

## Enhancement of Neuroprotection of Mulberry Leaves (*Morus alba* L.) Prepared by the Anaerobic Treatment against Ischemic Damage

Tong Ho KANG,<sup>a</sup> Hye Rim OH,<sup>b</sup> Sun Moon JUNG,<sup>a</sup> Jong Hoon RYU,<sup>b</sup> Mee Won PARK,<sup>c</sup> Yong Kon PARK,<sup>c</sup> and Sun Yeou KIM<sup>\*,a</sup>

<sup>a</sup> Graduate School of East-West Medical Science, Kyunghee University; Kyunggi 446–701, South Korea; <sup>b</sup> Department of Oriental Pharmaceutical Science, Kyunghee University; Seoul, 130–701, South Korea; and <sup>c</sup> Korea Food Research Institute; Kyunggi 463–420, South Korea. Received August 23, 2005; accepted November 25, 2005

Several neurological disorders such as Alzheimer's and Parkinson's diseases have been attributed to  $\gamma$ -aminobutyric acid (GABA) depletion in the brain. In order to provide a pharmacological basis for the neuroprotective actions of the enhanced accumulation of GABA in mulberry leaves (ML) against cerebral ischemia *in vitro* and *in vivo*, a process was developed to enhance the accumulation of GABA in mulberry leaves (GAML) as a result of the various anaerobic treatments. The GABA concentrations were changed by N<sub>2</sub> gas purging, the reaction temperature, reaction time, pH and the leaf size. GABA enhanced the potential of neuroprotection in the PC12 cells damaged by H<sub>2</sub>O<sub>2</sub>-induced oxidation. GAML reduced the cytotoxicity in the PC12 cells against oxygen glucose deprivation-induced cerebral ischemic condition. The neuroprotective effect of GAML was further demonstrated *in vivo* using middle cerebral artery occlusion brain injury model. GAML significantly decreased the infarct volume of the brain compared with than control group. Overall, these results suggest that the anaerobic treatment of ML makes GAML enhance the neuroprotection effect against *in vivo* cerebral ischemia such as *in vitro*.

**Key words** neuroprotection; mulberry leaf;  $\gamma$ -aminobutyric acid (GABA); anaerobic treatment; cerebral ischemia

Mulberry (*Morus alba* L., Moraceae) is cultivated in China, Japan and Korea, and their leaves have been used for a long time to feed silkworms (*Bombyx mori* L.). Chen reported that extracts from mulberry leaves have a potent anti-hyperglycemic activity in diabetic mice.<sup>1)</sup> N-containing sugars, plant hormones, moracetin and many antioxidative flavonoid compounds have been isolated from mulberry leaves.<sup>2,3)</sup> Recently, we reported that Mulberroside F isolated from the leaves of ML can inhibit melanin biosynthesis.<sup>4)</sup>

Among the nutritional components, the quantitative changes in carbohydrates, amino acids and adenine nucleotides in the stems of mulberry trees were followed from spring to early summer and from autumn to early spring.<sup>5)</sup> Among the amino acids,  $\gamma$ -aminobutyric acid (GABA) and glutamate are endogenous neurotransmitters in the central nervous system. The inhibitory amino acid neurotransmitter, GABA, and its relationship with neurological and psychiatric disorders have attracted a great deal of interest. GABA has been suggested to be involved directly and/or indirectly in the pathogenesis of several neurological and psychiatric disorders, such as Huntington's chorea, tardive dyskinesia, epilepsy, schizophrenia, depression, anxiety, and some other behavioral disorders.<sup>6)</sup> Several neurological disorders such as Alzheimer's and Parkinson's diseases have also been attributed to GABA depletion in the brain.<sup>7)</sup> We previously reported that Mulberry leaves contain many antioxidative flavonoid compounds and have the radical scavenging effects.<sup>3)</sup> Recent Zhao showed that GABA and glycine are protective to mature rat cortical neurons but toxic to immature rat cortical neurons under hypoxia conditions.<sup>8)</sup> Therefore we hypothesized that if we increase the GABA contents in Mulberry leaves, it may synergically enhance the neuroprotective effects against ischemic damage. The accumulation method of GABA-enriched mulberry leaves and their neuroprotection effects on the brain is not elucidated yet. In order to provide a pharmacological basis for the neuroprotective actions

of the enhanced accumulation of GABA in Mulberry leaves (ML) against cerebral ischemia, a process was developed, which enhanced accumulation of GABA in ML (GAML) as a result of various anaerobic treatments.

The neuroprotection of ML and GAML were compared in the transient middle cerebral artery occlusion in a mouse brain model as well as in the PC12 cells under the oxidative conditions.

### MATERIALS AND METHODS

**Materials** The RPMI 1640, DMEM, horse serum (HS), fetal bovine serum (FBS), penicillin–streptomycin (PS), and nerve growth factor (NGF) were purchased from Gibco BRL (Gland Island, NY, U.S.A.). The gamma-aminobutyric acid (GABA), monosodium glutamate (MSG), 2,3,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-triphenyltetrazolium chloride (TTC), and 3-methyl-1-phenyl-2-pyrazolium-5-one (MCI-186) were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.).

**Plant Materials** The Mulberry leaves (*Morus alba*) were collected in Mangpori, Korea in May 2004 and were identified by Prof. Sun Yeou Kim. And a voucher specimen (No. 20040501) was at the herbarium of the Graduate School of East-West Medical Science, Kyunghee University.

**Preparation of ML and GAML under Anaerobic Treatment and Determination of GABA** Mulberry leaves were dried in the air and were sieved in a 60 mesh. The powders were extracted with 85% MeOH for 1 h at 85 °C. After filtration, the solution was evaporated *in vacuo*. The dried extract was used as ML samples. The Mulberry leaves were dried in the air and were sieved in a 60 mesh. The powders were mixed with 3 volume of 1% monosodium glutamate (MSG) solution. The mixture solution was adjusted to pH 5.0. And anaerobic treatment was processed with N<sub>2</sub> gas purging into the mixture solution in a vinyl bag. The bag was

\* To whom correspondence should be addressed. e-mail: sunnykim@khu.ac.kr

incubated for 3 h at 40 °C in a shaking incubator. The bag was then boiled for 5 min at 100 °C in water bath to denature the enzymes. The powders were isolated by filtration and dried at 40 °C in a drying oven. The dried powders were extracted with 85% MeOH for 1 h at 85 °C. After filtration, the solution was evaporated *in vacuo*. The dried extract was used as GAML samples.

The determination of GABA in the samples has been performed by precolumn derivatization with phenylisothiocyanate using reverse-phase HPLC as described elsewhere.<sup>9)</sup>

**PC12 Cell Culture** Rat PC12 pheochromocytoma cells obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The PC12 cell lines were grown in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum, 10% heat-inactivated horse serum and 1% penicillin/streptomycin at 37 °C in 5% CO<sub>2</sub> and 95% air in a humidified incubator. The cells were grown on culture dishes precoated with poly-D-lysine (50 µg/ml in sterile water) overnight.

**Neurite Outgrowth Measurement** The neurotrophic effects of GAML, ML and NGF were evaluated by microscopically monitoring their potency in inducing the neurite outgrowth of PC12 cells. The compounds (50 µg/ml) were added to the media 24 h after PC12 cells (3 × 10<sup>5</sup> cells/well) were seeded onto 6 well plates (Corning, New York, U.S.A.). The medium and each compound were changed every 2 d. After 1, 2, 4, and 6 d, the randomly selected fields of neurites were photographed using a camera attached to an Olympus optical inverted phase-contrast microscope (model CK-2; ×100 magnification). Neurite extension was evaluated with lengths equivalent to one diameter of the cell body by the 3 observers. The neurite length at each data point was determined by measuring more than 10 cells.

**Hydrogen Peroxide Induced Lactate Dehydrogenase Release Assay** The PC12 cells were pretreated with each 1, 10, and 50 µg/ml of GAML and ML for 90 min before being exposed to H<sub>2</sub>O<sub>2</sub> (50 µM). After 24 h incubation, the lactate dehydrogenase (LDH) levels in the culture supernatants were measured as previously described.<sup>10)</sup> The medium from the control cells was used as a blank, and a standard curve was constructed using the known amounts of LDH added to the culture medium.

**Oxygen Glucose Deprivation** The PC12 cells (2.5 × 10<sup>4</sup> cells/well) were seeded on 96 well plates in RPMI (complemented media) supplemented with 10% (v/v) fetal bovine serum, 5% heat-inactivated horse serum and 1% penicillin/streptomycin, and incubated for 24 h at 37 °C in 5% CO<sub>2</sub> and 95% air in a humidified incubator. In order to generate the hypoxic conditions, the cells were washed three times with deoxygenated glucose-free RPMI, immediately placed into an anaerobic chamber (O<sub>2</sub> tension < 0.2%) (Anaerobic system 1025, Forma Scientific, Marietta, OH, U.S.A.) and then incubated at 37 °C within the chamber for 2 h. In order to reoxygenate the hypoxic cultures, the cells were transferred into a regular normoxic incubator (5% CO<sub>2</sub> and 95% air) and incubation continued for 24 h. The normoxic control cells were washed with normal serum-free RPMI and incubated under normoxic condition for the corresponding periods. After 24 h incubation, indication of cell viability was measured by the MTT reduction method.<sup>11)</sup>

**Middle Cerebral Artery Occlusion (MCAO)** All the experimental procedures were performed according to the

Principles of Laboratory Animal Care (NIH publication, #85-23, revised 1985) and the Animal Care and Use Guidelines of Kyunghee University, Korea. Adult male ICR mice (Jung-Ang Lab Animal, Seoul, Korea) weighing 24–32 g were used for the study. The animals were anesthetized in a chamber with a mixture of N<sub>2</sub>O and O<sub>2</sub> (70 : 30) containing 2.5% isoflurane, and were maintained by the inhalation of 1.5% isoflurane. A middle cerebral artery occlusion (MCAO) was induced using the intraluminal filament method, as described elsewhere.<sup>12)</sup> After 90 min of MCAO, the mice were again anesthetized and the occluding filament was withdrawn gently back into the common carotid artery to allow reperfusion to take place. The rectal temperature was maintained at 37 ± 0.5 °C throughout surgery using a heating pad (Biomed S.L., Spain). The heart rate, arterial blood oxygen saturation of arterial hemoglobin and ECG were monitored throughout the procedure (SurgiVet, U.S.A.).

The mice subjected to MCAO were randomly assigned to receive either the drugs or the vehicle treatment. Each drug was dissolved in either saline or 10% Tween 80 and administered orally (200 mg/kg) 30 min after the MCAO. MCI-186 was used as the positive control. MCI-186 (5 mg/kg) dissolved in saline was injected intravenously at the same time schedule. The mice in the vehicle group underwent the same experimental protocol, except that they received the same volume/weight of the vehicle only. After reperfusion for 22.5 h, the animals were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and sacrificed and their brains were carefully removed. The area of the cerebral infarction was identified using TTC staining.<sup>13)</sup> TTC is a marker for the mitochondrial function and can reliably indicate the ischemic areas for up to 3 d after ischemia.<sup>14)</sup> The area of the infarction (mm<sup>2</sup>) in each section was determined using a computerized image analysis system (TDI Scope Eye 3.0).

**Statistical Analysis** Data were analyzed using the Sigma Plot software. All data were expressed as mean ± standard deviation except an *in vivo* experiment. Statistical comparisons between different treatments were performed by one-way ANOVA with Dunnett's multiple comparison post test. Differences with *p* value less than 0.05, 0.01 and 0.001 were considered statistically significant.

## RESULTS

In order to optimize the suitable packaging form under anaerobic processes, the vinyl bag, the vacuum and N<sub>2</sub> treatment bag were evaluated by the content of GABA in ML for 30 h. Figure 1A shows that after 3 h, the GABA contents increased in the all packaging forms under the air-blocked state. The GABA contents in the N<sub>2</sub> purged bag was 1.4 times higher than that in the vacuum package form and 2.8 times higher than that in the vinyl bag package form. The package in the N<sub>2</sub> treatment bag showed the largest increase in the GABA contents in GAML. The appropriate temperature in the anaerobic process was optimized according to the contents of GABA in GAML. Figure 1B shows that 3 h and 6 h under the anaerobic process, the GABA contents in GAML at 40 °C was about 35% higher than that of the other temperature. The appropriate size of the leaves in the anaerobic process was optimized by determining the GABA content in GAML. Figure 1C shows that there was no significant

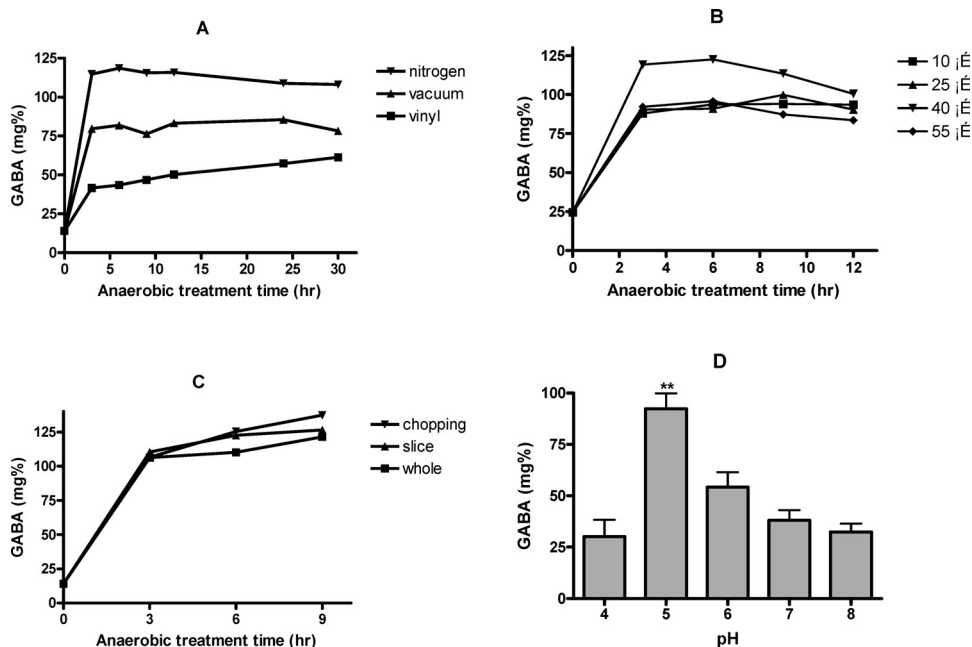


Fig. 1. GABA Contents by the Various Anaerobic Treatments

(A) The effect of packaging form on GABA content. Anaerobic treatment of the mixture of mulberry leaves powder and 1% MSG solution in pH 5 was performed at 40 °C for 0–30 h under the nitrogen; nitrogen gas purged in the vinyl package, vacuum; air vacuum in the vinyl package, and vinyl; in the vinyl package. (B) The effect of the incubation temperature on GABA content. Anaerobic treatment of the mixture of mulberry leaves powder and 1% MSG solution in pH 5 was performed for 0–12 h under the nitrogen gas purged in the vinyl package, at 10, 25, 40 and 55 °C respectively. (C) The effect of the leaf size on GABA content. Anaerobic treatment of the mixture of mulberry leaves and 1% MSG solution in pH 5 was performed at 40 °C for 0–9 h under the nitrogen gas purged in the vinyl package. chopping: chopped leaf size, slice: sliced leaf size, whole: whole leaf size. (D) The effect of pH on GABA content. Anaerobic treatment of the mixture of mulberry leaves powder and 1% MSG solution was performed for 3 h under the nitrogen gas purged in the vinyl package, at 40 °C. The values represent mean ± standard deviation of GABA contents (n=3) of three different experiments. \*\* Significantly different from the pH 4 group (p<0.01).

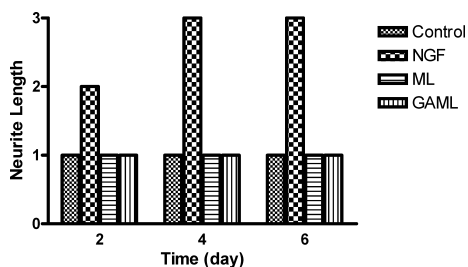


Fig. 2. Neurite Outgrowth in the PC12 Cells

Control group treated with vehicle; NGF group treated with nerve growth factor as positive control; ML group treated with Mulberry leaves 50 µg/ml; GAML group treated with GABA enriched Mulberry leaves 50 µg/ml. The value means 1; one time from diameter 2; two times from diameter 3; three times from diameter. The experiments was performed three different cultures (n=3).

change in GABA contents between chopping, slice, and whole leaves. The appropriate pH of mixture solution of Mulberry leaves and 1% MSG solution under the anaerobic process was optimized by the GABA contents. Figure 1D shows that pH 5 was the optimal pH for the enrichment of GABA.

The neurite outgrowth activities of the ML, GAML and nerve growth factor (positive control, NGF) were examined in the PC12 cells by measuring the length of neuritis (Fig. 2).

Both ML and GAML did not induce neurite outgrowth in the PC12 cells compared with the control group. NGF induced a neurite outgrowth in the PC12 cell from 2 to 6 d.

In order to understand the neuroprotective mechanism of ML and GAML, we investigated whether or not ML and GAML protect the PC12 cells from H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity. Both ML and GAML increased the cell viability

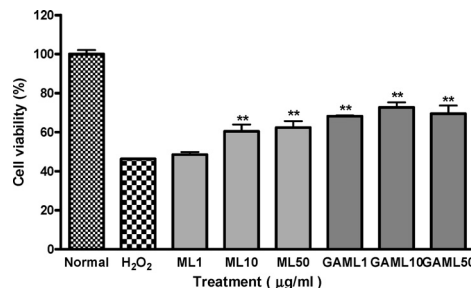


Fig. 3. Neuroprotection of GAML in PC12 Cells from H<sub>2</sub>O<sub>2</sub>-Induced Cytotoxicity

After pretreatment of PC12 cells with ML and GAML (1, 10 and 50 µg/ml) for 90 min, cells were exposed to hydrogen peroxide (150 µM) for 24 h, then cell viability were measured by LDH assay. The viability of hydrogen peroxide untreated cells as normal group was set to 100%. The values represent mean ± standard deviation of the percentage of the normal group (n=6) of three different cultures. \*\* Significantly different from the H<sub>2</sub>O<sub>2</sub> group (p<0.01).

from H<sub>2</sub>O<sub>2</sub>-induced injury at 10–50 µg/ml (Fig. 3). GAML showed the more effective neuroprotection than ML at concentration of 10 µg/ml.

PC12 cells were exposed to hypoxia for 2 h, and the cell viability was determined using a MTT assay. ML enhanced the cell viability 56% at 1 µg/ml, and enhanced the cell viability in dose dependent manner at higher concentrations. GAML also showed the neuroprotection at all concentrations, and the effect was particularly more effective than ML at 10–50 µg/ml (Fig. 4).

ML and GAML were further investigated to determine if they have neuroprotective effects against cerebral ischemia in an *in vivo* MCAO model. ML did not show a decrease the infarct volume of the brain than the control group against the

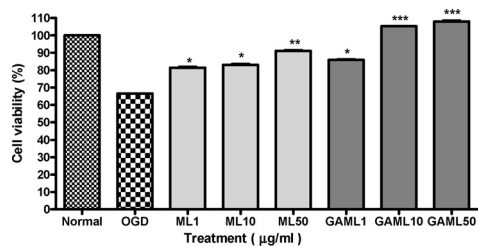


Fig. 4. Neuroprotection of GAML against Oxygen-Glucose Deprivation (OGD) Induced Cerebral Ischemia in PC12 Cells

Cell viability was measured by MTT assay. ML1, ML10, and ML50 are 1, 10, 50 μg/ml of ML. GAML1, 10, and 50 are 1, 10, 50 μg/ml of GAML. The OGD untreated group as normal group was set to 100%. The values represent mean ± S.D. of the percentage of the normal group ( $n=3$ ) of three different cultures. \* Significantly different from the OGD group ( $p<0.05$ ); \*\* Significantly different from the OGD group ( $p<0.01$ ); \*\*\* Significantly different from the OGD group ( $p<0.001$ ).

MCAO in mice. However, GAML 200 mg/kg *p.o.* group significantly decreased 31% infarct volume of the brain compared with control group ( $p<0.01$ ) (Fig. 5).

## DISCUSSION

This study provides the optimal enriching process of an anaerobic treatment for enhancing the accumulated GABA in ML. The GABA concentration of GAML was 9.8 times higher than that of ML. The optimal conditions are a  $N_2$  gas purged package form, a reaction temperature of 40 °C, a reaction time of 6 h, pH 5. The GABA is biosynthesized by glutamate decarboxylase (GAD) and is degraded by GABA transaminase (GABA-T).<sup>15</sup> Because the GABA concentration were increased with the glutamate concentration in a dose dependent manner (data not shown), it was assumed that the anaerobic treatment for enhancing the GABA concentration resulted from the potentiation of the GAD activity rather than by the inhibition of GABA-T. A further study will be needed to evaluate the GAD and GABA-T assay to determine the precise mechanism for the increasing GABA content. ML and GAML did not increase the neurite outgrowth activities in the PC12 cells. Therefore, it means that the neuroprotection of ML and GAML are not caused by the neurotrophic effects directly. Hydrogen peroxide is believed to be the major precursor for the highly reactive free radicals, and has been reported to induce apoptosis in the cells of the central nervous system.<sup>16</sup> Lactate dehydrogenase is an oxidoreductase, which catalyzes the conversion of lactate to pyruvate. In an effort to prevent or diminish the level of ROS-induced damage, this study evaluated the GAML that prevent the ROS generation and reduce neuronal damage. GAML had a higher antioxidant effect than ML at 10 μg/ml. The neuroprotection of ML and GAML resulted from the antioxidant activity against the  $H_2O_2$ -induced oxidative damage in PC12 cells. GABA can increase the activities of antioxidant enzyme under the hypoxia stress.<sup>17</sup> Neuronal ischemia is a pathological process that is caused by a lack of oxygen and glucose, resulting in neuronal death.<sup>18</sup> It is believed that apoptosis is one of the mechanisms involved in ischemic cell death.<sup>19</sup> These results showed that ML enhanced the cell viability 56% at 1 μg/ml, and enhanced the cell viability in a dose dependent manner at higher concentrations. Moreover, GAML showed better neuroprotective effects than ML at 10–50 μg/ml on OGD-induced cerebral ischemic cytotoxic-

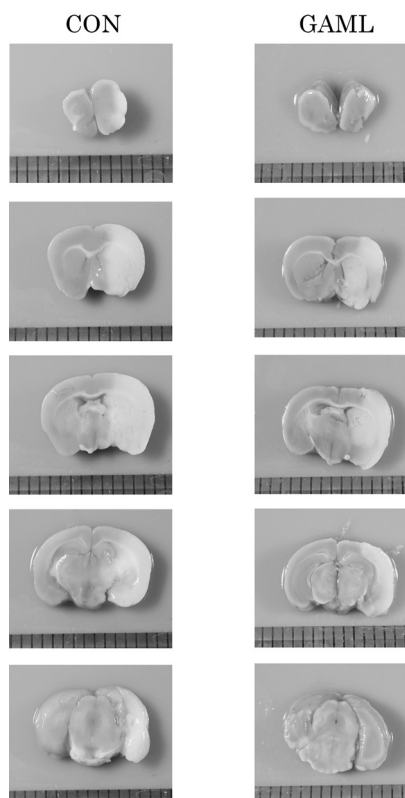
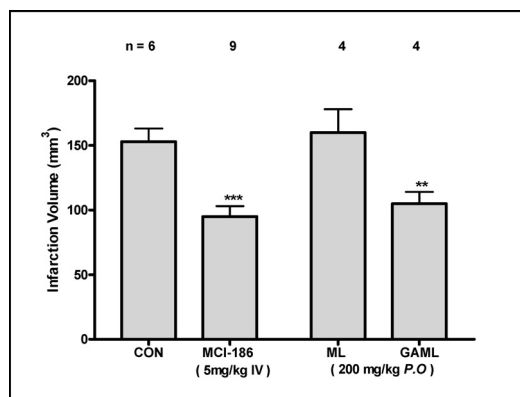


Fig. 5. Neuroprotection of GAML against Ischemic Damage in the Mouse Brain after the Transient Middle Cerebral Artery Occlusion (MCAO) CON

The control group treated with vehicle; the MCI-186 group as positive control treated with MCI-186 (5 mg/kg) by *i.v.*; sample group treated with ML and GAML 200 mg/kg by *p.o.* The data is represented as a mean ± standard error ( $n=4-9$ ). \*\* Significantly different from the control group ( $p<0.01$ ); \*\*\* Significantly different from the control group ( $p<0.001$ ).

ity in PC12 cells. GAML recovered the cell viability to a similar level as the normal cell group at each concentrations of 10 and 50 μg/ml. NO is a major toxin released from activated microglia and plays a deleterious role in brain inflammation and neuronal death.<sup>20</sup> The effects of ML and GAML on LPS-induced NO production in the BV2 microglial cells were examined. ML and GAML did not show a significant inhibition in NO production in LPS-induced BV2 cells (data not shown). The neuroprotective effect of GAML was not the result of the inhibition of NO production.

Experimental models of focal cerebral ischemia, which produce consistent lesions that are histologically similar to those observed in a human infarction, are widely used to

evaluate potential neuroprotective strategies.<sup>21)</sup> Although the neuroprotective effects of ML were elucidated in the *in vitro* experiments, ML did not decrease the infarct volume of the brain for neuroprotection in the *in vivo* mouse MCAO model. GAML significantly decreased the infarct volume of the brain compared with of the control group against transient mouse MCAO. Antagonists of excitatory neurotransmitters, such as glutamate, are effective in ischemic damage to the brain and spinal cord, but in clinical stroke limited by side effects. Agonists of inhibitory neurotransmitters, such as gamma-aminobutyric acid (GABA), may provide similar neuroprotection with less severe side effects.<sup>22)</sup> Madden have demonstrated that muscimol (a GABA-A agonist) or bicuculline (a GABA-A antagonist) was administered intravenously to groups of rabbits exposed to reversible spinal cord ischemia induced by temporary occlusion of the infra renal aorta.<sup>22)</sup> Johansen showed that enhancement of GABA neurotransmission after cerebral ischemia in the rat reduces loss of hippocampal CA1 pyramidal cells.<sup>23)</sup> And recently Zhao showed that GABA and glycine are protective to mature rat cortical neurons but toxic to immature rat cortical neurons under hypoxia conditions.<sup>8)</sup> Therefore overall investigations support that the increased GABA may enhance the neuroprotective effect against ischemic damage. These data demonstrate that the anaerobic treatment of ML may make GAML enhance the neuroprotection effect against cerebral ischemia such as *in vitro*.

In conclusion, our study is the first to demonstrate the process, which enhanced the accumulation of GABA in ML as a result of the anaerobic treatments and a comparison of the neuroprotective effects of ML and GAML *in vitro* and *in vivo* cerebral ischemia model.

**Acknowledgements** This research was support by grant of ARPC (GA 0502-0275), Ministry of Agricultural and Forestry.

## REFERENCES

- 1) Chen F, Nakashima N., Kimura I., Kimura M., *Yakugaku Zasshi*, **115**, 476—482 (1995).
- 2) Asano N., Tomioka E., Kizu H., Matsui K., *Carbohydr. Res.*, **253**, 235—245 (1994).
- 3) Kim S. Y., Gao J. J., Lee W. C., Ryu K. S., Lee K. R., Kim Y. C., *Arch. Pharm. Res.*, **22**, 81—85 (1999).
- 4) Lee S. H., Choi S. Y., Kim H., Hwang J. S., Lee B. G., Gao J. J., Kim S. Y., *Biol. Pharm. Bull.*, **25**, 1045—1048 (2002).
- 5) Yamashita T., *Tree Physiol.*, **6**, 191—200 (1990).
- 6) Sherif F. M., Ahmed S. S., *Clin. Biochem.*, **28**, 145—154 (1995).
- 7) Weiner M. F., Speciale S. G., Risser R. C., Kramer G. L., Petty F., *Biol. Psychiatry*, **40**, 933—934 (1996).
- 8) Zhao P., Qian H., Xia Y., *Eur. J. Neurosci*, **22**, 289—300 (2005).
- 9) Gunawan S., Walton N. Y., Treiman D. M., *J. Chromatogr.*, **503**, 177—187 (1990).
- 10) Koh J. Y., Choi D. W., *J. Neurosci. Methods*, **20**, 83—90 (1987).
- 11) Hansen M. B., Nielsen S. E., Berg K., *J. Immunol. Methods*, **119**, 203—210 (1989).
- 12) Kilic E., Bahr M., Hermann D. M., *Stroke*, **32**, 2641—2647 (2001).
- 13) Bederson J. B., Pitts L. H., Germano S. M., Nishimura M. C., Davis R. L., Bartkowski H. M., *Stroke*, **17**, 1304—1308 (1986).
- 14) Lin T. N., He Y. Y., Wu G., Khan M., Hsu C. Y., *Stroke*, **24**, 117—121 (1993).
- 15) Jayakumar A. R., Sujatha R., Paul V., Asokan C., Govindasamy S., Jayakumar R., *Brain Res.*, **837**, 229—235 (1999).
- 16) Chandra J., Samali A., Orrenius S., *Free Radic. Biol. Med.*, **29**, 323—333 (2000).
- 17) Gao H. B., Guo S. R., *Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao*, **30**, 651—659 (2004).
- 18) Siesjo B. K., *J. Neurosurg.*, **77**, 337—354 (1992).
- 19) Mattson M. P., Duan W., Pederson W. A., Culmsee C., *Apoptosis*, **6**, 69—81 (2001).
- 20) Park Y. J., Koketsu M., Kim J. M., Yeo J. H., Ishihara H., Lee K. G., Kim S. Y., Kim C. K., *Biol. Pharm. Bull.*, **26**, 1657—1660 (2003).
- 21) MacKenzie E. T., Duverger D., Gotti B., Nowiecki J. P., "Pharmacology of Cerebral Ischemia," Elsevier, Amsterdam, 1986, pp. 167—179.
- 22) Madden K., *Stroke*, **25**, 2271—2274 (1994).
- 23) Johanson F. F., Diemer N. H., *Acta Neurol. Scand.*, **84**, 1—6 (1991).