Effects of Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli-Tang) and Saiko-keishi-kankyo-to (Chaihu-Guizai-Ganjiang-Tang) on brain acetylcholine levels and on learning and memory in mice

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Abstract

In traditional Chinese medicine, Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli-Tang : SRT) and Saiko-keishi-kankyo-to (Chaihu-Guizai-Ganjiang-Tang : SKT) are both used for the treatment of common nervous conditions, although there is a difference in the clinical application of the two agents : SRT for a robust (sthenia) and nervous constitution, and SKT for a deficient (asthenia) and nervous constitution. We reported previously that SRT and SKT had influence on the levels of dopamine- and serotonin-related substances in mouse brain, although neither agent had a dose-related influence on any area of the brain. In the present study, to clarify an influence of SRT and SKT on the function of the central cholinergic nervous system, the effects of the agents on brain ACh levels and on deficits in learning and memory in mice were examined.

SRT increased ACh levels in the corpus striatum and hypothalamus and prolonged the shortened latency of the scopolamine-induced step-down passive avoidance response in mice. However, it had no influence on the learning and memory disorder in trimethyltin (TMT)-treated mice using a multiple maze task. On the other hand, SKT increased ACh levels in the hypothalamus, but had no influence on the behavioral task in the scopolamine-and TMT-treated mice. These results suggest that there is a difference in the mode of action between SRT and SKT on the central cholinergic nervous system in mice, although both agents are used in the Chinese medicine for the treatment of common nervous conditions.

Key words Kampo medicine, acetylcholine, learning and memory, mouse.

Abbreviations ACh, acetylcholine ; CMC, carboxy-methylcellulose ; SKT, Saiko-keishi-kankyo-to (Chaihu-Guizai-Ganjiang-Tang), 柴胡桂枝乾姜湯 ; SRT, Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli-Tang), 柴胡加竜骨牡蛎湯 ; TMT, trimetyltin chloride.

Introduction

In traditional Chinese medicine, Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli-Tang) is a well-known treatment for patients with a robust (sthenia) and nervous constitution, who have common subjective symptoms such as tenderness or discomfort of the hypochondrium, fullness of the upper abdomen and palpitation of the region above and below the umbilicus.[1,2] Saiko-keishi-kankyo-to (Chaihu-Guizai-Ganjiang-Tang) is used for patients with a deficient (asthenia) and nervous constitution,[1,2] who have common symptoms similar to those for which Saiko-ka-ryukotsu-borei-to is prescribed. Therefore, although both agents are used to treat common nervous condi-

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tions, there is a difference in their application depending on the pattern of the patient's symptoms.

We reported previously that Saiko-ka-ryukotsu-borei-to[3-5] and Saiko-keishi-kankyo-to[5,6] showed an increase in the levels of dopamine- and serotonin-related substances in mouse brain, although neither agent had a dose-related influence on brain region.

In the present study, to clarify an influence of Saiko-ka-ryukotsu-borei-to and Saiko-keishi-kankyo-to on the function of the central cholinergic nervous system, the effects of the agents on brain acetylcholine levels, and on deficits in learning and memory in mice were examined.

Materials and Methods

Animals : Four hundred forty male ddY mice, 6 weeks old, weighing 25~30 g, were purchased from the Shizuoka Laboratory Animal Center. The animals were housed, as a group of 10, in a room maintained at 22±2°C with 55±5 % humidity. Lights were on from 7:00 to 19:00. The animals were given free access to both water and standard laboratory chow. All experiments using animals were performed according to the Guide for Care and Use of Laboratory Animals at Iwate Medical University.

Chemicals : The agents used in the present experiment were extract powders (SRT and SKT) from Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli-Tang; Lot. No.890012001 p1) and Saiko-keishi-kankyo-to (Chaihu-Guizai-Ganjiang-Tang ; Lot. No. 1219300), and were provided by Tsumura & Co. SRT and SKT were suspended in a 5 % carboxymethylcellulose solution (CMC), and dosages of 50 and 400 mg/kg for SRT and of 75 and 750 mg/kg for SKT were prepared based on the dose per day for a human, respectively. In the single administration experiment, mice of each group were orally given a single dose of either CMC (10 ml/kg), SRT or SKT. In the repeated administration experiment, mice were orally given, once a day for 14 days, a dose of either CMC, SRT or SKT.

Chemicals were obtained from the following sources : Scopolamine hydrobromide from Kyorin Pharmaceutical Co. ; tetramethylammonium chloride, choline chloride and 70 % perchloric acid from Wako Pure Chemicals ; $Na_2HPO_4 \cdot 2H_2O$, Na_2EDTA, H_3PO_4 and trimethyltin chloride (TMT) from Kanto Chemicals ; acetylcholine perchlorate (ACh) from Sigma Chemicals ; and heptanesulfonic acid and 1-octanesulfonic acid sodium salt from Aldrich Chemicals. Ethylhomocholine was synthesized in our laboratory according to the method of Potter *et al.*[7] All other reagents and solvents were of analytical grade and used without further purification.

Experiment on step-down passive avoidance response :

1) Apparatus. The device consisted of a box (16W×14D×17H cm) made of a clear acrylic plate, a floor with steel grids of 2 mm in diameter at 8 mm intervals, and a wooden platform (5W×5D×3H cm) set on the grid in one corner. Electrostimulation was administered through the grid which was connected to an isolator (S5-202J, Nihon Kohoden).

2) Experimental procedure. (1) Acquisition trial : The trial was performed in the following order. (i) First a mouse was placed on the wooden platform. (ii) When the mouse stepped down to the grids from the wooden platform, electrostimulation (90 V, 50 Hz, 10 msec.) was given for 5 sec. (2) Retention trial : The trial was performed 24 hrs. after the acquisition trial. That is, the lapsed time (sec.) from placing a mouse on the wooden platform to stepping down from the platform was measured, with a cutting off time of 300 sec.

3) Enforcement. (1) Single administration of SRT and SKT : The acquisition trial was performed 30 min. after the i.p. administration of 10 ml/kg saline solution or 0.5 mg/kg scopolamine, which causes a dysfunction of the parasympathetic nervous system and a disorder of the step-down passive avoidance response, using mice that had received a dose of either CMC, SRT at 50 and 400 mg/kg, or SKT at 75 and 750 mg/kg, 120 min. prior to the beginning of the trial. The retention trial was performed 24 hrs. after the acquisition trial. (2) Repeated administration of SRT and SKT : The acquisition trial was performed 30 min. after the i.p. administration of 10 ml/kg saline solution or 0.5 mg/kg scopolamine, using mice that had received a dose of either CMC at 10 ml/kg, SRT at 50 and 400 mg/kg, or SKT at 75 and 750 mg/kg, 120 min. prior to the beginning of the trial on the day after the final administration of CMC, SRT or SKT. The

retention trial was performed 24 hrs. after the acquisition trial.

Experiment on learning and memory using a multiple maze task :

1) Apparatus. The apparatus (50W×110D×15H cm) developed in our laboratory,[8,9] as shown in Fig. 1, consisted of a starting area, four maze areas, a home area and a connecting tunnel. Each maze area had four alleys. A water tap was placed in the starting area and food was placed in the home area. The home area, which had a wooden tip, was covered with a black lid. The order of alleys opened in the maze was B-A-C-A, and this order was fixed during the experiment. When the mice were housed in this apparatus, they spontaneously learned the structure of the maze over a period of several days while they ran between the food area and the water area. The mice were continuously habituated in this apparatus and were trained there until the learning was completed.

2) Experimental procedure. While mice were housed in the maze apparatus, they were given a test session consisting of one trial every day. Sixteen hours before the next day's trial, mice were removed from the maze apparatus to other cages for fasting. In the trials, the number of errors (entering a blind alley with all four feet) and running time [lapsed time (sec.) from starting area to home area] were counted until the mouse entered the home area. If a mouse did not complete the learning of the maze within 5 min., the trial was stopped. At the end of each trial, the maze was cleaned to ensure that odour cues did not influence alley way selection. After the trials, all mice were again housed in their respective maze apparatus. The criterion level set for the achievement of learning was less than 4 errors for the mean number of errors and 90 sec. for the mean running time, for 4 consecutive days. The day after this criterion was achieved, the mice were orally treated with 10 ml/kg saline solution or with 3.5 mg/kg TMT, which is a potent neurotoxicant producing neuronal degeneration and disorders of short-term memory.[10-12] After treatment with TMT, the mice were removed from the maze apparatus to their respective breeding cages, which had been placed in another experimental room, until testing was performed.

3) Enforcement. (1) Single administration of SRT and SKT : After treatment with TMT, the mice were housed in breeding cages for 18 days, with free access to both water and standard laboratory chow. On the 19th day after treatment with TMT, a retention test of memory was performed and the number of errors and running time were counted, using mice that had individually received a dose of either CMC at 10 ml/kg, SRT at 50 and 400 mg/kg, or SKT at 75 and 750 mg/kg, 120 min. prior to the beginning of the test. (2) Repeated administration of SRT and SKT : After treatment with TMT, the mice were housed in breeding cages. From the 6th day after treatment with TMT, the mice were orally given a dose of either CMC at 10 ml/kg, SRT at 50 and 400 mg/kg, or SKT at 75 and 750 mg/kg, once a day for 14 days. On the 19th day after treatment with TMT, a retention test of memory was performed 120 min. after final administration of the agents and the number of errors and running time were counted.

Tissue preparation : Two hours after the single or final (repeated) administration of each agent, mice were irradiated with a microwave beam (5 kW, 0.7 sec., TMW-6402C, Toshiba) that was focused on the head. The brain was removed from each mouse and dissected into four regions : cerebral cortex, hypothalamus, corpus striatum and hippocampus, on an ice-chilled glass plate according to a modified method of Glowinski and Iversen.[13] The isolated tissues were quickly frozen on dry ice, and then weighed and stored in a 1.5 ml Eppendorf microtube at -80°C until extraction.

After thawing, the isolated tissues were homogenized with an ultrasonic cell disruptor (Model 200, Branson, USA) in 200 μl of an ice-chilled 0.1M perchloric acid solution containing 100 μl ethylhomocholine (as an internal standard for acetylcholine assay) and 0.1 mM Na_2EDTA for the corpus striatum, and in 160 μl of those for the hypothalamus, cerebral cortex and hippocampus. The homogenate was centrifuged at 12,000 g for 20 min at 4°C, and the clear supernatant was filtered through a 0.45 μm filter (Type HV, Nihon Millipore) and stored at -80°C until assay.

Chromatographic conditions for ACh : ACh was measured by an HPLC-ECD system according to the method described by Murai *et al.*[14] The HPLC system consisted of a delivery pump (Model L-5000,

Yanagimoto), a six-port injector (Model 7125, Rheodyne), RP-guard column (Eicom Perpak, 4.5 mm × 4 mm I.D., 7 μm, Eicom), an analytic column (Eicompak AC-GEL, 150 mm × 6 mm I.D., styrene polymer 10 μm, Eicom), an immobilized enzyme column (Eicom AC-Enzympak, 5 mm×4 mm I.D., 12 μm, silicagel, Eicom), a catecholamine-trap column (Eicom CA-Trap, 5 mm×4 mm I.D., 12 μm, styrene polymer, Eicom), and a Shimadzu CR-6A data processor (Shimadzu).

An immobilized enzyme column containing coveniently bound choline oxidase (EC 1. 1. 3. 17) and acetylcholinesterase (EC 3. 1. 1. 7) was inserted between the analytic column and working electrode. A catecholamine-trap (CA-T) column was connected just behind the immobilized enzyme column. The analytical column and the immobilized column were maintained at 33°C in a water bath. An electrochemical detector (Model VMD-5000, Yanagimoto) with a platinum working electrode (WE-PT, Eicom) was used at a voltage setting of +0.5 V versus an Ag/AgCl reference electrode. The mobile phase was a 0.07M phosphate buffer containing 0.06 % (wt/vol) Na_2EDTA, 0.065 % (wt/vol) tetramethylammonium chloride and 0.3 % (wt/vol) sodium 6-octanesulfonic acid. After adjusting to pH 8.3-8.5 with H_3PO_4, the mobile phase was filtered through a 0.45 μm filter and degassed by water aspiration. The conditions of measurement were set as follows: a flow rate of 2.0 ml/min. with a temperature of 33°C, which yielded a pressure of 60 kg/cm^2.

Statistical analysis : The results obtained were expressed as the mean values±S.E. Statistical analysis was performed with a Mann-Whitney two-tailed U-test for data of the step-down latency and learning and memory retrieval, and with a Dancan's new multiple comparison test for neurochemical data.

Results

Effects of SRT and SKT on brain ACh levels in mice

1) SRT (Table I). (1) Single administration of SRT : In the corpus striatum, SRT at 50 mg/kg produced a significant increase in ACh levels, but at 400 mg/kg, had no influence on ACh levels in the cerebral cortex, hypothalmus, corpus striatum or hippocampus. (2) Repeated administration of SRT : In the hypothalamus and corpus striatum, SRT at 50 and 400 mg/kg produced a significant increase in ACh levels. However, in the cerebral cortex and hippocampus, SRT at 50 and 400 mg/kg had no influence on ACh levels.

2) SKT (Table I). (1) Single administration of SKT : SKT at 750 mg/kg produced a significant increase in ACh levels in the hypothalamus, but at 75 mg/kg, had no influence on ACh levels in the cerebral cortex, hypothalmus, corpus striatum or hippocampus. (2) Repeated administration of SKT : SKT at 75 and 750 mg/kg had no influence on ACh levels in the cerebral cortex, hypothalmus, corpus striatum or hippocampus.

Effects of SRT and SKT on the latency of scopolamine-induced step-down passive avoidance response in mice

1) SRT (Fig. 2). (1) Single administration of SRT : SRT at 50 and 400 mg/kg had no effect on the shortened latency of scopolamine-induced step-down pas-

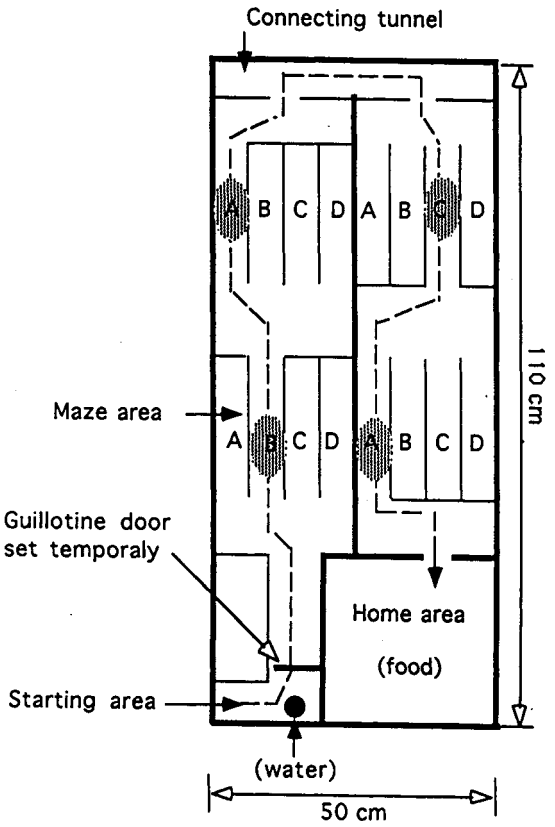

Fig. 1 Schematic drawing of the multiple maze apparatus.

Table I The contents of acetylcholine in the discrete brain areas of mice treated with a single or repeated administration of SRT and SKT.

1) SRT

Region	mg/kg	n	Single ad.	n	Repeated ad.
Cerebral cortex	Control	10	21.5±0.6	10	22.0±0.7
	50	10	21.8±0.8	10	25.3±1.2
	400	10	22.0±0.6	10	24.7±0.9
Hypothalamus	Control	10	29.7±1.3	10	29.9±1.0
	50	10	32.8±1.4	10	35.5±1.7*
	400	10	31.0±0.6	10	35.2±1.1*
Striatum	Control	10	81.2±2.2	10	78.6±3.4
	50	10	89.8±2.6*	10	91.4±4.5*
	400	10	88.2±2.1	10	92.0±1.4*
Hippocampus	Control	10	25.8±0.4	10	26.4±1.4
	50	10	27.4±0.7	10	29.9±1.7
	400	10	27.9±0.8	10	25.9±0.3

2) SKT

Region	mg/kg	n	Single ad.	n	Repeated ad.
Cerebral cortex	Control	10	20.8±0.5	10	20.7±1.0
	75	10	20.5±0.6	10	18.3±0.4
	750	10	21.0±1.4	10	20.6±1.0
Hypothalamus	Control	10	31.0±1.0	10	36.0±1.2
	75	10	32.9±0.9	10	40.5±1.6
	750	10	35.8±1.8*	10	40.5±2.4
Striatum	Control	10	80.9±1.9	10	82.6±1.8
	75	10	80.7±1.8	10	81.6±2.2
	750	10	82.4±1.6	10	86.2±3.1
Hippocampus	Control	10	26.0±0.5	10	26.4±0.9
	75	10	25.5±0.4	10	26.1±0.5
	750	10	25.8±0.4	10	28.0±0.5

SRT and SKT was suspended in a 5 % carboxymethylcellulose solution (CMC) and mice were orally given either single or repeated (once a day, for 14 days) administration of CMC at 10 ml/kg, SRT at 50 and 400 mg/kg, or SKT at 75 and 750 mg/kg, respectively. Two hours after the single or final (repeated) administration of each agent, mice were irradiated with a microwave beam focused on the head (5 kW, 0.7 sec.), and their brains were removed and dissected according to the text.
SRT : Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli-Tang). SKT : Saiko-keishi-kankyo-to (Chaihu-Guizhi-Ganjiang-Tang). Single ad. :single administration. Repeated ad. : repeated administration. *$p<0.05$ vs control group (CMC) by Dancan's new multiple comparison test. Values are presented as the mean ±S.E. (ng/g tissue). n=10.

sive avoidance response. (2) Repeated administration of SRT : SRT at 50 and 400 mg / kg significantly prolonged the shortened latency of scopolamine-induced step-down passive avoidance response.

2) SKT (Fig. 2). Neither single or repeated administration of SKT at 75 and 750 mg/kg influenced the shortened latency of the scopolamine-induced step-down passive avoidance response.

Effects of SRT and SKT on the TMT-induced deficit in learning and memory in mice

Neither single nor repeated administration of either SRT at 50 and 400 mg/kg (Fig. 3), or SKT at 75 and 750 mg/kg (data not shown), influenced the number of errors or running time, in mice with TMT-induced deficit in learning and memory.

Discussion

In traditional Chinese medicine, both SRT and SKT were used to treat common subjective symptoms

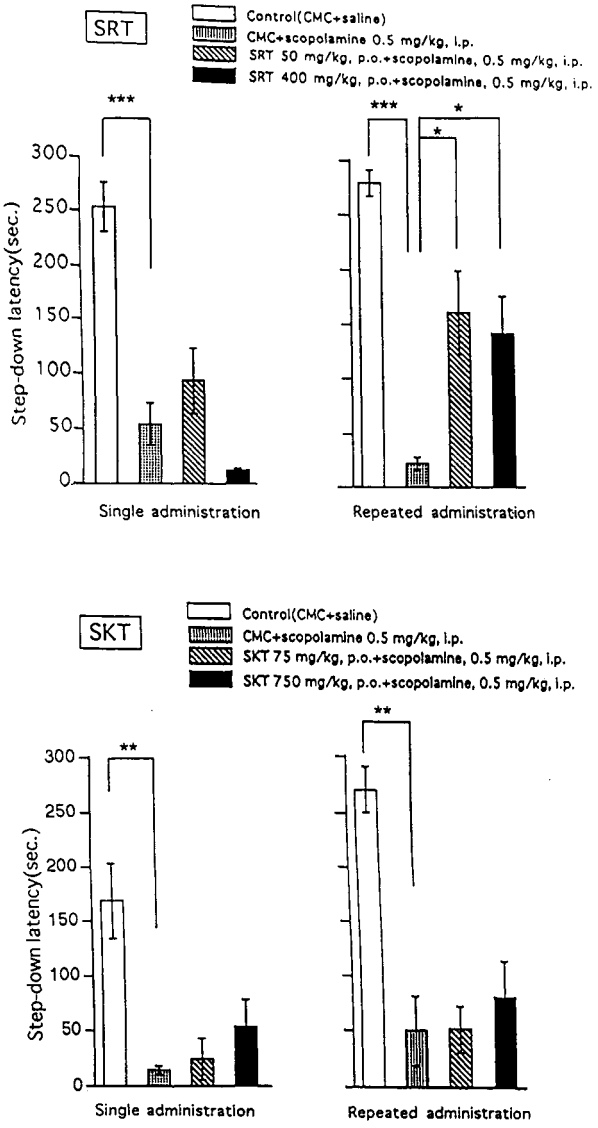

Fig. 2 Effects of SRT and SKT on the response to the step-down passive avoidance in mice treated with scopolamine (retention trial).
Each column (mean ± S.E.) expresses values (sec.) of step-down latencies. SRT and SKT were suspended in a 5 % carboxymethylcellulose solution and mice were orally given either single or repeated (once a day, for 14 days) administration of CMC at 10 ml/kg, SRT at 50 and 400 mg/kg, or SKT at 75 and 750 mg/kg. A half hour after the single or final (repeated) administration of each agent, mice were injected with 10 ml/kg saline solution or 0.5 mg/kg scopolamine, i.p. Thirty min. after the injection, mice were placed on a platform and when they stepped down from the platform, they were given electrostimulation at 90 V, 50 Hz, 10 msec. for 5 sec. The retention trial were conducted 24 hrs. after the acquisition trial.
SRT : Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli - Tang). $*p < 0.05$ as compared with CMC + scopolamine 0.5 mg/kg group, and $**p<0.01$ and ***0.001 as compared with control group (CMC) (Mann-Whitny two-tailed U-test). n=10.

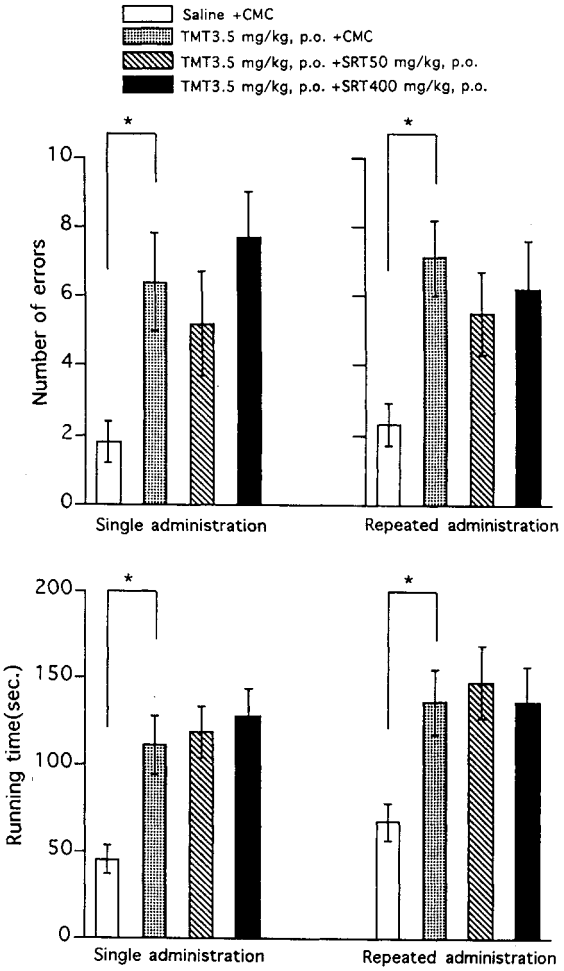

Fig. 3 Effect of SRT on the TMT-induced deficit in learning and memory in behavioral performance acquired in a multiple maze task mice.
Each column (mean ± S.E.) expresses the values of number of errors and running time (sec.). Mice were trained the learning in a multiple maze task for 4 days and on the 5 th day after they were orally given a saline solution at 10 ml/kg or TMT (trimethyltin chloride)at 3.5 mg/kg. Six days after TMT, mice were orally given either single or repeated (once a day, for 14 days) administration of a 5 % carboxy-methylcellulose solution (CMC) at 10 ml/kg, or SRT at 50 and 400 mg/kg. Two hours after the final administration of CMC or SRT. mice performed the re-trials, recording a number of errors and running time.
SRT : Saiko-ka-ryukotsu-borei-to (Chaihu-Jia-Longgu-Muli-Tang). $*p<0.05$ as compared with control group (CMC) (Mann-Whitny two-tailed U-test). n=10.

of nervous constitution, although there is a difference in the clinical application of the two agents : SRT for a robust (athenia) and nervous constitution, Shi Zheng, and SKT for a deficient (asthenia) and nervous constitution, Xu Zheng.[1,2] We reported previously that SRT[3-5] and SKT[5,6] had similar effects on the central dopaminergic and serotonergic nervous systems in

mice. In the present study, it became apparent that there is a difference in the mode of action between SRT and SKT on the central cholinergic nervous system.

Single administration of SRT at 50 mg/kg, but not at 400 mg/kg, produced an increase in ACh levels in the corpus striatum of mice. In the other regions of the brain, however, neither SRT at 50 nor 400 mg/kg produced an increase in ACh levels. Repeated administration of SRT at 50 and 400 mg/kg increased the ACh levels in the hypothalamus and corpus striatum. These findings suggest that frequency of administration may alter the effect of SRT on brain ACh levels as well as on the levels of monoamine-related substances.[3-5]

Dysfunction of the central cholinergic nervous system is known to be involved in the induction and extension of convulsions.[15,16] We reported previously that SRT had a slight inhibitory effect on the convulsions induced by electrostimulation and stimulants such as picrotoxin and cardiazol, and produced an increase in the levels of brain monoamine-related substances in mice.[17-19] Furthermore, we also suggested that the increase in brain ACh levels induced by SRT may have influence on the inhibition of the convulsions.[17-19] On the other hand, SKT only produced an increase in ACh levels in the hypothalamus, with a single administration of SKT at 750 mg/kg, as in our previous report,[5,6] suggesting that SKT may have little or no influence on convulsions in mice.

Working memory is important for the retention of information required in the short-term.[20] Therefore, we examined whether SRT and SKT are able to ameliorate the deficits in learning and memory induced by scopolamine, which causes a dysfunction of the parasympathetic nervous system and a disorder of step-down passive avoidance response in mice. Repeated administration of SRT at 50 and 400 mg/kg significantly prolonged the shortened latency in the response to scopolamine-induced step-down passive avoidance, whereas SKT had no effect, suggesting that SRT, but not SKT, has the ability to improve the disordered response in learning and memory. This is characteristic of a difference in the mode of action between SRT and SKT on the central cholinergic nervous system. Furthermore, effects of SRT and SKT on learning and memory deficits in mice treated with TMT also were examined using a multiple maze task. TMT is a potent neurotoxicant producing degeneration and irreversible lesions in the hippocampal Ammon's horn.[10-12,21] In mice treated with TMT, neither single nor repeated administration of SRT and SKT had influence on the number of errors or running time in a multiple maze task. These findings suggest that SRT and SKT have no influence on TMT-induced deficit in learning and memory.

In conclusion, SRT increased ACh levels in the corpus striatum and hypothalamus in mice and prolonged the shortening of step-down latency in scopolamine-treated mice, but did not improve the disordered learning and memory in TMT-treated mice. On the other hand, SKT increased ACh levels in the hypothalamus in mice, but had no influence on the behavioral task in the scopolamine- and TMT-treated mice. Thus, it is suggested that there is a difference in the mode of action between SRT and SKT on the central cholinergic nervous system, and that SRT has an ameliorative effect on memory deficit due to neuronal dysfunction in scopolamine-treated mice, but not in TMT-treated mice. However, it is very difficult to evaluate a cognitive enhancing and/or anti-demetic effect SRT from only the present results, and further studies are need to clarify the effect of SRT.

Acknowledgments

The Kampo preparations used in this work were supplied from Tsumura & Co., Japan.

和文抄録

柴胡加竜骨牡蛎湯（SRT）と柴胡桂枝乾姜湯（SKT）は，漢方臨床において，実証に対してSRT，虚証に対してSKTの適用の違いはあるものの，共通の精神・神経症状を有する患者に用いられている。我々は，先に，SRTおよびSKTがマウス脳において，それぞれの領野では用量依存的な影響は認められないが，ドパミンおよびセロトニン関連物質の含量を増大したことを報告した。今回は，SRTおよびSKTの中枢コリン作動性神経系の機能に及ぼす影響を明らかにするため，脳内ACh含量および学習・記憶に及ぼす影響についてマウスを用いて検討した。

SRTはマウスの線条体および視床下部のAch含量を

増大し，スコポラミンによるstep-down受動的回避反応潜時の短縮を延長したが，多重迷路法を用いたトリメチルスズ（TMT）による学習・記憶障害には影響を及ぼさなかった。一方，SKTはマウスの視床下部のACh含量を増大したが，スコポラミンやTMT処置マウスに対しては影響を及ぼさなかった。以上のことは，SRTとSKTは，漢方臨床において，共通の精神・神経症状を有する患者に用いられているが，マウスの中枢コリン作動性神経系に対する作用態度に相違があることを示唆する。

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