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Remedial effects of Yokukansan, a *Kampo* medicine, on social interactive deficits in Thiamine-deficient mice

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Abstract

The purpose of present study was to clarify remedial effects of *yokukansan*, a traditional Japanese medicine (*Kampo*), on social interactions such as aggressive and social behaviors observed in mice that were thiamine deficient (TD) mice due to feeding a TD diet. A significant increase in aggressive behavior and significant decrease in social behavior were observed in TD mice on the 21st day of TD feeding. TD diet was changed to control diet on the 27th day because TD mice began to die after the 27th day, and the animals were fed until the 35th day. Although the TD-induced decrease in body weights of TD mice was recovered by switching from TD diet to control diet, abnormal social interactive behaviors observed on the 21st day were still retained on the 35th day. Repeated oral administration of yokukansan (0.5 and 1.0 g/kg, once a day) for 14 days from the 22nd day to the 35th day ameliorated both aggressiveness and abnormal social behaviors on the 35th day. These results suggest that yokukansan has remedial effects on the increased aggressive behavior and decreased social behavior.

Key words: Yokukansan; Kampo; thiamine deficiency; aggressive behavior; social interaction

Introduction

Yokukansan is one of the traditional Japanese medicines called "*kampo*" medicines in Japan. This medicine has been approved by the Ministry of Health, Labor, and Welfare of Japan as a remedy for neurosis, insomnia, and irritability in children. Recently, yokukansan was reported to ameliorate behavioral and psychological symptoms such as hallucinations, agitation, and aggressiveness in patients with Alzheimer's disease, dementia with Lewy bodies, and other forms of senile dementia (Iwasaki *et al*., 2005, Mizukami *et al*., 2009). Until now, various dementia models have been used for research in the pathogenesis and therapy of dementia (Ikarashi *et al*., 2004, Maslish *et al*., 2001, Ishimaru *et al*., 1998, Given

et al., 1995). However, information regarding behavioral and psychological symptoms of dementia (BPSD) was few in the animal models, because most studies focused on deficits of the functions of learning and memory that are the main symptoms of dementia. Recently, it has been reported that not only impairment of learning and memory but also BPSD-like behaviors such as anxiety, depression, muricide, attacking, and startle responses are observed in thiamine-deficient (TD) rats and mice (Nakagawasai *et al*., 2000). TD is a critical factor in the etiology of Wernicke-Korsakoff's syndrome and a decrease in brain function is induced due to a decrease in thiamine-dependent energy metabolism (Butterworth *et al*., 1986). In addition to it, deficiencies in thiamine-dependent enzyme activities (Butterworth *et al*., 1990), selective neuronal loss (Langlais *et al*. 1990), cholinergic deficits (Barclay *et al*., 1981), and accumulations of the abnormal tau isoforms (Cullen *et al*., 1995) and APP (Calingasan *et al*., 1995) which resemble to Alzheimer's disease are reported. These findings suggest that TD animals may be a valuable tool for evaluation of pharmacotherapy for BPSD as well as dysfunction of learning and memory which is a core symptom of dementia. Recently, we demonstrated that yokukansan inhibited social interactive behaviors such as the increased aggressive behavior and decreased social behavior in TD rats that were produced by feeding a TD diet (Ikarashi *et al*., 2009). These findings suggest that yokukansan possesses a preventive or progression-controlling effect against abnormal social interactive behaveorrs in TD rats when yokukansan was administered together with the TD diet. However, it is still unclear whether yokukansan possesses remedial effects against these symptoms.

In the present study, a preventive effect of yokukansan on abnormal social interactive behaviors was first confirmed in TD mice, and then the remedial effect on these symptoms was investigated using the experimental conditions demonstrating the preventive effect.

Materials and Methods

Animals

Three-week-old male ddY mice were purchased from Japan SLC (Hamamatsu, Japan). Animals were group-housed (five mice in a cage) in plastic cages (230 x 310 x 155 mm) at a temperature of 23 ± 2°C, relative humidity of 55 ± 10% and 12-h light/dark cycle, with lights on from 07:00 to 19:00 daily, and allowed free access to water and standard laboratory food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). After habituation for 1 wk, 4-wk-old mice weighing 21-23 g at the beginning of the experiment were used in the present study. During experiment period, animals were housed individually in plastic cages (110 x 310 x 155 mm).

Conspecific male mice were also obtained from the same breeder to evaluate behaviors of subject mice in the social interaction test and were group-housed (five mice in a cage) in the same breeding environment until the animals were used in the tests.

TD diet and its control diet (AIN-93G) used in the present study were purchased from Oriental Yeast Co., Ltd. TD diet was the AIN-93G diet without thiamine. Control diet as well as standard laboratory chow (MF) contains 0.5 mg thiamine per 100 g of diet. All experimental procedures were performed according to the "Guidelines for the care and use of laboratory animals" approved by the Laboratory Animal Committee of Tsumura & Co.

Drugs and reagents

Yokukansan is composed of seven dried medicinal herbs: *Atractylodis lancea* rhizome (4.0 g, rhizome of *Atractylodes lancea* De Candolle), *Poria sclerotium* (4.0 g, sclerotium of *Poria cocos* Wolf), Cnidium rhizome (3.0 g, rhizome of *Cnidium officinale* Makino), Japanese Angelica root (3.0 g, root of *Angelica acutiloba* Kitagawa), Bupleurum root (2.0 g, root of *Bulpleurum falcatum* Linné), Glycyrrhiza root (1.5 g, root and stolon of *Glycyrrhiza uralensis* Fisher), and Uncaria thorn (3.0 g, thorn of *Uncaria rhynchophylla* Miquel).

Regarding ingredients contained in Yokukansan extract, 25 compounds have been identified by three-dimensional high-performance liquid chromatographic analysis (Mizukami *et al*., 2009). The dry powdered extract of yokukansan used in the present study was supplied by Tsumura & Co. (Tokyo, Japan). Doses (0.5 or 1.0 g/kg) of yokukansan were prepared by dissolving them in 10 ml of distilled water.

Measurement of thiamine content in yokukansan

We previously demonstrated that yokukansan did not include thiamine (Ikarashi *et al*., 2009). In brief, yokukansan (35 mg/ml) was dissolved in distilled water and centrifuged at 3,000 rpm for 10 min; then the supernatant was passed through a 0.45-μm membrane filter. An aliquot (20 μl) of the filtrate was injected into a HPLC (LC-10V system, Shimadzu Co., Kyoto, Japan) for determination of thiamine. The thiamine peak was not detected in the extract.

Experimental design

Evaluation of effect of yokukansan on social interactive behaviors in normal mice

Twenty mice were divided into two groups (n = 10/group): control, and 1.0 g/kg yokukansan. All animals were fed a control diet for 21 days. Distilled water (10 ml/kg body weight) was orally administered to the mice of the control group, and 1.0 g/kg yokukansan was orally administered to the mice of the yokukansan group. Effects of yokukansan on social interactive behaviors were evaluated 60 min after the final administration of the test substance on the 21st day by a social interaction test.

Evaluation of preventive effect of yokukansan on social interactive behaviors in TD mice

Forty mice were divided into four groups (n = 10/group): control, TD, TD + 0.5 g/kg yokukansan, and TD + 1.0 g/kg yokukansan. Animals in control group were fed a control diet for 35 days. Animals in TD and TD + yokukansan (0.5 g/kg and 1.0 g/kg) groups were fed a TD diet for the same period. Distilled water (10 ml/kg body weight) was orally administered to the mice of control and TD groups, and yokukansan (0.5 and 1.0 g/kg) was orally administered to the mice of yokukansan groups. Effects of yokukansan on social interactive behaviors were evaluated 60 min after the final administration of test substance on the 21st day by a social interaction test. Body weight, neurological symptoms, and survival rate (or mortality) were examined every day until the 35th day.

Evaluation of remedial effect of yokukansan on social interactive behaviors in TD mice

Thirty mice were fed a TD diet for 26 days. On the 21st day, social interactive behaviors of all animals were evaluated to select animals with aggressive behavior. The 23 mice selected were divided into three groups: TD (n=8), TD + 0.5 g/kg yokukansan (n = 7), and TD + 1.0 g/kg yokukansan (n = 8). These animals were fed the control diet for 9 days from the 27th day to the 35th day. On the other hand, animals (n = 10) in control group were fed control diet for 35 days. Yokukansan (0.5 g/kg or 1.0 g/kg) was orally administered to the mice of yokukansan groups for 14 days from the 22nd to 35th day. Distilled water (10 ml/kg body weight) was administered to the mice of control and TD groups during the same period. Social interaction test for evaluation of the remedial effect was performed 60 min after the final administration of test substance on the 35th day.

Social interaction test

Social interactive behaviors such as aggressive behavior and social behavior in mice were evaluated by a social interaction test (File, 1980, Lumley *et al*., 2004). A subject mouse and a non-treated (group-housed) control mouse were placed together in an open-field apparatus (50 cm x 50 cm x 40 cm; Neuroscience, Inc., Tokyo Japan). Interactions between the two animals were monitored with a video camera for 10 min, and the data were saved on a computer. Later, the total number of aggressive behaviors (tail rattling, chasing, and attacking) as the index of aggressiveness or social behaviors (sniffing, following, and contacting) as the index of sociability of the subject animal toward the control animal for 10 min were counted by two observers blind to the treatment.

Effect of yokukansan on thiamine concentration in the blood of TD mice

Fifty-four mice were divided into three groups (n = 18/group): control, TD and TD + 1.0 g/kg yokukansan. Animals in control group were fed a control diet for 21 days. Animals in TD and TD + yokukansan groups were fed a TD diet for the same period. Distilled water (10 ml/kg) was orally administered to the mice of control and TD groups, and yokukansan (1.0 g/kg) was orally administered to the mice of yokukansan group. To obtain blood samples, 6 mice fasted for overnight in each group were sacrificed on 0, 14th and 21st days, respectively: the blood samples were collected from ventral aorta of mice anesthetized with pentobarbital (50 mg/kg, i.p.). EDTA2Na was used as anticoagulant. Collected blood samples were used for measurement of thiamine concentration.

Determination of thiamine concentration in whole blood

Thiamine level in whole blood was measured using a microbiological assay kit (Thiamine BIO-5136, DRG International Inc., New Jersey, USA) in accordance with the manufacturer's instructions. In brief, an enzymatically pre-treated blood sample was transferred in the well of a microtiter plate coated with Lactobacillus fermentum. To make thiamine-dependent growth response of the bacteria, the plate was incubated at 37°C for 48 hours. Bacterial growth reflecting thiamine concentration was determined turbidimetrically at 610 nm using an ELISA-reader (Infinite 200, Tecan Group Ltd., Mànnedorf, Switzerland).

Statistical analysis

Body weight, behavioral and blood thiamine data were represented as the mean ± S.E.M. Statistical significance of all data was evaluated by one-way analysis of variance (ANOVA) or two-way ANOVA, followed by the Student's t-test, Bonferroni multiple comparison procedure or Fisher's protected least significant difference (PLSD) test. Survival data were represented the number of surviving mice in each group of 10. Statistical significance of the rate was evaluated by a Fisher's exact probability test because some expected values were less than 5 in a 2x2 contingency table. Significance level in each statistical analysis was accepted at $p<0.05$.

Results

Effect of yokukansan on social interactive behaviors in normal mice

Figure 1 shows the effects of yokukansan on aggressive behavior and social behavior on the 21st day in normal mice. Yokukansan had no significant effect on aggressive behavior (Fig. 1A) or social behavior (Figure 1B) in normal mice.

Preventive effect of yokukansan on social interactive behaviors in TD mice

Changes in body weights in each group are shown in Fig. 2A. Two-way ANOVA showed significant differences in factors for group ($F_{3,16} = 28.822$, $p<0.001$), period ($F_{3,35} = 95.774$, $p<0.001$), and group x period ($F_{3,105} = 92.387$, $p<0.001$). The weights of TD and TD + yokukansan groups decreased gradually after the 12th day compared with the control group. On the terminal day 35, weights of TD (18.8 ± 0.3 g), TD + 0.5 g/kg yokukansan (19.6 ± 0.3 g), and TD + 1.0 g/kg yokukansan (20.2 ± 0.4 g) groups were significantly decreased compared with that (39.9 ± 1.2 g) of control group. However, no significant differences in the decreased levels were observed between TD and TD + yokukansan (0.5 and 1.0 g/kg) groups.

Until the 21st day of this experiment, no abnormal symptom was observed in any group. However, all mice in TD group showed neurological symptoms characterized by opisthotonus, convulsion, seizure, staggering gait, and ataxia between the 24th and 35th day. The incidence of neurological symptoms was inhibited by treatment with yokukansan. In control group, neurological symptoms were entirely absent (data not shown here).

Figure 2B shows the survival rate in each group during experiment. From the 26th day, the deaths of several animals were observed in TD and TD + yokukansan groups. The survival rate in each group gradually decreased, i.e., the lethality increased. On the terminal day 35, the survival rate in TD, 0.5 g/kg yokukansan, and 1.0 g/kg yokukansan groups were 20% (2/10 animals), 50% (5/10), and 60% (6/10), respectively. In control group, no animal deaths were observed. Thus, the survival rate in yokukansan-treated groups attended to be higher than that in TD group, though a significant difference was not detected between these groups. Figure 2C shows effects of yokukansan on aggressive behavior and social behavior on the 21st day. In aggressive behavior data (Figure 2C-1), one-way ANOVA showed significant differences in group factor ($F_{3,36} = 5.278$, $p<0.05$). Post-hoc analysis revealed that

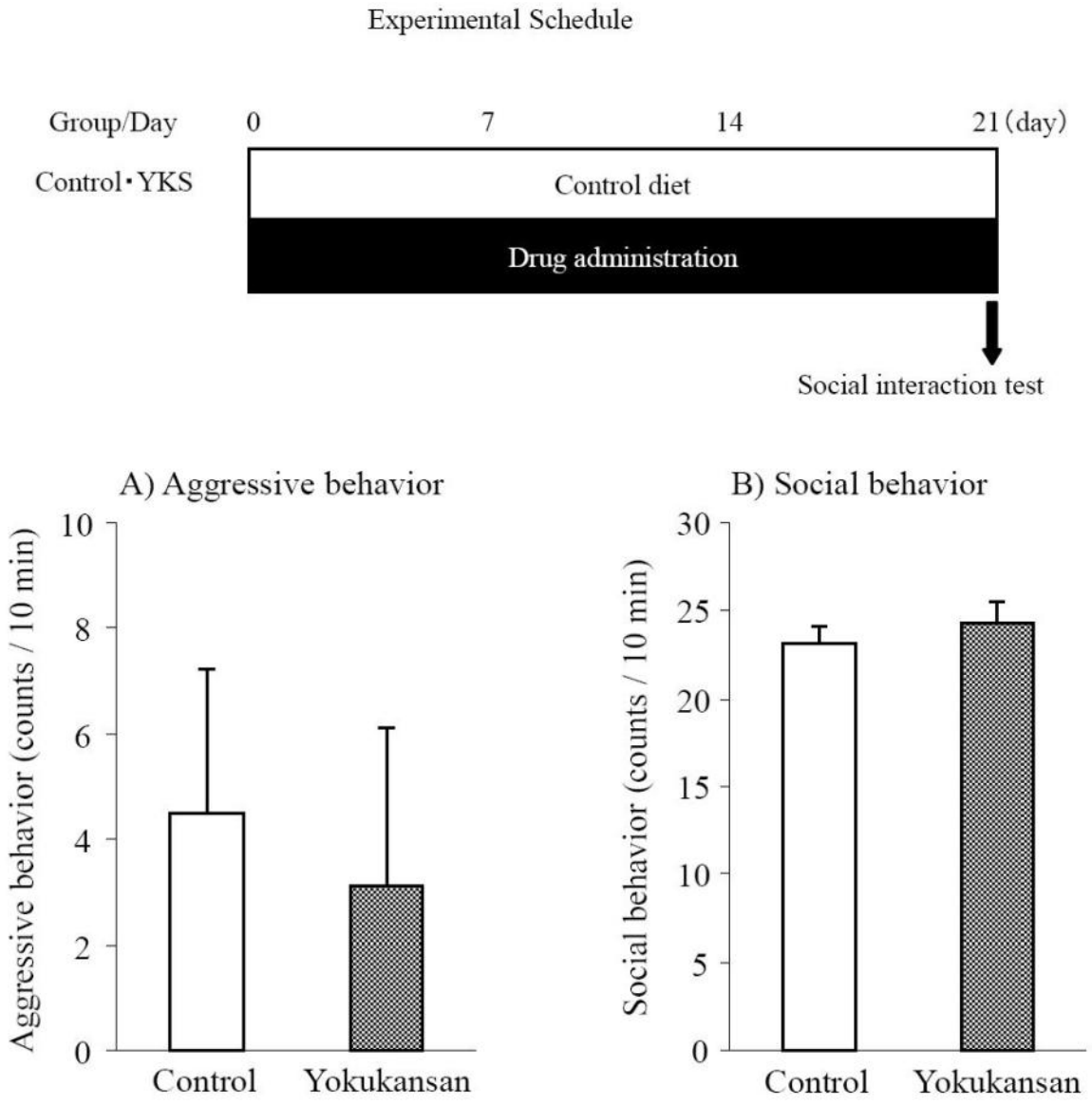


Figure 1. Effect of yokukansan on social interactive behaviors in normal mice. Animals in both control and yokukansan (YKS) groups were fed a control diet for 21 days. Distilled water (10 ml/kg, p.o.) or YKS (1.0 g/kg, p.o.) was administered to the mice of control or yokukansan group for the same period. Social interaction test was performed 60 min after the final administration of the test substance on the 21st day. Figure 1A or figure 1B shows the effects of YKS on aggressive behavior or social behavior. Each value represents the mean ± S.E.M. (n = 10). No significant differences in aggressive and social behaviors were observed between control and YKS groups (Student's t-test).

the number of aggressive behaviors in TD group significantly increased compared with control group. The TD-induced increase was significantly inhibited by treatment with yokukansan in a dose-dependent manner. In social behavior data (Figure 2C-2), one-way ANOVA shows significant differences in group factor ($F_{3,36}$=16.168, p<0.001). Post-hoc analysis revealed that the number of social behaviors in TD group decreased significantly compared with control group. The TD-induced decrease was significantly ameliorated by treatment with yokukansan in a dose-dependent manner.

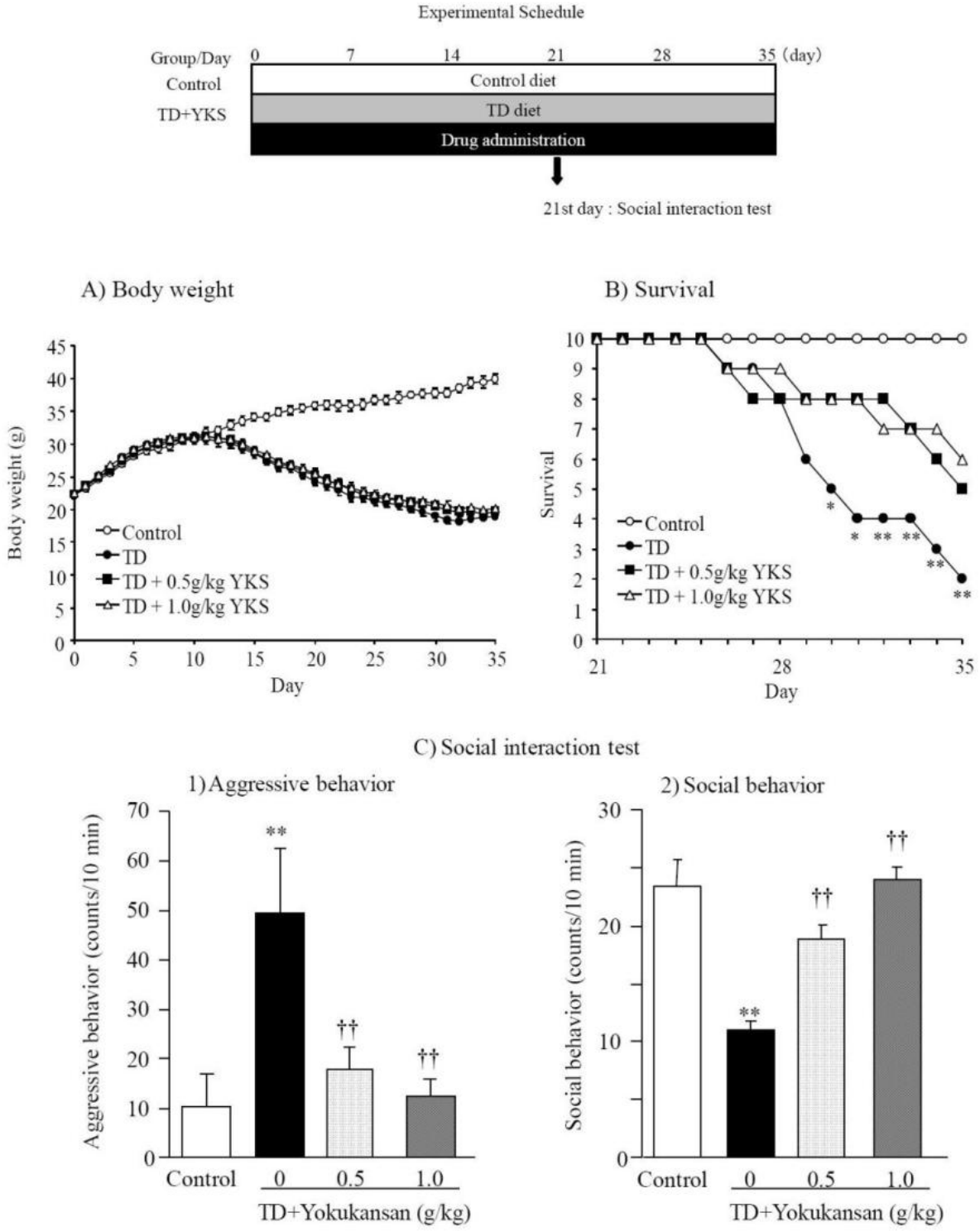


Figure 2. Preventive effect of yokukansan on social interactive behaviors in TD mice. Animals in control group were fed a control diet for 35 days. Animals in TD and TD + yokukansan (YKS, 0.5 g/kg and 1.0 g/kg) groups were fed a TD diet for the same period. Distilled water (10 ml/kg, p.o.) was administered to the mice of control and TD groups, and YKS (0.5 and 1.0 g/kg, p.o.) was administered to the mice of YKS groups for the same period. Social interaction test was evaluated 60 min after the final administration of the test substance on the 21st day. Figure 2A shows changes in body

weight. Each value represents the mean ± S.E.M. (n = 10). A significant difference was observ-ed between control and other three groups (two-way ANOVA, $F_{3,16} = 28.822$, $p<0.001$). However, no significant differences were observed among TD and YKS (0.5 and 1.0 g/kg) groups. Figure 2B shows the effect of YKS on survival rate. Each value represents the number of surviving mice in each group of 10. Statistical significance was evaluated by a Fisher's exact probability test: $^{*}p<0.05$ and $^{**}p<0.01$ vs the corresponding control group. Figure 2C shows the effects of YKS on social interactive behaviors. Each value represents the mean ± S.E.M. (n = 10). Statistical significance was evaluated by Fisher's PLSD after a one-way ANOVA: $^{**}p<0.01$ vs Control group, and $^{\dagger\dagger}p<0.01$ vs TD group.

Remedial effect of yokukansan on social interactive behaviors in TD mice

In the experiment to evaluate remedial effect of yokukansan on social interactive behaviors, TD diet given to animals in TD and TD + yokukansan (0.5 g/kg and 1.0 g/kg) groups was changed to control diet from the 27th day. Changes in body weights in each group according to the schedule are shown in Fig. 3A. Two-way ANOVA showed significant differences in factors for group ($F_{3,9} = 9.092$, $p<0.01$), period ($F_{3,35} = 50.666$, $p<0.001$), and group x period ($F_{3,105} = 15.096$, $p<0.001$). Body weights in the control group were gradually increased throughout the experimental period of 35 days. On the other hand, body weights in TD and TD + yokukansan groups increased as well as control group until the 12th day, but after the 12th day, body weights in these three groups decreased similarly until the 27th day. The decreased body weights in three groups were recovered to the control level on the 35th day by changing to control diet from TD diet.

Though neurological symptoms were observed in several animals of TD and TD + yokukansan groups from the 24th day as well as during the experiment to determine the preventive effect, these symptoms were recovered on the 35th day by changing to control diet from TD diet on the 27th day. In this experiment, no death was observed in any group.

Figure 3B shows the remedial effects of yokukansan that was administered for 14 days from the 22nd day to the 35th day, on aggressive behavior and social behavior on the 35th day. In aggressive behavior data (Fig. 3B-1), one-way ANOVA showed significant difference in group factor ($F_{3,29} = 4.809$, $p<0.01$). Post-hoc analysis revealed that the number of aggressive behavior in TD group significantly increased compared with that of control group. The TD-induced increase was significantly ameliorated by treatment with yokukansan in a dose-dependent manner.

In social behavior data (Figure 3B-2), one-way ANOVA shows significant difference in group factor ($F_{3,29} = 10.037$, $p<0.001$). Post-hoc analysis revealed that the number of social behavior in TD group significantly decreased compared with that of control group. The TD-induced decrease was significantly ameliorated by treatment with yokukansan in a dose-dependent manner.

Effects of yokukansan on blood thiamine concentration in TD mice

Changes in thiamine concentration in the whole blood of each group during the experimental period for 21 days are shown in Fig. 4. Two-way ANOVA showed significant differences in factors for group ($F_{2,15} = 175.297$, $p<0.001$), period ($F_{2,2} =1\ 35.265$, $p<0.001$), and group x period ($F_{2,30} = 31.596$, $p<0.001$). The thiamine concentration in control group

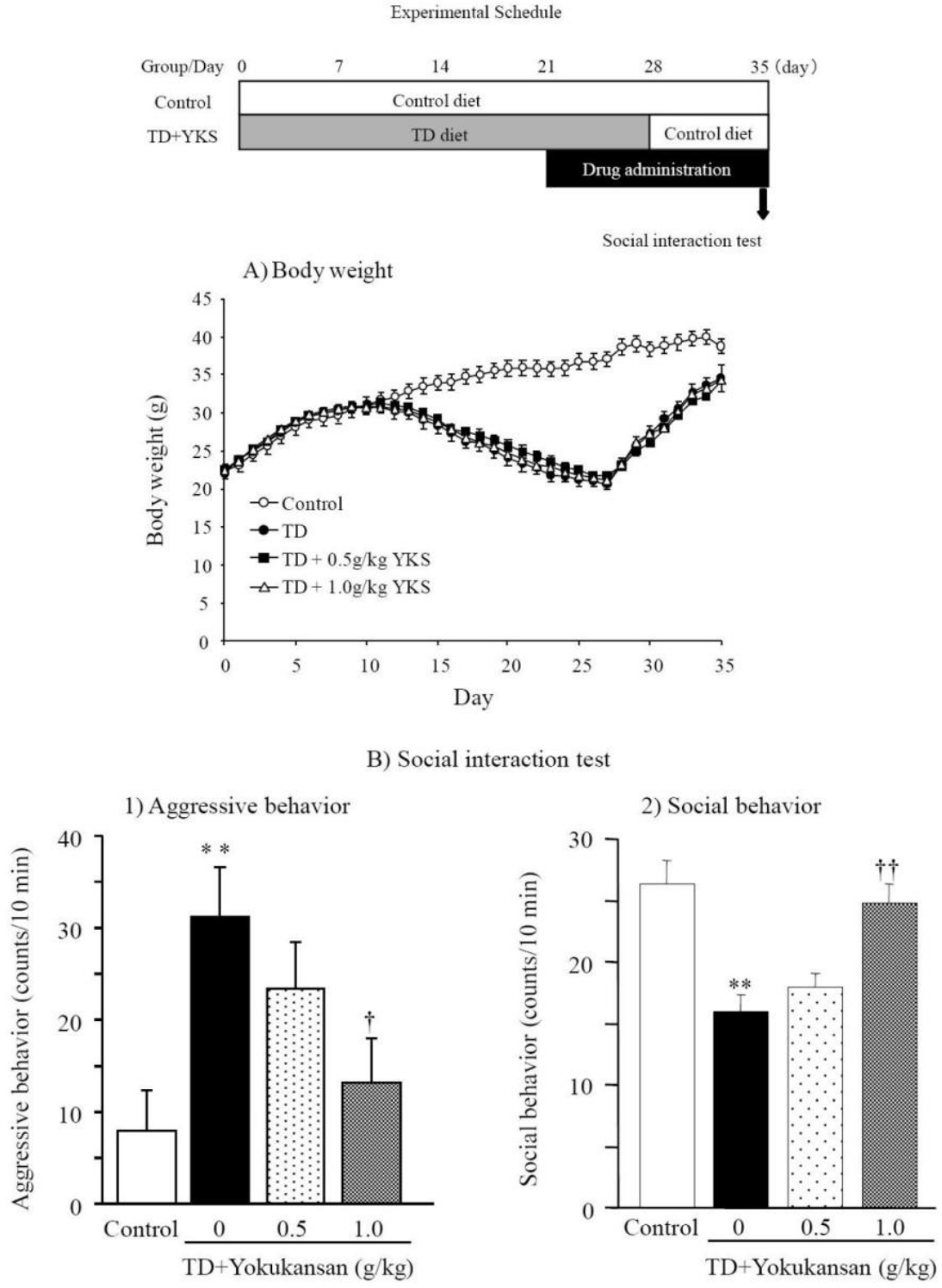
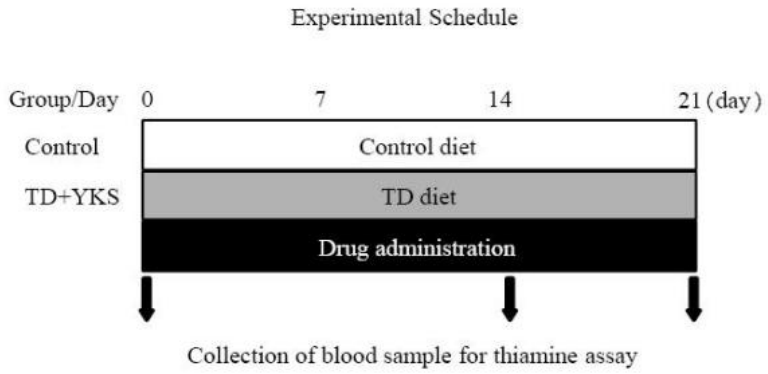


Figure 3. Remedial effect of yokukansan on social interactive behaviors in TD mice. Animals (n = 10) in control group were fed the control diet for 35 days. On the other hand, Animals were fed a TD diet for 26 days. On the 21st day, 23 animals with aggressive behavior were selected by social interaction test. The mice were divided into three groups: TD (n = 8), TD + 0.5 g/kg yokukansan (YKS, n = 7), and TD + 1.0 g/kg YKS (n = 8). TD diet was changed to control-diet for 9 days from the 27th day to 35th day. YKS (0.5 g/kg or 1.0 g/kg, p.o.) was administered to the mice of YKS

groups for 14 days from the 22nd to 35th day. Distilled water (10 ml/kg) was administered to the mice of control and TD groups during the same period. Social interaction test for evaluation of remedial effect was performed 60 min after the final administration of the test substance on the 35th day. Figure 3A shows changes in body weight. Each value represents the mean ± S.E.M. (n = 7-10). A significant difference was observed between control and other three groups (two-way ANOVA, $F_{3,9}$=9.092, p<0.01). However, no significant differences were observed among TD and YKS (0.5 and 1.0 g/kg) groups. Figure 3B shows the effects of YKS on social interactive behaviors. Each value represents the mean ± S.E.M. (n = 7-10). Statistical significance was evaluated by Fisher's PLSD after a one-way ANOVA: $^{**}p$<0.01 vs Control group, and $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01 vs TD group.



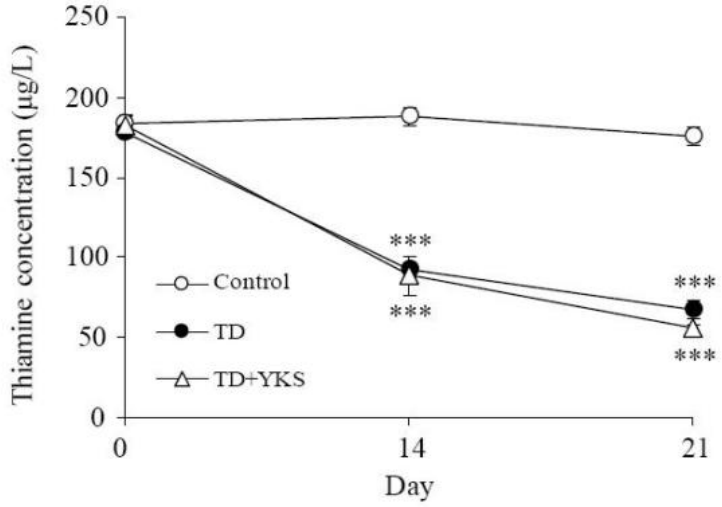


Figure 4. Effect of yokukansan on thiamine concentration in the blood of TD mice. Animals in the control group were fed a control diet for 21 days. Animals in TD and TD + 1.0 g/kg yokukansan (YKS) groups were fed a TD diet for the same period. Distilled water (10 ml/kg, p.o.) was administered to the mice of control and TD groups, and YKS (1.0 g/kg, p.o.) was administered to the mice of TD + 1.0 g/kg YKS group for the same period. Six mice fasted for overnight in each group were sacrificed on 0, 14th and 21st days to collect the blood samples for determination of thiamine concentration. Thiamine level in whole blood was measured using a microbiological assay. Each value represents the mean ± S.E.M. (n = 6). Statistical significance was evaluated by Fisher's PLSD after a two-way ANOVA: $^{***}p$<0.001 vs the corresponding control. However, no significant differences were observed between TD and TD + 1.0 g/kg YKS groups.

was constant throughout the experimental period. On the other hand, the concentrations of TD and TD + 1.0 g/kg yokukansan groups decreased gradually compared with those of the corresponding control group. On the terminal day 21, the concentrations in TD (67.3 ± 5.7 μg/L) and TD + 1.0 g/kg yokukansan (56.8 ± 2.2 μg/L) groups were significantly decreased compared with that (175.9 ± 5.9 μg/L) of control group. However, no significant difference was observed between TD and TD + yokukansan groups.

Discussion

The purpose of the present study was to clarify remedial effect of yokukansan on TD-induced abnormal behaviors such as increased aggressive behavior and decreased social behavior. To achieve this, the preventive effect of yokukansan on those behaviors was first verified. These abnormal behaviors were observed on the 21st day of TD feeding, and were inhibited by the co-administration of yokukansan. These results agree with our finding previously reported for TD rats (Ikarashi *et al*., 2009), suggesting that yokukansan possesses a preventive or inhibitory effect on the development of abnormal social interactive behaviors. In this experiment, animals were fed until the 35th day after social interaction test on the 21st day to examine neurological symptoms and lethality. Neurological symptoms (opisthotonus and loss of lighting reflex) and death appeared around the 26th day in TD mice. These symptoms interfere to the evaluation of social interaction test. Moreover, repeated administration for two or three weeks is necessary to demonstrate remedial effect of yokukansan on aggressiveness or sociability (Sekiguchi *et al*., 2009, Kanno *et al*., 2009). These suggest that an experimental condition in which animal does not die or develop neurological symptoms is necessary to evaluate remedial effect of yokukansan on TD-induced aggressive and social behaviors. Therefore, TD diet was changed to control-diet which contains thiamine for 9 days from the 27th day to 35th day, to avoid the development of neurological symptoms and death. It has been reported that learning and memory dysfunctions and emotional abnormalities including muricide in TD animals do not recover when thiamine is administered, though the appetite decrease, growth suppression, and neurological symptoms are recovered by thiamine treatment (Onodera *et al*., 1981, Murata *et al*., 2004). In the present study, when aggressive and social behavioral data in TD group were compared between the 21st day (Fig.3) and 35th day (Fig.4), abnormalities of both behaviors observed on the 21st day were still retained on the 35th day, although a recovery tendency was observed in aggressive behavior. The abnormal behaviors retained by TD mice were ameliorated in a dose-dependent manner by treatment with yokukansan for 14 days from the 22nd to 35th day, suggesting that yokukansan possesses a remedial effect on TD-induced abnormal behaviors. We recently demonstrated that three-week administration of yokukansan significantly ameliorated the aggressiveness induced by intracerebroventricular injection of amyloid β into mice in a dose-dependent manner (Sekiguchi *et al*., 2009). These results also support remedial effect of yokukansan on aggressiveness.

Social behaviors such as sniffing, following, and contacting between two animals have been evaluated as an index of anxiety or an anxiolytic effect because benzodiazepine- and serotonin(5-HT)-related anxiolytic drugs increase social behaviors, whereas anxiogenic agents decrease them (File *et al*., 2003). Kuribara and Maruyama (1996) demonstrated the anxiolytic effect of yokukansan treatment (0.25-2.0 g/kg, p.o., for seven days) by using a plus-maze test that is often used for evaluation of anxiety in mice. Taken together, our

present data regarding social behavior might suggest that yokukansan has an anxiolytic effect.

TD-induced behavioral and psychological symptoms including muricide, anxiety and depression are reported to be suppressed by administration of 5-hydroxytryptophan, a serotonin precursor (Onodera *et al.*, 1979), and chlorimipramine, a selective serotonin reuptake inhibitor (Onodera et al., 1981). In addition, extracellular glutamate concentrations have been demonstrated to increase in vulnerable regions of the brain such as the thalamus in TD (Langlais *et al*., 1993, Todd *et al*., 2001). A selective down-regulation of the astrocyte glutamate transporters GLT-1 and GLAST provides a rational explanation for the increase in interstitial glutamate levels (Hazell *et al*., 2001). In addition, the extent of cell death in the TD brain is reported to be reduced by treatment with the non-competitive N-methyl-D-aspartic acid receptor antagonist MK-801 (Langlais *et al*., 1990, Todd *et al*., 1998). These lines of evidence suggest that the developmental mechanism of TD diet-induced behavioral and psychological symptoms may involve the dysfunction or degeneration of central serotonergic and glutamatergic neurons.

On the involvement to serotonergic mechanism, we previously demonstrated *in vitro* that yokukansan and a constituent herb Uncaria hook showed a partial agonistic effect on 5-HT $_{1A}$ receptors (Terawaki *et al*., 2010). This *in vitro* finding was also supported by *in vivo* experiment demonstrating that yokukansan ameliorated abnormal aggressiveness and sociability observed in *para*-chloroamphetamine-induced cerebral 5-HT-depletion rats, and the ameliorative effect was counteracted by co-administration of a 5-HT_{1A} receptor antagonist, WAY-100635 (Kanno *et al*., 2009). Taken together, these findings suggest that a partial agonistic effect on 5-HT_{1A} receptors by Uncaria hook is involvement to ameliorative effects of aggressive and social behaviors by yokukansan. Uncaria hook has been demonstrated to contain some indole alkaloids such as geissoschizine methyl ether, corynantheine and dihydrocorynantheine having 5-HT_{1A} receptors agonistic effect (Kanatani *et al*., 1985, Pengs-uparp *et al*., 2001).

On the involvement to gulutamatergic mechanism, we previously demonstrated that yokukansan ameliorated BPSD-like symptoms together with an increase in cerebral extracellular glutamate in TD rats (Ikarashi *et al*., 2009, Iizuka *et al*., 2010). Furthermore, we demonstrated in cultured rat cortical astrocytes that yokukansan ameliorated the TD-induced decrease in glutamate uptake by astrocytes (Kawakami *et al*., 2009), implying that yokukansan may contain active herbs or compounds possessing this effect. Among the seven constituent herbs of yokukansan, significant effects were found in a constituent herb Glycyrrhiza root and its components (glycyrrhizin and 18β-glycyrrhetinic acid) (Kawakami *et al*., 2010). These evidence suggest that glycyrrhizin and 18β-glycyrrhetinic acid are likely responsible for amelioration of dysfunction of glutamate transport in astrocytes. Besides our evidence, oxyindole alkaloids such as isorhynchophylline, isocorynoxeine, and rhynchophylline, and indole alkaloids such as hirsuteine and hirsutine contained in Uncaria hook are known for their protective effects against glutamate-induced neuronal death (Shimada *et al*., 1999). Oral administration of geissoschizine methyl ether or hirsuteine inhibits glutamate-induced convulsion in mice (Mimaki *et al*., 1997).

A decline in the growth rate has been reported to be improved by giving thiamine to TD rats (Onodera *et al*., 1978a, Onodera *et al*., 1978b). If thiamine were contained in yokuk-

ansan, body weight in yokukansan-treated mice would have increased. However, no significant changes were observed in TD and yokukansan groups as shown in Figure 1. We confirmed that thiamine was not contained in the extract of yokukansan as described in the Materials section. In addition, yokukansan did not influence a decrease in blood thiamine concentration in TD mice. Taken together, the pharmacological effects of yokukansan on TD mice are, at least, not achieved by the effects of supplemental thiamine.

In conclusion, the present results suggest that yokukansan has remedial effects on the increased aggressive behavior and decreased social behavior.

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