Kampo medicine and neuroendocrine -From here to molecular biology

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Abstract

One oriental recipe, Toki-shakuyaku-san (当帰芍薬散 : TSS), has been known to treat amenorrhea, infertility and menopausal syndrome in China and Japan for many centuries. The study provides the evidence that administration of TSS facilitates the central autonomic nervous system together with neuroendocrine controlled anterior pituitary function and thus creates the restoration of ovarian and uterine function in women. Therefore, administration of TSS improves amenorrhea and infertility in women. A lack of estrogen in menopausal women seems to correlate with symptoms of undefined complains, however, estrogen is not only one to defined the undefined complains in menopausal women. Facilitation of ovarian function through activation of central autonomic nervous system together with neuroendocrine function by administration of TSS improves the symptoms of undefined complains in menopausal women.

Another major symptom which has positive correlation with a lack of estrogen in menopausal women is the dementia of Alzheimer type. The study demonstrated that (1) TSS reverses age-related declining of memory through activation of cholinergic and dopaminergic neurons, receptors, and protein synthesis ; (2) TSS prevents apoptosis of neurons at the level of the transcription and assists the maturation of neurons ; and (3) TSS has almost no adverse affect, and therefore, we are able to provide TSS for a long period of time. The study support the possibility of TSS being a remedy of therapeutics for Alzheimer disease. Overall, treatment with TSS provides the quiet passage of life in menopausal women.

Key words Toki-shakuyaku-san, neuroendocrine, menopause, dementia, anti-apoptosis, neuron growth, memory-improvement.

A : Kampo medicine and amenorrhea, infertility and menopausal symptoms

1. Introduction

The sensory stimuli regulate the neocortical function through modulation of the reticulo-thalamo-cortical activating system[1] and the neocortical function controls consciousness and orientation in man. The sensory stimuli also regulate the limbic cortical function through modulation of the periaqueductal - hypothalamo-septal-limbic activating system[2] and the limbic function controls memory, emotion and neuroendocrine function in man. The secretion of pituitary LH which is controlled by the hypothalamic LHRH regulates ovarian function, such as onset of puberty, ovulation and menstruation. The cell body of LHRH neuron is located in the anterior and middle regions of hypothalamus and the axon terminates in the outer layer of median eminence of the hypothalamus. From here the hypophyseal portal vein carries LHRH into the LH cell in the anterior pituitary. Ovulation and following menstruation appear cyclically after onset of puberty in healthy young women. A cyclic appearance of an elevation of serum estrogen, ovulation and menstruation are associated

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with the cyclic appearance of paradoxical EEG activity in the neocortex and limbic cortex.[3] It indicates that the function of limbic cortex and neocortex as well as sensory stimuli influences the onset of puberty, ovulation and menstruation in women. Therefore, dysfunction of limbic cortex, neocortex and hypothalamus will bring about amenorrhea, infertility and menopause in women. On the other hand, a lack of estrogen induced by ovarian surgery in young women also brings about similar symptoms like that of menopause in elderly women.[4] The menopausal symptoms represent not only undefined physical complaints, but they also exhibit the cloudiness of mind, such as dementia of Alzheimer type in women. One oriental recipe, Toki-shakuyaku-san (当帰芍薬散), has been known to treat amenorrhea, infertility and menopausal syndrome in China and Japan for many centuries. Recently, we have found that one oriental recipe, Toki-shakuyaku-san, has a therapeutic efficacy on treatment for the dementia of Alzheimer type. Therefore, the lecture on Today will review the recent pharmacokinetics and neuroendocrinology of Toki-shakuyaku-san, and we will also discuss the neurobehavioral and cellular and molecular aspect of Toki-shakuyaku-san concerning therapeutic efficacy on treatment for the dementia of Alzheimer type.

2. Pharmacokinetics of Kampo medicine

Toki-shakuyaku-san (TSS) is one of the natural products in the oriental recipes, and TSS has been widely used as therapeutics for long-term treatment of ovarian dysfunction and menopausal syndromes in women in Japan and China. TSS, one of their Japanese recipes, contains dried powder extract from the following six combined medicinal plants : 3.0 g of Angelica Root (Chinese Angelica Root, Radix Angelicae Sinensis), 4.0 g of Paeony Root (white peony root, Radix Paeonial), 4.0 g of Atractylodis Lancea Rhizome Japonica (Rhizome Atractylodis), 4.0 g of Hoelen (Fuling, Poria), 4.0 g of Alisma Rhizoma (Oriental Waterplantin Rhizoma, Rhizome Alismatis), and 3.0 g of Cniidium Rhizome (Chuan xiong Rhizome, Zingikeris Recents). The majority of the phytochemical constituents of TSS have been described elsewhere.[5] The extract of above recipe was obtained from Tsumura & Co., Tokyo, Japan. The Japanese recipe for TSS differs from that of the Chinese recipe.

TSS was administered orally and pharmacokinetic of major components of TSS, such as paeoniflorin and albiflorin in Paeony Root, and beta-eudismol in Atractylodis Lancea Rhizome Japonica, was observed. From the study of Takeda, *et al.* (1991),[6] they demonstrated that paeoniflorin was transported to blood immediately after administration of 2 g/kg body weight of TSS orally and reached a peak of paeoniflorin at 15 minutes and another paek at 45 minutes in blood and declined thereafter. Paeoniflorin remained in the circulation for 6 hr. Albiflorin and beta-eudismol behaved in a similar manner to paeoniflorin. Albiflorin was transported to blood immediately after administration of 2 g/kg body weight of TSS and reached a peak at 30 minutes after administration. Albiflorin remained in the blood circulation for 4 to 6 hr. However, beta-eudismol reached a peak at 20 minutes after administration of 2 g/kg body weight of TSS and remained in blood for 10 hr.

3. Kampo medicine on neuroendocrine controlled ovarian function

Toki-shakuyaku-san (TSS) is known to activate ovarian function in women who are suffering with amenorrhea and infertility. Therefore, we used immature female rat which has no development of ovarian follicle and ovum as an animal model for the study.

Administration of TSS (TJ-23, 500 mg/kg body wt) through drinking water at 23 days of age and continuing for 10 days brought about precocious ovulation in immature Sprague Dawley albino female rats at 33 days of age. However, regular onset of puberty (first ovulation) appeared at 39 days of age in rats. An anesthetization with pentobarbital (25 mg/kg body wt) prior to ovulation (at 2 : 00 pm on 32 days of age) blocked ovulation at 33 days of age, but ovulation occureed on the following day.[7] This suggests the involvement of neuroendocrine function in the action of TSS on ovulation.

In order to confirm the involvement of neuroendocrine function in the action of TSS on ovulation, TSS (TJ - 23, 500 mg / kg body wt) was administered

through drinking water at 23 days of age in Sprague Dawley albino female rats and continued for 10 days. The hypothalamic contents of LHRH and anterior pituitary contents of LH were examined at 29 days of age (7 days after administration of TSS) and at 33 days of age (one day after cessation of TSS administration) at the day of ovulation.

Treatment with TSS for 7 days increased the hypothalamic LHRH and anterior pituitary LH in immature female rats (Fig. 1 and 2). Further treatment with TSS for 3 days decreased hypothalamic LHRH and anterior pituitary LH (Fig. 1 and 2). Treatment with this regimen increased serum level of LH and brought about precocious ovulation in immature female rats. Moreover, treatment with this regimen for 10 days, but not 7 days, increased uterine

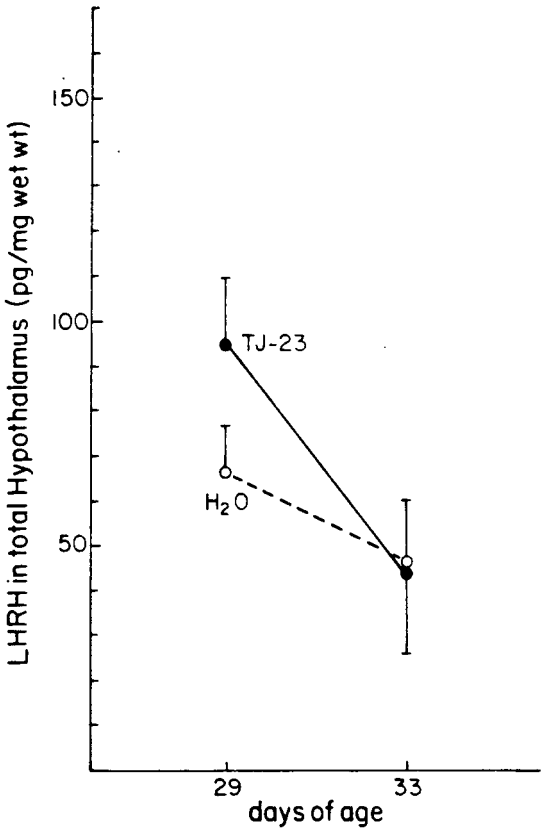


Fig. 1 Effect of Toki-shakuyaku-san (TJ-23) on hypothalamic LHRH.
Effect of Toki-shakuyaku-san (TJ-23) on synthesis and release of LHRH in the hypothalamus of the immature female rats. Opened circles represent the LHRH values in H_2O treated control rats and closed circles represent LHRH values in TJ-23 treated rats.

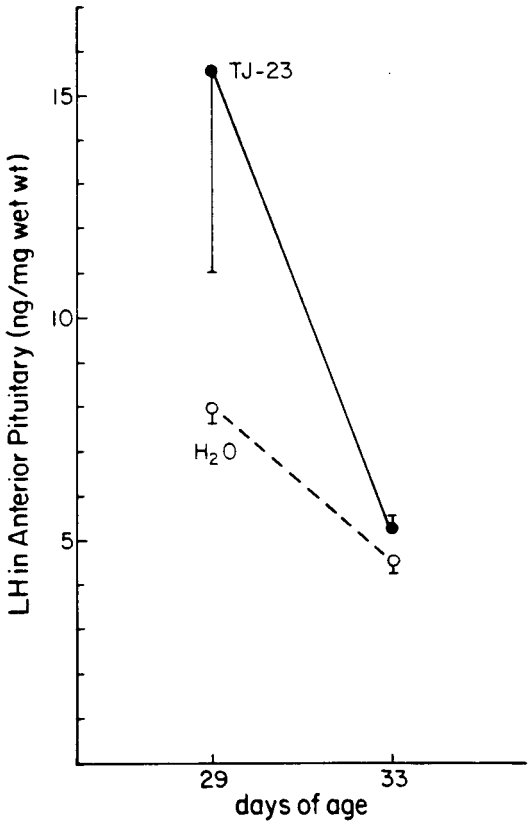


Fig. 2 Effect of Toki-shakuyaku-san (TJ-23) on anterior pituitary LH.
Effect of Toki-shakuyaku-san (TJ-23) on synthesis and release of anterior pituitary LH in the immature female rats. Opened circles represent the LH values in H_2O treated rats and closed circles represent TJ-23 treated rats.

estrogen receptors and uterine weight (Fig. 3).

Direct action of TSS on the ovarian tissues has been demonstrated.[8,9] However, our *in vivo* study with estrogen receptors in the uterus after treatment with TSS provides the evidence that TSS acts on the brain to stimulate synthesis of LHRH in the hypothalamus and subsequently it regulates synthesis and release of LH in the anterior pituitary. This stimulates ovarian function to secrete estrogen. Therefore, it was time laps between an increase in LHRH and LH and an increase in estrogen receptors in the uterus, an increase in LHRH and LH was observed at same day at 29 days of age, but an increase in estrogen receptors was observed at 32 days of age.

The study will explain how treatment with TSS

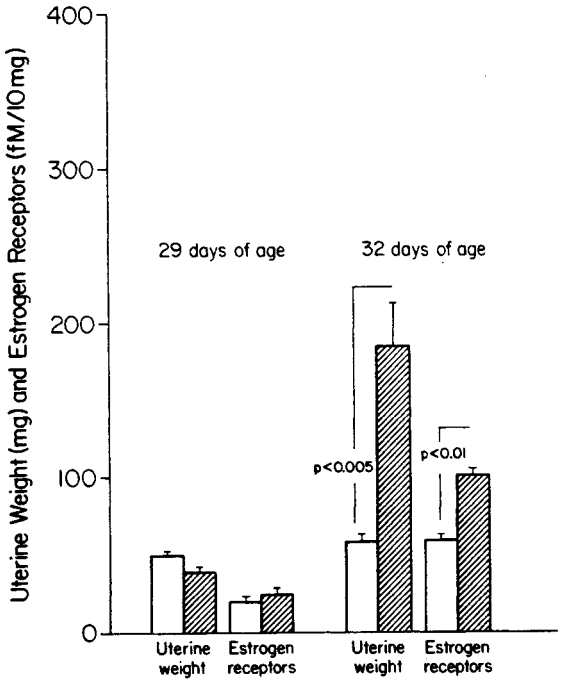


Fig. 3 Effect of Toki-shakuyaku-san (TJ-23) on uterine estrogen receptors and uterine weight.
Effect of Toki-shakuyaku-san on synthesis of estrogen receptors in the uterus of the immature female rats. Opened column represent the values of estrogen receptors and uterine weight in H_2O treated rats at 7 days treatment (at 29 days of age) and at 10 days treatment (at 32 days of age). Shaded column represent the values of estrogen receptors and uterine weight in Toki-shakuyaku-san treated rats at 7 days treatment (at 29 days of age) and at 10 days treatment (at 32 days of age).

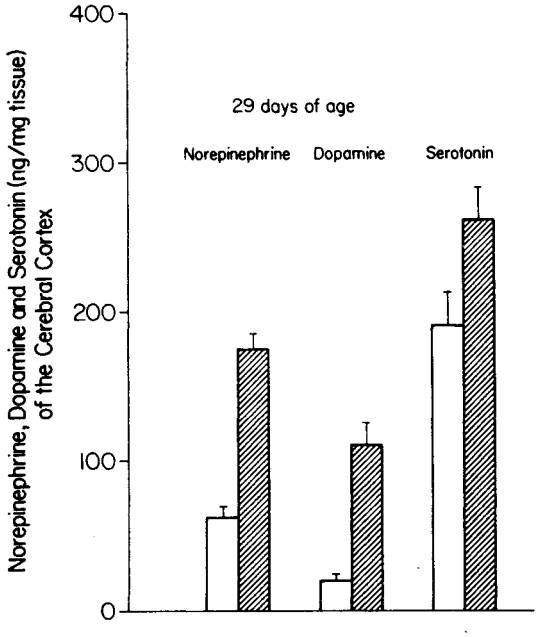


Fig. 4 Effect of Toki-shakuyaku-san on Tissue concentrations of norepinephrine, dopamine and serotonin.
Effect of Toki-shakuyaku-san on synthesis of norepinephrine, dopamine and serotonin in the brain after 7 days treatment (at 29 days of age). Opened column represent the values of tissue concentrations of neurotransmitters in H_2O treated rats. Shaded column represent the values of tissue concentrations of neurotransmitters in Toki-shakuyaku-san treated rats.

improves the amenorrhea and infertility in women through activation of neuroendocrine controlled ovarian function.

4. Kampo medicine on the activity of neurotransmitters

The mechanisms of induction of ovulation is the integrated action of autonomic nervous system in the limbic cortex and hypothalamus. The neurotransmitters of acetylcholine, dopamine and norepinephrine are involved in the induction of ovulation. Blocking of TSS-treated precocious ovulation by pentobarbital suggests that TSS would stimulate synthesis and release of neurotransmitters.

Administration of TSS (TJ-23 500 mg/kg body wt) through drinking water stimulates the activity of tyrosine hydroxylase in the dopaminergic neurons.[10]

Treatment with same regimens increase tissue concentrations of dopamine ($p<0.001$), norepinephrine ($p<0.001$) and serotonin ($p<0.01$) in the brain[11] (Fig. 4). Kataoka, *et al.* (1991)[12] demonstrated that infusion of TSS into the neuronal slices stimulates synthesis and release of dopamine. Using microdialysis technique, Oyama (1991)[13] reported that administration of TSS increased the concentrations of dopamine in the cerebral cortex. The study confirmed that TSS acts directly on neurons to synthesize and release of neurotransmitter. Moreover, the study provides the evidence that orally administered TSS (or components of TSS) pass through the brain-blood barrier to reach the neurons in the brain.

A brief summary of above discussion is that orally administered TSS facilitates brain function which in turn regulates neuroendocrine function on the ovary. A facilitation of the ovary by indirect or direct action of TSS improves the uterine function. Therefore, TSS has therapeutic efficacy on treatment

for amenorrhea and infertility in women.

5. Kampo medicine on menopause

Toki-shakuyaku-san (TSS) is known to use for treatment of menopausal syndrome. We used aged rat (menopausal rat) which shows cessation of ovulation and estrous cycles as an animal model for the study.

Aging female rats (menopausal rats) which exhibited cessation of the reproductive cycles demonstrated an increase of LHRH in the median eminence of the hypothalamus and of immunopositive FSH cells along with tissue concentrations of FSH in the pars tuberalis of the adenohypophysis (Fig. 5). However, there was no increase of FSH concentrations of FSH in the anterior pituitary.[14]

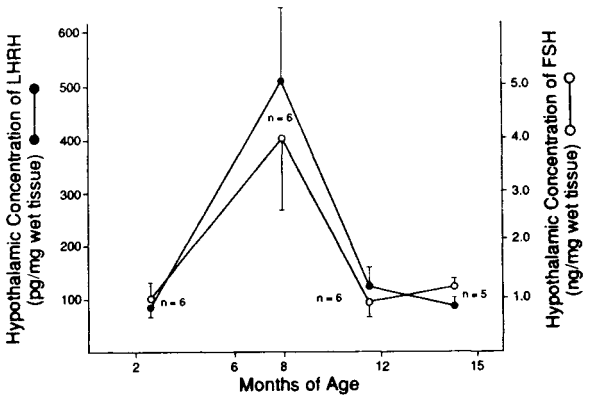


Fig. 5 Tissue concentrations of LHRH in the hypothalamus and FSH in the pars tuberalis of adenohypophysis in ages female rats.
Tissue concentrations of both LHRH in the median eminence of hypothalamus (closed circles) and FSH in the pars tuberalis of adenohypophysis (opened circles) at 8 months of age in menopausal rats. However, those LHRH and FSH values were declined at 12 months of age. n represents number of rats used for the study.

Treatment with TSS (500 mg / kg body wt) through drinking water in the menopausal rats (Fig. 6) decreased LHRH concentrations in the median eminence of hypothalamus and FSH concentrations in the pars tuberalis of adenohypophysis. However, treatment with this regimen did not change the tissue concentrations of LH in the pars tuberalis of adenohypophysis (Fig. 6). Moreover, treatment with TSS restored ovarian cyclicity in the menopausal rats ;

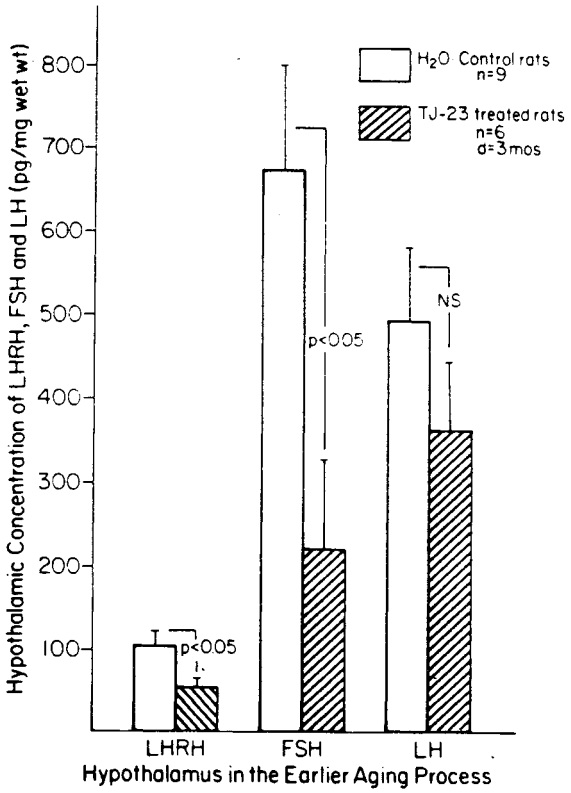


Fig. 6 Effect of Toki-shakuyaku-san on tissue concentrations of LHRH in the hypothalamus and FSH and LH in the pars tuberalis of adenohypophysis in menopausal rats. Effect of Toki-shakuyaku-san (TJ-23) on release of stored LHRH in the median eminence of hypothalamus and FSH in the pars tuberalis of adenohypophysis in menopausal rats. Opened column represent the values of tissue concentrations of LHRH and FSH in H_2O treated rats. Shaded column represent the values of tissue concentrations of LHRH and FSH in TJ-23 treated rats. n represents number of rats used for the study.

they exhibited cyclic elevation of serum estrogen at 330 days of age (Fig. 7) like that of young rats at 90 days of age showing proestrous (P), estrous (E) and diestrous (D) as compared with controls which show persistent estrous (PE) or persistent diestrous (PD) (Fig. 7). Histological examination revealed that treatment with TSS increased follicle maturation and corpus luteum formation in menopausal rats.[15]

Koyama (1991)[16] reported a clinical study of TSS for neuropsychiatric complaints in middle age and elderly women ; TSS is effective in various pre- and post-menstrual symptoms, climacteric disturbances, and in various symptoms in elderly women. However, it is not limited only to those related to hormones.

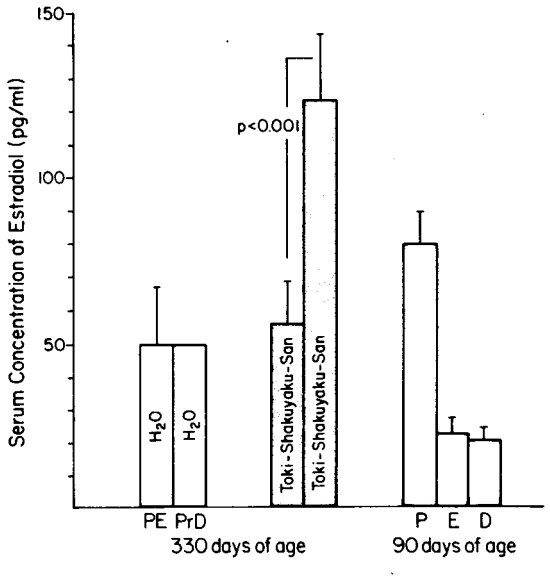


Fig. 7 Effect of Toki-shakuyaku-san on serum concentrations of estradiol in young cycling female rats and menopausal rats.
Effect of Toki-shakuyaku-san on serum concentrations of estradiol at 90 days of age (opened column in right side ; P : proestrous, E : estrous, D : diestrous) and no change of serum estradiol at 330 days of age (opened column in left side ; PE : persistent estrous, PD : persistent diestrous), however, base line values of serum estradiol was higher than that of young cycling rats. Treatment with Toki-shakuyaku-san (shaded column in the center) showed cyclic changes of serum estradiol.

Rather than directly improving the target organs, this therapy with TSS strives to improve the individual condition and it takes the patient's overall health in a better direction. A lack of estrogen in menopausal women seems to correlate with symptoms of undefined complaints. Administration of TSS brings about the brain activity and the ovarian function, and thus restores the secretion of estrogen. Estrogen replacement therapy is a proper manner to treat menopausal undefined symptoms, but Koyama (1991) indicates that TSS acts like estrogen replacement therapy. However, TSS has better therapeutic property to treat menopausal symptoms than that of estrogen. *TSS will provide quiet passage of life in menopausal women.*

B : Kampo medicine and dementia of Alzheimer type

1. Introduction

Another major symptom which has positive corelation with a lack of estrogen is the dementia of Alzheimer type (DAT). Presently, the largest and fastest growing population in the world is the elderly. An extension of our life span makes our life happier, but it creates medical problems related to aging, such as DAT. The rapid loss of brain cells (apoptosis) which creates rapid loss of memory and orientation is the major pathophysiology for DAT. These symptoms appear in the post - menopausal period. Presently available synthesized therapeutics attack DAT properly, but they exhibit extensive side effects which create more difficulty than benefit. Recently, we succeeded in using conjugated equine estrone (CEE) for adequate therapeutics to treat the DAT patient after long-term experiments with animals.[17] Basic research was initiated at the University of Texas Health Science Center at San Antonio in 1973, and the first phase of clinical trials was initiated in 1986 at Tokyo Tama Geriatric Center, Tokyo, Japan, and completed in 1993,[18] The second phase of clinical trials has continued in Japan. However, CEE is one of the female steroid hormones and limited to use on female patients. Moreover, we must consider the incidence of adverse effects of CEE.

In the past decade, therefore, our attention was focused on investigating possible therapeutics from natural products, hoping these would not have extensive side effects and that we would be able to provide these therapeutics for DAT patients over a long period of time. One of these natural therapeutics under current investigation is known as Toki-shakuyaku-san (TSS). TSS has been known to treat menopausal symptoms in women in China and Japan. The Japanese recipe for TSS has been described as follows ; 3.0 g of Angelica Root, 4.0 g of Paeony Root, 4.0 g of Atractylodis Lancea Rhizome Japonica, 4.0 g Hoelen, 4.0 g of Alisma Rhizoma and 3.0 g of Cniidium Rhizoma. The extract of above recipe was obtained from Tsumura & Co., Tokyo, Japan. The phytochemical constituents of TSS have been described elsewhere.[5]

I believe that the following three categories are

essential factors for the planning and development of medicine for treatment of dementia of Alzheimer type (DAT) : (1) medicine must reverse declining memory through activation of neurotransmitters, receptors, and protein synthesis ; (2) medicine must prevent apoptosis of brain cells as it stimulated cell growth and maturation ; and (3) medicine must not have adverse effects so medicine can be provided for long term use. Our research, therefore, was focused to examine the above three essential factors of TSS for treatment of DAT.

2. Kampo medicine on memory

Our initial study was focused on the examination using T-branch one way maze if TSS has a reversal effect on agerelated declining of memory through activation of neural activity. Learning trials were initiated at 5 weeks of age in mice from Charles River, and they reached 90 % of success rates in 25 % chance levels at 10-day trials. Delay time trials were begun immediately upon completion of the learning trials. An increasing delay time (a time interval between returning to starting door and opening of starting door) creates anxiety and it decreases success rates at 25 % chance level (Fig. 8). Male CD - strain mice maintained their memory which learned at 5 weeks of age up to 60 weeks, and their success rates in delay time was the same as that of young - aged mice. However, these mice began to demonstrate age-related declining of memory after 60 weeks of age ($q = 10.800$, $p < 0.0061$: further declining of success rates in delay time was observed at 95 weeks of age (q=17.914, $p<0.001$).

A declining of age-related memory was dependen upon the individual mice, however, frequent exercise of learning and memory retained their memory longer. We used aged mice that showed age - related declining of memory as a model for investigation of therapeutic efficacy of TSS for treatment of dementia (Fig. 9). TSS (500 mg/kg body wt) was given to aged mice at 68 weeks of age through drinking water and continued for four weeks. Administration of TSS reversed age-related declining of memory, and 72-

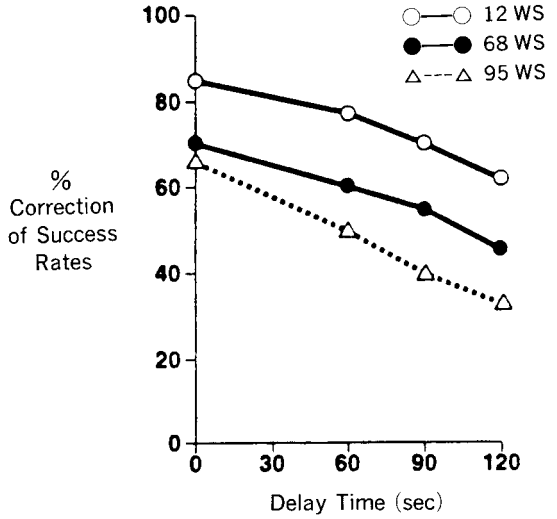


Fig. 8 Age-related declining of memory in male mice.
Age-related declining of memory in 20 male mice. The success rates in delay time was lower at 68 weeks of age than that of 12 weeks of age ($p<0.001$). Further decrease in success rates in delay time was observed at 95 weeks of age ($p<0.001$). Opened circles represent success rates in delay time on 12 weeks of age, closed circles represent that on 68 weeks of age, and opened triangles represent that on 95 weeks of age.

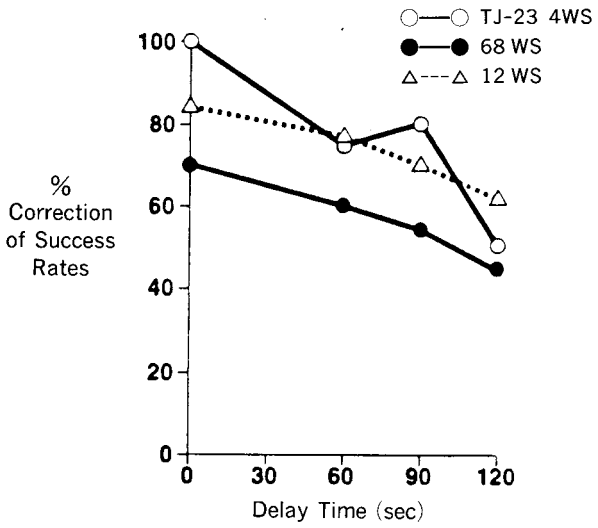


Fig. 9 Reversal effect of Toki-shakuyaku-san on age-related declining of memory.
Reversal effect of Toki-shakuyaku-san (TJ-23) on age-related declining of memory in 20 male mice. Treatment with 500 mg/kg body weight of Toki-shakuyaku-san for 4 weeks elevated success rates in delay time in 20 treated mice at 72 weeks of age as compared with that of 68 weeks of age ($p<0.05$). They behaved like that of 12 weeks of age. Opened circles represent success rates in delay time on 72 weeks of age after treatment with TJ-23 for 4 weeks. Closed circles represent success rates in delay time on 68 weeks of age and opened triangles represent that of 12 weeks of age.

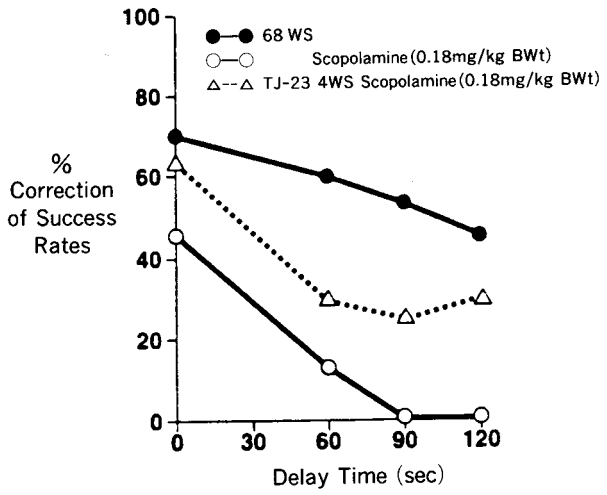


Fig. 10 Reversal effect of Toki-shakuyaku-san on scopolamine treated aged mice.
Reversal effect of Toki-shakuyaku-san (TJ-23) on scopolamine induced memory deficit in 20 aged mice. Treatment with 0.18 mg/kg body weight of scopolamine hydrobromide caused extensive reduction of success rates in delay time as compared with that of 68 weeks of age ($p<0.001$). Treatment with 500 mg/kg body weight of Toki-shakuyaku-san improved the success rates in delay time in scopolamine induced memory deficit aged mice ($p<0.05$). Opened circles represent success rates in delay time after treatment with scopolamine hydrobromide and closed circles represent that of 68 weeks of age. Opened triangles represent success rates in delay time in scopolamine hydrobromide treated aged mice after treatment with TJ-23 for 4 weeks.

week old mice behaved like 12-week old mice ($F(1,3)=11.340$, $p=0.0435$).

However, aged male mice became more sensitive to the scopolamine hydrobromide than young mice, and they showed more severe memory deficiency than young male mice (Fig. 10) ($q=11.330$, $p<0.001$). Treatment with TSS improved scopolamine-induced memory deficiency in a aged male mice (Fig, 10) ($q=6.135$, $p<0.05$).

As I mentioned above, estrogen replacement therapy in menopausal women improves their memory and daily life of DAT patients. However, estrogen is one of the female steroid hormones and limited to use on female patients. TSS has therapeutic efficacy on treatment of menopausal symptoms and DAT in women. Moreover, our study revealed that TSS administration reverses age-related declining of memory in male mice. This provides the evidence that TSS is not limited to use only in female patients, but TSS can also be used for male patients too.

In young adult rats, administration of TSS and/or Paeony root (one of the recipes of TSS) improved scopolamine-induced memory deficiency.[13,19-21] Furthermore, treatment with TSS improved the passive avoidance task in senescence-accelerated mice.[22] The administration of scopolamine hydrobromide blocked synthesis of acetylcholine and decreased acetylcholine centrally. Therefore, the improvement of scopolamine-induced memory deficiency by TSS suggests that administration of TSS increases synthesis of acetylcholine. Indeed it is that administration of TSS increased acetylcholine synthesis in young rats[13,19] and in aged rats.[14] The administration of TSS increased the activity of choline acetyltransferase in the hippocampus and cerebral cortex in aged rats.[23,24] Moreover, administration of TSS increased the activity of nicotine acetylcholine receptors in the cerebral cortex and hippocampus.[25]

3. Kampo medicine on Ca^{2+} flux

If TSS stimulates the activity of nicotine acetylcholine receptors, TSS administration would increase the intracellular concentration of Ca^{2+}. PC 12 cells are cultured in Ca^{2+} containing medium; images of intracellular concentration of Ca^{2+} are recorded every 10 sec. with a video camera (C-2400-80; Hamamatsu Photonic System Corp., Bridgeway, N.J.) equipped with an Argus-50/CA system (Hamamatsu Photonic System) which controlled image acquisition and display. The ratio of the fluorescence intensity by 340 nm over that by 380 nm was calculated. A stimulation of nicotine acetylcholine receptors by nicotine administration increased the intracellular concentration of Ca^{2+} in PC 12 cells. Using this same system, the effect of TSS on the intracellular concentration of Ca^{2+} in PC 12 cells was examined. Administration of TSS (0.2 mg / ml) into culture medium increases the intracellular concentration of Ca^{2+}[26] (Fig. 11).

An increase of Ca^{2+} flux stimulated the activity of tyrosine hydroxylase[27,28]; therefore, administration of TSS would stimulate the activity of tyrosine hydroxylase through the activation of Ca^{2+} flux *in vivo*. Indeed it is that administration of TSS *in vivo* increased the activity of tyrosine hydroxylase in the

ventro tegumental dopaminergic neurons[10] and increased synthesis and relrase of dopamine in the cerebral cortex in aged rats[14] (Fig. 12). Using microdialysis techniques, Oyama (1990)[13] demonstrated that administration of TSS increased the concentration of dopamine in the cerebral cortex. Moreover, Kataoka, *et al.* (1991)[12] used the infusion techniques with neuronal slices to demonstrate that an infusion of TSS into the slices containing dopaminergic neurons released dopamine continuously during infusion.

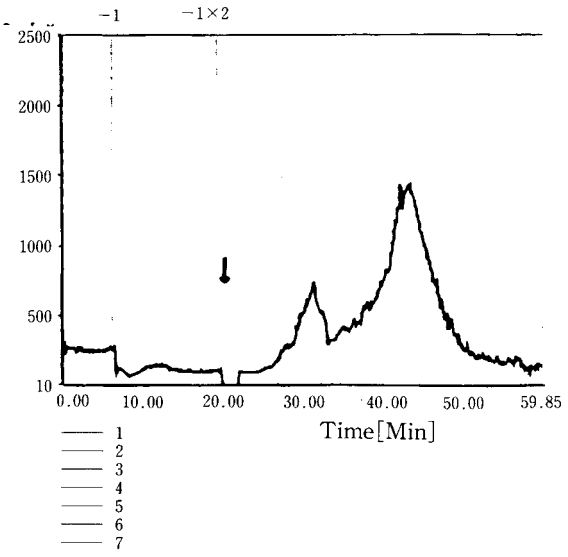


Fig. 11 Facilitation of calcium flux by treatment with Toki-shakuyaku-san.
Effect of Toki-shakuyaku-san on calcium flux in PCI2 cells. The vertical axis represents the intracellular concentrations of calcium and the horizontal axis represents the time after infusion of 0.2 mg/ml of Toki-shakuyaku-san into the perfusion medium (arrow). The line represents the average of seven PCI2 cells recorded individually at same time.

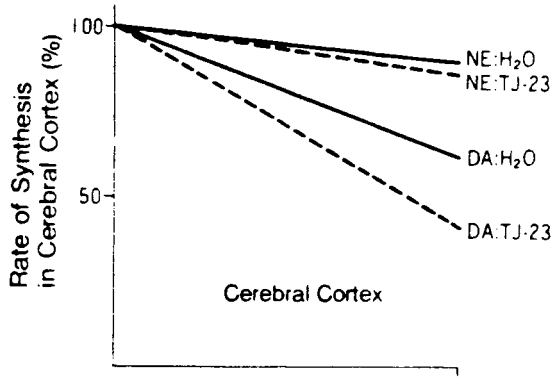


Fig. 12 Facilitation of synthesis and release of dopamine in cerebral cortex in aged animals by treatment with Toki-shakuyaku-san.
Facilitative effect of Toki-shakuyaku-san on synthesis and release of dopamine in cerebral cortex in aged rats. The vertical axis represets the rate of synthesis in cerebral cortex (%). The rate of synthesis and release of dopamine was accelerated by treatment with 500 mg/kg body weight of Toki-shakuyaku-san (DA : TJ-23) as compared with that of control (DA : H_2O).

Treatment with TSS in aged animals increases synthesis and release of acetylcholine. Furthermore, TSS increases Ca^{2+} flux into the cell through the activation of nicotine acetylcholine receptors. Thus creates synthesis and release of dopamine in the brain. An interconnection between dopaminergic neurons and cholinergic neurons is that it regulates the processes of learning and memory.[14, 29] The above studies demonstrated that TSS reverses age-related declining of memory through activation of cholinergic and dopaminergic neurons, receptors and protein synthesis. Now we need to know if TSS has an anti-apoptotic effect on neurons.

4. Anti-apoptotic effect of Kampo medicine

In order to examine if TSS has an anti-apoptotic effect on the neuron, we designed the following two experiments.[30] Experiment I : Postnatal cerebellar granule cells were cultured in either 25 mM K^+ or 5 mM K^+ BME medium for 8 days. The survival rates of cultured cells were examined using fluorescent microscopy counting the population of fluorescent diacetate (FDA) or propidium (PI) staining cells (live cells show green color of FDA staining and dead nuclei show red color of PI staining) and we also used phase microscopy to examine the growth and development of network of neurites and morphological changes of cells and nuclei while cells were cultured. The production of c-fos of transcription factors and DNA fragmentation were also examined daily for 8 days while cells were cultured in order to support the above observations. A 0.05 ml of TSS was added into the 5 mM K^+ BME medium at day 2 *in vitro* (2 DIV) and all the above examinations were carred out at 8 DIV. Experment II : In order to confirm the results of Experiment I, the postnatal cerebellar granule cells were cultured in 25 mM K^+ BME medium for 6 days and the BME medium changed to 5 mM K^+ at 7 DIV. The survival rates of cultured cells, network of neur-

ite, morphology of nuclei and DNA fragmentation were examined at 24 hr, 48 hr, and 72 hr later. 0.05 mg of TSS or 100 μg of Actinomycin D (an inhibitor of transcription factor) was added into the 5 mM K^+ BME medium at 7 DIV after the 25 mM K^+ BME medium was changed to 5 mM K^+ BME medium. All the above examinations were carried out at 24 hr, 48 hr and 72 hr later.

In control of Experiment I, 25 mM K^+ BME medium maintained cell growth and maturation. They brought about 80 %-90 % survival of cultured cerebellar granule cells which showed a well-grown and farmed network of neurite. The nuclei in 25 mM K^+ BME medium showed healthy cells with no nuclear condensation. On the other hand, the cells were able to grow up to 4 days in 5 mM K^+ BME medium, but a rapid neuronal death with DNA fragmentation occurred the following day and thereafter (Fig. 13, This shows daily observation of DNA fragmentation of cultured cerebellar granule cells in 5 mM K^+ BME medium. There was no trace of DNA at 5 DIV and thereafter).

At 8 DIV 80 % of cells showed nuclear condensation with extensive damage of neuritis network and there was only 20 % neuronal survival in 5 mM K^+ BME medium. Observations of phase-contrast microscopy and DNA electrophoresis indicated that neuronal death in 5 mM K^+ BME medium was through

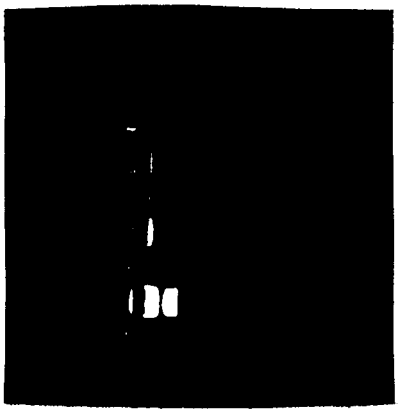


Fig. 13 Daily observation of DNA fragmentation of cultured cerebellar granule cells in 5 mM K^+ BME medium. Agarose gel electrophoresis of oligonucleosomal-length DNA fragments from cultured cerebellar granule cells in 5 mM K^+ BME medium. DNA fragmentation was examined at 2 DIV and continued for 6 days. Bottom line represents DNA marker (standard). DNA was presented at 2, 3 and 4 DIV, however, there was no trace of DNA at 5 DIV and thereafter.

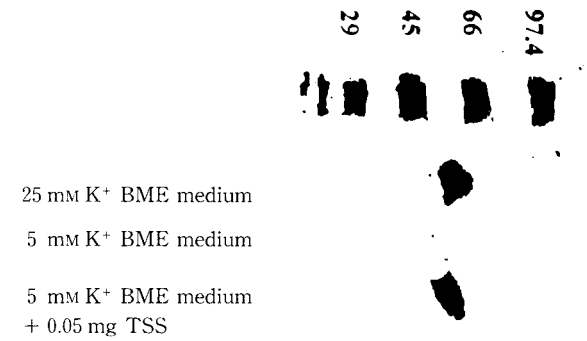
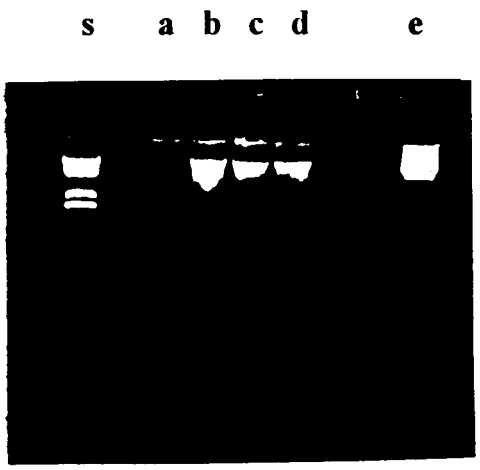


Fig. 14 Facilitation of c-fos protein production in cultured cerebellar granule cells by treatment with Toki-shakuyaku-san.
Precipitation of c-fos protein by antibodies raised against beta-gal-fos fusion protein. Lane I from top : marker protein (KD) ; Lane 2 (25 mM K^+ BME medium) : immunoprecipitation of c-fos protein from cultured cerebellar granule cells in 25 mM K^+ BME medium ; Lane 3 (5 mM K^+ BME medium) : no immunoprecipitation of c-fos protein from cultured cerebellar granule cells in 5 mM K^+ BME medium ; Lane 4 (5 mM K^+ BME medium + 0.05 mg TSS) : treatment with 0.05 mg/ml of Toki-shakuyaku-san (TSS) brought an immunoprecipitation of c-fos protein from cultured cerebellar granule cells in 5 mM K^+ BME medium.

apoptosis. Adding 0.05 mg/ml of TSS into the 5 mM K^+ BME medium at 2 DIV brought about 70 % neuronal survival and prevented nuclear condensation and DNA fragmentation. To compare the function of DNA in each cultured condition, we examined the production of c-fos transcription factor at 8 DIV with SDS-page (Fig. 14). In 25 mM K^+ BME medium cells produced c-fos protein (Fig. 7 : 25 mM K^+ BME medium) ; however, cells did not produce c-fos protein in 5 mM K^+ BME medium (Fig. 7 ; 5 mM K^+ BME medium). Adding 0.05 mg/ml of TSS into the 5 mM K^+ BME medium at 2 DIV brought about a production of c-fos protein in cultured cerebellar granule cells (Fig. 7 ; 5 mM K^+ BME medium + 0.05 mg TSS). Data provides the evidence that TSS prevented apoptosis and promoted growth and maturation of neurons.

To confirm those results, we designed the following Experiment II with matured cerebellar granule cells : the postnatal cerebellar granule cells were cultured in 25 mM K^+ BME medium for 6 days and BME medium changed to 5 mM K^+ from 25 mM K^+ BME medium at 7 DIV. The survival rates of cultured cells using FDA and PI staining and the formation of



s. standard for DNA
a. 25mM K^+-5mM K^+BME medium
b. 25mM K^+-5mM K^+BME medium+0.05mg TSS
c. 25mM K^+-5mM K^+BME medium+0.5mg TSS
d. 25mM K^+-5mM K^+BME medium+10 μg AD
e. 25mM K^+BME medium

Fig. 15 Intervention of DNA fragmentation of cultured cerebellar granule cells by treatment with Toki-shakuyaku-san.
Agarose gel electrophoresis of oligonucleosomal-length DNA fragments for cultured cerebellar granule cells. Lane 1 from left (s) : marker DNA ; Lane 2 (a) : there was no trace of DNA in cultured cerebellar granule cells at 48 hrs after changed 5 mM K^+ BME medium from 25 mM K^+ BME medium ; Lane 3 and 4 (b and c) ; treatment with 0.05 mg (b) or 0.5 mg (c) per ml of Toki-shakuyaku-san (TSS) intervened DNA fragmentation of cultured cerebellar granule cells at 48 hr after changed potassium (K^+) concentration in BME medium to 5 mM K^+ from 25 mM K^+ ; Lane 5 (d) : treatment with 10 μg of Actinomycin D (inhibitor of transcription factors) intervened DNA fragmentation of cultured cerebellar granule cells at 48 hr after changed 5 mM K^+ BME medium from 25 mM K^+ BME medium ; Lane 6 (e) : there was no DNA fragmentation in cultured cerebellar granule cells at 48 hr after changed 25 mM K^+ BME medium from 25 mM K^+ BME medium.

network of neurite and morphology of nuclei using phase microscopy were examined at 24 hr, 48 hr and 72 hr later. We observed also DNA fragmentation at 24 hr, 48 hr, and 72 hr later. Changing medium to 25 mM K^+ BME from 25 mM K^+ BME medium at 7 DIV cultured cerebellar granule cells did not show nuclear condensation, neurite damage, and DNA fragmentation at 24, 48, and 72 hours later (Fig. 15e). When culture medium was changed to 5 mM K^+ from 25 mM K^+ BME medium at 7 DIV, we observed only 35 % neuronal survival at 72 hr later. The cells showed nuclear condensation, extensive damage of neurite network, and DNA fragmentation (Fig.15a ; s represents standard of DNA). Adding either 0.5 mg/ml or 0.05 mg/ml of TSS to the 5 mM K^+ BME medium at 7 DIV brought about 65 % neuronal survival and prevented nuclear condensation and DNA fragmentation at 48 hr later (Fig. 15b and c respectively). Moreover, TSS-treated cells continued to mature in 5 mM K^+ BME medium and no DNA fragmentation was observed at 72 hr later. To investigate the site of action of TSS on prevention of DNA fragmentation, 10 μg of Actinomycin D (inhibitor of transcription factor) was added to 5 mM K^+ BME medium at 7DIV. Adding Actinomycin D prevented DNA fragmentation at 48 hr later in 5 mM K^+ BME medium (Fig. 15d) ; however, cells treated with Actinomycin D did not survive on the next day and all cells were dead at 72 hr later.

Results confirmed the anti-apoptosis effect of TSS in maturated neurons like that in Experiment I. The study provides the evidence that TSS prevents not only apoptosis, but also that TSS has a factor that stimulates synthesis and release of NGF to bring growth and maturation of neurons.

5. Adverse effect of Kampo medicine

We will now discuss the adverse effect of TSS in humans. The clinical application of TSS for dementia of Alzheimer type (DAT) is assessed and the results are obtained in open trials in women DAT patients. Treatment with TSS in DAT patients shows considerable improvement in arousal disorders, absentmindedness, eating disorders, delirium, easing of irritation, anxiety, depression and restlessness in the GBS dementia evaluation scale.[31] In Hasegawa simplified evaluation scale, a significant rise in the number of points is observed immediately after TSS administrations.[31] Moreover, a significant improvement in TSS-treated DAT patients is observed in conversation, removing/putting on clothes, writing ability, long-term memory, and even in intellectual factors as measured on the GBS scale.[32] These are significant changes observed in CT examination in the brain of DAT patients compared with senile dementia of Alzheimer type (SDAT). Further investigation reveals, however, that treatment with TSS improves

long-term memory, writing ability, and intellectual functions on the GBS scale in 50 % of TSS-treated DAT patients ($p<0.01$) compared with female HCI-treated DAT patients as controls, but SDAT patients do not respond to treatment with TSS.[33] Yamamoto (1991)[33] indicates that patients who have a shorter history of DAT show a better response to TSS treatment than patients who have a long-term history of DAT. Recently, Otsuka (1993)[34] expressed that he prescribes TSS combined with other medicinal plants for treatment and / or intervention of dementia in elderly patients. No one reported serious adverse effect in TSS in past few decades.

Note :

Before closing the manuscript the following idea came to my mind : I looked for the medicinal plants which treat only physical symptoms of menopausal symptoms and dementia in elderly patients ; however, I have neglected to investigate the medicinal plants which heal the stressed and strained mind in elderly patients. A healing of the stressed and strained mind in elderly patients is equally as important as the healing of their physical symptoms. Now we are focusing our research on investigating the medicinal plants which have a factor that heals the stressed and strained mind in elderly patients.

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