Introduction

Chotosan (CTS) is a Kampo formula that consists of 11 different medical herbs and *gypsum fibrosum*, which has long been used for the treatment of chronic headaches and hypertension, particularly in middle-aged or older patients who have weakened physical conditions, chronic headaches, painful tension in the shoulder and cervical muscles, vertigo, morning headaches, flushing, tinnitus, and insomnia (1). In a double-blind and placebo-controlled clinical study, CTS was shown to have an ameliorative effect on cognitive dysfunction in stroke patients (1). CTS also had a preventive effect on cognitive deficits in a mouse model of transient cerebral ischemia and improved learning and memory impairments in a mouse model of chronic cerebral hypoperfusion (2, 3). These findings indicated that CTS may be useful as an anti-dementia drug (4).

Diabetes mellitus (DM) is associated with various complications, including syndromes in the central nervous system (CNS) (5). A relationship between cognitive dysfunction and type 1 DM has been reported previously (6). Cognitive dysfunction in patients who developed type 1 DM in childhood or adolescence has been characterized by impaired memory and attention in cognitive tests relative to normal subjects (7, 8). Childhood-onset type 1 diabetic subjects, in particular, had poorer cognitive abilities than those of adolescence-onset type 1 diabetic subjects, independent of the duration of diabetes (9 – 11). It is therefore necessary to improve cognitive dysfunction associated with childhood-onset diabetes.

Type 1 diabetic animal models, generated by injecting streptozotocin (STZ), have been widely used (12 – 14). We previously demonstrated learning impairments and hippocampal LTD deficits in juvenile-onset DM (JDM)
rads (15, 16). NMDA-receptor function was shown to be enhanced in JDM rats (16), which may be more than that required to induce LTD (17). Moreover, hippocampal LTD was restored by glucagon-like peptide-1 (7 – 36) amide or a low concentration of an NMDA-receptor antagonist in JDM rats (15, 16). In the present study, we investigated whether CTS exerted effects on diabetes-induced cognitive deficits and NMDA-receptor subunit expression in JDM rats.

Materials and Methods

Drugs

The CTS extract used in this study was purchased from Tsumura Co. (Tokyo) 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 pericarpium (the skin of Citrus unshiu Markovich), 3.0 parts Pinelliae tuber (the tuber of Pinellia ternate Breitenbach), 3.0 parts Ophiopogonis tuber (the root of Ophiopogon japonicus Ker-Gawler), 3.0 parts Hoelen (the sclerotium of Poria cocos Wolf), 2.0 parts Ginseng radix (the root of Panax ginseng C.A. Meyer), 2.0 parts Saposnikoviae radix (the root and rhizome of Saposnokovia divaricata Schischkin), 2.0 parts Chrysanthemi flos (the flower of Chrysanthemum morifolium Ramatulle), 1.0 part Glycyrrhizae radix (the root of Glycyrrhiza uralensis Fisher), 1.0 part Zingiberis rhizoma (the rhizome of Zingiber officinale Roscoe), and 5.0 parts Gypsum fibrosum (CaSO₄·2H₂O). The yield of the CTS extract was 16.1%. Protocols for extraction and chemical profiling of CTS were the same as those described in the previous report (18) and mass spectrometry data obtained from the extract were stored in the Wakan-Yaku Database system (WakanDB ID: LCMS:Chotosan/11000001, http://wakandb.u-toyama.ac.jp/wiki/LCMS:Chotosan/11000001), Institute of Natural Medicine, University of Toyama. A voucher specimen (CTS: No. 2020047010) obtained from Tsumura Co., Ltd. has been deposited at the institute. CTS was dissolved in distilled water.

STZ and DL-2-amino-5-phosphonopentanoic acid (APV) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were from Wako Pure Chemical Industries (Osaka).

Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee at Tokyo University of Science and were conducted according to the guidelines of the National Institute of Health and Japan Neuroscience Society. We used Wistar rats (Japan SLC, Shizuoka) and made every effort to minimize the number of animals used as well as animal pain and distress. JDM was induced by STZ (85 mg/kg, i.p.) in 17-day-old rats. STZ was dissolved in phosphate-buffered saline (PBS). To confirm the induction of DM, we measured glucose levels in vein blood samples using the glucose test meter (Gunze Co., Kyoto). Animals were considered diabetic when glucose levels were more than 300 mg/dl. Vehicle-treated rats were used as controls. All animals were kept in a controlled environment, with a 12:12 h light schedule, temperature (23°C ± 1°C), and relative humidity (55% ± 5%), and were provided ad libitum access to food and water.

Y-maze test

Four experimental groups (n = 6 – 10 male rats per group) were designed as follows: 1) vehicle-treated control and diabetic groups and 2) CTS-treated control and diabetic groups. CTS (1 g/kg) was orally administered at approximately 4 p.m. The experimental schedule is shown in Fig. 1A. The Y-maze test was carried out between 6 and 7 p.m. and was performed as described previously with some modifications (15, 19). Each rat, new to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries were recorded visually. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The effect was calculated as the percent alternation according to the following formula: Percent alternation = (number of alternations / (total number of arm entries – 2)) × 100 (%).

Slice preparation

Transverse hippocampal slices (400-μm-thick) were prepared from 24- to 28-day-old male rats (Fig. 1A) using a vibratome (DTK-1000; Dosaka, Kyoto) in ice-cold dissection buffer (234 mM sucrose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 0.5 mM CaCl₂, 10 mM MgSO₄, 26 mM NaHCO₃, and 11 mM d-glucose) saturated with 95% O₂ and 5% CO₂. Slices were allowed to recover at 36°C for 50 min in normal artificial CSF (ACSF) (124 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgCl₂, 1.25 mM KH₂PO₄, 10 mM d-glucose, and 26 mM NaHCO₃) saturated with 95% O₂ and 5% CO₂ and were thereafter maintained at room temperature. Slices were left for a minimum of 30 min at room temperature before being transferred to a submerged recording chamber for experiments.
Extracellular field potential recordings

Extracellular field EPSPs (fEPSPs) from the stratum radiatum of the hippocampal CA1 region were recorded using glass micropipettes filled with ACSF. During these recordings, slices were maintained while being perfused at 3 – 4 ml/min with ACSF. Test stimuli (80 μs duration) were delivered once every 60 s by a bipolar tungsten electrode (150-μm pole separation; WPI, Sarasona, FL, USA), which was placed on the afferent fibers of the stratum radiatum. An evoked fEPSP was generated by an electric stimulator (SEN-3301; Nihon Koden, Tokyo) and isolater (SS-202J, Nihon Koden); and it was recorded with an amplifier (CEZ-2400; Nihon Koden), filtered at 5 kHz, digitized at 20 kHz, and analyzed in a personal computer using a PowerLab/4s system (AD Instruments, Castle Hill, Australia).

To induce LTP or LTD, the stimulation intensity was set to elicit 50% of the maximal fEPSP. After a stable baseline recording at 0.017 Hz for at least 15 min, LTP was induced by high-frequency stimulation (HFS: 100 stimuli at 100 Hz for 1 s), whereas LTD was induced by low-frequency stimulation (LFS: 900 stimuli at 1 Hz for 15 min). These responses were recorded for another 60 min. Statistical analysis for the normalized fEPSP amplitude at fixed times after the HFS or LFS was conducted with a two-factor (animal group × time) repeated measures analysis of variance (ANOVA).

To measure the paired-pulse ratio, paired pulses were delivered through a single stimulation electrode at varying interpulse intervals. The first pulse was set to elicit 50% of the maximal fEPSP. The various interstimulus intervals between successive pulses were 30, 50, 80, 120, 200, and 500 ms. The ratio of the maximum negative slope of the second pulse to the slope of the first pulse was computed as the paired-pulse ratio. The slope was measured by 30% – 80% of the fEPSP peak amplitude. APV (0.5 μM in ACSF) was perfused into the slice chamber perfusion line.

SDS-PAGE and western blotting

Tissue samples (Fig. 1A) were suspended in lysis buffer: 20 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 5% Triton X-100, 10% glycerol, 0.25%, v/v, protease inhibitor cocktail (Sigma-Aldrich). Protein content was determined with the BCA Protein Assay Kit (PIERCE, Rockford, IL, USA). An 8-μg sample of tissue was loaded and separated on a 10% SDS-polyacrylamide gel. After transfer to a polyvinylidene difluoride membrane, blots were blocked overnight with Tris-buffered saline (TBS) containing 5% nonfat dried milk and 0.1% Tween 20 at 4°C, and were then probed with the anti-NR1 (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-NR2A (1:500, Santa Cruz Biotechnology), anti-NR2B (1:5000; BD Biosciences, Bedford, MA, USA), anti-GluR1 (1:1000; Millipore Bioscience Research Reagents, Temecula, CA, USA), anti-GluR2 (1:1000, Millipore Bioscience Research Reagents), or anti-β-actin (1:20000, Sigma-
Aldrich) antibody at room temperature for 2 h. After three washes with TBS containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated bovine anti-goat IgG, anti-mouse IgG secondary antibody (Santa Cruz Biotechnology) or goat anti-rabbit IgG secondary antibody (BD Biosciences) diluted 1:5000 – 10000 with TBS containing 0.1% Tween 20 at room temperature for 1 h, followed by detection using enhanced chemiluminescence (Millipore Bioscience Research Reagents). The intensities of the bands were semiquantified using the public domain NIH Image program (ver. 1.6, NIH, USA).

Data analyses and statistics
Data analyses were performed using IgorPro (WaveMetrics, Lake Oswego, OR, USA). All values are given as the mean ± S.E.M.s. Statistical analyses were performed with a one-way factorial analysis of variance (ANOVA) followed by the Bonferroni-adjusted post hoc test or a two-way repeated measures ANOVA using Graphpad Prism (Graphpad Software, San Diego, CA, USA). In all cases, significance was set at $P < 0.05$.

Results
CTS treatment improved working memory-deficits in JDM rats
We recently described working memory-deficits in JDM rats (15). To assess whether the CTS treatment restored working memory in JDM rats, we examined spontaneous alternation behavior in the Y-maze test. The concentration of CTS used was based on a previous study (20).

Control rats had normal blood glucose levels (151.8 ± 4.9 mg/dl, n = 44) and all STZ-injected rats had hyperglycemia (595.6 ± 19.7 mg/dl, n = 40) (Table 1). Reductions in body weight were significantly higher in JDM rats than in control rats (n = 24, vehicle group + control rats; n = 20, vehicle group + JDM rats; $***P < 0.001$, Bonferroni-adjusted post hoc test) (n = 19, CTS group + control rats; n = 21, CTS group + JDM rats; $***P < 0.001$, Bonferroni-adjusted post hoc test). As indicated in Table 1, plasma glucose levels and body weight were not affected by the repeated CTS treatment.

As shown in Fig. 1B, the percent alternation was significantly lower in the vehicle group + JDM rats (52% ± 1.4%, n = 5) than in the vehicle group + control rats (68.9% ± 3.8%, n = 5) ($**P < 0.01$, the Bonferroni-adjusted post hoc test). The repeated treatment with CTS (1 g/kg p.o.) significantly restored the impairment in spontaneous alternation performance (vehicle group + JDM rats: 52% ± 1.4%, n = 5; CTS group + JDM rats: 76.5% ± 2.9%, n = 6, $***P < 0.001$, Bonferroni-adjusted post hoc test). No significant differences were observed in total arm entries among the groups ($P > 0.05$, Bonferroni-adjusted post hoc test) (Fig. 1C).

CTS treatment improved LTD deficits in JDM rats
We previously showed that 3- to 4-week-old JDM rats showed impaired LTD, but not impaired LTP at Schaffer collateral-CA1 (SC-CA1) synapses in hippocampal slices, relative to age-matched control rats (15). Impaired LTD was rescued by a low concentration of an NMDA-receptor antagonist (0.5 μM APV) in 3- to 4-week-old JDM rats ($F_{(1,11)} = 15.00$, $P < 0.01$, two-way repeated measures ANOVA) (Fig. 2C). We obtained similar results in 22- to 24-week-old JDM rats (16). Here, we showed the effects of CTS on synaptic plasticity in control and JDM rats.

LTP of CA1 fEPSPs was induced by a single episode of HFS (100 Hz, 1 s), and LTD was induced by LFS (1 Hz, 15 min). The average fEPSP amplitude during the 15 min prior to HFS or LFS induction was defined as 100%. After 15 min of baseline recordings, HFS or LFS induced LTP or LTD for 60 min. The level of LTP was not significantly different between control rats and

| Table 1. Body weights and blood glucose levels in control and JDM rats |
|-------------------------|----------------------|----------------------|
| Vehicle group + control rats (n = 24) | 60.2 ± 2.2 | 152.0 ± 8.0 |
| Vehicle group + JDM rats (n = 20) | 40.6 ± 1.7*** | 622.9 ± 29.2*** |
| CTS group + control rats (n = 19) | 57.5 ± 2.0 | 151.5 ± 5.1 |
| CTS group + JDM rats (n = 21) | 37.8 ± 1.4*** | 570.9 ± 26.2*** |

Significant reductions in body weight and elevations in blood glucose levels were observed in JDM rats (n = 24, vehicle group + control rats; n = 20, vehicle group + JDM rats; $***P < 0.001$, Bonferroni-adjusted post hoc test) (n = 19, CTS group + control rats; n = 21, CTS group + JDM rats; $***P < 0.001$, Bonferroni-adjusted post hoc test). On the other hand, body weight and plasma glucose levels in control and JDM rats were not affected by the repeated CTS treatment (n = 19 – 24, $P > 0.05$, Bonferroni-adjusted post hoc test). n, the number of animals. Data are shown as the mean ± S.E.M.
Fig. 2. Effect of CTZ on LTP and LTD. Hippocampal slices were obtained from vehicle group + control rats (white circle), vehicle group + JDM rats (black circle), CTS group + control rats (white triangle), or CTS group + JDM rats (black triangle). LTP was induced by HFS stimulation (arrow). A1: The magnitude of LTP in JDM rats was not significantly affected (n = 5 in vehicle group + control rats, n = 5 in vehicle group + JDM rats; *P > 0.05, two-way repeated measures ANOVA). A2: The magnitude of LTP was not affected by the administration of CTS (n = 6 in CTS group + control rats, n = 5 in CTS group + JDM rats; *P < 0.05, two-way repeated measures ANOVA). LTD was induced by LFS stimulation (dashed line). B1: The magnitude of LTD in JDM rats was significantly impaired (n = 9 in vehicle group + control rats, n = 6 in vehicle group + JDM rats; **P < 0.05, two-way repeated measures ANOVA). B2: No significant impairments in the magnitude of LTD were observed in control and JDM rats due to the administration of CTS (n = 6 in CTS group + control rats, n = 7 in CTS group + JDM rats; **P > 0.05, two-way repeated measures ANOVA). C: The magnitude of LTD in JDM rats was significantly restored in the presence of 0.5 μM APV (n = 7 in Veh + JDM rats, n = 6 in APV + JDM rats; **P < 0.01, two-way repeated measures ANOVA). Representative traces at the time points are shown on the top. Data are shown as the mean ± S.E.M.
JDM rats ($F_{(1,8)} = 1.313, P > 0.05$, two-way repeated measures ANOVA for the vehicle group; $F_{(1,9)} = 0.0135, P > 0.05$, two-way repeated measures ANOVA for the CTS group) (Fig. 2: A1 and A2). Although the vehicle group + JDM rats showed significantly impaired LTD relative to the vehicle group + control rats ($F_{(1,13)} = 12.01, P < 0.01$, two-way repeated measures ANOVA), the level of LTD was not significantly different between control rats and JDM rats within the CTS group ($F_{(1,11)} = 0.176, P > 0.05$, two-way repeated measures ANOVA) (Fig. 2: B1 and B2).

**Effect of CTS on synaptic transmission at the SC-CA1 pathway**

We measured excitatory synaptic transmission by making extracellular recordings in hippocampal SC-CA1 synapses. The relationship of the fEPSP slope to the stimulus intensity (input–output relation) of synaptic transmission was significantly stronger in JDM rats than in control rats ($n = 9$ in vehicle group + control rats; $n = 8$ in vehicle group + JDM rats; **$P < 0.01$, two-way repeated measures ANOVA). The treatment with CTS significantly restored the increased input–output relationship in JDM rats ($n = 8$ in vehicle group + JDM rats; $n = 10$ in CTS group + JDM rats; *$P < 0.05$, two-way repeated measures ANOVA) (Fig. 3: A).

Paired-pulse facilitation (PPF) is the facilitation of a second response when a synapse is stimulated twice with a short inter-stimulus interval (30 – 500 ms). This phenomenon has been attributed to an increase in the amount of neurotransmitter released in response to the second stimulus (21, 22). The strength of PPF in JDM was not significantly different from that in control rats ($F_{(1,15)} = 9.111, P < 0.01$, two-way repeated measures ANOVA) than in the vehicle group + control rats, but was not in the CTS group + JDM rats ($F_{(1,17)} = 1.466, P > 0.05$, two-way repeated measures ANOVA) (Fig. 3: A).

Figure 3. Effect of CTZ on synaptic transmission at the SC-CA1 pathway. A: The input-output relationship was obtained by plotting the fEPSP slope against the intensity of stimulation. The input-output relationship was significantly stronger in JDM rats than in control rats ($n = 9$ in vehicle group + control rats; $n = 8$ in vehicle group + JDM rats; **$P < 0.01$, two-way repeated measures ANOVA). The treatment with CTS significantly restored the increased input-output relationship in JDM rats ($n = 8$ in vehicle group + JDM rats; $n = 10$ in CTS group + JDM rats; *$P < 0.05$, two-way repeated measures ANOVA). B: Facilitation ratios were plotted against interstimulus intervals. PPF was not significantly different among the groups ($n = 8$ in vehicle group + control rats; $n = 9$ in CTS group + control rats; $n = 7$ in vehicle group + JDM rats; $n = 10$ in CTS group + JDM rats; $P > 0.05$, two-way repeated measures ANOVA). The insets show typical traces of fEPSP. Data are shown as the mean ± S.E.M.
These results suggest that the CTS treatment affected postsynaptic properties.

Effect of CTS on the expression of AMPA and NMDA receptor subunits

Since the CTS treatment restored enhanced synaptic transmission in JDM rats, we investigated the expression of AMPA- and NMDA-receptor subunits in the hippocampus by a western blot analysis. The quantity of immunoreactive bands of AMPA- and NMDA-receptor subtypes (GluR1, GluR2, NR1, NR2A, and NR2B) and β-actin protein was normalized to their expression levels in control rats. No significant changes were observed in the expression levels of the GluR1 subunit, GluR2 subunit, NR2A subunit, or NR1 subunit in the 4 groups (Fig. 4: A and B). However, expression levels of the NR2B subunit were higher in the vehicle group + JDM rats than in the vehicle group + control rats. The CTS treatment significantly down-regulated expression levels of the NR2B subunit in control and JDM rats (*P < 0.05, Bonferroni-adjusted post hoc test for control rats; ***P < 0.001, Bonferroni-adjusted post hoc test for JDM rats) (Fig. 4B).

Discussion

The present study demonstrated that the repeated treatment with CTS (1 g/kg per day) for 3 – 7 days restored working memory deficits and the impairment in synaptic plasticity in hippocampal SC-CA1 synapses in JDM rats. Furthermore, the 7-day treatment with CTS down-regulated expression levels of the NR2B subunit of the NMDA receptor in the hippocampus. These results suggest that CTS improved cognitive deficits by modulating the expression of NMDA receptors in JDM rats.

The behavioral task of the Y-maze test was used to elucidate spatial working memory in this study (15, 19, 23). The results obtained showed that cognitive learning and memory were impaired in JDM rats relative to control rats and were restored in CTS-treated JDM rats. Moreover, CTS-treated rats were unaffected in motor activity, serum glucose levels, and body weights. These findings in STZ-treated rats of a type 1 diabetes model are consistent with a previous report on C57BLKS/J-db/db mice of a type 2 diabetes model (18). The ameliorative effect of CTS on diabetes-induced cognitive deficits may involve a mechanism independent
of anti-hyperglycemia or anti-obesity. Although previous studies suggested that the effects of CTS were mediated in db/db mice or other animal models of vascular dementia by ameliorating dysfunctions in central cholinergic systems, which play an important role in learning and memory (2, 18, 24), we showed that CTS modulated the expression of NMDA receptors. However, whether the mechanisms of the CTS-induced ameliorative effects are common in type 1 and type 2 diabetes remains unclear. Further studies on central cholinergic systems in JDM rats are necessary to elucidate this possibility.

LTP and LTD are primary experimental models for investigating the synaptic basis of learning and memory (25 – 27). However, the effects of CTS on hippocampal synaptic plasticity have yet to be clarified. In the present study, we showed that the administration of CTS did not affect LTP and LTD in hippocampal SC-CA1 synapses. Moreover, the administration of CTS did not affect the input–output relationship or PPF. PPF is a well-established purely presynaptic phenomenon in the hippocampus (28). We showed impaired LTD and an increased input–output relationship in JDM rats, which were improved in CTS-treated JDM rats. Taken together, we suggest that CTS affects postsynaptic malfunctions in glutamate receptors, but not neurotransmitter release from presynaptic terminals in JDM rats.

We next elucidated that expression levels of the NR2B subunit of the NMDA receptor in the hippocampus were higher in vehicle-treated JDM rats than in age-matched vehicle-treated control rats and that the CTS treatment downregulated expression of the NR2B subunit of the NMDA receptor in both control and JDM rats. NMDA-subtype glutamate receptors play an important role in learning and memory (29). In a previous in vitro study, rhynchophylline and isorhynchophylline, oxindole alkaloid components of the Uncaria species, which is a major herb constituent of CTS, exhibited antagonistic properties for NMDA subtype glutamate receptors in a receptor expression model of Xenopus oocytes (30). A previous study showed that CTS ameliorated the down-regulation of vascular endothelial growth factor/platelet-derived growth factor (PDGF) in senescence-accelerated mice (31). Since PGDF has been implicated in the positive and negative modulation of NMDA-receptor function via the PKA and PKC pathways (32), the relevant mechanisms involved in the expression of NMDA receptors affected by the administration of CTS need to be examined in future studies.

Taken together, these results suggest that CTS improved cognitive deficits by modulating the expression of the NR2B subunit in JDM rats. However down-regulated expression of the NR2B subunit in CTS-treated control rats did not affect cognitive performance. Previous studies showed that NR2B antagonists, such as ifenprodil, Ro 25-6981, Ro 63-1908, and traxoprodil, did not impair performance in a task designed to measure avoidance learning or spatial/working memory (33 – 37). Therefore CTS may have no effect on cognition itself even if it reduced expression levels of the NR2B subunit in control rats. On the other hand, enhanced expression levels of the NR2B subunit in JDM rats might induce secondary changes that caused cognitive impairment, and hence CTS improved the cognitive impairment presumably by restoring expression levels of the NR2B subunit and/or the induced secondary changes. Further studies are required to determine whether CTS suppresses the induced secondary changes in JDM rats.

In conclusion, CTS attenuated cognitive deficits in STZ-treated type 1 diabetes model rats. The effects of CTS involved the reversal of malfunctions in NMDA receptors. Our findings provide further evidence for the anti-dementia effects of CTS.

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References
