Protective effect of the hooks and stems of *Uncaria sinensis* against nitric oxide donor-induced neuronal death in cultured cerebellar granule cells

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Abstract

We have previously shown that an aqueous extract of the hooks and stems of *Uncaria sinensis* (OLIV. HAVIL.), Uncariae Uncus Cum Ramulus, protects against glutamate-induced neuronal death in vitro. Nitric oxide (NO) free radicals are also implicated in the process of neuronal death. In this study, we investigated the protective effects of *Uncaria sinensis* extract (USE) and its phenolic and alkaloid fractions against NO donors, sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1),-induced neuronal death in cultured rat cerebellar granule cells. MTT assay showed cell viability to be significantly increased by the addition of USE (10, 30 and 100 µg/ml) compared with exposure (6, 12 and 24 h) to SNP (30 µM) only, and by the addition of USE (10 and 30 µg/ml) compared with exposure (6, 12 and 24 h) to SIN-1 (300 µM) only. Phenolic fraction of USE (10 and 30 µg/ml) significantly protected against SNP (30 µM, 24 hr)-induced cell death, and 3 and 10 µg/ml of this fraction significantly protected against SIN-1 (300 µM, 24 hr)-induced cell death. Alkaloid fraction of USE (30 and 100 µg/ml) significantly protected against SNP (30 µM, 24 hr) and SIN-1 (300 µM, 24 hr)-induced cell death. These results appear to indicate that *Uncaria sinensis* has a protective effect against NO-mediated neuronal death in cultured cerebellar granule cells and that its active components are included in phenolic and alkaloid fractions.

Key words *Uncaria sinensis*, phenol, alkaloid, nitric oxide donor, neuronal death, protective effect.

Abbreviations CGC, cerebellar granule cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NO, nitric oxide; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; US, *Uncaria sinensis*; USE, *Uncaria sinensis* extract.

Introduction

Nitric oxide (NO) plays an important role in the central nervous system as a messenger molecule. However, excessive generation of NO has been implicated in neurotoxicity. This form of neurotoxicity is thought to be concerned with a final common pathway of injury in a wide variety of neurological disorders including ischemia.1) During brain ischemia, excessive amounts of glutamate are released from the pre-synaptic site of glutamate neuron, overstimulate glutamate receptors, especially N-methyl-D-aspartate (NMDA) receptors of neurons, finally leading to a series of potentially neurotoxic events through excessive Ca2+ influx.2,3) One of these events is the activation of the neuronal form of NO synthase (nNOS) and the subsequent production of NO free radicals (NO·).1,2,4) Moreover, some kinds of cytokines overstimulate astroglia or microglia after the brain ischemia/reperfusion and this leads to excessive and uncontrolled production of NO· through a Ca2+-independent and inducible form of NO synthase.

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Another event is the generation of superoxide anion (O_2^•−) due to the release of arachidonic acid. NO can react with O_2^•− to form peroxynitrate (ONOO•), which results in dose-dependent neuronal cell death. The mechanisms proposed for NO-mediated neurotoxicity include inactivation of the mitochondrial respiratory chain, nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), inhibition of cis-aconitase, activation of poly (adenosine 5′-diphosphoribose (ADP-ribose)) synthetase, and DNA damage. Many investigators have reported that the application of various kinds of NO donors can produce cell death in cultured neurons. Sodium nitroprusside (SNP) generates NO that reacts with endogenous O_2^•−, 3-morpholinosydnonimine (SIN-1) has been shown to produce NO and O_2^•−, thereby producing peroxynitrate (ONOO•) further hydroxyl radicals (‘OH).

The hooks and stems of Uncaria sinensis (OLIV.) HAVIL. (US), Uncariae Uncus Cum Ramulus, comprise the main medicinal plant of Choto-san (Diao-Teng-San in Chinese). Choto-san, which is composed of 11 crude drugs, has long been administered as a decoction to relatively aged patients suffering from headache, dizziness, vertigo, tinnitus, shoulder stiffness and so forth in China and Japan. We recently revealed the effectiveness of Choto-san on patients with vascular dementia by a well-controlled study and a double-blind, placebo-controlled study. We also demonstrated that the Uncaria sinensis water extract (USE) and its phenolic and alkaloid compounds have protective effects against glutamate-induced neuronal death in cultured cerebellar granule cells (CGCs). However, the effect of US against NO-mediated neurotoxicity has not been clarified. In this study, we investigated the neuroprotective effect of US from the view point of NO-mediated toxicity and found that US and its phenolic and alkaloid fractions have protective abilities against NO donor-induced neuronal death in CGCs.

Materials and Methods

Plant material and extraction: The US used in this study was obtained as described previously. It was purchased from a Chinese market in the Guangxi district in 1996, and voucher specimens (No. 16130) have been deposited in the herbarium stock room of the Tsumura Laboratory.

Plant materials (1 kg) were chopped and extracted with boiling water (5 l) for 2 h three times. Each extract was combined and lyophilized to give a brown mass (114.6 g, yield 11.5%). The water extract was chromatographed on polysacryl polymer gel (Diaion HP-20, 2 l), eluted with water (2 l, 58.2 g, yield 5.8 %; water eluate (USE-W)), H₂O-methanol (1:1) (2 l, 29.4 g, yield 2.9 %; phenolic fraction (USE-P)), and then methanol (2 l, 2.4 g, yield 0.2 %; alkaloid fraction (USE-A)), successively.

CGCs culture: Cerebellar granule cells from 8-day-old Wistar rats were cultured in poly-L-lysine (Sigma) coated 35-mm culture dishes with basal Eagle medium (Sigma) containing 10% fetal bovine serum (Sigma) and 25 mM KCl at a density of 6 × 10⁵ cells/ml (2 ml/dish) as described previously. The cultures were maintained at 37°C with 5% CO₂ in a humidified incubator, and cytosine arabinoside (Sigma) (10 μM) was added 18 h after plating to prevent proliferation of glial cells. The culture medium was not changed thereafter. The cells were used at 8-9 days in vitro for the experiments. All animal use procedures were approved by the Committee on Animal Experimentation at Toyama Medical and Pharmaceutical University.

Drug treatment and evaluation of cell viability: SNP, SIN-1 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma. USE, USE-W, USE-P and USE-A were dissolved in Locke’s solution (in mM: 154 NaCl, 5.6 KCl, 1.2 Mg, 3.6 NaHCO₃, 5.0 HEPES, 2.3 CaCl₂, 5.6 glucose, pH 7.4) as × 100 concentrated stock solution, and added (20 μl) to the original culture medium. Only the same amount of Locke’s solution was added to untreated and NO donor only (control) culture medium. Ten minutes later, the NO donors, SNP and SIN-1, were dissolved in Locke’s solution as × 100 concentrated solution and added (20 μl) to culture medium. The same amount of Locke’s solution was added to only untreated cultures. The cell cultures were incubated with drugs at 37°C with 5% CO₂ in a humidified incubator for certain periods. Cell viability was evaluated by MTT assay as previously described. After incubation with drugs, MTT (500 μg/ml) was applied and incubated for 30 min at 37°C.
tometer and expressed as percentage of the untreated cultures.

Statistical analysis: Values were expressed as mean ± S.D. from 4 experiments. The data were analyzed by Kruskal-Wallis test followed by Bonferroni's t-test. A p value < 0.05 was considered statistically significant.

Results

Concentration relationship of NO donor-induced cell death and protective effect of USE

To establish a model of NO donor-induced neurotoxicity and examine the effect of USE in CGCs, we examined the concentration-response relationship of neuronal cell death at 24 h after exposure to NO donors. SNP (10, 30 and 100 μM) decreased cell viability dose-dependently (10 μM, 53%; 30 μM, 15%; 100 μM, 11%) (Fig. 1a). SIN-1 (300 μM) also decreased cell viability (35%) (Fig. 1b). USE had significant protective effects against SNP (10 and 30 μM)-induced cell death at concentrations of 10, 30 and 100 μg/ml, and against SNP (100 μM)-induced cell death at concentrations of 30 and 100 μg/ml, in a dose-dependent manner (Fig. 1a). Furthermore, USE (10 and 30 μg/ml) significantly prevented SIN-1 (300 μM)-induced cell death in a dose-dependent manner (Fig. 1b). SNP or SIN-1-induced neuronal degeneration appeared as destruction of the soma and neurite network as well as a bright pyknotic mass of nuclei (Fig. 2a,b). These indications of neuronal death were prevented by USE (Fig. 2c,d).

Time course of NO donor-induced cell death and protective effect of USE

Next, we examined the time course of NO donor-induced cell death and the protective effect of USE. Based on the results from the concentration relationship of SNP and SIN-1-induced cell death, we decided on concentrations of 30 μM of SNP and 300 μM of SIN-1. Application of SNP or SIN-1 for 3 h did not influence cell viability (SNP, 90%; SIN-1, 99%), but longer applications decreased cell viability (SNP 6 h, 44%; 12 h, 33%; 24 h, 24%; SIN-1 6 h, 61%; 12 h, 33%; 24 h, 29%) (Fig. 3a,b). USE (10, 30 and 100 μg/ml) signifi-
Neuroprotective effect of *Uncaria sinensis*

Fig. 4 Effects of water eluate (USE-W), phenolic fraction (USE-P) and alkaloid fraction (USE-A) of *Uncaria sinensis* extract against SNP (30 μM, 24 h) (a) and SIN-1 (300 μM, 24 h) (b) -induced neuronal death evaluated by MTT assay in cultured cerebellar granule cells. Each fraction was added to the culture medium 10 min before NO donors application. Cells were incubated in drugs for 24 h. Values are mean ± S.D. from 4 experiments. *p<0.05, **p<0.01 compared with control.

Significantly prevented SNP-induced cell death at 6, 12 and 24 h (Fig. 3a). Furthermore, USE (10 and 30 μg/ml) provided significant protection against neuronal death induced by SIN-1 at 6, 12 and 24 h (Fig. 3b).

**Protective effects of phenolic and alkaloid fractions against NO donor-induced cell death**

We examined the protective effects of USE-W, USE-P and USE-A against SNP and SIN-1-induced neuronal death. USE-W had no protective ability (Fig. 4a,b). USE-P (10 and 30 μg/ml) and USE-A (30 and 100 μg/ml) provided protection against neuronal death induced by SNP (30 μM, 24 h) in a dose-dependent manner (Fig. 4a). USE-P (3 and 10 μg/ml) and USE-A (30 and 100 μg/ml) also had protective effects against neuronal death induced by SIN-1 (300 μM, 24 h) in a dose-dependent manner (Fig. 4b).

Finally, in order to examine whether USE and its fractions were toxic to CGCs, we evaluated the effects of them on cell viability in the absence of NO donors. As a result, USE and its fractions (24 h-incubation) did not show any toxicity at least at the concentrations of 100 μg/ml or less (data not shown).

**Discussion**

In the present study, we showed that USE has a neuroprotective effect against NO donor-induced cell death in CGCs and that its active components were included in phenolic and alkaloid fractions. Previously, we reported that USE and its phenolic and alkaloid compounds protected against glutamate-induced neuronal death in CGCs. It is known that NO mediates glutamate neurotoxicity. The excessive amounts of glutamate, which are released from the pre-synaptic neurons during brain ischemia, overstimulate glutamate receptors in the post-synaptic neurons, especially NMDA receptors. This event leads to the excessive Ca²⁺ influx into neurons through voltage-dependent and more glutamate-regulated Ca²⁺-channels, then the activation of nNOS and subsequent production of NO. Therefore the present and our previous studies suggest a possibility that the protective effect of USE against glutamate-induced neuronal death is at least in part due to its protective ability against NO-mediated neurotoxicity.

Gliai cells also play important roles in the process of the neuronal death induced by brain ischemia. Gliai cells take in glutamate and metabolize it to glutamine by glutamine synthetase. This ability is thought to act neuroprotectively in brain ischemia. On the other hand, in the later phase of brain ischemia/reperfusion, several stimuli involving activation of some kinds of cytokines, strongly induce iNOS expression in glial cells, allowing excessive and uncontrolled production of NO, and leading to delayed neuronal death. In cultured neurons, neuronal cell death produced by a low level of NO donor is characterized by delayed neuronal death, which is apoptosis rather than necrosis. In our present study, application of NO donors did not influence cell viability until 3 h, but longer application decreased cell viability and produced nuclear condensation and fragmentation in CGCs, a feature of apoptosis, and these findings were protected by USE. Whereas we prevented the proliferation of glial cells by application of cytosine arabinoside in the present cultures, further studies need to focus on whether USE has any effect on glial cells in the process of neuronal death.

In the process of brain ischemia/reperfusion, large amounts of O₂⁻ are generated resulting from the mitochondrial dysfunction, the accumulation of hypoxanthine, the release of arachidonic acid, the activation of leukocytes, and so forth. Increasing evidences obtained from *in vivo* studies suggest that formation of ONOO⁻ from the reaction of NO' with O₂⁻, and further 'OH generation, is a mechanism through which neurotoxicity is induced. In the present study, we used...
two kinds of NO donors, SNP and SIN-1. At least on
condition that SNP (30 μM, 24 hr) and SIN-1 (300 μM,
24 hr) were applied, phenolic fraction of US showed a
protective effect at relatively lower concentration com-
pared with alkaloid fraction.
Uncaria genus is known to have antioxidant and
free radical scavenging activities from evaluation using
the electron spin resonance technique and the inhibitory
effect on lipid peroxidation in the brain of iron-induced
epileptic rats. Further, Uncaria genus has an inhibitory
effect against convulsion induced by the in vivo admin-
istration of excitatory amino acids, glutamate and
kainate, the neuronal damages of which are thought
to be caused in part by oxidative stress produced by
NO and O₂⁻ generation. These findings suggest that the
protective effect of US against NO donor-induced
neurotoxicity was at least partly a result of its free radical
scavenging activity.

It is well known that phenolic compounds isolated
from natural products possess antioxidant and free radical
scavenging properties. The present data, and those
obtained from other neuronal cell types, such as cultured
hippocampal cells, demonstrated that phenolic com-
ounds can inhibit the neuronal damage from the oxida-
tive stress produced by NO generation. Phenolic
compounds also have protective effects against oxidative
stress-induced cell damage in cultured endothelial cells
and ischemia/reperfusion injury by their antioxidant
abilities.

The alkaloid compounds of Uncaria genus have
various pharmacological activities, such as vasodilative
activity, anti-convulsive activity against glutamate-
induced convulsion, and so forth. In the present study,
we demonstrated that not only phenolic fraction but also
alkaloid fraction of US have protective abilities against
NO-mediated neurotoxicity. We showed previously that
an alkaloid fraction of USE suppressed vaso-contraction
induced by oxidative stress in vitro, supporting the no-
tion that alkaloid fraction of US possess free radical
scavenging properties.

The neuroprotective effects of traditional Chinese/
Japanese (Kampo) medicines besides US were investi-
gated in vitro. Watanabe et al. reported that the extracts
from Toki-shakuyaku-san, a Kampo formula, and its
comprised six medicinal plants, Angelica Radix,
Paeoniae Radix, Hoelen, Atractylodis Lanceae Rhizoma,
Alismatis Rhizoma and Cnidii Rhizoma, protected gluta-
mate-induced neuronal damages in CGCs. Liu et al. re-
ported that Angelicae Radix and Cnidii Rhizoma
prevented the methionine sulfoximine-reduced glutamine
synthetase activity in mixed CGC-gliial cell cultures, and
suggested their protective abilities against glutamate-
induced neuronal dysfunction. Moreover we revealed
that the extract from the bark of Cinnamomum cassia
Blume, Cinnamomum Cortex, has a protective effect
against glutamate-induced neuronal death in CGCs.

In conclusion, the results of the present study sug-
gest that US has neuroprotective effect against NO-
mediated neurotoxicity and that its active components
are included in phenolic and alkaloid fractions. This abil-
ity of US may have the potential to contribute to protec-
tion against NO-related neuronal damage, such as against
that occurring in ischemic cerebral diseases.

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和文抄録

培養ラット小脳顆粒細胞を用いて、NO donor 誘導神
経細胞死に対する釈藤鎖エキスの保護作用を検討した。
NO donor には sodium nitroprusside (SNP) と 3-
morpholinosydnonimine (SIN-1) を用い、細胞生存率の
評価には MTT 法を用いた。釈藤鎖エキス（10, 30,
100 μg/ml）は、SNP（30 μM）6, 12, 24時間添加によ
る神経細胞死を有意に抑制した。釈藤鎖エキス（10, 30
μg/ml）は、SIN-1（300 μM）6, 12, 24時間添加によ
る神経細胞死を有意に抑制した。釈藤鎖エキスのフェノ
ール画分は、10, 30 μg/mlの濃度で SNP（30 μM, 24時間）
添加による神経細胞死を有意に抑制し、同じく3, 10 μg/
ml の濃度で SIN-1（300 μM, 24時間）添加による神経
細胞死を有意に抑制した。釈藤鎖エキスのアルカロイド
画分（30, 100 μg/ml）は、SNP（30 μM, 24時間）およ
び SIN-1（300 μM, 24時間）添加による神経細胞死を有
意に抑制した。以上より、釈藤鎖エキスは NO donor
によって誘導される神経細胞死に対して保護作用を有し、
その活性は釈藤鎖のフェノール画分およびアルカロイド
画分にあることが示唆された。
Neuroprotective effect of Uncaria sinensis

References


