Effects of Kampo medicines on choline acetyltransferase activity in rat embryo septal cultures

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Abstract

Effects of Kampo medicines with 52 formulas on choline acetyltransferase (ChAT) activity of rat embryo septal cultures were investigated in this study. Yoku-kan-san-ka-chimpi-hange, Kihi-to and Kami-untan-to remarkably increased ChAT activity of septal cultures. At maximum effective dose, Kami-untan-to (250 μg/ml) and Yoku-kan-san-ka-chimpi-hange (250 μg/ml) increased about 85 % and 63 % of the ChAT activity induced by the maximum effective dose of nerve growth factor (50 ng/ml), respectively. The memory deficit rats induced by scopolamine significantly increased latency on passive avoidance test of step through type by oral administration of one dose of Kami-untan-to (200 mg/kg b.w.).

These results suggested that Kami-untan-to may be available as a potential therapeutic formula for treating dementia such as Alzheimer disease.

Key words ChAT, septal culture, passive avoidance behavior, Kami-untan-to.

Abbreviations ChAT, choline acetyltransferase ; CNS, central nervous system ; NGF, nerve growth factor.

Introduction

Basal forebrain contains abundant cholinergic neurons which belong to the medial septal nucleus and the diagonal band of Broca. Cholinergic neurons in this area may involve deeply with the cognitive function. Therefore neuronal degeneration in this area has been considered to be responsible for several types of dementia including Alzheimer disease. The neurotransmitter, acetylcholine is synthesized from acetyl coenzyme A and choline by the action of choline acetyltransferase (ChAT ; EC2.3.1.6). Consequently ChAT has been considered as a specific maker for the activity of cholinergic neurons. Alzheimer disease significantly decreases the ChAT activity in the cerebral cortex and hippocampus.[1,2] Therefore induction of ChAT activity in cholinergic neuron may improve the damaged cognitive function in Alzheimer disease.

Recently, some Kampo medicines have been demonstrated to improve the damaged cognitive function of animal model for dementia.[3-7] These findings suggest that some Kampo medicines may be useful for the treatment of dementia.

In the present paper, we screened 52 kinds of Kampo formulas on ChAT activity in rat septal cultured cells in order to find effective formulas for dementia due to cholinergic deficits.

Materials and Methods

Materials : Fetal bovine serum (FBS) was purchased from Bioserum., Australia, horse serum from Cell Culture Laboratories, U.S.A., [1-^{14}C] Acetyl CoA (0.15 GBq / mmol) from NEN, U.S.A., and nerve growth factor 2.5 S from Biomedical Technologies Inc. U.S.A.. All medicinal herbs were obtained from Uchida Wakan-Yaku Co. Ltd. (Tokyo, Japan).

Preparation of Kampo medicines : Fifty two kinds of Kampo formulas were examined in this study.

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Forty six formulas which are not available as pharmaceutical extract prepartion covered by national health insurance were selected from the prescription book of the pharmacy in Oriental Medicine Research Center of the Kitasato Institute. The other 6 formulas were selected from the prescriptions which have been expected to have the efficacy for several types of dementia by the animal experiments and clinical studies. Each Kampo medicine was prepared according to above prescription book. Certain amounts of component herbs for each prescription were mixed with 600 ml of distilled water, and decocted to half volume. Then the decoction was centrifuged at 7,000 rpm for 30 min, and the supernatant was lyophilized. For the *in vitro* assay, each lyophilized extract of Kampo medicine (2 mg) was dissolved in distilled water (2 ml), and the solution or suspension was passed through cellulose acetate membrane (0.2 μm, DISMIC-25 cs, Toyo Roshi Kaisya, Ltd.). Then the sterilized filtrate was used for the assay.

Preparation of primary cultured cells : Septal cells were cultured by the modified method of Muramoto *et al.*.[8] The septal area of rat embryos (17-19 days old) was removed and dissected into small pieces with scissors under sterile conditions. The dissected tissue was then digested at 37°C for 30 min in a solution of phosphate buffered saline (pH 7.4) containing 180 U of papain, 0.02 % L-cystein-HCl, 0.02 % bovine serum albumin, and 0.5 % glucose. The precipitate of the digest was obtained by centrifugation at 900 rpm for 5 min and washed with 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF medium). Washed precipitate was resuspended in DF medium containing 5 % fetal bovine serum and 5 % horse serum. After gently pipetting through a plastic tip, the dissociated cells were plated in the same medium at a density of 1×10^6 cells/well in 24-well plates (Primaria, Becton Dickinson, U.S.A.). After preincubating for 7 days, the septal cells were cultured for 3 days further in the presence of sterilized extract of Kampo medicines and then measured for ChAT activity.

Determination of ChAT activity : Rat embryo septal cells in the primary culture were washed with phosphate buffered saline (PBS) and then solubilized in 210 μl of 50 mM Tris buffer (pH6.8) containing 1 % Triton X-100. The solubilizate was taken for the determination of ChAT activity according to the method of Fonnum.[9]

Passive avoidance test (step through type) for scopolamine treated rats : Male Wistar rats weighing 150-180 g were used in this experiments. Once each rat entered from a small light room ($11\times26\times29$ cm) into a large dark room ($31\times31\times34$ cm), he would receive an electric shock (5 mA) for 3 sec. After only one acquisition trial of learning, the animals were returned to their own cage. Twenty four hrs after the acquisition test, the retention trial was performed again. Each rat was placed in the light room and was left there for 300 sec. The latency and the number of rat which did not enter the dark room were recorded. Rats were treated with scopolamine (6 mg / kg) intraperitoneally 30 min before the beginning of the test trial. Kami-untan-to (200 or 400 mg/kg) was orally administered 2 hr before the test session.

Statistics : For analyzing *in vitro* data, ANOVA followed by Dunnett's post hoc procedure was employed. The step through latencies were analyzed with the Kruskal-Wallis test follwed by Scheffe's F test.

Results

Six kinds of Kampo formulas (Choto-san, Yoku-kan-san, Yoku-kan-san-ka-chimpi-hange, Kihi-to, Toki-shakuyaku-san and Oren-gedoku-to), which have been expected to have the efficacy for several types of dementia from the results of animal experiments[3-7] and clinical effects,[10-13] were tested on ChAT activity in septal cultures. When the septal cells were cultured for 3 days in the presence of each Kampo formula at the concentrations ranging from 50 to 200 or 250 μg/ml after 7 days of preincubation, Yoku-kan-san-ka-chimpi-hange and Kihi-to showed increased ChAT activity of septal cultured neurons (Fig. 1). However, Oren-gedoku-to decreased ChAT activity at the range of 50-200 μg/ml due to death of neuronal cells observed microscopically (data not shown).

In order to find another newly available Kampo formula for the treatment of dementia, the other 46 kinds of Kampo formulas, which are not available as pharmaceutical extract preparation covered by

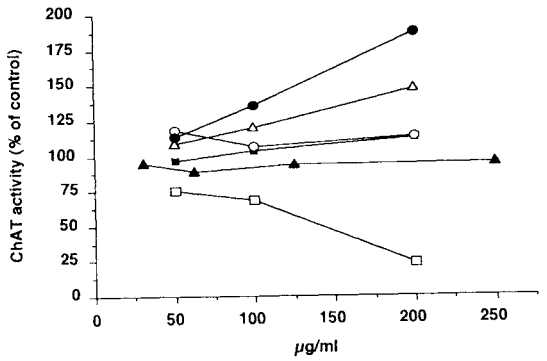

Fig. 1 Effects of 6 kinds of Kampo formulas on ChAT activity in rat embryo septal cells *in vitro*. Septal cells were treated for 3 days in the presence of Kampo formulas [□ ; Oren-gedoku to (黃連解毒湯 ; Huang Lian-Jie Du-Tang), ■ ; Choto-san (釣藤散 ; Diao-Teng San), ○ ; Yoku kan-san (抑肝散 ; Yi-Gan San), ● ; Yoku kan-san ka chimpi hange (抑肝散加陳皮半夏 ; Yi-Gan San-Jia Chen Pi-Ban-Xia), △ ; Kihi to (歸脾湯 ; Gui Pi-Tang), ▲ ; Toki-shakuyaku-san (当歸芍藥散 ; Dang-Gui-Shao Yao San)] . Values are expressed as % of control and represent the means of 2 culture wells.

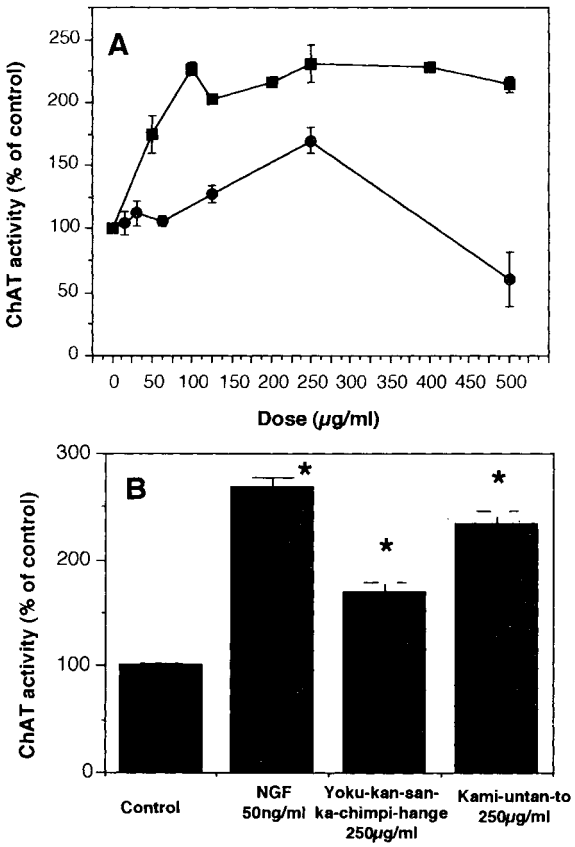

Fig. 2 Effects of Kami untan-to and Yoku-kan-san ka chimpi-hange on ChAT activity in rat embryo septal cells. (A) Dose response (0-500 μg/ml) of Kami untan to (■) and Yoku-kan-san ka chimpi hange (●). (B) Comparison of effects of NGF (50 ng/ml) with Kami-untan-to or Yoku-kan san ka-chimpi hange (250 μg/ml) on ChAT activity in septal cells. Values are expressed as % of controls and represent the means of 3 culture wells ± S.D. *$p < 0.01$ versus control.

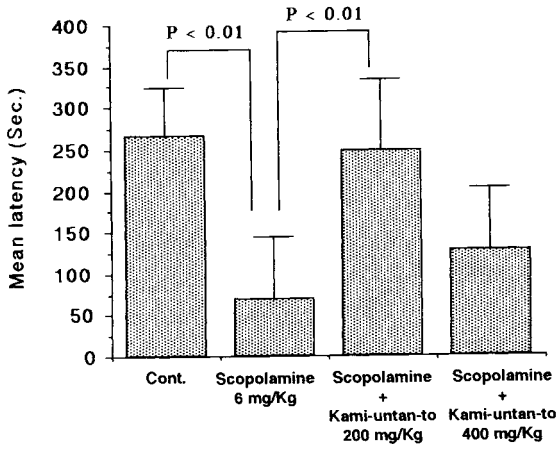

Fig. 3 Effect of Kami-untan to on the passive avoidance test in scopolamine treated rats. Rats were treated with scopolamine (6 mg/kg) intraperitoneally 30 min before the beginning of test trial. Kami-untan-to was orally administered 2 hr before test trial. The latency periods were measured for control group, scopolamine treated group and Kami-untan-to (200 or 400 mg/kg) plus scopolamine treated groups (n=10 each). Values are expressed as mean latency ± S.D..

national health insurance and have not been tried to treat on any types of dementia, on ChAT activity in septal cells were examined. Then septal cells were incubated for 3 days in the presence of 200 μg/ml of each Kampo formula, only Kami-untan-to remarkably increased the ChAT activity, and the increment was 158 % of control (Table. I). However, some of Kampo formulas decreased ChAT activity.

From above results, Kami-untan-to and Yoku-kan-san-ka-chimpi-hange (Table II) exhibited a stimulatery effect on ChAT activity in septal cells, the dose response effects of these two formulas on ChAT

Table I Effects of 46 Kampo medicines which are not covered by national health insurance on ChAT activity in septal cultured neuron.

Kampo formula (Japanese ; Chinese)	ChAT activity*	% of control**
Bakumondo inshi (麦門冬飲子 ; Mai-Men Dong Yin-Zi)	4.92	110
Bushi richu to (附子理中湯 ; Fu-Zi Li-Zhong-Tang)	4.53	101
Chu-kenchu to (中建中湯 ; Zhong Jian-Zhong-Tang)	3.34	101
Ennen hange-to (延年半夏湯 ; Yan-Nian-Ban-Xia-Tang)	5.60	109
Goko-nichin to (五虎二陳湯 ; Wu-Hu Er-Chen-Tang)	5.20	101
Hachimotsu koka-to (八物降下湯 ; Ba-Wu Jiang Xia-Tang)	0.79	18
Hoyo-kango to (補陽還五湯 ; Bu-Yang-Huan-Wu Tang)	3.86	50
Ifu-to (胃風湯 ; Wei-Feng-Tang)	5.43	106
Isho-ho (痿証方 ; Wei Zheng Fang)	1.00	19
Jijin-tsuji-to (滋腎通耳湯 ; Zi Shen Tong-Er Tang)	0.15	2
Jinrei byakujutsu-san (参苓白朮散 ; Shen-Ling Bai Zhu-San)	3.80	115
Jurokumi ryuki in (十六味流気飲 ; Shi Liu-Wei Liu-Gi Yin)	7.70	89
Kakkon koka-to (葛根紅花湯 ; Ge Gen Hong-Hua-Tang)	0.13	3
Kami hassen to (加味八痓湯 ; Jia-Wei Ba-Xian Tang)	7.53	87
Kami untan-to (加味温胆湯 ; Jia Wei Wen-Dan Tang)	13.66	158
Kanzo-shashin-to (甘草瀉心湯 ; Gan-Cao Xie-Xin-Tang)	0.16	2
Karo-gaihaku hakusyu to (瓜呂薤白白酒湯 ; Gua-Lu Jiu-Bai Bai Jiu-Tang)	6.02	79
Kagai san (華蓋散 ; Hua Gai San)	5.91	115
Kei kyo so-so o sin-bu to (桂姜棗草黄辛附湯 ; Gui Jiang Zao Cao-Huang Xin-Fu Tang)	6.49	85
Keishi ka ogi-to (桂枝加黄耆湯 ; Gui Zhi-Jia Huang Gi-Tang)	5.69	74
Keishi ka-ryo jutsu-bu to (桂枝加苓朮附湯 ; Gui Zhi-Jia Ling Shu-Fu-Tang)	3.68	111
Keishi mao-kakuhan to (桂枝麻黄各半湯 ; Gui Zhi-Ma Huang-Ge-Bang Tang)	3.86	116
Keishi shakuyaku chimo-to (桂枝芍薬知母湯 ; Gui Zhi-Shao-Yao-Zhi Mu-Tang)	3.35	101
Kenchu to (堅中湯 ; Jian-Zhong-Tang)	3.19	96
Keppu-chikuo to (血府逐瘀湯 ; Xue-Fu Zhu-Yu Tang)	3.58	108
Kigi kenchu to (帰耆建中湯 ; Gui Gi-Jian Zhoug Tang)	7.83	91
Kosha rikkunshi-to (香砂六君子湯 ; Xiang-Sha-Liujun-Zi-Tang)	7.37	85
Kumi binro-to (九味檳榔湯 ; Jia Wei-Bing-Lang-Tang)	7.87	91
Mibaku-ekki-to (味麦益気湯 ; Wei-Mai-Yi Qi-Tang)	5.74	112
Oren-akyo-to (黄連阿膠湯 ; Huang-Lian A-Jiao Tang)	0.11	1
Renju-in (連珠飲 ; Lian Zhu-Yin)	5.70	111
Ryo-kan-kyo-mi shin-ge to (苓甘姜味辛夏湯 ; Ling Gan-Jiang Wei Xia-Ren-Tang)	6.49	127
Saiko sokan-to*** (柴胡疎肝湯 ; Chai-Hu-Shu Gam-Tang)	8.11	94
Saiko sokan to**** (柴胡疎肝湯 ; Chai-Hu Shu Gam-Tang)	8.31	96
Sai-shaku-rikkunshi-to (柴芍六君子湯 ; Chai-Shao-Liu-Jun Zi-Tang)	4.99	111
Seijo kentsu-to (清上蠲痛湯 ; Qing Shang Jian Tong-Tang)	3.51	78
Senkan meimoku-to (洗肝明目湯 ; Xi-Gan Ming Mu-Tang)	0.23	5
Sessho in (折衝飲 ; Zhe-Heng-Yin)	3.86	116
Shaku-kan o-shin bu-to (芍甘黄辛附湯 ; Shao-Gan-Huang-Xin Fu-Tang)	4.14	92
Sho-joki-to (小承気湯 ; Xia Cheng-Tang)	4.84	108

Kampo formula (Japanese ; Chinese)	ChAT activity*	% of control**
Sho-zokumei-to (小続命湯 ; Xia-Xu-Ming-Tang)	3.50	78
Toki-nentsu-to (当帰拈痛湯 ; Dang-Gui-Zhu-Yu-Tang)	2.94	86
Toki-shigyaku-to (当帰四逆湯 ; Dang-Gui-Si-Ni-Tang)	3.58	108
Untan-to (温胆湯 ; Wendan-Tang)	5.82	113
Uyaku-junki-to (烏薬順気湯 ; Wu-Yao-Shun-Gi-Tang)	6.05	118
Zen-shikunshi-to (喘四君子湯 ; Chuan-Si-Jun-Zi-Tang)	5.70	74

Septal cells were treated for 3 days in the presence of Kampo formulas (200 μg/ml) and then measured the ChAT activity.
* The data are expressed in terms of pmols of [^{14}C] acetylcholine/well/min. Each ChAT activity can not compare each other because several 24 well plates were used for the assay.
** Values are expressed as % of control of each plate and represent the means of 2 wells.
*** The formula was prescribed according to Kampo-ikkando-igaku (漢方一貫堂医学).
****The formula was prescribed according to Igaku-tosi (医学統旨).

Table II Composition of Kami-untan-to and Yoku-kan-san-ka-chimpi-hange.

Kampo formula	Herbs (g)
Kami-untan-to	Hange ; 半夏 ; tuber of *Pinellia ternata* BREI. (5.0 g)
	Chikujo ; 竹筎 ; stalk of *Phyllostachys nigra* MUNRO. (3.0 g)
	Kijitsu ; 枳実 ; immature fruit of *Citrus aurantium* L. (3.0 g)
	Bukuryo ; 茯苓 ; fungus of *Poria cocos* WOLF. (3.0 g)
	Chimpi ; 陳皮 ; peel of *Citrus unshiu* MALKOV. (3.0 g)
	Kanzo ; 甘草 ; root of *Glycyrrhiza glabra* L. (2.0 g)
	Onji ; 遠志 ; root of *Polygala tenuifolia* WILLD (2.0 g)
	Genjin ; 玄参 ; root of *Scrophularia ningpoensis* HEMSLEY (2.0 g)
	Ninjin ; 人参 ; root of *Panax ginseng* C.A. MEYER (2.0 g)
	Jio ; 地黄 ; root of *Rehmannia glutinosa* LIB. (2.0 g)
	Sansonin ; 酸棗仁 ; seed of *Zizyphus jujuba* MILLER (2.0 g)
	Taiso ; 大棗 ; fruit of *Zizyphus jajuba* MILLER. var (2.0 g)
	Shokyo ; 生姜 ; rhizome of *Zingiver officinale* ROSCOE. (0.5 g)
	(Total 31.5 g)
Yoku-kan san ka-chimpi-hange	Toki ; 当帰 ; root of *Angelica acutiloba* KITAGAWA (3.0 g)
	Chotoko ; 釣藤鉤 ; thorn of *Uncaria rhynchophylla* MIQ. (3.0 g)
	Senkyu ; 川芎 ; rhizome of *Cnidium officinale* MAKINO (3.0 g)
	Bukuryo ; 茯苓 ; fungus of *Poria cocos* WOLF. (4.0 g)
	Sojutsu ; 蒼朮 ; rhizome of *Atractylodes lancea* DC. (4.0 g)
	Saiko ; 柴胡 ; root of *Bupleurum falcatum* L. (2.0 g)
	Kanzo ; 甘草 ; root of *Glycyrrhiza glabra* L.var. (1.5 g)
	Chimpi ; 陳皮 ; peel of *Citrus unshiu* MARKOV. (3.0 g)
	Hange ; 半夏 ; tuber of *Pinellia ternata* BREIT. (3.0 g)
	(Total 26.5 g)

activity were examined. Fig 2A shows that Kami-untan-to to the septal cells caused a significant increase in ChAT activity at the range of 50-500 μg/ml and Yoku-kan-san-ka-chimpi-hange increased significantly at the range of 125-250 μg/ml. However 500 μg/ml of Yoku-kan-san-ka-chimpi-hange reduced ChAT activity to 60.0±21.75 % of control because of cell death. The most effective concentration of Kami-untan-to (250 μg/ml) on ChAT activity increased about 85 % of the response obtained by a maximum

dose of NGF (50 ng/ml), and that of Yoku-kan-san-ka-chimpi-hange (250 μg/ml)induced about 63 % (Fig. 2B).

In order to examine the effect of Kami-untan-to *in vivo*, the single trial passive avoidance test was performed. Scopolamine showed a significant decrease both in the mean latency and the number of animals staying (data not shown). Kami-untan-to administered orally improved this disruption of learning ability at a dose of 200 mg/kg b.w. (Fig. 3).

Discussion

Alzheimer disease is the most popular dementia in the elderly people and is characterized by memory loss and a progressive global impairment of intellect. Nerve growh factor (NGF) is a target derived neurotrophic factor (NTF) which affects the survival and function of septal cholinergic neurons.[14] Therefore, it has been suggested that the administration of NGF improves some of the biological abnormalities that occur in the dementia due to cholinergic deficits and the associated symptoms.[11] However, the oral administration of NGF has no effect on the central nervous system (CNS) neuron, because NGF is a large molecular weight protein that can not pass through the gastro-intestinal tract and even the blood-brain barrier.[15] Recently it has been known that some Kampo medicines are useful for the treatment of dementia. Therefore, we screened neurotrophic activity of 46 kinds of Kampo formulas which have not been suggested the efficacy for dementia by the effect on ChAT activity of septal cultured neurons. As a result, Kami-untan-to was found to have a remarkable increase in ChAT activity and negligible cell toxicity (50-500 μg/ml). Although Kami-untan-to has been used clinically as CNS effective drug for insomnia and neurosis *etc.*, no clinical study of this formula for the treatment of dementia has been reported up to now. Present results suggested that Kami-untan-to is useful as a potential therapeutic formula to diseases exhibiting cholinergic deficits such as Alzheimer disease.

Of another 6 kinds of Kampo formulas (Choto-san, Yoku-kan-san, Yoku-kan-san-ka-chimpi-hange, Kihi-to, Toki-shakuyaku-san and Oren-gedoku-to), which have been expected the efficacy for several types of dementia from the results of animal experiments[3,7] and clinical studies,[10-13] Yoku-kan-san-ka-chimpi-hange and Kihi-to showed a significant increase of ChAT activity, but other formulas did not induce ChAT activity. Choto-san and Oren-gedoku-to have been mainly expected the clinical efficacy for the dementia of cerebrovascular type, but not Alzheimer type. Therefore effects of these formulas on septal cells seemed to confirm clinical studies. Because of Yoku-kan-san had no stimulatory effect on ChAT activity, it was expected that the effect of Yoku-kan-san-ka-chimpi-hange was due to that of chimpi (陳皮 ; the ripe fruit of *Citrus unshiu* MARKOV.) and/or hange (半夏 : tuber of *Pinellia ternata* BREIT.). However, hot water-extracts of chimpi and hange did not alter the effect on ChAT activity (data not shown). Some additive effects of the component herbs may be involved in the action of Yoku-kan-san-ka-chimpi-hange. Isho-ho, Jijin-tsuji-to, Kanzo-shashin-to, Kakkon-koka-to, Hachimotsu-koka-to, Oren-akyo-to, Senkan-meimoku-to and Oren-gedoku-to showed a considerable decrease of ChAT activity in septal cells. As a result of morphological study using microscope, cell death was apparently observed in these formulas treated cells (data not shown). These formulas include Oren (黄連 ; rhizome of *Coptis japonica* MAKINO) or Obaku (黄柏 ; bark of *Phellodendron amurense* RUPRECHT) which are known to contain a large amount of berberine. Therefore, it was suggested that alkaloids such as berberine may have strong toxicity for cholinergic neuron.

Oral administration of Kami-untan-to demonstrated to improve passive avoidance behavior of scopolamine treated rats. However the effect was not in a dose dependent manner and an optimal effective dose may be existed. In the present study, other Kampo formulas were not examined passive avoidance test. However, Egashira *et al.* have reported that Yoku-kan-san-ka-chimpi-hange improved scopolamine-induced cognitive deficit.[7] Oren-gedoku-to improves the disruption of spatial cognition induced by cerebral ischaemia rather than scopolamine-induced disruption.[5] These observations seemed to agree with our screening results.

Toki-shakuyaku-san has been reported to increase

ChAT activity in cerebral cortex of aged rats[16] and to recover scopolamine-induced deficit of spatial cognition[5] and cognitive function of patients of senile dementia of Alzheimer type.[11] However, present results showed that Toki-shakuyaku-san did not induce ChAT activity in septal cells. This observation suggested that *in vivo* effect of Toki-shakuyaku-san on CNS may be not due to direct effects for cholinergic neuron. A further study is needed required to prove this hypothesis.

The present results suggested that ChAT activity in embryo septal culture is a good target for the screening of therapeutic drugs which affect to the cholinergic neurons directly.

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和文抄録

アセチルコリン合成酵素であるCholine acetyltransferase (ChAT) 活性に及ぼす52種類の漢方方剤の煎液エキスの影響を調べた。中隔野初代培養細胞に漢方方剤エキス (250 μg/ml) を添加し3日後のChAT activityを測定したところ抑肝散加陳皮半夏，帰脾湯，加味温胆湯のエキスの添加により著明なChAT activityの上昇が認められた。その上昇効果は50 ng/mlのNerve growth factorのChAT activity上昇作用と比べて250 μg/mlの濃度で加味温胆湯でその85％，抑肝散で63％であった。また加味温胆湯の経口投与 (200 mg/kg) によりScopolamine誘発記憶障害ラットの受動的回避学習試験の成績は有意に改善された。これらの結果より，アルツハイマー症のようなコリン作動系の障害を伴う痴呆疾患に対して加味温胆湯が有効な治療薬となり得る可能性が示唆された。

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