Short communication

Kamishoyosan reduces conditioned fear-induced freezing behavior in socially isolated ovariectomized rats

Nobuaki Egashira a, b, *, Hikari Iba a, Haruna Kuwano a, Rikako Kawanaka a, Masaki Nagao a, Hiroshi Moriyama a, Takuya Watanabe a, Kaori Kubota a, c, Shutaro Katsurabayashi a, Katsunori Iwasaki a, c

a Department of Neuropharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180, Japan
b Department of Pharmacy, Kyushu University Hospital, Fukuoka 812-8582, Japan
c A.I.G. Collaborative Research Institute for Aging and Brain Sciences, Fukuoka University, Fukuoka 814-0180, Japan

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A B S T R A C T

In the present study, we investigated the effect of kamishoyosan (KSS) on conditioned fear-induced freezing in ovariectomized (OVX) rats. Socially isolated OVX rats showed the longest freezing time among the following four groups: group-housed sham-operated (Sham), isolated Sham, group-housed OVX, and isolated OVX rats. Repeated oral administration of KSS (30–300 mg/kg) reduced conditioned fear-induced freezing in socially isolated OVX rats. The reduction of freezing by KSS was reversed by flumazenil (3 mg/kg) and bicuculline (3 mg/kg). These findings suggest that the GABA_A-benzodiazepine receptor complex is involved in the anxiolytic effect of KSS in socially isolated OVX rats.

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Kamishoyosan (KSS), a Kampo medicine, is widely used to treat menopausal psychiatric symptoms in women. KSS has been reported to improve anxiety and depression in midlife women with psychological symptoms (1). Moreover, KSS improves sleep disturbance in Japanese peri- and post-menopausal women (2). Although it is likely that KSS improves various psychiatric symptoms in midlife female patients, the mechanisms of these effects remain unclear. KSS has been shown, in social interaction testing, to exert an anxiolytic effect through γ-aminobutyric acid A (GABA_A)-benzodiazepine receptor stimulation in male mice (3). However, the anxiolytic effect of KSS in a menopausal experimental model has not been reported.

During menopause, women are more likely to develop anxiety and/or depressive symptoms (4). Midlife changes in hormonal and psychosocial environments contribute to the risk of perimenopausal depression (5). Moreover, stress exacerbates somatic symptoms of menopause, increasing the risk of recurrence of mood disorder in perimenopausal patients (6). Especially, the loneliness during female midlife (45–65) and older (over 65) can function as the serious psycho-social stressor raised by diminishing self-esteem with the launching of children (e.g., empty-nest syndrome), grief caused by the loss of parents/close persons, or limited contacts with acquaintances or friends as the empirical life events (7). These observations indicate that psychosocial environments and stress, as well as hormonal changes, play an essential role in the development and exacerbation of anxiety and/or depressive symptoms in perimenopausal patients. Ovariectomized (OVX) rats are the most common model used in research on menopausal symptoms. The conditioned fear stress test is an established murine anxiety model. Therefore, we investigated the effect of KSS on conditioned fear-induced freezing in socially isolated OVX rats.

Female Sprague–Dawley rats, aged 8 weeks, were obtained from the Kyudo Co. (Tosu). Animals were housed either in social isolation (one rat per cage) or in social groups (four rats per cage) under standardized lighting conditions (lights on 07:00–19:00) at a constant temperature (23 ± 2 °C) with food and water available ad libitum. All procedures regarding animal care and use were carried out based on regulations established by the Experimental Animal Care and Use Committee at Fukuoka University, Japan.

KSS (Lot. No. 2020024010) was a generous gift from Tsumura & Co. (Tokyo) and was a dried extract of the following raw materials:
Bupleuri Radix (*Bupleurum falcatum*, 3.0 g), Paeoniae Radix (*Paeonia lactiflora*, 3.0 g), Atractyloids Rhizoma (*Atractylodes ovate*, 3.0 g), Angelicae Radix (*Angelica acutiloba*, 3.0 g), Hoelen (*Poria cocos*, 3.0 g), Gardeniae Fructus (*Gardenia jasminoides*, 2.0 g), Moutan Cortex (*Paeonia suffruticosa*, 2.0 g), Glycyrrhizae Radix (*Glycyrrhiza uralensis*, 1.5 g), Zingiberis Rhizoma (*Zingiber officinale*, 1.0 g), and Mentheae Herba (*Menthae arvensis*, 1.0 g). Each plant material was authenticated by identification of external morphology and marker compounds of plants specimens, according to the methods of the Japanese Pharmacopoeia and Tsumura & Co.’s standard. The medical herbs were extracted with purified water at 95 °C for 1 h, and the extraction solution was separated from the insoluble waste and concentrated by removing water under reduced pressure. Spray drying was used to produce a dried extract powder, which was subsequently suspended in distilled water. The yield of the extract was about 17.8%, and extracts were manufactured in compliance with the Japanese Pharmacopoeia (Sixteenth Edition, JP16) under Good Manufacturing Practice (GMP).

Diazepam (Wako Pure Chemical Industries Ltd., Osaka) was suspended in 0.5% sodium carboxymethylcellulose solution. Flumazenil (Wako Pure Chemical Industries, Ltd.) and bicuculline (Sigma–Aldrich, St. Louis, MO, USA) were suspended in 1% Tween 80 solution.

At 8 weeks of age, rats were anesthetized with intra-peritoneal (i.p.) sodium pentobarbital (50 mg/kg; Tokyo Kasei, Tokyo) and underwent bilateral ovariectomy. In sham-operated (Sham) groups, rats underwent the same incisions and the ovaries and fallopian tubes were exposed and then replaced in the abdominal cavity before the muscle and skin were closed. For recovery, animals were housed for 3 weeks prior to testing.

On the first test day, each rat was placed in a shock chamber (30 × 30 × 30 cm) with a grid floor and allowed to habituate for 5 min. The next day, rats were individually subjected to inescapable electric foot shock (1 mA of scrambled shock, duration of 4 × 5 s and interval of 15 s) in the same chamber, and were removed from the shock chamber after receiving the electric shock. The electric shock was provided by a shock generator (Model SGS-002; Murakami Kikai Co., Ltd., Tokyo). Twenty-four hours after the shock, rats were again placed in the shock chamber and the duration of freezing behavior within a 5-min period was recorded. Freezing was defined as the absence of all observable movements of the skeleton and the vibrissae, except for those related to respiration.

Fig. 1. Effect of social isolation on freezing behavior (A), locomotor activity (B), and uterine weight (C) in sham-operated (Sham) and ovariectomized (OVX) rats. Values are expressed as the mean ± SEM. #P < 0.05, **P < 0.01 compared with group-housed Sham rats, ***P < 0.01 compared with isolated Sham rats. The number of rats per group is shown at the base of each column.
The following day, locomotor activity was measured using an automated activity counter (NS-AS01; Neuroscience, Tokyo) placed 17 cm above a plastic box (30 × 35 × 17 cm). At the end of the experiment, rats were sacrificed by decapitation and their uteri were weighed to confirm the success of ovariectomy. Among the rats receiving ovariectomy, the rats without reduction of marked uterine weight were excluded from the present experiment. Only data from animals have successful ovariectomy was analyzed.

KSS and diazepam were administered orally once daily for eight days, and these drugs were administered 60 min before habituation or testing on days 5–8. Flumazenil and bicuculline were administered i.p., immediately before administration of KSS.

Results were statistically analyzed by one-way or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer post-hoc test (StatView; Abacus Concepts, Berkeley, CA, USA) to determine differences between the groups. Values are expressed as the mean ± SEM. The criterion for statistical significance was considered to be \( P < 0.05 \).

Socially isolated OVX rats showed the longest duration of freezing among the four groups tested: group-housed Sham, isolated Sham, group-housed OVX, and isolated OVX rats [an effect of surgery \( (F(1,39) = 9.603, P < 0.01) \), an effect of housing \( (F(1,39) = 1.855, P > 0.1) \), and a surgery × housing interaction \( (F(1,39) = 6.091, P < 0.05) \) by two-way ANOVA, \( P < 0.05, 0.01 \) by Tukey–Kramer post-hoc test, Fig. 1A]. However, no difference in locomotor activity was observed between the four groups [an effect of surgery \( (F(1,39) = 0.017, P > 0.1) \), an effect of housing \( (F(1,39) = 0.239, P > 0.1) \), and a surgery × housing interaction \( (F(1,39) = 5.549, P < 0.05) \) by two-way ANOVA, \( P > 0.1 \) by Tukey–Kramer post-hoc test, Fig. 1B]. Uterine weight of group-housed and isolated OVX rats was lower than that of group-housed and isolated Sham rats [an effect of surgery \( (F(1,39) = 480.195, P < 0.0001) \), an effect of housing \( (F(1,39) = 0.075, P > 0.1) \), and a surgery × housing interaction \( (F(1,39) = 0.147, P > 0.1) \) by two-way ANOVA, \( P < 0.01 \) by Tukey–Kramer post-hoc test, Fig. 1C].

![Figure 2](image-url)

**Fig. 2.** Effects of diazepam and kamishoyosan on freezing behavior (A), locomotor activity (B), and uterine weight (C) in isolated ovariectomized (OVX) rats. Diazepam (0.1 mg/kg) and kamishoyosan (10–300 mg/kg) were administered orally once daily on days 1–4 from 2 weeks after surgery, and were administered 60 min before habituation or testing on days 5–8. Values are expressed as the mean ± SEM. \*P < 0.05, \**P < 0.01 compared with group-housed Sham rats, \*P < 0.05, \**P < 0.01 compared with isolated OVX rats treated with distilled water. The number of rats per group is shown at the base of each column.
As shown in Fig. 2A, repeated administration of 0.1 mg/kg diazepam, a benzodiazepine receptor agonist (as a positive control) significantly reduced conditioned fear-induced freezing in socially isolated OVX rats [comparison among Sham, OVX and diazepam (F(2,22) = 4.947, P < 0.05) by one-way ANOVA, P < 0.05 by Tukey-Kramer post-hoc test]. However, diazepam did not significantly affect locomotor activity [comparison among Sham, OVX and diazepam (F(2,22) = 1.274, P > 0.1) by one-way ANOVA, Fig. 2B] or reduced uterine weight [comparison among Sham, OVX and diazepam (F(2,22) = 32.835, P < 0.0001) by one-way ANOVA, P > 0.05 by Tukey-Kramer post-hoc test, Fig. 2C]. Similarly, repeated oral administration of KSS (30, 100, or 300 mg/kg) significantly reduced conditioned fear-induced freezing in socially isolated OVX rats [comparison among Sham, OVX and KSS (F(5,46) = 6.843, P < 0.0001) by one-way ANOVA, P < 0.05 or P < 0.01 by Tukey-Kramer post-hoc test, Fig. 2A] without affecting locomotor activity [comparison among Sham, OVX and KSS (F(5,46) = 3.078, P < 0.05) by one-way ANOVA, P > 0.05 by Tukey-Kramer post-hoc test, Fig. 2B] or reduced uterine weight [comparison among Sham, OVX and KSS (F(5,46) = 32.616, P < 0.0001) by one-way ANOVA, P < 0.05 by Tukey-Kramer post-hoc test, Fig. 2C].

The selective benzodiazepine receptor antagonist flumazenil (3 mg/kg, i.p.) significantly reversed the reduction of freezing by KSS (300 mg/kg) in socially isolated OVX rats [F(2,22) = 5.955, P < 0.01 by one-way ANOVA, P < 0.05 by Tukey-Kramer post-hoc test, Fig. 3A]. Similarly, the GABAA receptor antagonist bicuculline (3 mg/kg, i.p.) completely reversed the reduction of freezing by KSS [F(2,21) = 11.689, P < 0.001 by one-way ANOVA, P < 0.01 by Tukey-Kramer post-hoc test, Fig. 3B]. However, these drugs had no effect on locomotor activity or uterine weight in socially isolated OVX rats (data not shown).

In group-housed OVX rats, the duration of freezing induced by conditioned fear did not differ from that in group-housed Sham rats. Recently, Li et al. (8) showed that ovariectomy did not alter electric foot-shock-induced conditioned place avoidance, suggesting that ovariectomy does not affect aversive stimulation-related apprehension in group-housed rats. The result of the present study was essentially consistent with a previous observation. In socially isolated rats, however, conditioned fear-induced duration of freezing in OVX rats was markedly prolonged compared with Sham rats. Iizuka et al. (9) reported that electric foot shock stress shortened the duration of pentobarbital-induced sleep in OVX mice but not in Sham mice. The results of the present study suggest that social isolation stress does not influence conditioned fear-induced freezing in female rats with intact ovaries, but that ovariectomy renders the rats more susceptible to the influence of social isolation stress, resulting in prolonged duration of freezing. Therefore, this experimental model may be useful in studying the menopausal psychological symptoms related to psychosocial environments and stress.

In the present study, we observed that KSS reduced conditioned fear-induced freezing in socially isolated OVX rats. Our data support that KSS is useful for the treatment of menopausal psychological symptoms in middle-age women. On the other hand, repeated administration of KSS did not affect locomotor activity or uterine weight in socially isolated OVX rats. Levels of plasma estradiol and progesterone are decreased in OVX rats (10). Estradiol replacement markedly increases reduced uterine weight with an increase in levels of plasma estradiol but not progesterone in OVX rats (9, 10). Therefore, it is unlikely that the anxiolytic effect of KSS on conditioned fear-induced freezing can be attributed to increased locomotion or estradiol replacement.

Moreover, we found that flumazenil and bicuculline reversed the reduction of freezing caused by KSS. Social isolation alters the subunit expression of GABAA receptor (11). Our findings suggest that activation of the GABAA-benzodiazepine receptor complex is involved in the anxiolytic effect of KSS on freezing enhanced by social isolation. This theory is supported by a previous report that extracts of some ingredients of KSS bind to GABAA receptors (12).

Gardeniae Fructus, one such ingredient, has been reported to play a role in the anxiolytic effect of KSS in social interaction testing (13). Therefore, it might also be an active ingredient of KSS in the anxiolytic effect.

In rodents, conditioned fear-induced freezing is closely associated with emotional learning and memory (14). Hippocampal lesions or scopolamine decreases freezing behavior depending on impairment of learning and memory (14, 15). The benzodiazepines (e.g., diazepam) have limited usage due to their potential side effects such as anterograde amnesia (16). Though KSS is in widespread clinical use, there is no information that KSS causes memory impairment. Moreover, there are no reports that KSS affects the learning and memory in animals. Therefore, it is unlikely that the anxiolytic effect of KSS on conditioned fear-induced freezing is due to impairment of learning and memory.

![Graph](image-url)
In conclusion, this study demonstrated, for the first time, that KSS reduces conditioned fear-induced freezing in socially isolated OVX rats, and that the GABA<sub>B</sub>-benzodiazepine receptor complex is involved in the anxiolytic effect of KSS.

**Conflicts of interest**

The authors indicated no potential conflicts of interest.

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**References**


