Kososan, but not milnacipran, elicits antidepressant-like effects in a novel psychological stress-induced mouse model of depression

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ABSTRACT

Aim: Mild or moderate depressive symptoms are often resistant to treatment with currently available antidepressants. We constructed a novel animal model of depression induced by the exposure of mice to psychological stress and evaluated the antidepressant efficacy of kososan and milnacipran.

Methods: Depressive state was elicited in mice by repeated rat-exposure stress over 10 days. Kososan or milnacipran were administered orally during the stress paradigm. Alterations in behavioral indices (evaluated using forced swimming test and open-field test), cell proliferation (evaluated on bromodeoxyuridine immunohistochemistry), and orexin-A production were evaluated.

Results: Mice exposed to psychological stress had increased immobility time on forced swimming test, and elevated serum corticosterone. Additionally, decreased bodyweight gain during the stress exposure and reduced food or water intake following the stress were observed. Although oral kososan improved the immobility time on forced swimming test without affecting the locomotor activity in open-field test, treatment with milnacipran did not alter the immobility time. The number of cells staining positive for bromodeoxyuridine in the dentate gyrus or for orexin-A in the lateral hypothalamic area was not affected by the stress exposure.

Conclusion: Psychological stress-induced mouse model of depression was successfully implemented, with animals exhibiting symptoms of mild or moderate depressive state. This model differs from previous physical stress-based models, as evidenced by bromodeoxyuridine or orexin-A staining. Kososan, but not milnacipran, was found to elicit antidepressant-like effects in this model.

KEY WORDS: antidepressant-like effect, depression-like model, forced swimming test, Kampo, kososan, milnacipran

INTRODUCTION

Depression is a serious and highly prevalent disease that is associated with long-lasting and debilitating symptoms [1]. Individuals with depression may suffer from symptoms that include depressed mood (sadness, emptiness) and anhedonia (loss of interest or pleasure) [2]. A large body of research has attempted to elucidate the pathogenesis of the disease, with several groups reporting that stressful life events can cause depression in humans [3–5]. Most of these stressful experiences have been classified as psychological stressors, but the relationship between the stressors and depression is complex [6]. Additionally, the molecular mechanisms by which psychological stress evokes depression are not fully understood. A number of research groups have attempted to establish animal models of depression that could be used to investigate these mechanisms [7].

Stressful events have been reported to activate the hypothalamic-pituitary-adrenal (HPA) axis and stimulate the monoamine signaling systems in the brain [8]. A number of antidepressant treatments have been developed on the basis of these proposed mechanisms [9]. In most cases, patients with major depressive disorder are treated with antidepressants such as selective serotonin re-uptake inhibitors (SSRI) and serotonin and norepinephrine re-uptake inhibitors (SNRI) [10], but the effectiveness of these drugs is limited, with inadequate effectiveness of current treatments reported in certain types of depressive disorders [11]. In particular, their therapeutic effects are

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not sufficient for the treatment of patients with mild or moderate depressive symptoms [12]. Furthermore, a fundamental issue that remains to be explained is the discrepancy between the timing of the changes in brain monoamine level and the onset of therapeutic effect of antidepressants. Given that it is impossible to obtain brain tissues directly from the depressive patients to elucidate the pathology of the disease, neuropharmacological research into depression relies on the use of animal models. Several different models and useful methods for evaluating depression have been developed using existing antidepressant agents [7,13–16], but the dependence on currently available drugs to validate models makes it difficult to construct new models for depressive disorders in which antidepressants are not effective, such as drug-resistant depression. With this in mind, the aim of the present study was to construct a novel mouse model of depression using only psychological (rat-exposure) stress, which would not depend on the existing antidepressant drugs for validation.

Kososan (Xiang-Su-San in Chinese) is a Kampo medicine that has been used in the treatment of depressive mood. In previous reports, we constructed a model of depression triggered by physical stress and showed that oral kososan improves depressive behavior due to a mechanism that differs from that of an SNRI antidepressant, milnacipran, and have reported that physical stress downregulates orexin-A (OX-A), a neuropeptide produced specifically in the lateral hypothalamic area (LHA) and in the posterior hypothalamus, while kososan or milnacipran normalizes OX-A levels [17,18]. Additionally, physical stress was observed to reduce the number of cells staining positive for bromodeoxyuridine (BrdU), a marker of cell proliferation, in the dentate gyrus. Reduction in the number of BrdU-positive cells was attenuated by oral kososan or milnacipran. In this study, we investigated whether treatment with kososan alters the number of OX-A- and BrdU-positive cells in mice exposed to psychological stress.

METHODS

Animals

Seven-week-old male ddY mice (30–38 g) and adult male Wistar rats (past breeding age) were purchased from Japan SLC (Hamamatsu, Japan). The animals were housed under constant temperature (23 ± 2°C), humidity (55 ± 10%), and a 12 h light cycle (lights on at 08:00 hours), with food and water available ad libitum. Two–five mice were housed per cage, except for the periods of rat-exposure stress and the evaluation of food consumption. The rats were housed separately in breeding cages ($225 \times 338 \times 140$ mm^3), except during the period of rat-exposure stress. All animals were habituated for at least 1 week after arrival. All animal experiments were performed according to the Guidelines for Care and Use of Laboratory Animals at the Kitasato Institute and Kitasato University after approval of animal experiments. Every effort was made to minimize the number of animals used and their suffering.

Drugs

Kososan was composed of Cyperi Rhizoma (4.0 g, Tsumura, Tokyo, Japan), Aurantii Nobilis Pericarpium (3.0 g, Tsumura), Perillae Herba (2.0 g, Tsumura), Glycyrrhizae Radix (2.0 g, Uchida Wakan-yaku, Tokyo, Japan), and Zihgiberis Rhizoma (0.5 g, Tsumura) and formulated by the authors. The Kampo formulation was decocted with 600 mL of distilled water until the volume was reduced by half. The extract was immediately filtered under vacuum and the filtrate was lyophilized. The yield of kososan extract was approximately 28% from the herbal mixture, based on dry weight.

Milnacipran hydrochloride (trade name Toledomin) was purchased from Asahi Kasei Pharma (Tokyo, Japan).

Drug treatment

The kososan extract or milnacipran were dissolved or suspended in distilled water, respectively. Kososan (1.0 g/kg) or milnacipran (60 mg/kg) were administered orally immediately before the rat-exposure stress, as described in our previous studies [17,18].

Bromodeoxyuridine (Roche Diagnostics, Indianapolis, IN, USA), a marker of proliferating cells, was dissolved in saline containing 0.007 mol/L NaOH. For experiments measuring cell proliferation, BrdU (200 mg/kg) was injected i.p. into kososan- and milnacipran-treated mice 4 h before blood collection.

Rat-exposure stress-induced mouse model of depression

In a rat-exposure stress paradigm (Fig. 1a), a mouse was introduced into the home cage of a rat and kept in the cage for 1 h. Mice were subjected to two 1 h exposures per day for 10 days. During the stress paradigm, both the mouse and the rat were permitted free access to food and water. In order to avoid habituation, each mouse was exposed to a different rat on two consecutive days. Mouse bodyweight was measured before (day −2) and after the stress period (day 13).

Food and water consumption test

Mice were placed individually in feeding cages and allowed free access to food and water. The consumption of food and water was measured for 24 h. Bodyweight was measured before this test.

Open field test

The spontaneous locomotor activity was measured using the open field test (OFT) under non-stressed conditions. Briefly, mice were placed individually in an opaque open-field box ($40 \times 40 \times 40$ cm^3) and were allowed to move freely for 5 min. The total distance and duration of movement were measured using a video tracking system (Etho Vision; Noldus, Wageningen, Netherlands). This behavioral experiment was carried out between 09:00 and 13:00 hours.

Mice were grouped based on bodyweight gain, food and water consumption, and locomotor activity, so that the mean bodyweight, food consumption, water consumption, and total

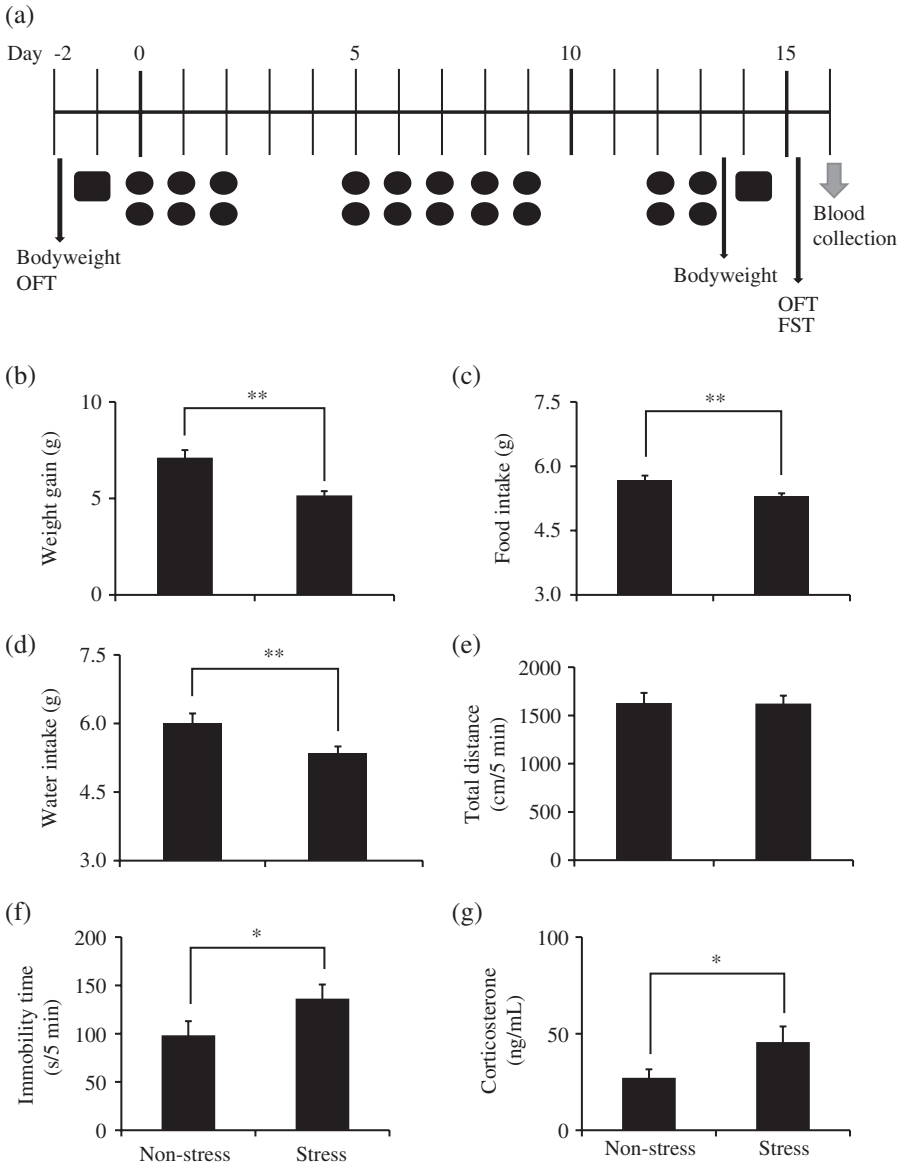

Figure 1 | Depressive-like states induced by psychological (rat-exposure) stress. (a) Experimental design for rat-exposure stress. (●) Rat exposure (1 h); (■) food and water consumption test. (b) Bodyweight was measured on days 2 and 13, and weight gain during the stress paradigm was calculated. After the stress period, (c) food and (d) water intake were measured over 24 h. (e) The total distance traveled in a 5 min open-field test (OFT) was measured on day 15. (f) The duration of immobility in a 5 min forced-swim test (FST) was measured following OFT. (g) Blood samples were collected after decapitation on day 16. Serum corticosterone was measured using an ELISA kit. Data given as mean ± SEM ($n = 20$). $^{*}P < 0.05$, $^{**}P < 0.01$.

distance and duration of movement were similar between the groups.

Forced swimming test

Forced swimming test (FST) was performed using a modification of a previously described procedure [19]. Briefly, a mouse was placed in a 5 L beaker (height 27 cm, diameter 18 cm) filled with 4 L of water (23 ± 1°C) for 5 min, and the total duration of the mouse's immobility was measured. A mouse was judged immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. This behavioral experiment was carried out between 13:00 and 15:00 hours.

Serum corticosterone

Blood samples were collected 1 day after the behavioral tests. In the untreated group, blood samples were collected after decapitation. In kososan- and milnacipran-treated animals, blood

samples were collected from the iliofemoral artery under anesthesia with isoflurane.

Blood samples were centrifuged at 4°C, and the obtained sera were stored at −20°C until the corticosterone assay was performed. Serum corticosterone was measured using an Assay-Max Corticosterone ELISA Kit (Assaypro, St Charles, MO, USA), according to manufacturer instructions.

Brain fixation and tissue storage

After the blood was collected, kososan-treated mice were perfused transcardially with cold phosphate-buffered saline (PBS) and subsequently with cold 4% paraformaldehyde solution. Brains were collected and stabilized in 4% paraformaldehyde solution at 4°C for 1 day. Serial coronal 50 μm sections were obtained throughout the hippocampus (bregma −1.2 to −2.5 mm) using a vibratome (Technical Products International, St Louis, MO, USA) and were stored in PBS/NaN_3 at 4°C until subsequent experiments.

Immunohistochemistry

Immunohistochemistry for BrdU and OX-A was performed under free-floating conditions in 24-well plates as described previously [18].

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using the unpaired Student *t*-test. Statistical significance was defined as $P < 0.05$.

RESULTS

Induction of a depression-like model in mice

Bodyweight gain in mice exposed to the stress paradigm was significantly lower, compared to the non-stressed mice (Fig. 1b). Additionally, mice exposed to stress consumed significantly less food and water than non-stressed mice (Fig. 1c,d).

In the assessment of spontaneous locomotor activity, total distance traveled in 5 min in the OFT (day 15) did not significantly differ between stress-exposed and control mice (Fig. 1e).

Stress exposure significantly prolonged immobility in the FST, compared to the non-stressed mice (Fig. 1f). Serum corticosterone in stress-exposed mice was found to be higher than in the non-stressed mice on day 16 (Fig. 1f).

Effects of kososan on duration of immobility and serum corticosterone

Kososan treatment (1.0 g/kg, Fig. 2a) reduced the duration of immobility in the FST, compared with water (Fig. 2b). Conversely, neither locomotion in the OFT nor serum corticosterone level was affected by kososan treatment (Fig. 2c,d). Despite significant differences in bodyweight and in the consumption of food and water between the control and stress-exposed groups, no difference was observed between kososan- and water-treated mice, regardless of the presence of stress (data not shown).

Effects of rat-exposure stress and kososan on BrdU- and OX-A-positive cells

The number of BrdU-positive cells in the dentate gyrus of the hippocampus in non-stressed mice and stressed mice was not significantly different (Fig. 3a). Similarly, no significant difference was found in the number of OX-A-positive cells between non-stressed and stressed mice (Fig. 3b). Kososan did not alter the number of BrdU- or OX-A-positive cells.

Effects of milnacipran on duration of immobility

Neither duration of immobility during the FST nor locomotion in the OFT was affected by milnacipran treatment (Fig. 4a,b).

DISCUSSION

In this study, we developed a novel animal model of depression based on psychological (rat-exposure) stress. Additionally, we demonstrated that kososan, but not milnacipran, exhibits antidepressant-like efficacy in this animal model.

We chose rat-exposure stress as the psychological stressor in our model, with a single mouse housed in a cage with a rat during the stress paradigm. Although the mouse and rat were not separated, mice did not suffer any detectable injury from the rats. Therefore, the stressor in this model appears to mimic psychological stress, which might be composed of the threat from, and anxiety due to, the presence of a rat and segregation from the cage mate [20].

The decrease in bodyweight gain observed after the stress period was accompanied by decreased food and water intake, suggesting that rat-exposure stress could cause a loss of feeding motivation in mice. FST, an easy-to-perform and reliable test commonly used for screening antidepressant agents, was used to further evaluate the induced depressive state [16]. Rat-exposure stress was observed to increase mouse immobility time. The measurement of locomotor activity in the OFT [21], showed that the total distance of movement was not affected by the stress. This suggests that the stress-evoked increase in immobility in the FST was not caused by a reduction in locomotor activity, and shows that the stressed mice exhibit behavior that mimics depressive symptoms. Stress-exposed animals have an activated HPA axis, resulting in increased corticosterone level, a well-recognized biomarker of the depressive state [22]. In the present study there was an upregulation of corticosterone following stress, suggesting that the stress-exposed mice were in a depression-like state. Blood samples for corticosterone measurement were obtained the day after the FST, because corticosterone level could be affected immediately following the FST [23]. Based on the present findings of depression-like behavior and elevated blood corticosterone, we can conclude that the rat-exposure stress induced a depression-like state in mice, and this psychological stress model may be used as an animal model mimicking depression in humans.

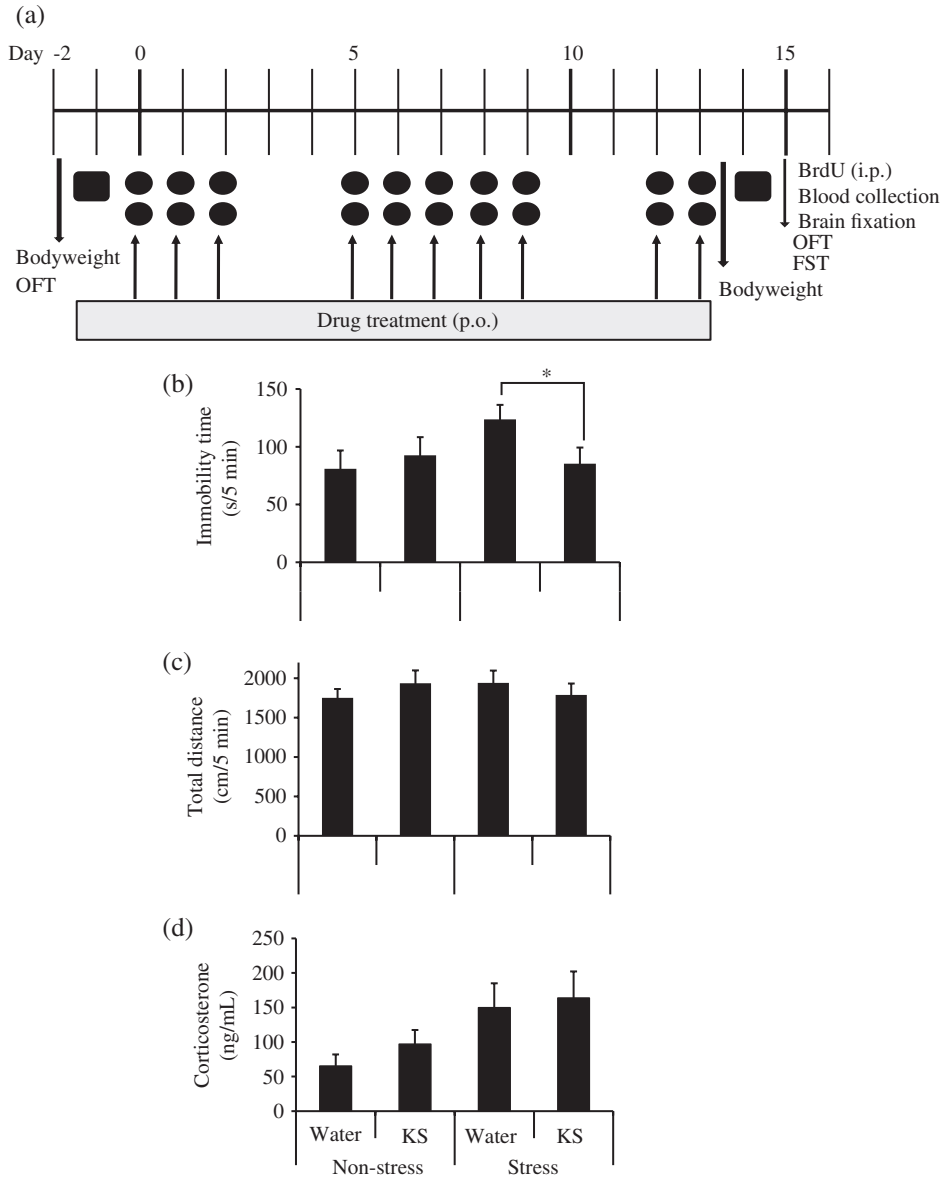

Figure 2 | Antidepressant-like effects of kososan (KS) on the psychological stress-induced mouse model of depression. (a) Experimental design for rat exposure stress and drug treatment. (●) Rat exposure (1 h); (■) food and water consumption test. KS (1.0 g/kg) was administered orally for 10 days to the mice following the psychological stress paradigm. (b) Duration of immobility was measured during a 5 min forced-swim test (FST) conducted on day 15. (c) Total distance traveled was measured during a 5 min open-field test (OFT) performed before FST. (d) Blood samples were collected from the iliofemoral artery under anesthesia with isoflurane on day 16. Serum corticosterone was measured using an ELISA kit. Data given as mean ± SEM ($n = 15$). $^*P < 0.05$. BrdU, bromodeoxyuridine.

One of the advantages of the model used in this study is that it is induced using only a psychological stressor. Most animal models require the use of physical stressors, which are not the major stressors in humans. From this perspective, the present model parallels more closely the depressive state in patients. Although some groups reported psychological stress models, these models often utilized unique devices or animal species [24,25], and other groups faced difficulty in replicating same conditions [26]. Additional advantage of the present proposed model is the widespread availability of animals used, because the model animals are widely available and no atypical apparatus is needed. These characteristics make the model easy to use, and allow this mouse model of depression to be used for investigating the molecular and neurochemical mechanisms underlying depression. Furthermore, the magnitude of the effect of rat-exposure stress on immobility time

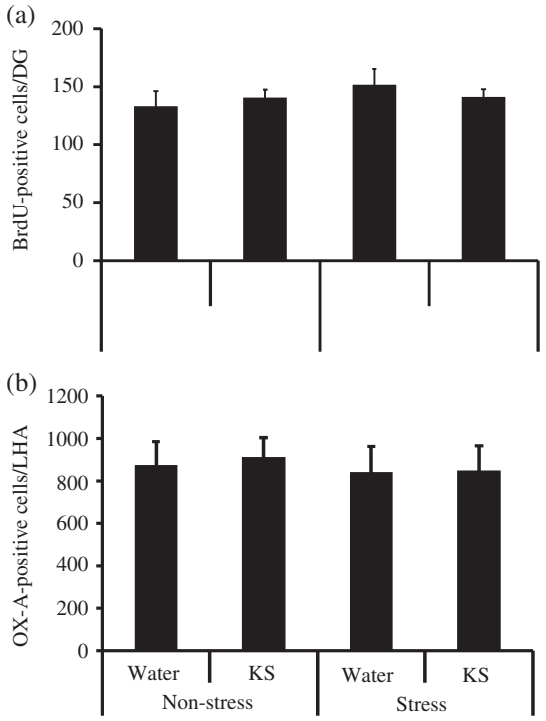

Figure 3 | Effects of psychological stress or kososan (KS) treatment on hippocampal cell proliferation and hypothalamic orexin-A (OX-A) level. KS (1.0 g/kg) was administered orally for 10 days to the stress-induced depression-like model mice. Bromodeoxyuridine (BrdU; 200 mg/kg) was injected 4 h before brain fixation. Immunohistochemistry staining for BrdU and OX-A was performed. (a) BrdU-positive cells in the dentate gyrus (DG) and (b) OX-A positive cells in the lateral hypothalamic area (LHA) were counted. Data given as mean ± SEM (n = 15).

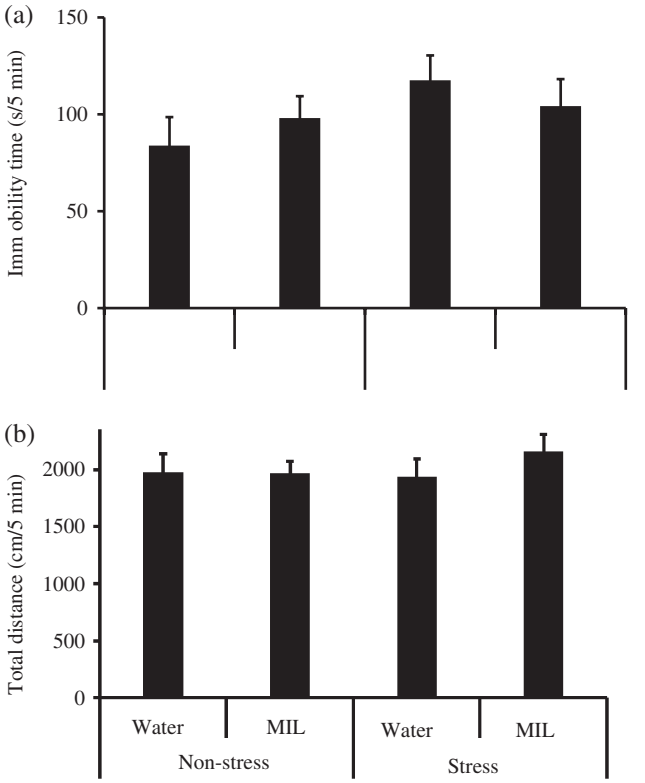

Figure 4 | Effects of milnacipran (MIL) on the psychological stress-induced depression-like model mice. (a) MIL (60 mg/kg) was administered orally for 10 days to the stress-induced depression-like model mice. The duration of immobility was measured during a 5 min forced swimming test (FST) conducted on day 15. (b) The total distance was measured during a 5 min open-field test before FST. Data given as mean ± SEM (n = 16–18).

and corticosterone level was not drastic, suggesting that this model may mimic moderate depressive condition, which is difficult to treat using currently available drugs.

We have shown that treatment with kososan improves the depression-like state in mice that experienced rat-exposure stress. These findings support a published case report demonstrating that kososan alleviates depressive mood in humans [27]. Conversely, the SNRI antidepressant milnacipran did not affect immobility in FST. It has been reported, however, that patients in a slightly depressive state often fail to improve on treatment with existing drugs, such as SSRI and SNRI [12]. This observation, along with the present results, suggests that kososan and milnacipran may exert their antidepressant activity via different mechanisms.

Bodyweight, food consumption and corticosterone level were not affected by kososan. This implies that the immobility time in FST is induced by different mechanisms from those factors. It was reported that bodyweight and food consumption were controlled by peptides, such as neuropeptide Y and ghrelin, in the brain [28]. Treatment of kososan may not alter these peptides in the present model mice. Further studies are, however, needed to clarify the relationships.

We measured the number of BrdU and OX-A positive cells, and showed that rat-exposure stress did not alter either positive cell. The present observations are in contrast to the physiological stress model, in which the numbers of BrdU- and OX-A-positive cells were decreased [17]. This difference may be attributed to the different type of stress involved (physical vs psychological stress) as well as differences in stress intensity. Additionally, kososan may exert its antidepressant effect via mechanisms independent of hippocampal cell proliferation or OX-A production in the present moderate stress mouse model.

Conclusion

We report a method for inducing a novel mouse model of depression based on psychological stress. Furthermore, we provide evidence showing kososan to be superior to an existing antidepressant for treatment of mild depressive states. Further studies using this model are warranted to clarify the mechanism of action underlying the antidepressant effects of kososan.

CONFLICT OF INTEREST

The authors declare no conflict of interests for this article.

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