Effects of Kamikihi-To, a Traditional Chinese Medicine, on Behavioral Changes Induced by Methyl-β-carboline-3-carboxylate in Mice and Rats

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ABSTRACT—The effects of Kamikihi-To (KMK), a traditional Chinese medicine, on behavioral changes induced by methyl-β-carboline-3-carboxylate (β-CCM) were evaluated in mice and rats. β-CCM, an anxiogenic benzodiazepine receptor inverse agonist (3.0 mg/kg, i.v. administered 1 min before the test), decreased the locomotor activity of mice in a novel environment. Furthermore, β-CCM (0.1 mg/kg, i.v. administered 10 min before the test) facilitated the suppression of drinking behavior induced by punishment in the water lick conflict test in rats. KMK (1.0 and 2.0 g/kg, p.o. administered 1 hr before the test) antagonized the decreased locomotor activity in the β-CCM-treated mice. KMK (2.0 g/kg, p.o.) also recovered the suppression of drinking behavior in the β-CCM-treated rats. KMK (2.0 g/kg, p.o.) had no effect on β-CCM-untreated mice and rats in these tests. These findings suggest that KMK has a protective effect against β-CCM-induced behavioral changes.

Keywords: Kamikihi-To, Anxiety, Methyl-β-carboline-3-carboxylate, Locomotor activity, Water-lick test

Benzodiazepines have been widely employed as anxiolytics, sedative/hypnotics and anticonvulsants. They are known to act by binding to benzodiazepine receptor sites in the central nervous system (1, 2). Ligands of central benzodiazepine receptors are classified into three overlapping groups: agonists (full and partial), antagonists and inverse agonists (3). Among them, benzodiazepine receptor inverse agonists such as methyl-β-carboline-3-carboxylate (β-CCM), ethyl-β-carboline-3-carboxylate and FG-7142 produce anxiogenic behavior in animals (4–6).

When a rodent is put into a new environment, it tends to move around and investigates the features of its surroundings (7). Anxiolytic benzodiazepine receptor agonists have been reported to increase the ambulatory activity of animals (8). In contrast, anxiogenic drugs such as β-CCM (6), yohimbine (9) and FG-7142 (10) decrease the locomotor activity in rodents. These results suggest that the anxiolytic activity of drugs can be evaluated by measuring the locomotor activity of rodents in a novel environment.

Vogel's punishment drinking test (11) is a widely-used animal test for anxiety. In this test, water-deprived rats are given electric shocks while drinking. Anxiolytic benzodiazepines increase the number of shocks taken. On the other hand, Corda et al. (12) found that anxiogenic β-CCM enhanced the suppression of drinking under a lower shock intensity in rats. This test using β-CCM-treated rats is also thought to be useful for evaluating the anxiolytic activity of drugs.

Kamikihi-To (KMK), a traditional Chinese medicine described in "Nai-Ke-Zhai-Yao" (1529 A.D.), has been used clinically to treat neuroses such as anxiety (13) and amnesia (14). In previous studies, we have shown that KMK ameliorates memory deficits in some animal models (15–17). We also found that KMK had protective effects against cerebral ischemic disorders in mice and gerbils (18). These results suggest that KMK affects some psychological and neurological functions in the central nervous system.

In the present study, we investigated the anxiolytic effects of KMK in comparison with a typical benzodiazepine receptor agonist, diazepam, on β-CCM-induced deficits of locomotor activity in mice and on drinking suppression in the water lick conflict test in β-CCM-treated rats.

MATERIALS AND METHODS

Animals
 Male ddY mice (Japan SLC Inc., Hamamatsu; 20–31
g) and male Wistar rats (Japan SLC Inc., 165–200 g) were used. They were housed in a temperature- and humidity-controlled (23 ± 2°C, 55 ± 10%) room, and given standard diet and tap water ad libitum before being used. Mice and rats were fasted for 17–22 hr prior to the experiments.

**Drugs**

KMK is prepared as a spray-dried powder from a hot-water extract (10 parts of water at 95–100°C for 1 hr, yield approximately 19%) of a mixture of 14 constituent herbs: Astragalus, Ginseng, Atractylodes, Hoelen, Polygala, Jujube, Longan, Zizyphus, Angelica, Licorice, Ginger, Saussurea, Bupleurum and Gardenia (16). To verify the quality of the KMK, the quantities of geniposide and glycyrrhizin, which are derived mainly from Gardenia and Licorice respectively, were measured by HPLC. KMK was suspended in water (10 ml/kg for mice, 5 ml/kg for rats). Diazepam was synthesized by Kanebo, Ltd. (Tokyo) and suspended in water (10 ml/kg for mice, 5 ml/kg for rats) containing 0.5% polyoxyethylene sorbitan monooleate (Wako Pure Chemical Industries, Ltd., Osaka). β-CCM (Sigma Chemical Company, St. Louis, MO, USA) was dissolved in 20 μl of dimethyl sulfoxide (Wako Pure Chemical Industries) and suspended in saline containing 0.5% polyoxyethylene sorbitan monooleate (10 ml/kg for mice, 3 ml/kg for rats).

**Locomotor activity**

Locomotor activity in a novel environment was measured using an animal movement analyzing system (Scanet SV-10; Matys, Toyama). Each mouse was individually placed in a plastic case (27 x 18 x 20 cm) equipped with 144 pairs of photosensors for measuring locomotor activity. Locomotor activity was calculated from the scanning data every 10 min for 20 min.

**The effects of KMK, diazepam and β-CCM on novel environment locomotor activity in mice**

KMK (0.5, 1.0 and 2.0 g/kg, p.o.), diazepam (0.5, 1.0, 2.0, 4.0 and 8.0 mg/kg, p.o.) and β-CCM (0.1, 0.3, 1.0 and 3.0 mg/kg, via a tail vein) were administered 1 hr, 30 min and 1 min before the measurement of locomotor activity, respectively. Vehicle was administered to the control mice using the same schedule as above.

**Water lick conflict test**

A modified Vogel type conflict procedure (12) was performed using a plastic chamber (22 x 30 x 22 cm) enclosed in a sound-attenuated ventilated box. It was equipped with a stainless steel grid floor and a drinking bottle containing water. An electroshock (ES) could be applied between a spout of the drinking bottle and the grid floor. Rats were deprived of water for 72 hr prior to the test. Each animal was placed in the test chamber and allowed to drink water through the spout. The test started automatically when the rat received the first ES. Rats received one ES every 20 licks in the test period. The number of ES received in the 3-min test period was recorded automatically (SC-8101-B; O’Hara & Co., Ltd., Tokyo) and was defined as the “drinking response”.

**The effects of KMK and diazepam on the drinking response of rats in the water lick conflict test**

KMK (0.5, 1.0 and 2.0 g/kg, p.o.) and diazepam (0.5, 1.0 and 2.0 mg/kg, p.o.) were administered to rats 1 hr and 30 min before the test, respectively. Vehicle was administered to the control and the treated control rats using the same schedule as above.

**The effects of KMK and diazepam on drinking suppression in the water lick conflict test in the β-CCM-treated rats**

KMK (0.5, 1.0 and 2.0 g/kg, p.o.) and diazepam (0.3 and 1.0 mg/kg, p.o.) were administered 1 hr and 30 min before the test, respectively. Vehicle was administered to the control and the treated control rats using the same schedule as above. Subsequently, β-CCM (0.1 mg/kg, i.v.) was injected into each rat 10 min before the test. Low shock intensity (5 V AC, duration of 0.5 sec) was given to rats while they were licking. Rats in the control group were placed into the chamber using the same schedule but received no shock.

**The effects of KMK and diazepam on the spontaneous water drinking behavior of the β-CCM-treated and the vehicle-treated rats**

In order to measure spontaneous water intake, we used the apparatus described above without ES. Rats were deprived of water for 72 hr. KMK at a dose of 2.0 g/kg, p.o. and diazepam at a dose of 1.0 mg/kg, p.o. were administered 1 hr and 30 min before the test, respectively. Vehicle was orally administered to the vehicle-treated
group using the same schedule as above. Subsequently, the \( \beta \)-CCM-treated groups and the vehicle-treated groups were intravenously injected with \( \beta \)-CCM (0.1 mg/kg) and vehicle, respectively, 10 min before the test.

**Statistics**

Data are expressed as the means±S.E. Analysis of variance followed by the Dunnett's test was used to analyze the locomotor activity. Student’s t-test was used for analysis of the locomotor activity between the control and the treated control group. The Kruscal-Wallis test followed by the Scheffe type test was used to analyze the drinking response in the water lick conflict test. The Mann-Whitney U-test was used only for analysis of the drinking response between the control and the treated control group in the water lick conflict test. Statistical analyses were performed by using the computer program "Yukms Statistical Library I" (Yukms Corp., Tokyo). A value of \( P<0.05 \) was considered to be indicative of statistical significance.

**RESULTS**

**The effects of KMK, diazepam and \( \beta \)-CCM on novel environment locomotor activity in mice**

The effects of KMK, diazepam and \( \beta \)-CCM on novel

<table>
<thead>
<tr>
<th>Drugs</th>
<th>N</th>
<th>Locomotor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>4721±404</td>
</tr>
<tr>
<td>KMK 0.5 g/kg, p.o.</td>
<td>9</td>
<td>5013±512</td>
</tr>
<tr>
<td>1.0</td>
<td>9</td>
<td>4994±313</td>
</tr>
<tr>
<td>2.0</td>
<td>9</td>
<td>4361±452</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>5379±209</td>
</tr>
<tr>
<td>Diazepam 0.5 mg/kg, p.o.</td>
<td>10</td>
<td>6455±472</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>6731±464</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>8437±521**</td>
</tr>
<tr>
<td>4.0</td>
<td>10</td>
<td>6823±716</td>
</tr>
<tr>
<td>8.0</td>
<td>10</td>
<td>5202±472</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>3936±336</td>
</tr>
<tr>
<td>( \beta )-CCM 0.1 mg/kg, i.v.</td>
<td>10</td>
<td>4352±173</td>
</tr>
<tr>
<td>0.3</td>
<td>10</td>
<td>4006±273</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>2756±544</td>
</tr>
<tr>
<td>3.0</td>
<td>10</td>
<td>1831±378*</td>
</tr>
</tbody>
</table>

KMK (p.o.), diazepam (p.o.) and \( \beta \)-CCM (i.v.) were administered 1 hr, 30 min and 1 min before the measurement of locomotor activity, respectively. Vehicle was administered to the control mice using the same schedule as above. Each value represents the mean±S.E. and N indicates the number of mice used. \( ^*P<0.05 \) and \( ^{**}P<0.01 \), significantly different from the control group (Dunnett’s test).

**The effects of KMK and diazepam on \( \beta \)-CCM-induced deficits of novel environment locomotor activity in mice**

The effects of KMK and diazepam on \( \beta \)-CCM-induced deficits of novel environment locomotor activity in mice are shown in Figs. 1 and 2, respectively. \( \beta \)-CCM decreased locomotor activity in each experiment. KMK at doses of 1.0 and 2.0 g/kg and diazepam at doses of 1.0 and 2.0 mg/kg prevented the \( \beta \)-CCM-induced deficits of locomotor activity in the first 10 min, significantly. KMK as well as diazepam had no influence on locomotor activ-

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**Fig. 1.** Effect of KMK on \( \beta \)-CCM-induced deficits of novel environment locomotor activity in mice. KMK was orally administered 1 hr before the test. Distilled water was orally administered to the control mice and the treated control mice using the same schedule as above. Subsequently, \( \beta \)-CCM was intravenously injected into the treated control mice and KMK-treated mice 1 min before the test. Vehicle was injected into the control mice using the same schedule as above. Locomotor activity was measured every 10 min for 20 min. \#0–10 min, \#10–20 min. Each value represents the mean±S.E. (N=15). \(^{**}P<0.01\), significantly different from the control group (Student's t-test). \(^*P<0.05\), significantly different from the treated control group (Dunnett's test).
The effects of KMK and diazepam on the drinking response of rats in the water lick conflict test are shown in Table 2. ES punishment significantly decreased the drinking response in each experiment. KMK did not affect the suppression of the drinking response by punishment. Diazepam reversed the suppression of the drinking response in a dose-dependent manner. Diazepam at a dose of 10 mg/kg, p.o. significantly increased the drinking response.

Table 2. Effects of KMK and diazepam on the drinking behavior of rats in the water lick conflict test

<table>
<thead>
<tr>
<th>Drugs</th>
<th>N</th>
<th>Drinking response/3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>11.0 ± 2.4</td>
</tr>
<tr>
<td>Treated control</td>
<td>7</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>KMK 0.5 g/kg, p.o.</td>
<td>8</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>1.0</td>
<td>8</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td>2.0</td>
<td>8</td>
<td>4.6 ± 0.9</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>11.0 ± 3.4</td>
</tr>
<tr>
<td>Treated control</td>
<td>8</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>Diazepam 1.0 mg/kg, p.o.</td>
<td>9</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>3.0</td>
<td>9</td>
<td>7.1 ± 1.6</td>
</tr>
</tbody>
</table>
| 10.0               | 9 | 18.2 ± 3.0             **

KMK and diazepam were orally administered 1 hr and 30 min before the experiment, respectively. Vehicle was administered to the control and the treated control rats using the same schedule as above. Electroshock (20 V, duration of 0.5 sec) was given to rats while they were licking. Rats in the control group were placed into the chamber using the same schedule but received no shock. Each value represents the mean ± S.E. and N indicates the number of rats used. *P < 0.05 and **P < 0.01, significantly different from the control group (Mann-Whitney U-test). *P < 0.05 and **P < 0.01, significantly different from the treated control group (Kruskal Wallis test-Scheffe type test).
Fig. 4. Effect of diazepam on drinking suppression in the water lick conflict test in the β-CCM-treated rats. Diazepam was orally administered 30 min before the test. Vehicle was administered to the control rats and the electroshock (ES)-treated control rats using the same schedule as above. Subsequently, β-CCM was intravenously injected into all the rats 10 min before the test. The control rats received no ES and the other rats received ES (5 V). The drinking response was counted during the 3 min session. Each value represents the mean±S.E. (N=16). ##P<0.01, significantly different from the control group (Mann-Whitney U-test) *P<0.05, significantly different from the treated control group (Kruscal Wallis test-Scheffe type test).

Table 3. Effects of KMK and diazepam on the spontaneous drinking behavior of rats in the water lick test

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Spontaneous drinking response/3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle i.v.</td>
</tr>
<tr>
<td>Vehicle p.o.</td>
<td>18.6±1.9</td>
</tr>
<tr>
<td>KMK 2.0 g/kg, p.o.</td>
<td>20.3±1.6</td>
</tr>
<tr>
<td>Vehicle p.o.</td>
<td>22.1±0.9</td>
</tr>
<tr>
<td>diazepam 1.0 mg/kg, p.o.</td>
<td>27.1±3.0</td>
</tr>
</tbody>
</table>

KMK and diazepam were orally administered 1 hr and 30 min before the experiment, respectively. Vehicle was orally administered to the vehicle (p.o.)-treated group using the same schedule as above. Subsequently, the β-CCM (i.v.)-treated group and the vehicle (i.v.)-treated group were intravenously injected with β-CCM (0.1 mg/kg) and vehicle, respectively, 10 min before the experiment. There were no differences between each group. Each value represents the mean±S.E. (N=10).

The effect of diazepam on drinking suppression induced by low ES punishment in the water lick conflict test in the β-CCM treated rats is shown in Fig. 4. Diazepam also increased the drinking response in a dose-dependent manner. Diazepam at a dose of 1.0 mg/kg, p.o. significantly increased the drinking response.

The effects of KMK and diazepam on the spontaneous water drinking behavior of the β-CCM-treated and the vehicle-treated rats

The effects of KMK (2.0 g/kg, p.o.) and diazepam (1.0 mg/kg, p.o.) on the spontaneous drinking behavior of the β-CCM (0.1 mg/kg, i.v.)-treated and the vehicle (i.v.)-treated rats are shown in Table 3. β-CCM (i.v.), compared with vehicle (i.v.), tended to decrease the spontaneous drinking response in the vehicle (p.o.)-treated rats. In contrast, there were no differences between the vehicle (i.v.)- and the β-CCM (i.v.)-treated groups in the KMK (p.o.)- and the diazepam (p.o.)-treated rats. KMK and diazepam each tended to increase the spontaneous drinking response of the β-CCM (i.v.)-treated rats, but the trends were not significant. KMK and diazepam had no influence on the spontaneous drinking response of the vehicle (i.v.)-treated rats.

DISCUSSION

Pharmacological and neurochemical studies have suggested that benzodiazepine-GABA receptor-ionophore complex (19), serotonergic (20) and noradrenergic (21) systems play important roles in anxiety. Among these systems, the effects on anxiety of benzodiazepine receptor-related drugs have been most intensively investigated. Benzodiazepine receptor agonists are thought to act at benzodiazepine binding sites that are associated with the GABA complex to produce an anxiolytic effect (19). In contrast, benzodiazepine receptor inverse agonists, such as β-CCM, are thought to produce anxiety by binding to benzodiazepine receptors and decreasing the frequency of Cl⁻-channel opening generated by GABA binding (5). In the present study, the behavioral changes induced by β-CCM, a benzodiazepine inverse agonist, and the protective effect of KMK against β-CCM-induced behavioral changes were studied using mice and rats.

In the present experiment, we considered that the affectable period of β-CCM would be short, because β-CCM was administered intravenously, and also that the timing of β-CCM administration was very important. Therefore we administered β-CCM to mice and rats at different times in each experiment. In the water lick test, the timing of β-CCM administration was the same as that reported by Corda et al. (12). β-CCM was administered 10 min before the experiment, and it took about 15 min from the administration to the end of the experiment. On the other hand, it took 20 min even for the locomotor experiment. The timing of β-CCM administration in the locomotor experiment was set at 1 min before meaure-
ment since its effect would not be maintained for long.

Nakamura and Ozawa (8) have reported that anxiolytic diazepam at a low dose increases novel environment locomotor activity in mice, and this effect was thought to be caused by the drug's anxiolytic action, although high doses of diazepam decreased the locomotor activity caused by myo-relaxant activity. In the present experiment, a low dose of diazepam increased the locomotor activity of mice in a novel environment for the first 10 min. In contrast, anxiogenic 3-CCM decreased the novel environment locomotor activity of the mice in a dose-dependent manner. Jones et al. (6) have reported that 3-CCM reduces exploratory activity and social interaction and that Ro 15-1788, a central type benzodiazepine receptor antagonist, reverses the suppression of exploratory activity and social interaction induced by 3-CCM in rats, although we failed to show that Ro 15-1788 at a dose of 1.0 mg/kg, i.v. antagonized the effects of 3-CCM and diazepam on novel environment locomotor activity of mice, which might be caused by using a low maximum dose (data not shown). These results suggest that the 3-CCM-induced decrease in locomotor activity in the present study was caused by its anxiogenic action. Furthermore, KMK and diazepam antagonized the 3-CCM-induced deficits of locomotor activity in mice. KMK at the effective dose did not affect the locomotor activity of 3-CCM-untreated mice, whereas diazepam at the effective dose stimulated the locomotor activity of naive mice. These results suggest that the inhibitory effect of KMK may be more specific to 3-CCM-induced anxiogenic behavioral changes than that of diazepam.

The first water lick conflict test in the present experiment is a widely used type for anxiety. Electroshock (20 V in this study) is given as punishment. KMK had no anti-conflict effect, although diazepam induced significant anti-conflict behavior. In contrast, the second water lick test in the present experiment is the other type reported by Corda et al. (12) and Hill et al. (22). A low intensity of electroshock (5 V in this study) is given as punishment under the 3-CCM administration. They reported that anxiogenic 3-carbolines elicited a proconflict action of rats in the water lick test and that its action was inhibited by Ro 15-1788. These results showed that the proconflict action of 3-CCM was induced via central type benzodiazepine receptors. In the present study, we observed drinking suppression in response to low intensity shock in 3-CCM-treated rats. KMK as well as diazepam reversed the drinking suppression. KMK at the effective dose did not significantly stimulate the spontaneous drinking response. These results suggest that the protective effect of KMK against drinking suppression in 3-CCM-treated rats was not due to stimulation of the spontaneous response. The results of the water lick test as well as the locomotor test suggest that KMK only has a protective effect against 3-CCM-induced behavioral changes, without influencing normal behavior.

It is possible that the effects of KMK may be mediated by direct and/or indirect action on the benzodiazepine receptor. If KMK had a direct effect, it would act as an antagonist and not a full agonist on the benzodiazepine receptor because there was a difference in profile between KMK and diazepam, a full agonist, and KMK had no effect in normal animals. The binding studies of KMK on the benzodiazepine receptor and other receptors have been reported. Yamada et al. (23) reported that long term administration of KMK increased [3H]flunitrazepam binding, which labels central benzodiazepine receptors, in the brain of aged rats. Hayashi et al. (24) recently demonstrated that KMK modulated the binding of [3H]-quinuclidinyl benzilate and [3H]N-(1-(2-thienyl)cyclohexyl)-3,4-piperidine, which label muscarinic receptors and N-methyl-d-aspartate receptors, respectively, to rat brain slices. Egashira et al. (25) showed that KMK enhanced both the Bmax and Vmax of [3H]quinuclidinyl benzilate binding and choline acetyltransferase activity in aged rats. These results suggest that KMK affects not only benzodiazepine receptors but also other receptors in the central nervous system and that these receptors, except for benzodiazepine receptors, may be indirectly responsible for the 3-CCM-induced behavioral changes in the present experiments.

In conclusion, the results of the present study suggest that 3-CCM produces anxiogenic behavior in mice and rats and that KMK ameliorates these behavioral changes without influencing normal behavior. This anxiolytic action of KMK may be produced via benzodiazepine receptors and/or the related receptors. However, further experiments are required to characterize the mechanism of this effect.

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