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Short communication

Neuroprotection by *chotosan*, a Kampo formula, against glutamate excitotoxicity involves the inhibition of GluN2B-, but not GluN2A-containing NMDA receptor-mediated responses in primary cultured cortical neurons

Sachie Sasaki-Hamada [a], Azusa Suzuki [a], Emi Sanai [a], Kinzo Matsumoto [b], Jun-Ichiro Oka [a, *]

[a] *Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Japan*
[b] *Division of Medicinal Pharmacology, Institute of Natural Medicine, University of Toyama, Japan*

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ABSTRACT

Chotosan (CTS), a traditional herbal formula called *Kampo* medicine, was shown to be effective in the treatment of vascular dementia in a clinical study, and exerted protective effects against transient cerebral ischemia-induced cognitive impairment in mice. In the present study, we investigated the neuroprotective effects of CTS using primary cultured rat cortical neurons. CTS (250–1000 μg/mL) inhibited neuronal death induced by 100 μM glutamate. This glutamate-induced neuronal death was blocked by a GluN2B-, but not GluN2A-containing NMDA receptor antagonist. Therefore, the neuroprotective effects of CTS were related to an inhibition of GluN2B-containing NMDA receptor-mediated responses.

Chotosan (CTS) is a Kampo formula that consists of 11 different medical herbs and *gypsum fibrosum*, which is generally prescribed to middle-aged and elderly patients with a weak physical constitution and symptoms related to hypertension and chronic headaches.[1] CTS exhibits antioxidant and free radical scavenging activities.[2] Although the effects of its major component herb, Chotoko (*Uncaria geneus*), or indole alkaloids contained in Chotoko on glutamate-induced neuronal death have already been examined,[3–5] it currently remains unclear whether CTS exerts neuroprotective effects.

Glutamate is a physiological excitatory amino acid transmitter in the central nervous system. Glutamate-mediated toxicity has been suggested to be involved in several neurological disorders including ischemia[6] and neurodegenerative diseases such as Alzheimer's disease.[7] Excitotoxicity is mainly associated with the excessive release of glutamate and subsequent Ca^{2+} influx via NMDA-subtype glutamate receptors, leading to an intracellular cascade of cytotoxic events; i.e., the excessive activation of glutamate receptors evokes neuronal dysfunction and damage or even death.[8]

We herein investigated the neuroprotective effects of CTS against glutamate-induced cell death in cultured rat cortical neurons. The effects of antagonists selective for NMDA receptors containing GluN2A or GluN2B subunits on glutamate-induced cell death were also examined.

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Tokyo University of Science, and conducted according to the guidelines of the National Institute of Health and the Japanese Pharmacological Society. E18 Wistar rats were purchased from Japan SLC, Inc. (Shizuoka), and efforts were made to minimize the number of animals used as well as animal pain and distress. A primary culture was performed according to our previous study[9] with minor modifications. Cortical neurons were dissociated from E18 Wistar rats with 6 U/mL papain and 0.1 mg/mL DNase I at 37 °C for 15 min. The brain tissue suspension was centrifuged for 5 min at 1000 rpm, and the resultant pellet was resuspended in feeding medium, which contained DMEM with 5% fetal bovine serum, 5% horse serum, and 1% penicillin-streptomycin solution. The cell suspension was seeded at 1×10^6 cells/mL into 24-well plates precoated with polyethyleneimine (Sigma–Aldrich,

* Corresponding author. Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan.
E-mail address: okaji@rs.noda.tus.ac.jp (J.-I. Oka).
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St. Louis, MO, USA). Cells were incubated at 37 °C in a 9:1 mixture of atmospheric air and CO_2, and were treated for 24 h with 10 μM cytosine arabinoside (Sigma–Aldrich) 2 days after plating to remove actively dividing cells. Medium was changed on the next day, and then once a week. Cultures were used for experiments on the 10th day *in vitro*.

The CTS extract used in this study was purchased from Tsumura Co. (Japan) in the form of a spray-dried powder extract prepared according to the standardized extraction method of medical plants registered in *Japanese Pharmacopoeia XV*. CTS was extracted from a mixture of 3.0 parts *Uncariae Uncis cum Ramulus* (the hooks and branch of *Uncaria rhynchophylla* MIQUEL), 3.0 parts *Aurantii Nobilis*

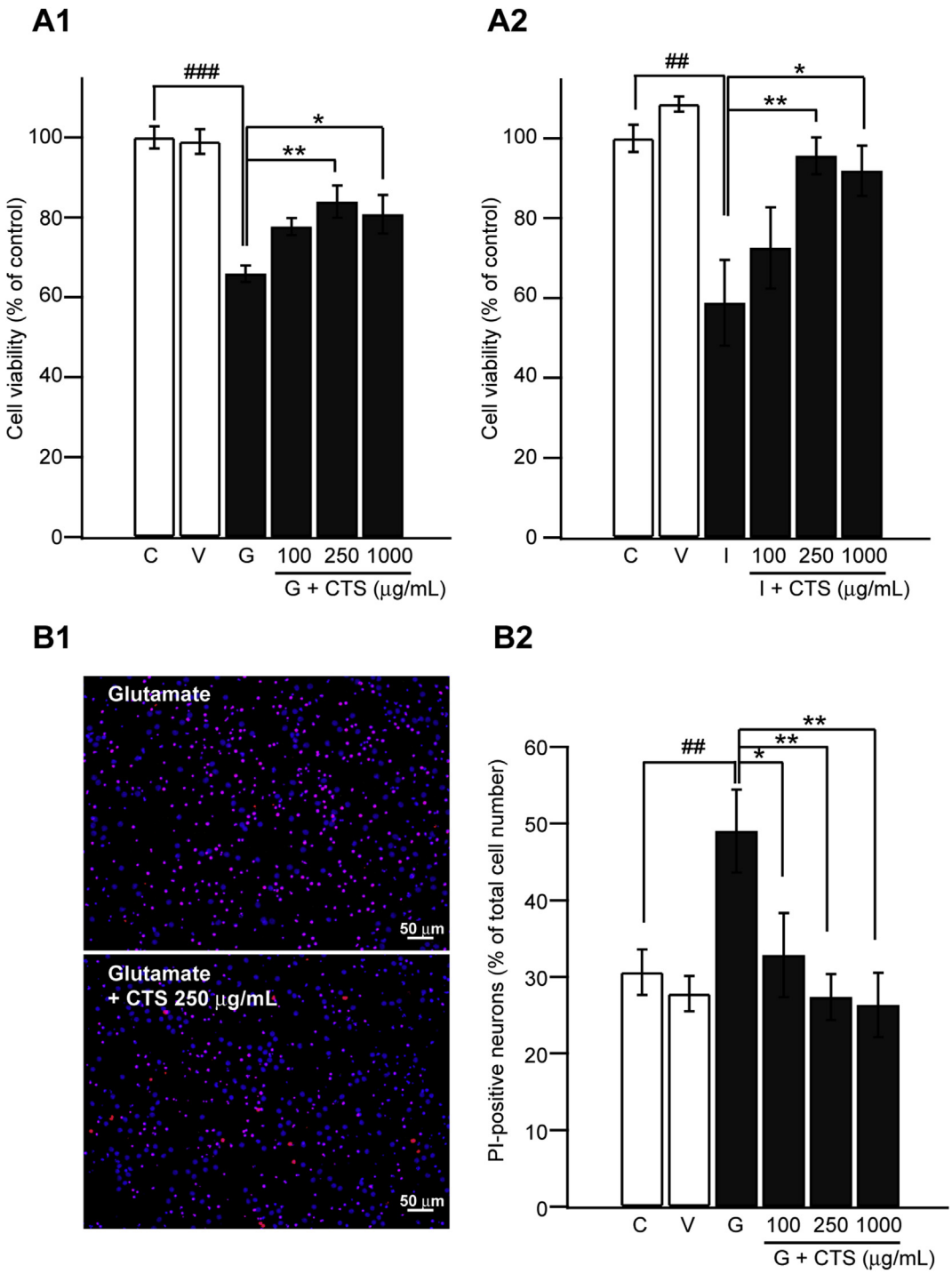

Fig. 1. Effects of CTS on glutamate- or ionomycin-induced toxicity. (**A**) The MTT assay for cell viability in glutamate- or ionomycin-treated primary cultured cortical neurons. Cortical neurons were pre-incubated with 100, 250, and 1000 μg/mL CTS for 48 h, followed by exposure to 100 μM glutamate (**A1**) or 5 μM ionomycin (**A2**) for 24 h. (**B**) Hoechst 33342 and PI double-staining in cultured cortical neurons. The glutamate treatment increased the number of PI-positive neurons that was decreased in neurons pretreated with CTS (**B1**). Scale bar = 50 μm. Quantitative analysis of histograms expressed as a percentage of apoptotic neurons in all neurons observed under each microscopic field (**B2**). Results are expressed as means ± SEM. ## $P < 0.01$, ### $P < 0.001$ vs. the control group, *$P < 0.05$, **$P < 0.01$ vs. the glutamate- or ionomycin-treated group (a one-way ANOVA followed by Bonferroni's multiple comparison test). n = 7–16.

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