Protection of glutamate induced neuronal damages in cultured cerebellar granule cells by Chinese herbal medicine, Toki-shakuyaku-san and its comprised six medicinal herbs

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(*Received January 9, 1995. Accepted March 10, 1995.*)

Abstract

The neuroprotective properties of the Chinese herbal medicine, Toki-shakuyaku-san, and its comprised six Chinese herbs (indicated as concentrations), Toki (Angelicae sinensis Radix) 12.5 %, Shakuyaku (Paeoniae Radix) 25.0 %, Bukuryo (Hoelen) 16.7 %, Sojutsu (Atractylodis lanceae Rhizoma) 16.7 %, Takusha (Alismatis Rhizoma) 16.7 %, and Senkyu (Ligustici wallichii Rhizoma) 12.5 %, were investigated in cultured cerebellar granule cells. Measurement of the lactate dehydrogenase (LDH) activity was used as an index to assess the survival of neurons after exposure to glutamate (Glu). Exposure of 1 mM Glu to the 6 days *in vitro* neural cultures for 6 hours led to a 7-fold increase in released LDH activity. These increases in released LDH activity were significantly reduced by treatment with the examined extracts of Chinese medicine and its comprised six herbs. In order to evaluate the protective mechanism of these extracts, the mobilization of intracellular free calcium ion ($[Ca^{2+}]i$) in cultured cerebellar granular cells was examined by means of the fura-2 method. Fifty millimoler KCl increased the $[Ca^{2+}]i$ levels up to 8-fold higher than the steady state levels (100±8 nM). The extracts of Toki-shakuyaku-san and its six herbs, except Shakuyaku, blocked such increases in a dose-dependent manner from dosages of 10 μg/ml to 1 mg/ml. Twenty five micromoler Glu, in the absence of Mg^{2+}, enhanced the $[Ca^{2+}]i$ levels to 400 nM, and the tested extracts also inhibited the Glu increased $[Ca^{2+}]i$ levels in a dose-dependent manner. In conclusion, the protection of extracts of Chinese herbs on the neural dysfunction induced by excessive amounts of Glu might be related to their inhibitory effects on inordinate increases of cytosolic Ca^{2+} levels.

Key words Chinese herbal medicine, cultured cerebellar granule cells, cytosolic calcium ion levels, Ca^{2+} channel inhibition, glutamate, lactate dehydrogenase (LDH) activity.

Introduction

The Chinese prescription of Toki-shakuyaku-san had been mainly applied for women with menoxenia and disorders caused by pregnancy, childbirth, or abortion. In the last several decades, it has been widely used as a tonic for both men and women to improve anemia, fatigability, and poor circulation, *etc.*[1)]

Recent clinical reports showed that Toki-shakuyaku-san can cause improvements in neural dysfunctions, such as Alzheimer's disease, senile dementia and memory-loss.[1,2)] In support of these clinical effects of this Chinese medicine, pre-clinical studies including both *in vivo* and *in vitro* experiments have indicated the improvement of learning and memory functions by using Toki-shakuyaku-san,[3-5)] as well as its neuroprotective properties in glutamate (Glu) induced neu-

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ral death.[6] However, the mechanisms of this Chinese medicine on the effects in the central nervous system, especially its protective effects against neural dysfunction, have not been well evaluated. An evaluation of these mechanisms might find a key to the usefulness of Chinese herbal medicine for the treatments of neurological disorders.

In this paper, we studied the protective qualities of each extract of Toki-shakuyaku-san and its six components, Toki (Angelicae sinensis Radix), Shakuyaku (Paeoniae Radix), Bukuryo (Hoelen), Sojutsu (Atractylodis lanceae Rhizoma), Takusha (Alismatis Rhizoma) and Senkyu (Ligustici wallichii Rhizoma), on the neural dysfunction induced by a large dosage of Glu (1 mM) in cultured cerebellar granule cells, using LDH activity assay.[7] We also examined the inhibitory effects of these extracts on the amounts of cytosolic Ca^{2+} ($[Ca^{2+}]i$) increased by excessive dosage of Glu using the fura-2 method.[8] In the case in which both results can be correlated, the possible protective mechanism of these Chinese herbs in the central nervous system can be discussed. It may be useful to examine the protective effects demonstrated by the aforementioned Chinese herbs in the presence of the mechanism of neural dysfunction due to the cytotoxicity of Glu and the subsequent excessive accumulation of $[Ca^{2+}]i$.[9,15]

Materials and Methods

Materials : The aqueous extracts of Toki-shakuyaku-san and its six composed Chinese herbs, which are massproduced as for clinical use, were kindly supplied by the Kanebo Chinese Traditional Medicine Research Institute (Osaka, Japan). Trypsin, tyrosin inhibitor, DNAase, glutamine, gentamycin, polylysine and cytosine arabinoside were obtained from Sigma Chemical Corp. (St. Louis, MO, U.S.A.). Basal Medium Eagle solution (BME) was purchased every four weeks from Kyokuto Pharm. Ind. Corp. (Tokyo, Japan). Fetal bovine serum was obtained from Bockneck Laboratories (Toronto, Canada). And the other chemicals were purchased from Wako Pure Chemical Corp. (Tokyo, Japan). Glass coverslips (thickness 0.12 to 0.17 mm) were obtained from Matsunami Inc. Corp. (Tokyo, Japan). The thirty-five millimeter pre-coated culture dishes were obtained from Nunc Corp. (Roskilde, Denmark).

Cell cultures : Cultures of cerebellar granule cells were prepared using our previously reported method.[16] Briefly, ten cerebella of 8-day-old Wistar rats were chopped bi-directionally into 400-μm blocks, then incubated at 37°C for 13 min in calcium-free Krebs-Ringer buffer solution containing 0.025% trypsin. The tissue blocks were collected by centrifugation and triturated in the presence of 0.004 % trypsin inhibitor and 0.008 % DNAase. Finally, the collected cells were suspended in BME supplemented with 10 % fetal bovine serum, 5 mM glutamine, 25 mM KCl and 180 μM gentamycin. Then a 2-ml aliquot of the cell suspension was seeded into each 35 mm pre-coated culture dish at a concentration of 1.25×10^6 cells/ml. Cells used for the measurement of $[Ca^{2+}]i$ levels were applied onto the polylysine pre-coated 25 mm glass coverslips. Ten micromol of cytosine arabinoside (final concentration) was added to each dish at 16 hr after suspension to inhibit the proliferation of glia cells.

Assessment of neural damage using LDH activity assay : The Glu-induced neural toxicity was induced by adding a 1 mM Gly (final concentration) to the culture medium.[17] The exposure of Glu for either 15 min, 6 hr or 24 hr was terminated by removal of the incubation medium. Five hundred microliters of the media was assayed for extracellular LDH activity. The remaining media and cells after treatment of two freezings were assayed for the total LDH activity. LDH activity was measured spectrophotometrically by following NADH-oxidation at 340 nm.[18] LDH activity in International Unit (IU) of each aliquot was determined by Wróblewski-La Due method using COBAS/FARA II (Roche, Switzerland). The released LDH ratio was recorded as a percentage of extracellular LDH activity to total LDH activity.

Measurement of $[Ca^{2+}]i$ levels : The measurement of $[Ca^{2+}]i$ in cultured cerebellar granule cells using the fura-2 method and the effects of either KCl or Glu on $[Ca^{2+}]i$ levels were described in our previously published paper.[19] Briefly, the coverslip glass containing neurons was washed twice with Krebs' HEPES (KH) buffered solution (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES, pH 7.4). Then cells were incubated with

5 μM fura 2-AM in KH at 37°C for 45 min. Loaded cells were then transferred into the measuring chamber of the fluorimeter (CAF 100, Jasco, Tokyo) and were superfused with KH (1 ml/min at 37°C). A group of cells was alternately illuminated with 340 / 380 nm excitation. Emitted light was collected at 500 nm and the fluorescences were monitored and recorded by means of computer. The value of $[Ca^{2+}]i$ was determined by the method as described by Grynkiewicz *et al.*[8] The resting $[Ca^{2+}]i$ level in the cultured cells was 100±8.6 nM. The superfusion of 50 mM KCl and 25 μM Glu in Mg^{2+} omitted KH, for 90 sec, maximally enhanced the $[Ca^{2+}]i$ levels to about 800 nM and 400 nM, respectively. These maximum levels were discussed for the pharmacological effects of the examined herbs, since 50 mM KCl and 29 μM Glu were chosen as the stimulating agents in this experiment.

Application of Chinese herbs on cells with Glu-induced neural toxicity : To examine how the Chinese medicine affects the 6 hr exposure of Glu-induced neural toxicity, the treatment of neural cultures with sterilized aqueous extracts was required. The sterilized Chinese herb extract solutions were prepared by the following procedures : each 2 mg/ml of extract was dissolved in BME, and sonicated for 10 min by a ultrasonic machine (Bronson, Seattle, U.S.A.), then the solution was filtered with a 22 μm filter (Millipore, MA, U.S.A.). Each filtered solution was applied at 2 and 0.5 hr prior to Glu treatment by the replacement of 1 ml from the incubation medium. Others were also applied at 0.5 hr after Glu exposure, and then incubated for 5.5 hr. In this case the Chinese herb extract was dissolved in BME containing 1 mM Glu. To determine whether each filtered extract effects either the extracellular or total LDH activity, the dosage of 1 mg/ml of each extract was incubated for 8 hr in sister cultures. The obtained results showed no effects on either LDH release or total LDH activity.

Consideration of self-fluorescence measured by Chinese medicines : The extracts used in the Ca^{2+} measurements were dissolved at the concentration of 10 mg/ml in KH and incubated at 60°C for 1 hr. Then the solution was centrifuged at 3,000 g for 15 min. The supernatant was filtered with Advantec-2 filter paper (Toyo, Tokyo, Japan) and stocked at 4°C for later assay.

The examined aqueous extracts showed self-fluorescence at excitation wavelengths of 340 and 380 nm. These self-fluorescent intensities were examined in both fura 2-AM loaded and unloaded cells. The fluorescent intensity of the unloaded cell was increased at both wavelengths under the superfusion of Chinese medicines, although cells without Chinese medicines did not display any selffluorescence. Such fluorescence disappeared when the Chinese medicines were washed out of the cells with KH. The ratio of 340/380 was not remarkably changed throughout this process. Similar phenomena were seen in the case when Chinese medicines were exposed to fura 2-AM loaded cells. Furthermore, we examined whether the Chinese medicines affect the resting $[Ca^{2+}]i$ or not. The preincubated cells with Chinese medicines were washed and subsequently loaded with 5 μM fura 2-AM for 45 min as described in *"Measurement of $[Ca^{2+}]i$ levels"*. Compared to the resting fura-2 fluorescence in the non-treated cultures, none of the examined Chinese medicines had any effects on the resting fura-2 fluoresence.

Application of Chinese medicines on the 6DIV cells with $[Ca^{2+}]i$ measurement : In experiments on the inhibitory effects of Chinese medicines on the stimulating agents which increase $[Ca^{2+}]i$, the $[Ca^{2+}]i$ levels were determined by the calculated fura-2 ratio which was the subtraction of both background (neuron-free condition) and self-fluorescence of the tested aqueous extracts from the fluorescence obtained at 340 nm and 380 nm.

The application of Chinese medicines onto a fura 2 loaded cell was carried out by the following five steps : 1) 90-sec perfusion with each stimulating agent with no Chinese medicine present, 2) 5-min perfusion with each concentration of Chinese medicine without any stimulating agent, 3) 90-sec perfusion with both Chinese medicine and the stimulating agent, 4) 5-min perfusion with KH containing neither Chinese medicine nor stimulating agent, and 5) repeat of step 1). The inhibitory potentiation of the examined Chinese medicine on the stimulating agents which increased $[Ca^{2+}]i$ was determined by comparing to the results of 1) and 3). The percentage of inhibition was calculated by dividing the results of 3) by the results of 1). Furthermore, the magnitude of $[Ca^{2+}]i$ increase by

either 50 mM KCl or 25 μM Glu was almost the same as that seen in step 1) and in step 5). This means that the influence of Chinese medicine could be abolished by washing.

Statistical analysis : The statistical significance was established as follows. The ANOVA one-way analysis of variance followed by pairwise comparisons using the Scheff test was used for the multigroup comparisons. The statistical analysis between two groups was evaluated by the F-t test. A probability value of 5 % or less was considered indicative of a significant effect.

Results

Neural toxicity induced by 1 mM Glu

To determine the suitable experimental conditions of Glu-induced neural toxicity in cultured cerebellar granule cells, we examined the extracellular LDH activity (Fig. 1A) and released LDH ratio (Fig. 1B) using 3, 6 and 10 DIV cultures with three different exposure times to 1 mM Glu (15 min, 6 hr and 24 hr as shown in Fig. 1). The extracellular LDH activity in the non-treated culture was 4.3±0.9 (IU, mean±S.E. M., n=7). In the case of 3 DIV cultures, exposure to 1 mM Glu for 15 min did not increase the extracellular LDH activity significantly. However, 6 and 24 hr exposures to 1 mM Glu did enhance the extracellular LDH activity compared to the magnitude of the non-treated cell. In 6 DIV cultures, a 15 min exposure to 1 mM Glu elevated the extracellular LDH activity significantly. The extracellular LDH activities of six and twenty four hour exposures to 1 mM Glu were 7-fold higher than each control value. In 10 DIV cultures, the enhanced extracellular LDH levels were significant and these values were the same as those seen in the 6 DIV cultures. Furthermore, we compared the released LDH ratio (Fig. 1B) of each culture in order to identify the actual damage to the cultured cells. As shown in Fig.1B, the highest ratio was seen at 6 hrs exposure to 1 mM Glu in 6 DIV cultures. This result was chosen as the most appropriate experimental condition for examining the protective effects of Chinese medicine on Glu-induced neural toxicity.

Protective effects of Chinese medicine on LDH release induced by 1 mM Glu

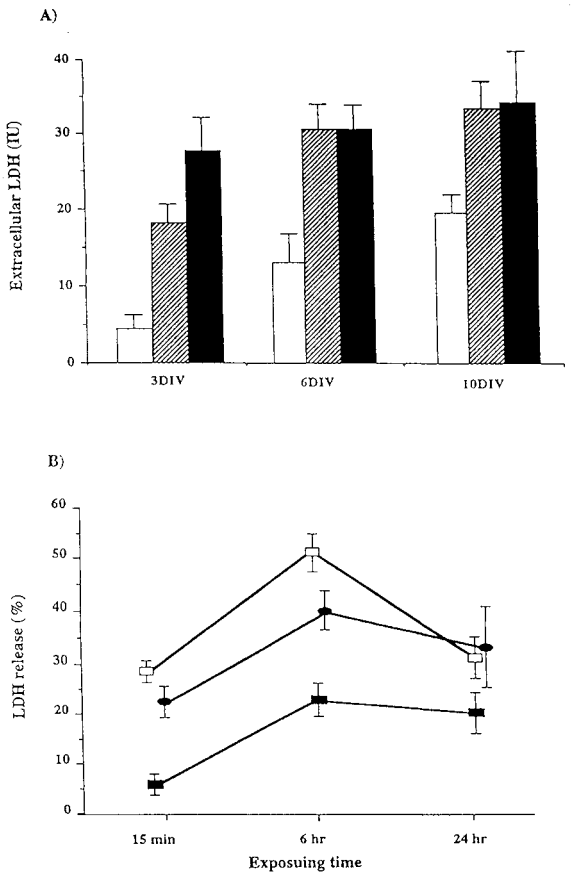

Fig. 1 A) The extracellular LDH activity enhanced by exposure to 1 mM Glu for 15 min (open bar), 6 hr (oblique bar) and 24 hr (closed bar) in 3 DIV, 6 DIV and 10 DIV cultured cerebellar granule cells. Each data represents the mean±S.E.M. of 6 to 8 experiments. B) The percentage of extracellular LDH to total LDH in each examined 3 DIV (■ – ■), 6 DIV (□ – □) and 10 DIV (● – ●) sample. Six hours exposure to 1 mM Glu of 6 DIV cultures shows the highest ratio.

Protective effects of the extracts against 1 mM Glu-induced neural toxicity are shown in Table I. Data represent the released LDH ratio as the mean± S.E.M. of 4 ~ 8 separate experiments. One mg/ml (final concentration) of Chinese medicine reduced the Glu-induced LDH release in all treated cultures. The cultures treated with the Chinese medicine prior to Glu exposure showed a significant inhibition of Glu-induced LDH release. Whereas the inhibition of Shakuyaku, Bukuryo and Sojutsu were slightly weaker in the case of 30 min prior to Glu treatment than those seen in the 2 hr pretreated cultures, there were no significant differences between the 2 hr and 30 min

Table I Inhibition by the Chinese herbs on 1 mM Glu induced LDH release in cultured cerebellar granule cells.

	2 hr before	30 min before	30 min after
Vehicle/1 mM Glu	51.0±2.1 (%)	48.6±3.3 (%)	46.9±5.8 (%)
Toki-shakuyaku-san/1 mM Glu	27.6+1.7**	25.2±1.6**	27.3±1.8*
Toki/1 mM Glu	23.2±1.6**	26.0±1.5**	34.9±2.5
Shakuyaku/1 mM Glu	26.0±1.7**	34.1±1.7*	44.6±5.9
Bukuryo/1 mM Glu	24.6±3.1**	30.7±1.4*	37.2±3.5
Sojutsu/1 mM Glu	25.6±1.8**	30.5±4.7*	38.4±6.1
Takusha/1 mM Glu	24.4±1.1**	21.1±0.9**	24.3±2.6*
Senkyu/1 mM Glu	36.8±1.8**	26.7±1.6**	24.2±2.4*

Data represent the percentage of extracellular LDH activity/total LDH activity (mean±S.E.M. of 4 to 8 experiments). The application of Chinese herb extract was described in *"Material and Methods"*. The exposure to 1 mM Glu to sister cultures, as a control, was replaced with half of the BME incubation medium in the same process as the Chinese herb treated cultures were done. Significant difference were obtained to compare the "Vehicle/1 mM Glu" v.s. "each Chinese herb/1 mM Glu" ; $^{**}p<0.01$, $^{*}p<0.05$.

Chinese herb preincubation groups. In the application of Chinese medicine at 30 min after Glu treatment, Toki, Shakuyaku, Bukuryo and Sojutsu did not show any significant inhibition. However, the treatments of Toki-shakuyaku-san, Takusha and Senkyu showed significant inhibition of Glu induced LDH release in three different application periods.

Ca^{2+} antagonistic effects of Toki-shakuyaku-san and its components

The inhibition curves of Toki-shakuyaku-san to the 50 mM KCl and 25 μM Glu-induced $[Ca^{2+}]i$ increase are shown in Fig. 2A and Fig. 2B, respectively. In Fig. 2A, the dose of 10 μg/ml Toki-shakuyaku-san showed about 10 % inhibition to the 50 mM KCl induced $[Ca^{2+}]i$ increase, and 1 mg/ml Toki-shakuyaku-san completely blocked such $[Ca^{2+}]i$ increase. In the case of the 25μM Glu enhanced $[Ca^{2+}]i$ increase, 200 μg/ml Toki-shakuyaku-san showed about 10 % inhibition of such a $[Ca^{2+}]i$ increase, and 1 mg/ml of this medicine inhibited more than 90 % of the $[Ca^{2+}]i$ levels increased by Glu (Fig. 2B). Fig. 3 shows the inhibitory potency of the six components, Toki, Shakuyaku, Senkyu, Bukuryo, Sojutsu and Takusha, to the 50 mM KCl (solid bars) or 25 μM Glu (closed bars) induced $[Ca^{2+}]i$ increase. The six components, except Shakuyaku, inhibited the 50 mM KCl enhanced $[Ca^{2+}]i$ increase in a dose-dependent manner from 100 μg/ml to 1 mg/ml. And inhibition of all six components by

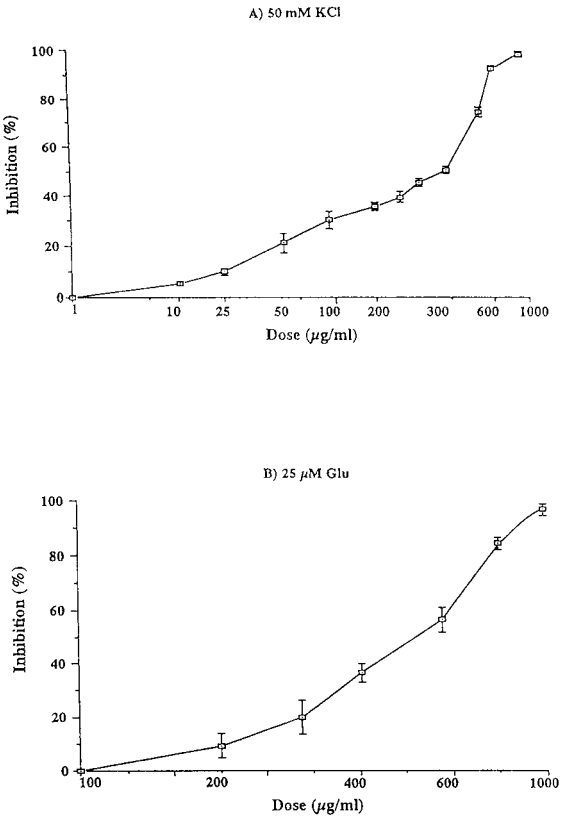

Fig. 2 A) The inhibitory effects of Toki-shakuyaku-san on 50 mM KCl induced $[Ca^{2+}]i$ levels increase. B) The inhibitory effects of Toki-shakuyaku-san on 25 μM Glu-induced $[Ca^{2+}]i$ levels increase. Each data represents mean ±S.E.M. of 3 to 6 experiments.

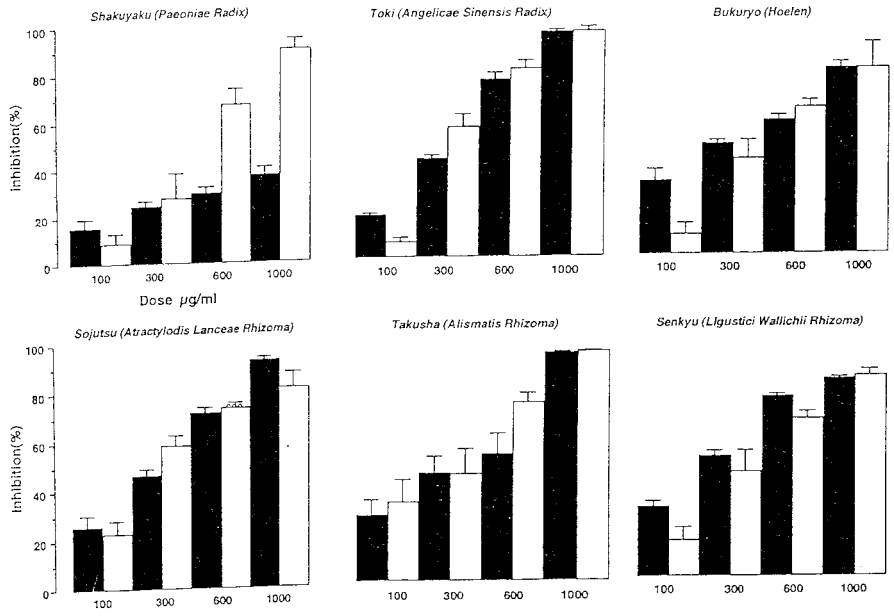

Fig. 3 Inhibitory effects of the six components in Toki shakuyaku-san on either 50 mM KCl (dark dar) or 25μM Glu (open bar) increased $[Ca^{2+}]i$ levels in cultured cerebellar granule cells. Each data represents the mean±S.E.M. of 3 to 6 experiments.

Table II Fifty percent inhibition concentration (IC_{50}) by Toki shakuyaku-san and its six components on 50 mM KCl and 25 μM Glu induced $[Ca^{2+}]i$ increase in cultured cerebellar granule cells.

	50 mM KCl	25 μM Glu
Toki-shakuyaku san	300±14 μg/ml	550±45 μg/ml
Toki	230±10	361±41
Shakuyaku	>2000	291±14
Bukuryo	279±27	411±49
Sojutsu	308±19	410±51
Takusha	231±21	326±33
Senkyu	349±31	238±38

Each data represents mean±S.E.M. of 6 experiments.

the 25 μM Glu increased $[Ca^{2+}]i$ levels were seen in a corresponding manner. The fifty percent inhibition values (IC_{50}, mean±S.E.M., n=3~6) of the examined Chinese medicines are shown in Table II. The IC_{50} value of Shakuyaku to the 50 mM KCl induced $[Ca^{2+}]i$ increase was above 2 mg/ml, and this value was significantly larger than those of the other components. In the inhibition of the 25 μM Glu induced $[Ca^{2+}]i$ increase, Toki-shakuyaku-san was weaker than its components, but this was of no significance.

Discussion

In general, the prescription of Chinese herbal medicine has often been prepared with the mixture of several Chinese herbs as empirical knowledge. And such a "cocktail" of Chinese herbal medicines appeared to be much more potent in the treatment of diseases than each isolated chemical composition of those Chinese herbs. Therefore, it might be important to examine the pharmacological properties of this Chinese herbal medicine using the entire "cocktail".

In the goal of preventing inconsistent results due to the extraction of Chinese herbs, we used the extracts of one Chinese herbal medicine and its six comprised herbs which are routinely prepared in the pharmaceutical company as medicine on the market.

The experiments presented in this article identified : 1) Pre-treatments of Toki-shakuyaku-san and its six components inhibited 1 mM Glu-induced neural damage in cultured cerebellar granule cells, and post-treatments of Toki shakuyaku-san, Takusha and Senkyu also blocked these neural damages. 2)

Toki-shakuyaku-san and five of its components inhibited both the 50 mM KCl and 25 μM Glu-induced $[Ca^{2+}]i$ increases in a dose-dependent manner, although the inhibition of Shakuyaku on the 50 mM KCl induced $[Ca^{2+}]i$ increase much weaker than that on the 25 μM Glu increased $[Ca^{2+}]i$.

In experiments of Glu-induced neural toxicity (Fig. 1A), our results were slightly different from the data of others.[7, 17] They observed that immature neurons in cortical cultures were apparently resistant to the Glu neural toxicity, and the basis for the resistance of immature neurons to excitotoxin was explained by reasoning that the immature neurons have fewer membrane receptors for Glu than the mature neurons. In our experiments, exposure of 3 DIV cultures to 1 mM Glu for 6 and 24 hrs did significantly increase the extracellular LDH activity, although the LDH activity was not apparently changed in 3 DIV cultures which were exposed to 1 mM Glu for 15 min. The discrepancies between our results and the others can be explained by the different ontogenetic development of Glu receptors in cerebellar granule cell cultures as opposed to cortical cultures.[20, 21] For instance, our data obtained from the experiment of Glu-induced $[Ca^{2+}]i$ increases in cultured cerebellar granule cells showed that the responses of such $[Ca^{2+}]i$ increases induced in 3 and 5 DIV cultures were as good as those obtained in 10 to 14 DIV cultures. Furthermore, the maximum response of $[Ca^{2+}]i$ obtained by larger dosages than 25 μM Glu was of similar levels, although the dose of more than 25 μM Glu prolonged the recovery time of the $[Ca^{2+}]i$ levels to their unstimulated level.[19] Therefore the inhibition of the initial increase of $[Ca^{2+}]i$ might be significant in protecting the vulnerability of neural systems.

Exposure of 1 mM Glu to 6 and 10 DIV cultures induced significant neural toxicity. Six and twenty-four hour exposures of 1 mM Glu showed similar extracellular LDH activity in both cultures. In the 6 DIV culture, and this ratio was the hightest value in the examined cultures, the LDH release ratio in the 6 hr exposed cultures was higher than that seen in the 24 hr exposed cultures. This difference might be explained as an increase of total LDH activity due to the proliferation of glia in the extra 18 hr incubation with 1 mM Glu.[22] In particular, we have previously reported that the growth of glial cells stained by GFAP in the 6 DIV cultuures could be much faster than those in the 3 and 10 DIV cultures.[16] The increased LDH release ratio was related to the magnitude of neural damages in cultured cells, because the study of trypan blue dye in the sister cultures identified the number of stained cells (data are not shown).

In 6 DIV cultures which were exposed to 1 mM Glu for 6 hrs, the pre-treatment of one mg/ml of Toki-shakuyaku-san and its components protected against the Glu-induced neural damages indicated by LDH release ratio (Table I). This examined dosage of each extract was seen to block the increase of Glu-induced $[Ca^{2+}]i$ levels completely (Fig. 2B, Fig. 3). In this blocking effect, Toki-shakuyaku-san and Shakuyaku, but not others, demonstrated different pharmacological properties with respect to the inhibition of $[Ca^{2+}]i$ increased by 50 mM KCl and as to the inhibition of $[Ca^{2+}]i$ increased by 25 μM Glu. The IC_{10} values of Toki-shakuyaku-san to high-K-and Glu-increased cytosolic Ca^{2+} levels were about 10 and 200 μg/ml, respectively (Fig. 2), although the IC_{50} values of this Chinese medicine to both channels were about 1.8-fold different. Furthermore, the IC_{50} value of Shakuyaku to high-K-increased $[Ca^{2+}]i$ levels was more than 2000 μg/ml, but the IC_{50} value to Glu-increased $[Ca^{2+}]i$ levels was about 300 μg/ml. At present, it is difficult to explain the exact reason why Shakuyaku showed the different effects between Glu and high-K increased $[Ca^{2+}]i$ levels. But the antagonistic action of Chinese herbs on the voltage-dependent and/or receptor-operated Ca^{2+} channels has been reported in several papers.[23-25] And these papers discussed that their antagonistic actions might be due to their membrane stabilizing effects. The pharmacological mechanisms of Chinese herbs on neural cells can be discussed in regards to several possibilities because of their "natural" elements. In the present paper, IC_{50} values of examined medicinal herbs, except for Shakuyaku, in respect to 50 mM KCl increased $[Ca^{2+}]i$, were similar. These results document that a low dose of Toki-shakuyaku-san readily blocks voltage-dependent calcium channel (VOC) activities, and higher dosages completely inhibit both VOC and the Glu related Ca^{2+} channels (e,g., NMDA receptor operated Ca^{2+} channel and/or the subsequent

increased activities of VOC), although the pharmacological effects of Shakuyaku mainly appeared through the inhibition of the Glu related Ca^{2+} channel activity. These antagonistic effects on VOC and the Glu related Ca^{2+} channel might be related to the blocking effects of pre-treatments of Chinese medicine on Glu induced neural toxicity.

In addition, as in our previously reported paper,[19] the organic Ca^{2+} antagonists, flunarizine, SM6586, SM12565 and nifedipine, also blocked the Glu increased $[Ca^{2+}]i$ levels, and the IC_{50} values were 3 to 35 μg/ml. Furthermore, these antagonists can block the Glu induced neural toxicities. Although the examined Chinese medicines were much weaker inhibitors in the case of the Glu related Ca^{2+} channels than these Ca^{2+} antagonists, as shown in Table II. These extracts of herbs appeared to exhibit the same pharmacological properties in relation to the excitotoxicities (see Table I).

Given the calcium hypothesis on Glu neural toxicity,[12] an excessive $[Ca^{2+}]i$ increase induced by Glu might be an important factor in Glu neural toxicity. Specifically, the inhibition of Ca^{2+} influx through either N-methyl-D-aspartate (NMDA) receptors, some of the non-NMDA receptors, or VOC by the antagonists could improve the neural dysfunction and protect against neural death.[7,23-25] In our experiments, Toki-shakuyaku-san and its six components are considered to have blocking effects on both VOC and the receptor operated calcium channels (Fig. 2, Fig. 3). Additionally, Toki-shakuyaku-san inhibited 100 μM NMDA induced $[Ca^{2+}]i$ increases in the same manner as seen in the inhibition of the 25 μM Glu induced $[Ca^{2+}]i$ increase (data are not shown).

Some of the examined Chinese medicine, Toki-shakuyaku-san, and two of its' components, Takusha and Senkyu, blocked the increase of LDH induced by Glu even though they were applied at 30 min after Glu treatment of the cell. These results appear to be unclear, since it can not be deduced by only their Ca^{2+} antagonistic actions. In the recent decade, it has not been clearly proven that the NMDA antagonists and Ca^{2+} antagonists can be useful for the treatment of apoplepsy. However, in our most recent results, some modulators of immuno-active substances blocked the hippocampul neural cell death in the hypoglycemia two vessel occulusion model, in spite of the fact that these were treated at 30 min after occlusion. It has been well-discussed that the Chinese herbal medicines have immuno-active or suppressive actions.[29] Therefore Toki-shakuyaku-san, Takusha and Senkyu may modulate the immuno-active substances which regulate the cellular functions, and these herbs appear to exhibit protective effects on Glu-increased excessive LDH release.

In conclusion, it might be suggested that one of the mechanisms of Toki-shakuyaku-san in blocking excessive amounts of Glu induced neural toxicity is the inhibition of a sustained increase of $[Ca^{2+}]i$ induced by Glu. Therefore, Toki-shakuyaku-san may be useful in prophylasis and/or in emergency therapy involving neural damage induced by Glu and this property might more specifically related to the pharmacological action of Takusha and Senkyu.

Acknowledgments

We thanks Drs. Jon Stocker and Kirk Mower for their helpful advice in preparing the manuscript. Partial financial supports from the Grant-in-Aid for Scientific Research and the Japan Health Sciences Foundation (Y.W. & T.S.) is gratefully acknowledged. X.Q.Z. is a Ph.D. student with financial support from the Sasakawa Peace Foundation, Japan (93012).

和文抄録

和漢薬の神経保護作用をラット培養小脳顆粒細胞を用いて検索した。和漢薬として当帰 (Angelicae scinensis Radix) 13 %, 芍薬 (Paeoniae Radix) 25 %, 茯苓 (Hoelen) 18 %, 蒼朮 (Atractylodis Lanceae) 18 %, 沢瀉 (Alismatis Rhizoma) 18 %, 川芎 (Ligustici wallichii Rhizoma) 15 % を含有する当帰芍薬散及び6種の生薬の抽出成分を 10 μg/ml から 1000 μg/ml の濃度に調節して実験に供した。1 mM glutamate (Glu) を6日目の培養細胞に6時間処置すると LDH 活性は7倍に上昇した。この Glu による LDH 活性の上昇は，2時間及び30分前処置をした当帰芍薬散及び6つの構成生薬によって著明な抑制が認められた。さらに，Glu 処置 30 分後に適用した当帰，芍薬，沢瀉，及び川芎は，Glu 誘発の LDH 活性上昇を著明に抑制した。この和漢薬の Glu 誘発神経障害抑制機序の一端を明らかにするため，細胞内

Ca^{2+} 上昇阻害作用を Fura-2 法により検索した。50 mM KCl 誘発の細胞内 Ca^{2+} 上昇は定常状態（100±8 nM）と比較して約8倍であった。芍薬を除いた5種類の生薬及び当帰芍薬散は用量依存的な，しかも著明な抑制作用を有することが明らかとなった。25 μM Glu 誘発によって細胞内 Ca^{2+} 濃度は約 400 nM まで上昇した。この上昇に対して，本研究に供した方剤及び6種の生薬はいずれも用量依存的で，且つ著明な抑制作用を示した。しかも，この阻害濃度は，LDH 活性上昇抑制濃度と同程度であった。以上の研究成績から，本研究で用いた和漢薬の神経保護作用として，Glu 誘発細胞内 Ca^{2+} 過剰増量に対する抑制効果は機序の一つと成り得ることが示唆された。

References

1) Chen, Z.Q. and Gao, Z.G.: Clinic application of Tang Kuei and Peony Formula. *Mintong Med. Journal* **155**, 17-21 (abstract in English), 1989.
2) Narita, H.: Kampo therapy for psychiatric region. *Jpn. J. Neuropsychopharmacol.* **12**, 165-172 (abstract in English), 1990.
3) Hagino, N.: The mode of action of Toki-Shakuyaku-San (TJ-23) on the brain: With special reference to Alzheimer's disease. *Jpn. J. Neuropsychopharmacol.* **12**, 229-234 (abstract in English), 1990.
4) Fujiwara, M.: Toki-Shakuyaku-San (TJ-23) improves the cognitive disruption caused by central cholinergic dysfunction. *Jpn. J. Neuropsychopharmacol.* **12**, 217-227 (abstract in English), 1990.
5) Watanabe, H., Satoh, T., Matsumoto, K., Ohta, H., and Shimada, H. and Shimada, T.: Psychopharmacological effects of Shimotsu-to and Shimotsu to components, Toki, Shakuyaku, Jio and Senkyu, in the mice. *J. Med. Pharm. Soc. WAKAN-YAKU* **8**, 102-107, 1991.
6) Kataoka, Y., Kouzuma, M., Koizumi, S., Niwa, M. and Taniyama, K.: Neuroprotective effects of Toki Shakuyaku-San (TJ-23) on hypoglycemia/hypoxia-induced neuronal damage in rat striatal slices. *Phytotherapy Res.* **7**, 67-69, 1993.
7) Koh, J. Y. and Choi, D.W.: Quantitative determination of glutamate mediate cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J. Neurosci. Methods* **20**, 83-90, 1987.
8) Grynkiewicz, G., Poenie, M. and Tsien, R.Y.: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450, 1985.
9) Schanne, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L.: Calcium dependence of toxic cell death: a final common pathway. *Science.* **206**, 700-702, 1979.
10) Miller, R.J.: Multiple calcium channels and neuronal function. *Science* **235**, 46-52, 1987.
11) Collingrige, G.L. and Bliss, T.V.P.: NMDA receptors: their role in long-term potentiation. *Trends Neurosci.* **10**, 288-293, 1987.
12) Choi, D.W.: Calcium-mediated neurotoxicity: relationship to speciffic channel types and role in ischemic damage. *Trends Neurosci.* **11**, 465-469, 1988.
13) Watanabe, Y., Zhang, X Q. and Shibuya, T.: Practicability of calcium channel blockers for the treatment of highly brain dysfunction. *Jpn. Pharmacol. Ther.* **18**, 3565S-3572S, 1990.
14) Michael, R.L. and Rothman, S.M.: Glutamate neurotoxicity in vitro: antagonist pharmacology and intracellular calcium concentration. *J.Neurosci.* **10**, 2545-2551, 1991.
15) Crowder, J.M., Croucher, M.J., Bradford, H.F. and Collins, J.F.: Excitatory amino acid receptors and depolarization induced Ca^{2+} influx into hippocampal slices. *J. Neurochem.* **48**, 1917-1924, 1991.
16) Watanabe, Y., Endo, T., Zhang, X-Q., Masuda, M., Hasegawa, K. and Shibuya, T.: A newly developed method for quantal measurement of glia cells in cultured neuronal cells using a flow cytometry. *Neurosciences (Japan)* **17**, 261-269, 1991.
17) Lustig, H.S., Chan, J. and Greenberg, D.A.: Comparative neurotoxic potential of glutamate, endothelines, and platelet-activating factor in cerebral cortical cultures. *Neurosci. Lett.* **139**, 15-18, 1992.
18) Bergmeyer, H.U.: Biochemical reagents: In "Methods of enzyme analysis" (Bergmeyer H.U.Ed) Academic Press, New York, 2nd Ed, p.480, 1974.
19) Zhang, X Q., Watanabe, Y. and Shibuya, T.: Comparative studies on the inhibitory effects of calcium antagonists on cytosolic Ca^{2+} levels increased by high potassium or glutamate in cultured rat cerebellar granular cells. *Jpn. J. Pharmacol.* **62**, 411-414, 1993.
20) Balazs, R., Regan, C.M., Gordon, R.D., Annunziata, P., Kingsbury, A.E. and Meier, E.: Certain surface properties of isolated and cultured cerebellar cells. In "Neurotransmitter interaction and compartmentation." (Bradford, H.F.Ed). Plenum press, New York and London, pp.515-534, 1981.
21) Pearce, B.R. and Dutton, G.R.: Amino acid neurotransmitter release. In "Neuroscience approached through cell culture." (Pfeffer S.E.Ed) CRC Press, Florida, pp.143-156, 1982.
22) Uchihori, Y. and Pour, D.G.: Glutamate as a neuron to glia for mitogenesis: role of glia N-methyl-D aspartate receptors. *Brain Res.* **613**, 212-220, 1993.
23) Yamahara, J. Miki, S., Matsuda, H., Kobayashi, G. and Fujimura, H.: Screening test for calcium antagonist in natural products. The active principles of Uncariae ramulus et uncus. Folia *Pharmacol. japan.* **90**, 133-140 (Abstract in English), 1987.
24) Hidaka, S., Abe, K., Takeuchi, Y. and Liu, S.Y.: Inhibition of the formation of oral calcium phosphate precipitates: beneficial effects of Chinese traditional (kampo) medicines. *J. Perio. Res.* **28**, 27-34, 1993.
25) Aiko, S., Tadashi, T., Kunitsugu, K., Masahiro, M and Eiichi, S.: Inhibitory effects of peony root extract on pentylenetetrazol induced intracellular calcium increase. *Bioimages* **1**, 115-123, 1993.
26) Ciardo, A. and Meldolesi, J.: Regulation of intracellular calcium in cerebellar granule neurons: effects of depolarization and of glutamatergic and cholinergic stimulation. *J. Neurochem.* **56**, 184-191, 1991.
27) Dubinsky, J.M. and Rothman, S.M.: Intracellular calcium during "chemical hypoxia" and excitotoxic neuronal injury. *J. Neurosci.* **11**, 2545-2551, 1991.
28) Dubinsky, J.M.: Intracellular calcium levels during the period of delayed excitotoxity. *J. Neurosci.* **13**, 623-631, 1993.
29) Chang, R.S. and Yeung, H.W.: Inhibition of growth of human immunodeficiency virus *in vitro* by crude extracts of Chinese medicinal herbs. *Antiviral Res.* **9**, 163-176, 1989.