

## Full-length article

## Neuroprotective effects of roasted licorice, not raw form, on neuronal injury in gerbil hippocampus after transient forebrain ischemia<sup>1</sup>

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### Key words

transient ischemic attack; roasted licorice; raw licorice; neurons; PC12 cells; hippocampus; pyramidal cells; Gerbillinae; superoxide dismutase

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### Abstract

**Aim:** To observe neuroprotective effects of raw and roasted licorice against hypoxia and ischemic damage. **Methods:** When elucidating the protective effects of raw and roasted licorice, we analyzed the lactate dehydrogenase (LDH) release using PC12 cells after hypoxia in an *in vitro* study and after transient forebrain ischemia in an *in vivo* study on Mongolian gerbils. **Results:** Raw and roasted licorice significantly reduced LDH release from PC12 cells exposed to an hypoxic chamber for 1 h. In the roasted licorice-treated group, the decrease of LDH release was more pronounced compared to that of the raw licorice-treated group. In roasted licorice-treated animals, approximately 66%–71% of CA1 pyramidal cells in the ischemic hippocampus were stained with cresyl violet compared to the control group. However, in the raw licorice-treated animals, no significant neuroprotection against ischemic damage was shown. In addition, ischemic animals in roasted licorice-treated group maintained the Cu, Zn-superoxide dismutase (SOD1) activity and protein levels compared to the control group, while in raw licorice-treated group SOD1 activity and protein levels were reduced significantly. High pressure liquid chromatography analysis showed that non-polar compounds containing glycyrrhizin-degraded products, such as glycyrrhetic acid (GA) and glycyrrhetic acid monoglucuronide (GM), were increased in roasted licorice. **Conclusion:** Roasted licorice had neuroprotective effects against ischemic damage by maintaining the SOD1 levels. In addition, the difference in protective ability between raw and roasted licorice may be associated with non-polar compounds, such as GA and GM.

### Introduction

Several studies have reported that plant extracts have protective effects against ischemic damage in several organs such as the brain, heart and kidneys<sup>[1–3]</sup>. Licorice or *Glycyrrhiza inflata*, which is a commonly used herb, has a history of consumption for a few thousand years in both Eastern and Western cultures. It has been reported that major bioactive components of licorice are saponins, such

as glycyrrhizin and glycyrrhetic acids. In previous studies, it has been reported that glycyrrhizin has various desirable pharmacological properties such as anti-inflammatory effects<sup>[4]</sup>, anti-viral effects<sup>[5]</sup> and free radical scavenging activity<sup>[6]</sup>. In recent studies it has been shown that 18- $\beta$  glycyrrhetic acid, a major component of licorice, reduces infarct size in isolated rabbit hearts<sup>[7]</sup>, and that glycyrrhizin shows a protective effect on ischemia/reperfusion or nephrotoxic injuries<sup>[8,9]</sup>. However, in some studies it was found that licorice

interfered with steroid metabolism and might cause edema and hypertension<sup>[10]</sup>.

The roasting process of licorice modifies the chemical composition and reduces the toxicity level<sup>[11]</sup>. Roasting of licorice under controlled conditions, including the roasting temperature and duration, results in the conversion of glycyrrhizin to 18- $\beta$  glycyrrhetic acid<sup>[12]</sup>. Despite the considerable number of physiological studies that have been done on licorice, a comparative study between licorice and roasted licorice during *in vivo* ischemia has not been carried out. In the present study, therefore, we investigated the neuroprotective effects of licorice and roasted licorice on neuronal injury in the gerbil hippocampus induced by transient ischemia.

## Materials and methods

**Reagents** Dried licorice roots were obtained from Dea Guang Medical (Chunchon, South Korea). Acetic acid was obtained from Merk (Ger, Germany). Glycyrrhetic acid (GA), glycyrrhetic acid monoglucuronide (GM) and glycyrrhizin (GL) were obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile was of HPLC grade, and all other chemicals were of analytical grade.

**Roasting of licorice and GL** Roasting of licorice was performed in a controlled manner using an oil bath. For dry-roasting, 20 g of sliced licorice was weighed into a rotary glass bottle (30 r/min) and placed in the heated oil bath at 150 °C for 100 min. To determine whether roasting of licorice caused the thermal decomposition of GL, the decomposition of GL in powder form was also studied by heating. For roasting, 10 mg of powder was weighed into a glass vial and placed in the heated oil bath at 150 °C for 30 min.

**Preparation of samples of licorice and GL before and after roasting** The GL samples (raw and roasted) were dissolved in methanol and the solutions were membrane-filtered (0.45  $\mu$ m). After dilution with methanol, the final concentrations of the prepared raw and roasted GL solutions for HPLC analysis were 1 and 5 mg/mL, respectively. Roasted licorice roots were first ground into fine powder using a laboratory blender (Waring Model 51BL30). The licorice powder (10 g) was refluxed in 50 mL of 95% ethanol for 2 h. Boiling stones were used during reflux to minimize bumping and sample loss. This extraction procedure was repeated three times. For comparison, 10 g of unprocessed licorice (raw licorice) was refluxed similarly. The ethanol extract was dried under vacuum at a low temperature (below 40 °C). The final concentrations of the prepared licorice solutions for HPLC analysis were 5 mg/mL.

**HPLC analysis** HPLC system consisted of two models of P-580 pumps, a model of ASI-100 Automated sample injector, a model of STH-585 column oven, and a model of UVD 170S UV-detector (Dionex, USA). The sample was separated on a reverse-phase Phenomenex Luna C<sub>18</sub> column (4.6 mm $\times$ 250 mm ID; 5  $\mu$ m). The mobile phases consisted of 2% acetic acid and acetonitril as mobile phase A and B, respectively, which were degassed ultrasonically prior to use. The gradient was as follows: an isocratic elution with 20% acetonitril for 5 min, followed by a linear gradient elution with 20%–90% acetonitril for 50 min. The overall analysis time including re-equilibration was 65 min. The column was thermostated at 28 °C and a flow-rate of 0.8 mL/min was used. UV detection was operated at 254 nm.

**Culture of PC12 cells** PC12 cells were grown in Dulbecco's modified Eagle's medium.

(DMEM) supplemented with 7% fetal calf serum, 7% horse serum, 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin. The cell cultures were incubated at 37 °C in an atmosphere of 6% CO<sub>2</sub>. Medium was changed twice weekly, and the cultures were split at a 1:6 ratio once a week. Cells were washed twice with warm DMEM (without phenol red), then treated with serum-free medium. In all experiments, cells were treated with licorice before hypoxia.

**Hypoxia** On the day of experiment, culture media were replaced with glucose-free DMEM, then gassed with 85% N<sub>2</sub>, 10 % H<sub>2</sub>, and 5 % CO<sub>2</sub> for various periods in the absence or presence of various doses of licorice extract (raw and roasted).

**Lactate dehydrogenase (LDH) release assay** After hypoxia for 1 or 2 h, the supernatant of cultured PC12 cells was collected for assay of LDH release. The reaction was initiated by mixing 0.1 mL of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 0.2 mL in a 96-well plate. The rate of absorbance was read at 490/630 nm on an automated SpectraMAX 340 microtiter plate reader. Data were expressed as the mean percent of viable cells versus hypoxia control.

**Experimental animals** The progeny of male Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were housed in a temperature (23 °C) and humidity (60%) controlled room with a 12-h light/12-h dark cycle and provided with food and water *ad libitum*. Experimental procedures and animal care conformed to the Institutional Guidelines that are in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No 85–23, 1985,

revised 1996) and are approved by the Hallym's Medical Center Institutional Animal Care and Use Committee. All of the experiments were conducted to minimize the number of animals used and the amount of suffering.

**Induction of ischemia** The gerbils were divided into 4 groups: normal group, vehicle (saline)-treated group, raw licorice-treated group and roasted licorice-treated group. At least 21 d before surgery, 50 and 100 mg/kg of the extract of raw or roasted licorice were injected orally using the jonde every day until gerbils were killed. Gerbils weighing 65–75 g were anesthetized with a mixture of 2.5% isoflurane (Baxter, USA) in 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck. Both common carotid arteries were isolated, freed of nerve fibers, and occluded with non-traumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in the eyeball using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. Restoration of blood flow (reperfusion) was observed under the ophthalmoscope. We maintained the body (rectal) temperature under free-regulating or normothermic ( $37\pm 0.5$  °C) conditions with a rectal temperature probe (TR-100; YSI, USA) and thermometric blanket before, during, and after the surgery until the animals fully recovered from anesthesia. Normal animals served as controls.

**Histological analysis by cresyl violet staining** Seven animals in each group were anesthetized with pentobarbital sodium and perfused transcardially with 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) at the designated times after the surgery. Brains were removed and post-fixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose solution overnight. Thereafter the tissues were frozen and serially cut into 30- $\mu$ m thick coronal sections on a cryostat and the sections were collected in 6-well plates containing PBS. The sections were stained with cresyl violet acetate according to the previously published procedures<sup>[13]</sup>.

The number of cresyl violet-positive neurons was counted by two blinded observers at the same time using an image analyzing system equipped with a computer-based CCD camera (Software: Optimas 6.5, CyberMetrics, USA). The number of cresyl violet-positive neurons in a 1-mm diameter of the hippocampus was counted in 10 sections for each animal. The number of cresyl violet-positive neurons was compared to that of the sham-operated group.

**Assay of SOD1 activity** Seven animals in each group were used for SOD1 activity measurement. The activity was measured by monitoring the capacity to inhibit the reduc-

tion of ferricytochrome c by xanthine/xanthine oxidase as described by McCord and Fridovich<sup>[14]</sup>. The samples were separated by electrophoresis in 10% native polyacrylamide gels and visualized as described by Beauchamp and Fridovich<sup>[15]</sup>. Briefly, the gel was soaked in 2.45 mmol/L nitroblue tetrazolium solution for 15 min, and then in 28 mmol/L *N,N,N',N'*-tetramethylethylene diamine, 28  $\mu$ mol/L riboflavin, and 0.36 mmol/L potassium phosphate buffer (pH 7.8) for 30 min. The gel was then exposed to a fluorescence light source until the bands showed maximum resolution.

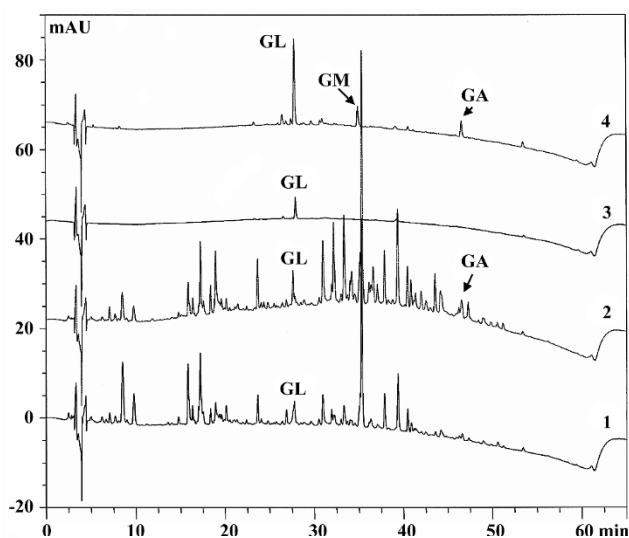
**Western blotting of SOD1 protein** Seven animals in each group (the same animals as in the analysis of SOD1 activity) were used for an immunoblotting study. The hippocampus were removed and sectioned into 400- $\mu$ m thick coronal slices on a Vibratome (Leica, Germany), and the hippocampal CA1 region was dissected with a surgical blade. The tissues were homogenized using an electrical homogenization machine in 50 mmol/L PBS (pH 7.4) containing 0.1 mmol/L egtazic acid (pH 8.0), 0.2% NP-40, 10 mmol/L edetic acid (pH 8.0), 15 mmol/L sodium pyrophosphate, 100 mmol/L  $\beta$ -glycerophosphate, 50 mmol/L NaF, 150 mmol/L NaCl, 2 mmol/L sodium orthovanadate, 1 mmol/L PMSF, and 1 mmol/L DTT. After centrifugation at 10 000 $\times$ g, the protein concentration was determined in the supernatants by using the Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, USA). Aliquots containing 20  $\mu$ g total protein were boiled in loading buffer containing 150 mmol/L Tris (pH 6.8), 3 mmol/L DTT, 6% SDS, 0.3% bromophenol blue, and 30% glycerol. Each aliquot was then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell, USA). To reduce background staining, the filters were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rat anti-mouse SOD1 antiserum (1:400) with peroxidase conjugated horse anti-mouse IgG (Sigma, USA), and then with ECL kit (Amersham, USA).

For quantitative analysis of the Western band of SOD1 in the hippocampus, video images were digitized into an array of 512 pixels  $\times$  512 pixels. Each pixel resolution was of 256 gray levels. The intensity of western band of SOD1 was expressed as a relative optical density (ROD) value which was transformed from mean gray values using the formula:  $ROD = \lg(256/\text{mean gray})$ . A background parameter was obtained from each section out of the immunolabeled structures and subtracted from obtained ROD values of each group. ROD values are informed as ROD units. The bands of Western blot study were scanned and a ROD value was obtained using Scion Image software (Scion Corp, USA).

**Statistical analysis** Inter-animal differences in each group, as well as inter-experiment differences, were not statistically significant. Values shown represent the mean of experiments performed for each hippocampal area. All data obtained from the quantitative data are expressed as mean $\pm$ SD and analyzed using one-way ANOVA to determine statistical significance. Bonferroni's test was used for *post-hoc* comparisons.  $P < 0.05$  or  $0.01$  was considered statistically significant.

## Results

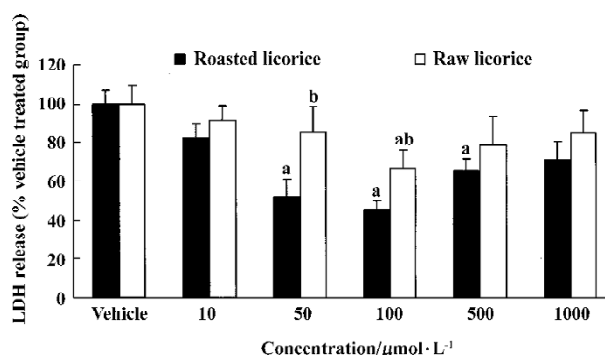
**Effects of roasting on licorice and GL** The major peaks in licorice extracts were completely separated by HPLC analysis. The comparative non-polar components which were eluted after 30 min were significantly increased. The untreated GL showed only the GL peak. After being roasted at 150 °C for 30 min, two new peaks appeared (Figure 1). Upon spiking with standard solution (GA and GM), the new peak was identified to have resulted from GA and GM (data not shown). The appearance of GM and GA was also confirmed by the UV contour plot obtained by the PDA detector (data not shown). The fact that the formation of GM and GA was attributed to thermal decomposition of GL was the same as previous reports<sup>[12,15]</sup>. It was reported that sugar chains in the saponin and glycosidic flavonoid constituents in lico-



**Figure 1.** HPLC diagram for chemical constituents of licorice (*G inflata*) extracts. (1) Ethanol extract of licorice root; (2) Ethanol extract of roasted licorice root; (3) Glycyrrhizin (GL), (4) Roasted GL. GM, glycyrrhetic acid monoglucuronide; GA, glycyrrhetic acid. Column: Phenomenex Luna 5  $\mu$ m C18 (250 mm $\times$ 4.6 mm); Solvents: 2% acetic acid, 20% acetonitrile at 0–5 min and 20%–90% acetonitrile at 5–55 min. Detection wavelength: 254 nm.

rice were hydrolyzed step-by-step during roasting through hydrothermolysis<sup>[16]</sup>.

**LDH release inhibition by raw and roasted licorice** Raw and roasted licorice effectively protected PC12 cells from hypoxic damage. In the raw licorice-treated group, LDH release was decreased by 9%–33% at concentrations of 10–1000  $\mu$ mol/L, while in the roasted licorice-treated group, LDH release was decreased by 17%–49% at concentrations of 10–1000  $\mu$ mol/L (Figure 2).



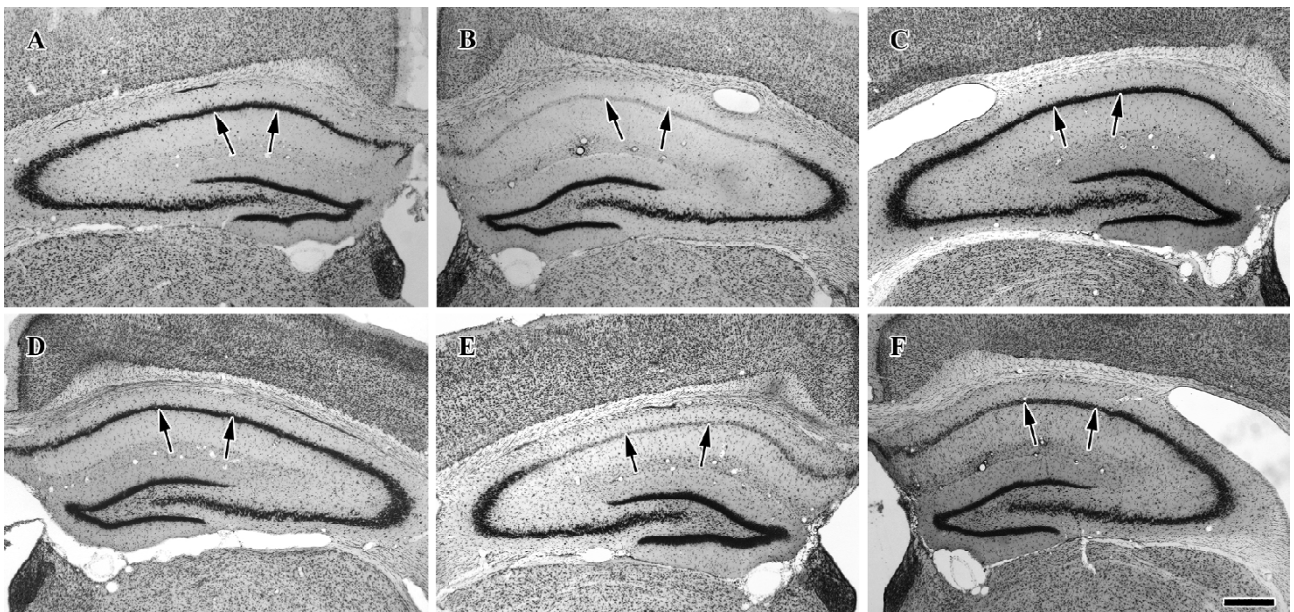
**Figure 2.** Effects of the raw and roasted licorice on LDH release in PC12 cells after hypoxia.  $n=7$ . Mean $\pm$ SD. <sup>b</sup> $P < 0.05$  vs vehicle. <sup>a</sup> $P < 0.05$  vs roasted licorice treated group.

### Protective effects of raw and roasted licorice on ischemic pyramidal cells

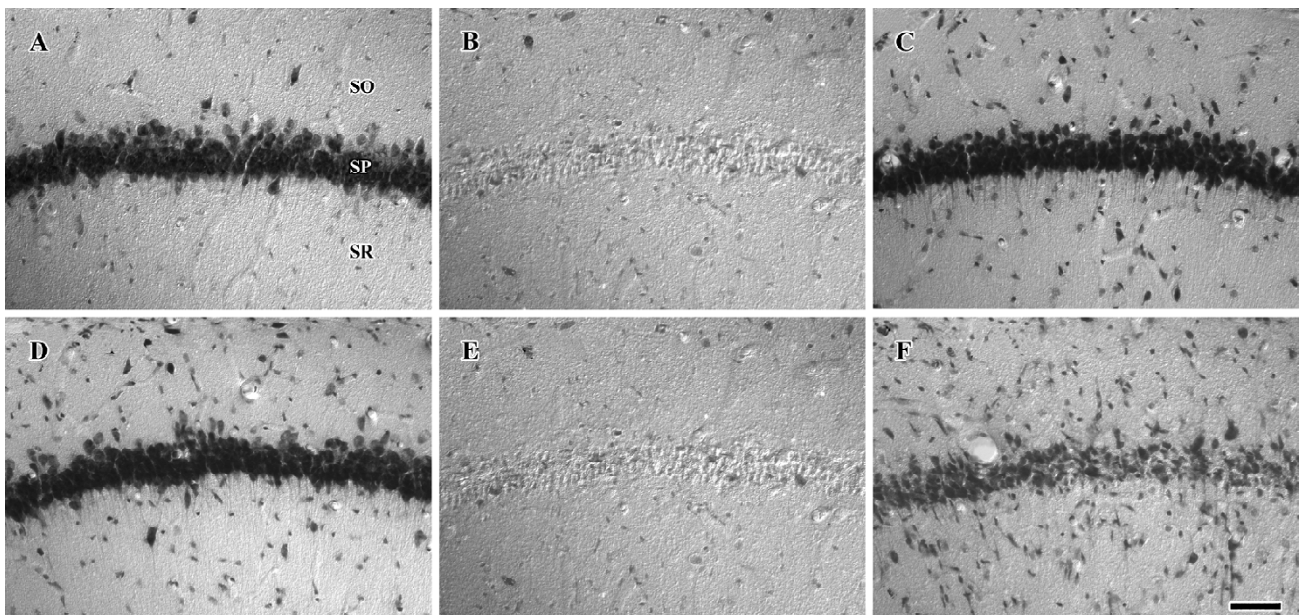
In the vehicle-treated group, the percentage of cresyl violet-positive pyramidal cells in the CA1 region was 11.5% compared with the control group at d 4 after ischemia/reperfusion (Figures 3B, 4B, 5). In 50 mg/kg raw licorice-treated group, the number of cresyl violet-positive neurons showed no difference to that of the vehicle-treated group ( $P > 0.05$ ; Figures 3E, 4E, 5). In 100 mg/kg raw licorice-treated group, the number of cresyl violet-positive neurons was slightly increased ( $P < 0.05$ ; Figures 3F, 4F, 5).

In contrast, in the roasted licorice-treated group, abundant CA1 pyramidal cells were detected in the hippocampal CA1 region after ischemia/reperfusion. In the 50 mg/kg roasted licorice-treated group, approximately 71.4% of CA1 pyramidal cells were stained with cresyl violet (Figures 3C, 4C, 5). In the 100 mg/kg roasted licorice-treated group, approximately 66.4% cresyl violet-positive neurons were detected in the striatum pyramidal of the CA1 region (Figures 3D, 4D, 5).

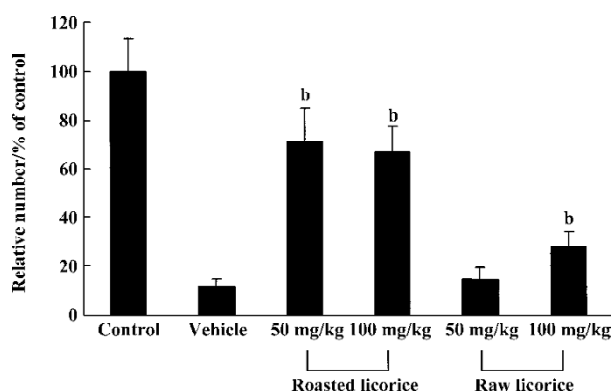
**Changes in SOD1 activity** In the vehicle-treated group, SOD1 activity was significantly decreased compared with the control group. The SOD1 activity was slightly increased in the raw licorice-treated groups and significantly increased in the roasted licorice-treated groups compared with that in



**Figure 3.** Low magnification of the hippocampus stained with cresyl violet in control (A), vehicle-treated (B), 50 mg/kg (C) and 100 mg/kg (D) of roasted licorice-treated, 50 mg/kg (E) and 100 mg/kg (F) of raw licorice-treated group 4 d after ischemic insult. In the vehicle-treated, 50 mg/kg and 100 mg/kg of raw licorice-treated group, the striatum pyramidale (arrows) of the hippocampal CA1 region was poorly stained with cresyl violet. In the 50 mg/kg (C) and 100 mg/kg (D) of roasted licorice-treated group, the striatum pyramidale (arrows) of the hippocampal CA1 region was well stained with cresyl violet. Bar=800  $\mu$ m.

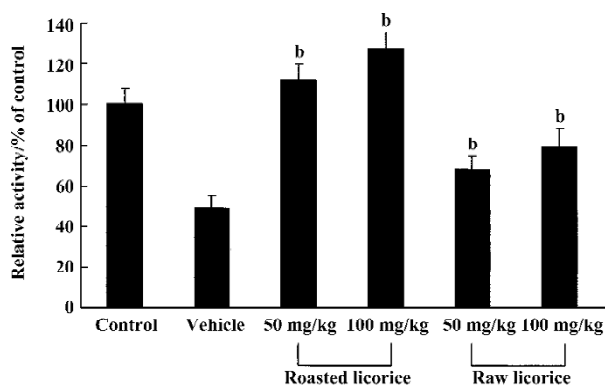


**Figure 4.** High magnification of the hippocampal CA1 region stained with cresyl violet in control (A), vehicle-treated (B), 50 mg/kg (C) and 100 mg/kg (D) of roasted licorice-treated, 50 mg/kg (E) and 100 mg/kg (F) of raw licorice-treated group 4 d after ischemic insult. In the vehicle-treated, 50 mg/kg and 100 mg/kg of raw licorice-treated group, a few pyramidal cells were stained with cresyl violet in the striatum pyramidale of the hippocampal CA1 region. In the 50 mg/kg and 100 mg/kg of roasted licorice-treated group, many pyramidal cells were stained with cresyl violet in the striatum pyramidale of the hippocampal CA1 region. Bar=50  $\mu$ m.



**Figure 5.** Relative number of cresyl violet-positive CA1 pyramidal cells in vehicle-treated, 50 mg/kg and 100 mg/kg of raw licorice-treated, 50 mg/kg and 100 mg/kg of roasted licorice-treated group.  $n=7$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs vehicle.

vehicle-treated group (Figure 6).

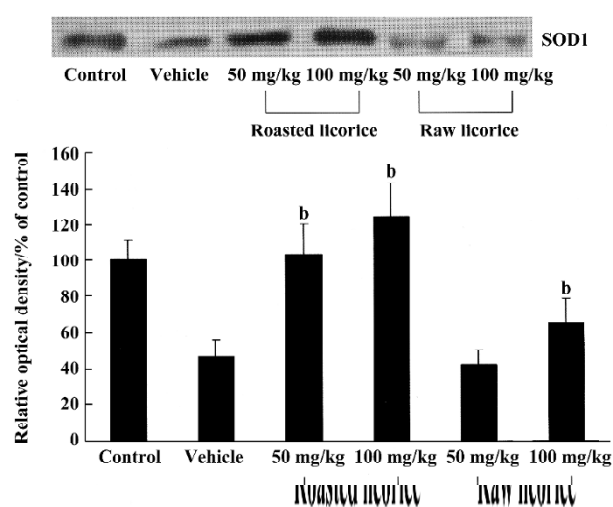


**Figure 6.** Analysis of specific SOD1 activity in the hippocampus in control, vehicle-treated, 50 mg/kg and 100 mg/kg of raw licorice-treated, 50 mg/kg and 100 mg/kg of roasted licorice-treated group after ischemia/reperfusion.  $n=7$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs vehicle.

**Changes in SOD1 protein contents** In the vehicle-treated and raw licorice-treated groups, SOD1 protein contents were significantly decreased compared with the control group ( $P<0.05$ ). But in the roasted licorice-treated groups, SOD1 protein contents were not significantly altered compared with the control group ( $P>0.05$ ; Figure 7).

## Discussion

In the present study, we examined the effects of raw or roasted licorice on LDH release in normoxic and hypoxic PC12 cells because the release of LDH implied neuronal damage. We found that roasted licorice at 50–500  $\mu\text{mol/L}$  significantly reduced LDH release in hypoxic PC12 cells. A



**Figure 7.** Western blot analysis of SOD1 in the hippocampus derived from control, vehicle-treated, 50 mg/kg and 100 mg/kg of raw licorice-treated, 50 mg/kg and 100 mg/kg of roasted licorice-treated group after ischemia/reperfusion.  $n=7$ . Mean $\pm$ SD. <sup>b</sup> $P<0.05$  vs vehicle.

weak, but statistically significant protection was also observed in hypoxic PC12 cells treated with 100  $\mu\text{mol/L}$  of raw licorice. In addition, 100–1000  $\mu\text{mol/L}$  of raw or roasted licorice caused cytotoxic effects in normoxic PC12 cells. Therefore, the optimal concentration of neuroprotection for raw and roasted licorice was 50–100  $\mu\text{mol/L}$ . In addition, we found that 50 mg/kg or 100 mg/kg of raw or roasted licorice, which is the commonly used dose in herbal medicine, exerted neuroprotective effects in an *in vivo* ischemic model.

We found that roasted licorice had a neuroprotective effect against transient forebrain ischemia. However, raw licorice had no significant effect against ischemic damage. In the process of roasting licorice, almost all polar components were not altered, while the non-polar components were significantly increased. In previously published studies, it has been reported that licorice root has an effect on the anti-apoptotic protein Bcl-2, which is a 26-kDa protein that blocks cell death by inhibiting cytochrome c release from mitochondria, a critical event in the apoptotic pathway<sup>[17, 18]</sup>. In our study, SOD1 activity and protein content in the roasted licorice-treated group were significantly increased compared to those in the vehicle-treated group. This result suggests that roasted licorice has neuroprotective effects through antioxidant activity.

The biopharmaceutical properties of roasted licorice have been previously examined to clarify the influence of roasting on the bioavailability of glycyrrhizin after oral administration of the extract. Among polar components of licorice,

glycyrrhizin is a major component. It is taken orally and is transformed (hydrolysed) by intestinal bacteria into the active metabolite glycyrrhetic acid, non-polar component<sup>[19]</sup>. This glycyrrhetic acid is widely used as a gap junction inhibitor that is effective in the micromolar concentration range. Glycyrrhetic acid seems to act through changes in phosphorylation and/or connexin assembly and inhibition of sarcolemmal Ca<sup>2+</sup> currents and of mRNA synthesis. In our study, we also observed that the increased non-polar compounds found in roasted licorice contained glycyrrhizin-degraded products such as glycyrrhetic acid and glycyrrhetic acid monoglucuronide.

We observed in the present study that 18-β glycyrrhetic acid strongly protected hippocampal CA1 pyramidal neurons from ischemic damage. This result is supported by a previous report that carbenoxolone, the succinyl ester of 18-β glycyrrhetic acid has protective effects against ischemic damage in middle cerebral artery occlusion models<sup>[20]</sup>.

In conclusion, roasted licorice has a significant neuroprotective effect against ischemic damage through antioxidant effects. In addition, the neuroprotective effect of roasted licorice is associated with non-polar compounds including glycyrrhetic acid monoglucuronide and its degradation product, glycyrrhetic acid.

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