 ± 0.20 pg/mg, $n = 13$, Fig. 1). Treatment with *H. erinaceum* 300 mg/kg for 1 day had no effect on NGF levels in any of the three regions in intact mice (data not shown).

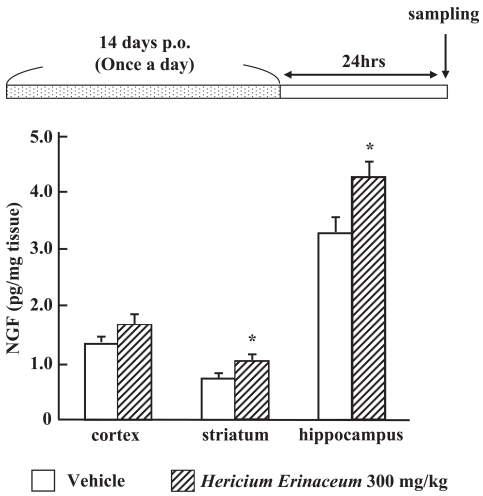

Fig. 1. Effect of 14-day Repeated *H. erinaceum* Treatment on NGF Levels in Intact Mice

NGF levels in the cortex, striatum and hippocampus were measured by ELISA after 14 days of repeated oral *H. erinaceum* treatment at a dose of 300 mg/kg. Values represent the mean ± S.E.M. $^*p < 0.05$ compared with vehicle (Student's *t*-test).

Effect of Acute Treatment with *H. erinaceum* on Cerebral Infarction Induced by MCA Occlusion

H. erinaceum administered 1 hr before and 2 hr after MCA occlusion had no effect on the size of the infarct at 24 hr after occlusion (vehicle, 107.2 ± 11.6 mm^3, $n = 9$; *H. erinaceum* 100 mg/kg, 112.9 ± 7.7 mm^3, $n = 9$; *H. erinaceum* 300 mg/kg, 104.1 ± 9.2 mm^3, $n = 9$, Fig. 2A).

Effect of 14-day Pre-ischemic *H. erinaceum* and β-1,3-glucan Treatment on Cerebral Infarction Induced by MCA Occlusion

Treatment with *H. erinaceum* for 14 days prior to MCA occlusion significantly and dose-dependently reduced the infarct volume induced by ischemia. *H. erinaceum* 300 mg/kg significantly reduced the infarct volume (vehicle, 87.8 ± 4.9 mm^3, $n = 9$; *H. erinaceum* 30 mg/kg, 93.4 ± 8.6 mm^3, $n = 5$; *H. erinaceum* 100 mg/kg, 67.4 ± 8.6 mm^3, $n = 6$; *H. erinaceum* 300 mg/kg, 52.4 ± 15.9 mm^3, $n = 6$, $p < 0.05$ vs. vehicle, $F(3, 22) = 3.804$, $p < 0.05$, one-way ANOVA followed by Dunnett's test, Fig. 2B). Treatment with β-1,3-glucan treatment 300 mg/kg for 14 days prior to MCA occlusion, however, had no effect on infarct volume (β-1,3-glucan, 90.9 ± 17.6 mm^3, $n = 6$, Fig. 2B).

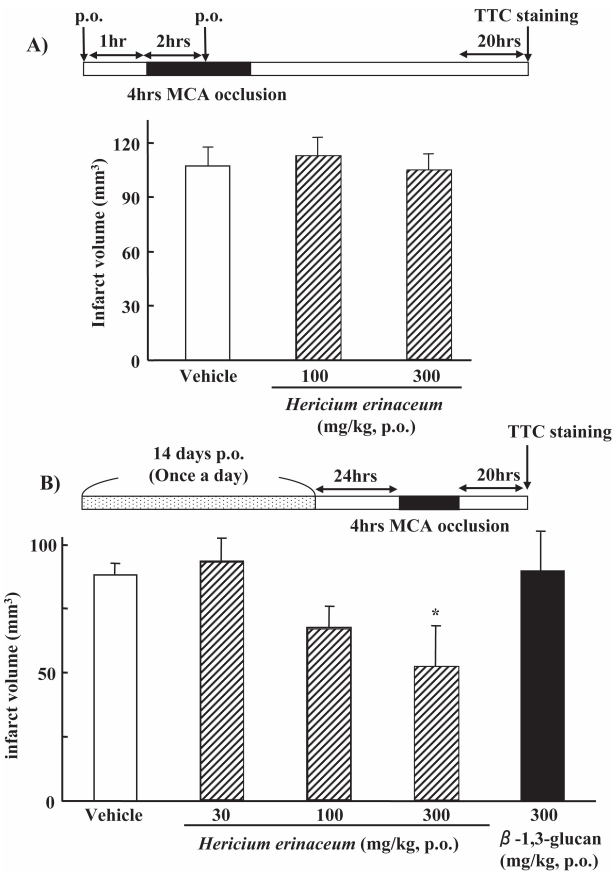

Fig. 2. Effect of *H. erinaceum* on Cerebral Infarction Induced by MCA Occlusion

Infarct volumes were analyzed by TTC staining. A) Effect of acute *H. erinaceum* treatment. *H. erinaceum* was administrated orally 1 hr before and 2 hr after MCA occlusion. B) Effects of 14-day pre-ischemic oral *H. erinaceum* and β-1,3-glucan treatment. *H. erinaceum* and β-1,3-glucan were administrated orally for 14 days before MCA occlusion. Values are expressed as the mean ± S.E.M. $^{*}p < 0.05$ compared with vehicle (one-way ANOVA test followed by Dunnett's test).

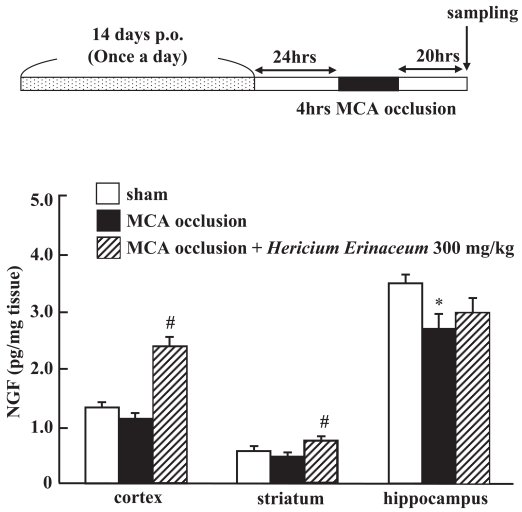

Fig. 3. Effect of 14-day Repeated Pre-ischemic *H. erinaceum* Treatment on Ipsilateral NGF Levels in Mice Subjected to MCA Occlusion

NGF levels were measured in the ipsilateral cortex, striatum and hippocampus by ELISA after 14 days of repeated pre-ischemic oral *H. erinaceum* treatment at a dose of 300 mg/kg. Values represent the mean ± S.E.M. $^{*}p < 0.05$ compared with vehicle, $^{\#}p < 0.05$ compared with MCA occlusion (one-way ANOVA test followed by Tukey-Kremer test).

Effect of 14-day Pre-ischemic *H. erinaceum* Treatment on Ipsilateral NGF Levels in Mice Subjected to MCA Occlusion

Treatment with *H. erinaceum* 300 mg/kg for 14 days prior to MCA occlusion significantly increased NGF levels in the ipsilateral cortex (sham, 1.42 ± 0.08 pg/mg, $n = 17$; MCA, 1.13 ± 0.09 pg/mg, $n = 8$; MCA + *H. erinaceum* 300 mg/kg, 2.44 ± 0.16 pg/mg, $n = 8$, $p < 0.05$ vs. MCA, $F(2, 30) = 31.559$, $p < 0.01$, one-way ANOVA followed by Tukey-Kramer test, Fig. 3) and striatum (sham, 0.64 ± 0.06 pg/mg, $n = 17$; MCA, 0.53 ± 0.05 pg/mg, $n = 10$; MCA + *H. erinaceum* 300 mg/kg, 0.80 ± 0.09 pg/mg, $n = 8$, $p < 0.05$ vs. MCA, $F(2, 32) = 3.456$, $p < 0.01$, one-way ANOVA followed by Tukey-Kramer test, Fig. 3). There was no significant difference in NGF levels in the ipsilateral hippocampus between the MCA-treated group and the MCA + *H. erinaceum*-treated group (sham, 3.59 ± 0.18 pg/mg, $n = 16$; MCA, 2.573 ± 0.26 pg/mg, $n = 10$; MCA + *H. erinaceum* 300 mg/kg, 3.07 ± 0.43 pg/mg, $n = 8$, $p < 0.05$ vs. sham, $F(2, 31) = 4.984$, $p < 0.05$, one-way ANOVA followed by Tukey-Kramer test, Fig. 3).

Effect of 14-day Pre-ischemic *H. erinaceum* Treatment on CBF in the Cortex of Mice Subjected to MCA Occlusion

CBF was decreased in the cortex immediately after MCA occlusion, and the decrease in CBF was maintained during the 4 hr of MCA occlusion. Immediately after reperfusion, the CBF returned to 72.0 ± 3.5% in the vehicle-treated group (Fig. 4).

Treatment with *H. erinaceum* 300 mg/kg for 14 days prior to MCA occlusion had no effect on CBF during and after 4 hr MCA occlusion, compared with the vehicle-treated group (average CBF during the 4 hr MCA occlusion, vehicle 18.2 ± 0.5%, $n = 5$; 300 mg/kg *H. erinaceum* 23.2 ± 1.0%, $n = 4$, average CBF after the 4 hr MCA occlusion, vehicle 72.0 ± 3.5%, $n = 5$; 300 mg/kg *H. erinaceum* 71.2 ± 5.6%, $n = 4$, Fig. 4).

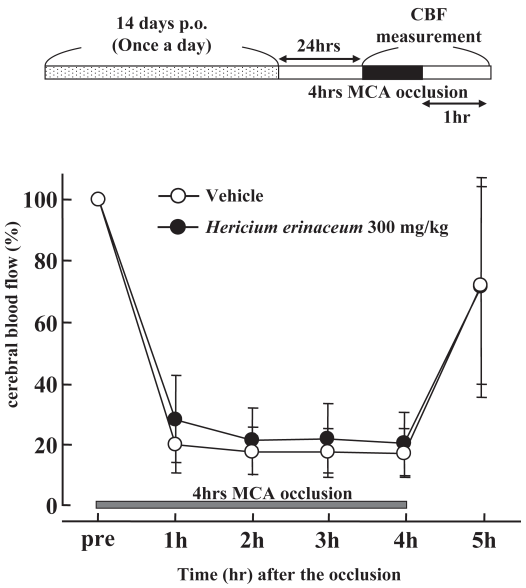

Fig. 4. Effect of 14-day Repeated Pre-ischemic *H. erinaceum* Treatment on CBF in the Cortex of Mice Subjected to MCA Occlusion

CBF in the cortex was measured by laser Doppler flowmetry during MCA occlusion, after oral administration of *H. erinaceum* for 14 days. Values are expressed as the mean ± SEM.

DISCUSSION

Treatment with *H. erinaceum* for 14 days prior to MCA occlusion showed a neuroprotective effect against cerebral ischemia. In addition, 14 days of *H. erinaceum* treatment also increased NGF levels in both intact mice and mice subjected to MCA occlusion. However, the 14 days of *H. erinaceum* pretreatment had no effect on CBF in mice subjected to MCA occlusion. These results suggest that treatment with *H. erinaceum* 300 mg/kg for 14 days prior to MCA occlusion could protect against cerebral ischemia by stimulating NGF, without influencing CBF.

It has previously been reported that NGF synthesis in astrocytes was stimulated by both hericenone and erinacine isolated from *H. erinaceum*.[10–12] Oral administration of *H. erinaceum* for 7 days increased NGF mRNA expression in the mouse hippocampus,[20] and oral administration of erinacine A significantly increased the levels of NGF in the rat locus coeruleus and hippocampus.[21] We therefore measured NGF levels to confirm the mechanism of action of *H. erinaceum* in the present study. Fourteen days of repeated oral treatment with *H. erinaceum*, but not acute treatment (twice, 1 hr interval), increased NGF levels in the cortex, striatum and hippocampus in intact mice, though the difference in cortex levels was not significant. These results suggest that active components of *H. erinaceum* could be absorbed into the blood and delivered to the brain across the blood-brain barrier.

In the present study, 14 days of pre-ischemic *H. erinaceum* treatment at a dose of 300 mg/kg significantly reduced the volume of the infarct induced by MCA occlusion. *H. erinaceum* contains various components, such as polysaccharides, proteins, lectins, erinacol and terpenoids, in addition to hericenone, erinacine and dilinoleoylphosphatidylethanolamine.[22] β-1,3-glucan, which has demonstrated anti-tumor activity against Sarcoma 180, is the most abundant component and constitutes approximately 20% of the total components. In addition, peripherally applied lentinan, a branched β-glucan, has been shown to facilitate the synaptic efficacy of the dentate gyrus neurons *in vivo*.[23] Thus the β-1,3-glucan component of *H. erinaceum* may play an important role in its neuroprotective effects. However, 14 days of repeated pre-ischemic treatment with β-1,3-glucan failed to reduce the volume of infarcts induced by MCA occlusion, suggesting that β-1,3-glucan in *H. erinaceum* had no effect on the central nervous system. The neuroprotective effects of *H. erinaceum* thus seem to be related to compounds including hericenone and erinacine other than β-1,3-glucan. Previous study was reported that hot water extracts of *Pleurocybella porrigens* (*P. porrigens*) caused shock and acute toxic death in mice because the extracts of *P. porrigens* was recently suspected to cause acute encephalopathy including coma and spasm in some patients with nephrosis.[24] *H. erinaceum* may include new low-molecular compound can penetrate the blood-brain barrier because the contained amount of hericenone and erinacine for *H. erinaceum* powder was lower than 1%.

We also examined the effects of *H. erinaceum* on NGF levels in mice subjected to MCA occlusion. NGF levels were reduced in the ipsilateral cortex, striatum and hippocampus in mice subjected to MCA occlusion, and treatment with *H. erinaceum* 300 mg/kg for 14 days prior to ischemia significantly attenuated this decrease in NGF levels, in both the cortex and striatum. This result, together with the effects of *H. erinaceum* on NGF levels in the cortex, striatum, and hippocampus of intact mice, supports the hypothesis that *H. erinaceum* acts on the central nervous system in mice subjected to MCA occlusion. This suggests that the neuroprotective effects of *H. erinaceum* are related to active

compounds that are low-molecular weight and relatively lipid soluble, and able to pass through the blood brain barrier and stimulate NGF synthesis.

Several studies have reported on the use of pharmacological blood pressure augmentation and restoration of blood flow for acute phase clinical treatment of ischemia,[25, 26] and mild hypertension and increased blood flow have been reported to improve cerebral ischemic injury.[27–29] Fourteen days of repeated treatment with *H. erinaceum* 300 mg/kg had no effect on CBF in the cortex of either intact mice (data not shown) or those subjected to 4-hr MCA occlusion. These results suggest that *H. erinaceum* might act directly on the brain cells and provide its neuroprotective effect against focal cerebral ischemia without affecting CBF.

In the present study, 14 days of pre-ischemic *H. erinaceum* treatment at a dose of 300 mg/kg significantly reduced the volume of infarcts induced by MCA occlusion. However, *H. erinaceum* had no effect on infarct volume 24 hr after occlusion when it was administered twice (1 hr before and 2 hr after MCA occlusion). Moreover, 14 days of repeated *H. erinaceum* treatment, but not acute treatment, also increased NGF levels in both intact mice and mice subjected to MCA occlusion. Mori *et al.* also reported that feeding with a diet containing 5% dried *H. erinaceum* powder for 7 days, but not 1 day, increased NGF mRNA levels in the hippocampus.[20] These results indicate that continuous pre-ischemic treatment with *H. erinaceum* is required to realize its neuroprotective effects.

It is also possible that unknown derivatives with NGF-inducing activity, other than hericenones, exist in the fruiting bodies of *H. erinaceum*.[20] Moreover, erinacines have been reported to demonstrate agonistic activity towards the κ opioid receptor.[1, 30] A κ opioid receptor agonist has been shown to improve learning and memory ability[31, 32] and to have neuroprotective effects in a MCA occlusion model in rats.[33, 34] Further studies are therefore required to identify any unknown constituents of *H. erinaceum*, and to investigate the existence of any mechanisms, other than its NGF-stimulating activity.

In conclusion, the present study demonstrated that 14 days of repeated pre-ischemic treatment with *H. erinaceum* had neuroprotective effects in mice subjected to MCA occlusion, through the stimulation of NGF synthesis, but with no effect on CBF. It is possible that *H. erinaceum* and its components could be useful as preventative agents for cerebral infarction.

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