Short Communication

Protective Effect of Methanol Extract of *Uncaria rhynchophylla* Against Excitotoxicity Induced by N-Methyl-D-Aspartate in Rat Hippocampus

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Abstract. *Uncaria rhynchophylla* is a medicinal herb used for convulsive disorders in Oriental medicine. In this study, the effect of the methanol extract of *Uncaria rhynchophylla* against N-methyl-D-aspartate (NMDA)-induced excitotoxicity was investigated. Pretreatment with the extract of *Uncaria rhynchophylla* reduced the degree of neuronal damage induced by NMDA exposure in cultured hippocampal slices. In the patch clamp study, *Uncaria rhynchophylla* significantly inhibited NMDA receptor-activated ion current in acutely dissociated hippocampal CA1 neurons. These results indicate that *Uncaria rhynchophylla* offers protection against NMDA-induced neuronal injury and inhibitory action on NMDA receptor-mediated ion current may be a mechanism behind the neuroprotective effect of *Uncaria rhynchophylla*.

Keywords: *Uncaria rhynchophylla*, N-methyl-D-aspartate, hippocampus

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS). Its action on cells is mediated by two classes of glutamate receptors: the ionotropic glutamate receptors and the metabotropic glutamate receptors. Ionotropic glutamate receptors are further classified according to their preferred agonists as N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate receptors. The ionotropic glutamate receptors mediate not only normal intercellular communications but also neuronal injury and death (1). Exposure to agonists of glutamate receptors is known to lead to increased neuronal death in most cases, whereas antagonists of glutamate receptors appear to offer neuroprotection (2, 3).

In ischemic attacks, depletion of oxygen and glucose bring about rapid losses in ATP and consequently depolarization of the membrane, leading to increased synaptic release of glutamate (4). Increased extracellular concentration of glutamate overstimulates NMDA receptors, resulting in increased Ca²⁺ influx, which in turn disables mitochondrial functions (5), rapidly increases the concentration of cytoplasmic reactive oxygen species (ROS) (6), and ultimately causes neuronal cell death. Because NMDA receptors play a crucial role in glutamate-induced acute neuronal damage, NMDA receptor antagonists are thought to reduce neuronal cell death during and following ischemic attacks (7).

*Uncaria rhynchophylla* (Uncariaceae Ramulus cum Uncis) has been used for suppression of liver hyperfunction, relief of dizziness, and treatment of tremors and convulsions in Oriental medicine. It is known to have sedative and anticonvulsive effects and has thus been applied in the treatment of epilepsy (8). The active components of *Uncaria rhynchophylla* are mainly alkaloids: rhynchophylline, isorhynchophylline, corynoxeine, hirsutine, and hirsuteine. Hirsutine is reported to produce concentration-dependent relaxation of isolated rat aorta stimulated by norepinephrine or high potassium solution through inhibition of voltage-
dependent Ca²⁺ channels (9).

Glutamate plays an important role in various physiological functions including learning and memory formation, development, and synaptic plasticity. In contrast, abnormal high exposure to glutamate induces neurotoxicity, known as excitotoxicity. In the present study, the protective effect of the methanol extract of Uncaria rhynchophylla against excitotoxicity induced by NMDA and its effects on the ion currents induced by glutamate and other agonists of glutamate subtypes receptors were investigated.

The stems and roots of Uncaria rhynchophylla (Miq.) Jack (Uncariaceae Ramulus cum Uncis) were purchased from the Bowhadong Oriental drug store (Seoul, Korea). The ground plant material (5.0 kg) was extracted with 100% methanol three times at room temperature for 7 days. The methanol filtrate was evaporated in vacuo to give the methanol extract. The extract was concentrated under reduced pressure to yield a thick dark brown residue (820 g). The freeze-dried extract of Uncaria rhynchophylla was dissolved in saline solution at 400 mg/ml.

In the first part of the experiment, the protective effect of Uncaria rhynchophylla was investigated using hippocampal slice cultures. Hippocampal slice cultures were made according to the methods of Noraberg et al. (10). Sprague-Dawley rats (9-day-old) were decapitated, the brains were removed, and transverse dorsal hippocampal slices (400-µm-thick) were made using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). The hippocampal slices were then placed on porous insert membranes (0.4 µm) (Millipore Corp, Bedford, MA, USA). The inserts were transferred to 6-well plates, each well containing 1.2 ml culture medium composed of 50% Mg²⁺-free modified Eagle Medium (Gibco-BRL, Renfrewshire, UK), 25% horse serum, and 25% Hank’s Balanced Salt Solution (Gibco-BRL) supplemented by 25 mM of d-glucose. The medium was changed every 3 days, and the experiment was commenced 14 days after formulation of the culture. The cultures were divided into 6 groups: the control group, the NMDA-treated group, the NMDA-and 1 µg/ml Uncaria rhynchophylla-treated group, the NMDA- and 10 µg/ml Uncaria rhynchophylla-treated group, the NMDA- and 100 µg/ml Uncaria rhynchophylla-treated group, and the NMDA-and MK-801-treated group. The concentrations of NMDA and MK 801, a NMDA receptor antagonist, used in this study were based on the study of Kristensen et al. (11); EC₅₀ values of NMDA following 2 days exposure in a hippocampus slice was 10 µM, and 3 µM of MK-801 protected completely against NMDA-induced damage. Cell death was assessed by propidium iodide (PI) staining, and PI fluorescence was measured at 514 nm under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Goettingen, Germany). The digital photographs were analyzed densitometrically using the NIH Image 1.62 analysis program (National Institute of Health, Bethesda, MD, USA).

In the second part of the experiment, the effect of Uncaria rhynchophylla on the ion currents induced by glutamate and other agonists of glutamate subtypes receptors were investigated via patch clamp study. The rat hippocampal CA1 neurons were dissociated as previously described (12). Sprague-Dawley rats of 10 to 15 days of age were decapitated under Zoletil 50™ (Vibac Laboratories, Carros, France)-induced anesthesia (10 mg/kg, i.m.). The brains were removed, and transverse slices (400-µm-thick) were made using a microslicer (DTK-1000; DSK, Tokyo). The slices were then preincubated in incubation solution saturated with 95% O₂ and 5% CO₂ for 30 min. Then the slices were treated with 1 mg/6 ml of pronase (protease XIV; Sigma Chemical Co., St. Louis, MO, USA) for 40 – 80 min at 32°C and subsequently with thermolysin (proteinase X, Sigma Chemical Co.) for 10 – 20 min under the same conditions. The CA1 region of a slice in a 60-mm culture dish coated with silicone was identified through a binocular microscope (SZ-ST; Olympus, Tokyo) and was micropunched out from the slices with an electrolytically polished injection needle. The micropunched CA1 portions were mechanically dissociated with fire-polished fine glass Pasteur pipettes in 35-mm plastic culture dishes filled with standard solution. The dissociation procedure was carried out under an inverted phase-contrast microscope (CK-2, Olympus). For the study of NMDA-activated current, Mg²⁺-free standard solution with 10⁻⁴ M glycine was used. Electrical recordings were performed in the nystain-perforated patch recording mode with the voltage clamped at −50 mV. Patch pipettes were prepared from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PB-7; Narishige, Tokyo). The resistance between the recording electrode filled with internal pipette solution and the reference electrode was 6 – 8 MΩ. The series resistance ranged from 16 to 25 MΩ. Electrical stimulation, current recordings, and filtration of currents (at 2.9 kHz) were obtained using an EPC-7 patch-clamp amplifier (List-Electronic, Darmstadt/Eberstadt, Germany). The current and voltage values were monitored on a pen recorder (Recti-Horiz-8K; NEC San-ei, Tokyo).

Results were presented as the mean ± S.E.M. Statistical analysis was made by one-way ANOVA and Dunnett’s multiple comparisons, and differences were considered significantly for P<0.05.
Neuronal damage induced by NMDA was visualized by PI staining, which is preferentially taken up into non-viable cells. In the control group, weak PI staining was observed. After 48 h of exposure to NMDA, the level of PI uptake was markedly increased, and most of the cells in the pyramidal layer of the hippocampal CA1 were stained with PI. In the cultures treated with extract of *Uncaria rhynchophylla* for 48 h, PI uptake was significantly reduced. PI uptake in the groups treated with *Uncaria rhynchophylla* at the concentrations of 1, 10, and 100 μg/ml was 68.5 ± 2.6%, 43.9 ± 5.6%, and 39.0 ± 3.3%, respectively, of the level in the NMDA-treated group. Very low level of PI staining was observed in the cultures treated with MK-801 (Fig. 1).

In the patch-clamp experiment, inward currents were recorded following the application of 10⁻⁵ M glutamate, 10⁻⁴ M NMDA, 10⁻⁵ M AMPA, and 10⁻⁵ M kainate in almost all of the CA1 neurons tested. Application of 1 and 10 μg/ml of *Uncaria rhynchophylla* alone to CA1 neurons did not elicit ion currents. Glutamate at a concentration of 10⁻² M was applied every 2 min, and the magnitude of the resulting current used as the control current. Application of 1 and 10 μg/ml of *Uncaria rhynchophylla* inhibited the glutamate-activated ion current by about 27.2 ± 4.6% and 16.5 ± 0.9% of the control value (n = 6). The ion current induced by 10⁻⁴ M NMDA was used as the control value, and application of 1 and 10 μg/ml of *Uncaria rhynchophylla* markedly inhibited the NMDA-activated current by about 37.3 ± 3.0% and 45.0 ± 1.7% of the control value (n = 6). On the other hand, the current induced by 10⁻⁵ M kainate was not inhibited significantly, and the current induced by 10⁻⁵ M AMPA was increased by *Uncaria rhynchophylla* application (Fig. 2).

*Uncaria rhynchophylla* is reported to suppress kainic acid-induced neuronal damage in rats and to inhibit the increases in lipid peroxide levels in rats following ferric chloride-evoked seizures. Recently, phenolic compounds have been isolated from *Uncaria sinensis* that protect against glutamate-induced neuronal death in cultured cerebellar granule cells (13). NMDA receptors-induced neuronal damage following ischemic insults is most prominent among the various subtypes of glutamate receptors (4 – 7). However, the effect of methanol extract of *Uncaria rhynchophylla* on NMDA-induced neurotoxicity in slice culture has not been reported yet. In the present study, NMDA treatment was shown to induce significant neuronal injury in hippocampal slice cultures and MK-801 significantly alleviated the neuronal damage induced by NMDA. Likewise, treatment with the methanol extract of *Uncaria rhynchophylla* significantly decreased neuronal damage caused by NMDA.

In addition, a patch clamp experiment showed that *Uncaria rhynchophylla* significantly suppresses NMDA receptor-activated ion current in acutely dissociated hippocampal CA1 neuron, documenting that *Uncaria rhynchophylla* directly inhibits the NMDA receptor-activated channel. The NMDA receptor-channel, because of its high permeability to Ca²⁺, controls several important physiologic processes including long-term potentiation and depression. The most widely accepted hypothesis on how NMDA receptor antagonists salvage neurons states that they do so by inhibiting Ca²⁺ entry through NMDA receptors following glutamate release from presynaptic neurons; Ca²⁺-dependent nitric oxide
Neuroprotection of Uncaria rhynchophylla

It has been reported that fast synaptic transmission is mediated mainly by AMPA receptors and that activation of AMPA receptors exerts a neuroprotective effect. In this study, increased AMPA receptor-induced current by Uncaria rhynchophylla may contribute to the neuroprotective effect of this herb.

In the present study, it was shown that Uncaria rhynchophylla has a protective effect against NMDA induced excitotoxicity. This suppressive effect of Uncaria rhynchophylla on ion current induced by NMDA may be one of the mechanisms behind the neuroprotective effect of this herb.

Acknowledgments

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References