BPB Reports

Regular Article

Potential Ameliorative Effects of Kampo Medicines Ninjin'yoeito and Kamikihito on Frailty-Like Behavior in Naturally Aged C57BL/6J Mice

Masahiro Tabuchi, Yosuke Matsubara,* Shinji Miyazaki, Keita Mizuno, Yuji Omiya, and Yoshiki Ikeda

Kampo Research & Development Division, Tsumura & Co., Ibaraki, Japan
Received March 30, 2022; Accepted June 3, 2022

Frailty is defined as an age-related decline in physiological reserve and increased vulnerability to stress. As frailty is a multifaceted condition, there is no effective pharmacotherapy for it yet. Ninjin'yoeito (NYT) and Kamikihito (KKT), traditional Japanese medicines (Kampo medicines), are promising in treating multifaceted conditions of frailty including fatigue and mental anxiety. However, their effects are still unclear. In this study, the effects of NYT and KKT on different types of frailty in naturally aged mice were explored by survival, physical aspects (Frailty Assessment Scores and muscle strength measurements), psychological aspects (sucrose splash test for motivation-related behavior), and social aspects (rescue-like behavior test for prosocial behavior). Mice aged 22-months were fed a diet containing 3% NYT or 3%KKT for 13 weeks until the age of 25 months. Behavioral alterations in old mice were compared with those in adult mice (five months old). Throughout the study period, Old-control mice showed frailty-like symptoms, including elevation of frailty assessment score, reduction of muscle strength and motivation for self-care, and rescue-like behavior compared to adult mice. NYT increased the survival rate of old mice and suppressed the declines in their frailty assessment score, muscle strength, and motivation for self-care. KKT reduced decreases in the frailty assessment score, motivation for self-care, and rescue-like behavior in old mice. These results suggest that NYT and KKT alleviate general frailty-like symptoms in old mice. Additionally, NYT may extend lifespan. These findings suggest that NYT and KKT may be helpful for improving the multifaceted symptoms of frailty.

Key words frailty, aged mice, ninjin'yoeito, kamikihito, kampo medicine

INTRODUCTION

Frailty is a clinical condition in which susceptibility to stress decreases because of the cumulative reduction in physical and mental functions associated with aging.[1,2] It is closely related to the onset and progression of many geriatric diseases. The multifaceted symptoms of frailty include physical symptoms, such as muscle weakness and fatigue; psychiatric symptoms, such as depression and reduced motivation; psychological symptoms, such as cognitive decline; and social symptoms, including loneliness and social deprivation. All these facets interact with each other.[3] For the prevention of the onset and progression of frailty, exercise and nutritional interventions (e.g., calorie restriction) have been reported to be effective.[4,5] Alternatively, the pharmacological treatment for frailty has been attempted using existing drugs (e.g., metformin, rapamycin, and angiotensin-converting enzyme inhibitors) and sirtuin activators as well as senescent cell removal agents. However, these interventions have not yet been established as suitable treatments since the possibility for side effects remains.[6]

Animal models that shed light on human frailty pathology are essential for establishing pharmacotherapies for frailty. Whitehead *et al*. developed a frailty index that is primarily limited to motor and neuromuscular functions for findings in naturally aged C57BL/6J mice.[7] This index corresponds to human frailty symptoms. The age-associated alterations in the cumulative score of the 31 items of this index with aging closely approximate alterations in human frailty scores. Later, Rizzo *et al*. partially simplified and modified it into a non-invasive protocol (Frailty Assessment Scores) that can be used over time and showed that the cumulative scores of 27 items increased with aging.[8] Additionally, naturally aged mice show muscle weakness and reduced motivation-related behavior, making them a useful animal model for *in vivo* evaluation of phenotypes that reflect multiple aspects of frailty.[9,10]

Traditional Japanese medicines (Kampo medicines), which comprise many naturally occurring ingredients, have shown promise as a treatment for frailty with multifaceted symptoms because each crude drug included in Kampo medicines can exert various effects. Ninjin'yoeito (NYT) is a Kampo formula used in clinical practice to improve declined constitution after recovery from disease, fatigue and malaise, anorexia, perspiration during sleep, cold limbs, and anemia. NYT ameliorates anorexia, apathy, and cognitive decline in patients with Alzheimer's disease.[11] It also improves nutritional status and enhances rehabilitation effects in patients with hip fracture and sarcopenia.[12] Animal studies have shown that NYT relieves the reduced food intake and apathy-like behavior observed in an apathy model.[13] Kamikihito (KKT) is a clinical Kampo formula used to improve anemia, insomnia, mental anxiety, and neurosis. KKT effectively treats depression, anxiety, and fatigue in patients with prostate cancer.[14] It also

*To whom correspondence should be addressed. e-mail: matsubara_yousuke@mail.tsumura.co.jp

improves anxiety and gastrointestinal QOL in female patients with chronic constipation.[15] Animal studies have reported that KKT improves anhedonia and cognitive function in naturally aged mice. These drugs hold promise as effective therapeutics against many aspects of frailty.[16] However, there are insufficient pharmacological studies on the subject.

In this study, we evaluated the effects of NYT and KKT on frailty assessment scores of naturally aged C57BL/6 mice (old mice) showed relatively low mental and physical stress compared with adult mice as negative control for frailty of naturally aging. We also examined the effects of NYT and KKT on motivation-related behavior (self-care behaviors), prosocial behavior (rescue-like behaviors), skeletal muscle function (forelimb grip strength), and survival rate (life span).

MATERIALS AND METHODS

Animals All mice (male C57BL/6J, 4 or 22 months at the start of the study) were purchased from the Jackson Laboratory Japan, Inc. (Yokohama, Japan), and four mice per cage were allowed free access to water and a CRF6 diet (Oriental Yeast Co., Ltd., Tokyo, Japan). The cages were maintained at a room temperature of 23°C ± 3°C, relative humidity of 50% ± 20%, and with a light/dark cycle of 12 h/12 h throughout the study. Every week, the number of surviving mice during the 13-week treatment period was counted.

Drugs KKT and NYT extract powders were manufactured by Tsumura & Co (Tokyo, Japan). in compliance with good manufacturing practice requirements.

KKT (lot.361095300) used in our study was made using the following 14 kinds of crude drugs that were mixed in the proportions indicated in parentheses: Astragali Radix (3 g, The roots of *Astragalus propinquus* Schischkin or *Astragalus mongholicus* Bunge), Bupleuri Radix (3 g, The roots of *Bupleurum falcatum* L.), Ziziphi Semen (3 g, The seeds of *Ziziphus jujuba* var. *spinosa* [Bunge] Hu ex H.F.Chow), *Atractylodis Lanceae* Rhizoma (3 g, The rhizome of *Atractylodes lancea* [Thunb.] DC. or *Atractylodes chinensis* [Bunge] Koidz.), Ginseng Radix (3 g, The roots of *Panax ginseng* C.A.Mey.), Poria (3 g, sclerotium of *Wolfiporia cocos* Ryvarden et Gilbertson), Longan Arillus (3 g, The arillus of *Dimocarpus longan* Lour.), Polygalae Radix (2 g, The roots of *Polygala tenuifolia* Willd.), Gardeniae Fructus (2 g, The fruits of *Gardenia jasminoides* J.Ellis), Ziziphi Fructus (2 g, The fruits of *Ziziphus jujuba var. inermis* [Bunge] Rehder), Angelicae Radix (2 g, The roots of *Angelica acutiloba* [Siebold & Zucc.] Kitag. or *Angelica acutiloba* var. *sugiyamae* Hikino), Glycyrrhizae Radix (1 g, The roots and stolons of *Glycyrrhiza uralensis* Fisch. ex DC. or *Glycyrrhiza glabra* L.), Zingiberis Rhizoma (1 g, The rhizome of *Zingiber officinale* Roscoe), and Saussureae Radix (1 g, The roots of *Aucklandia costus* Falc.). The mixture was then extracted using hot water and dried using the spray drying method. The quality of KKT was guaranteed by measuring following ingredients according to the Japanese Pharmacopoeia: saikosaponin b2, geniposide, and glycyrrhizic acid. In addition, the chemical profiling of KKT via three-dimensional HPLC was previously reported.[17]

NYT (lot.362113100) used in our study was made using the following 12 kinds of crude drugs that were mixed in the proportions indicated in parentheses: Rehmanniae Radix (4 g, The roots of *Rehmannia glutinosa* Liboschitz var. *Purpurea* Makino or *Rehmannia glutinosa* Liboschitz), Angelicae Acutilobae Radix (4 g, The roots of *Angelica acutiloba* Kitagawa or *Angelica acutiloba* Kitagawa var. *sugiyamae* Hikino), Atractylodis Rhizoma (4 g, The rhizome of *Atractylodes japonica* Koidzumi ex Kitamura or *Atractylodes macrocephala* Koidzumi (*Atractylodes ovata* De Candolle)), Poria (4 g, sclerotium of *Wolfiporia cocos* Ryvarden et Gilbertson), Ginseng Radix (3 g, The roots of *Panax ginseng* C. A. Meyer (*Panax schinseng* Nees)), Cinnamoni Cortex (2.5 g, The bark of *Cinnamomum cassia* Blume), Polygalae Radix (2 g, The roots of *Polygala tenuifolia* Willdenow), Paeoniae Radix (2 g, The roots of *Paeonia lactiflora* Pallas), Citri Unshiu Pericarpium (2 g, The pericarp of *Citrus unshiu* Markowicz or *Citrus reticulata* Blanco), Astragali Radix (1.5 g, The roots of *Astragalus membranaceus* Bunge or *Astragalus mongholicus* Bunge), Glycyrrhizae Radix (1 g, The roots and stolons of *Glycyrrhiza uralensis* Fischer or *Glycyrrhiza glabra* Linn´e), and Schisandrae Fructus (1 g, The fruits of *Schisandra chinensis* Baillon). The mixture was then extracted and dried in the same manner as KKT. The quality of NYT was guaranteed by measuring following ingredients according to the Japanese Pharmacopoeia: glycyrrhizic acid, paeoniflorin, and hesperidin. The chemical profiling of NYT via three-dimensional HPLC was previously reported.[18]

Experimental Schedule The mice were fed a powdered CRF-6 diet containing 3% powdered KKT or 3% powdered NYT. Old mice (22 months old, 36 mice) were divided into the following groups without any difference in body weight (34.4 ± 0.4 g) and without changing their cage mates; the Old-control (CRF6, 12 mice), Old-KKT (CRF6+KKT, 12 mice), and Old-NYT groups (CRF6+NYT, 12 mice). The adult mice group (4 months old, 10 mice) was fed the CRF6 diet. The experimental schedule is shown in Fig. 1. This study was conducted according to the Code of Ethics for Animal Experiments with the approval of the Animal Experiment Committee of Tsumura & Co. Food intake of each mouse during the experimental schedule was calculated and averaged as below; 3.45 ± 0.10 g/day in the adult group, 3.39 ± 0.20 g/day in the Old-control group, 3.27 ± 0.21 g/day in the Old-KKT group and 3.25 ± 0.20 g/day in the Old-NYT group. Although those of each group might be inaccurate due to group housing and natural mortality of old mice, we considered that there was no difference in food intake between the experimental groups.

Frailty Assessment Scores Frailty-like symptoms in old and adult mice were measured at week 4 of drug administration using the frailty assessment score for mice according to Rizzo *et al*.[8] (Supplementary Table 1). A trained observer assigned all animals a score on a 3-point scale (0, 0.5, and 1.0) following the degree of disability (aging) for 27 items, including sensory, motor, and cognitive functions (Supplementary Table 2). The individual and cumulative scores for the 27 items were evaluated.

Motivation-Related Behavior The sucrose splash test evaluates self-grooming motions shown in mice which categorized as the self-care behavior. They are sprayed with a viscous solution on the dorsal neck as a method to assess motivational, anhedonia-like, or apathy-like behavior.[19] After spraying 10% sucrose solution on the dorsal neck of adult mice (6 months old) and old mice (24 months old) during the sixth week of drug administration, the mice were placed in a transparent cylindrical tube (12-cm diameter and 18-cm height), and the grooming frequency was measured for 5 min. The inner surface of the transparent cylindrical tube was wiped using 70% alcohol after each measurement.

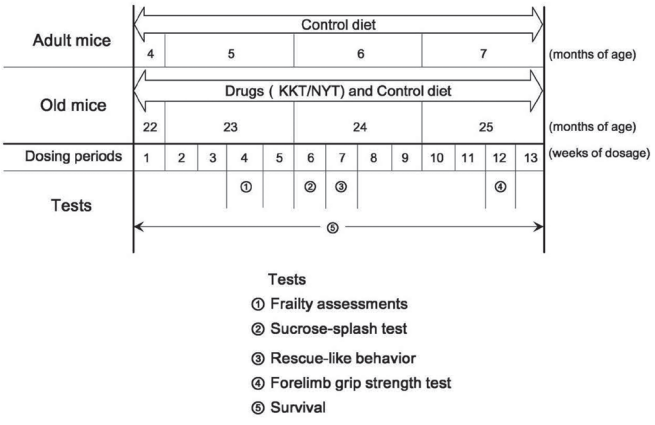

Fig. 1. Experimental Schedule

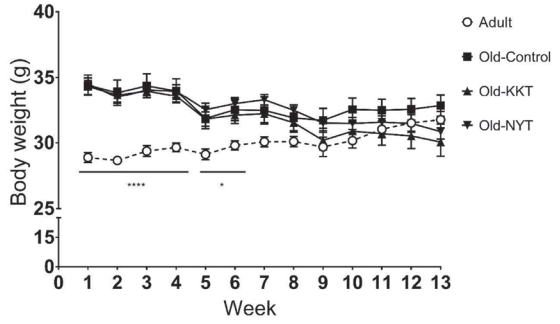

Fig. 2. Effects of Ninjin'yoeito (NYT) and Kamikihito (KKT) on Body Weight of Old Mice

Temporal comparison of body weight for 13 weeks. Data are presented as the mean ± SEM. Two-way repeated measures analysis of variance + Bonferroni's multiple comparison test was used to calculate significance. *; P < 0.05, ****; P < 0.0001 vs. Old-control group at each time point.

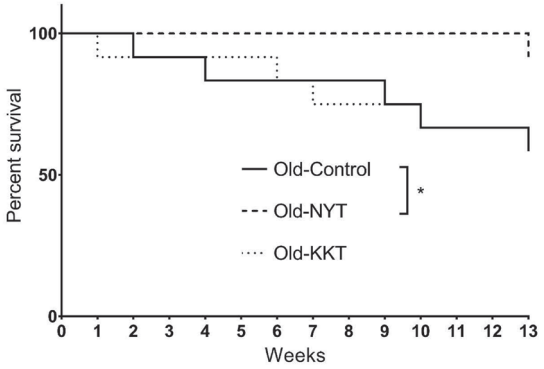

Fig. 3. Effect of Ninjin'yoeito (NYT) and Kamikihito (KKT) on Survival Rate of Old Mice

Kaplan-Meier curves indicated the survival rates of each old mice groups. The generalized Wilcoxon test was used to compare the Old-control group with the Kampo medicine groups. P < 0.05 was considered statistically significant.

Prosocial Behavior The rescue-like behavior test is used as one of the methods to evaluate prosocial behavior (willingness to make social contact or empathy) that benefits others. This test followed the method described by Ueno *et al*..[20] Adult mice (6 months old) and Old mice (24 months old) during the seventh week of drug administration were placed in 50-mL plastic centrifuge tubes, with their lids covered with tissue paper. They were trained to break through the tissue paper and go outside thrice a day for 2 days. As a result, all of the mice learned to break the tissue paper lid and get out. Next, nontreated normal mice (4 months old) were placed in a cage with the mice restrained in a 50-mL plastic centrifuge tube covered with tissue paper. Simultaneously, a paper-lidded tube containing no animals was also placed in the cage. The test mice were then placed in the cage, and the time needed for the test mice to break the paper lid of the tube from the outside within 90 min was measured. The test was conducted once a day for each test animal.

Forelimb Grip Strength Test Forelimb grip strength was measured in adult mice (seven months old) and old mice (25 months old) during the 12th week of drug administration. The measurement method was that described by Aartsma-Rus *et al*..[21] Both forelimbs of mice were placed on the stainless steel net of a "Saito-type" grip strength meter for mice (MK-380M, Muromachi Kikai Co., Ltd.), and the maximum traction force when the mouse tail was pulled was measured. Each test was conducted in triplicate, with the average grip force being used as a data point and a break of at least 5 min between tests.

Statistical Analysis All statistical analyses were conducted using the GraphPad Prism software version 7.04 (GraphPad Software, San Diego, CA, USA). Behavioral test results were analyzed using either one-way analysis of variance with post hoc-Bonferroni's multiple comparison test for parametric data or Kruskal Wallis test with post hoc-Dunn's multiple comparison test for non-parametric data. Body weight was analyzed by two-way repeated-measures analysis of variance followed by Bonferroni's multiple comparison test. Kaplan–Meier curves indicated survival rates of old mice groups, and the generalized Wilcoxon test was used to compare the Old-control group with the Kampo medicine groups. $P < 0.05$ was considered statistically significant.

RESULTS

Body Weight There was no difference in body weight between the Old-KKT group or the Old-NYT group and the Old-control group during the treatment period (Fig. 2).

Survival Rates The rates of surviving mice in the old mice groups during the 13-week treatment period is indicated in Fig. 3. The mortality rate at 13 weeks was 42% in the Old-control group and 8% in the Old-NYT group, which was significantly different. Meanwhile, the survival trend of the Old-KKT group was similar to that of the Old-control group. There were no deaths in the adult control group during the observation period (data not shown).

Frailty Assessment Scores The cumulative scores of frailty-like symptoms in the adult control, Old-control, Old-KKT, and the Old-NYT groups at week 4 are indicated in Fig. 4-A. The score value of the Old-control group was significantly higher than that of the adult control group, and the frailty-like symptoms had enhanced. The score values of the Old-KKT and NYT groups were significantly lower than that of the Old-control group ($p < 0.05$ and $p < 0.01$, respectively), and the frailty-like symptoms were ameliorated. All score values of individual items are indicated in Supplementary Table 2. Loss of fur color (Fig. 4-B, $p < 0.05$ for the Old-NYT group), coat

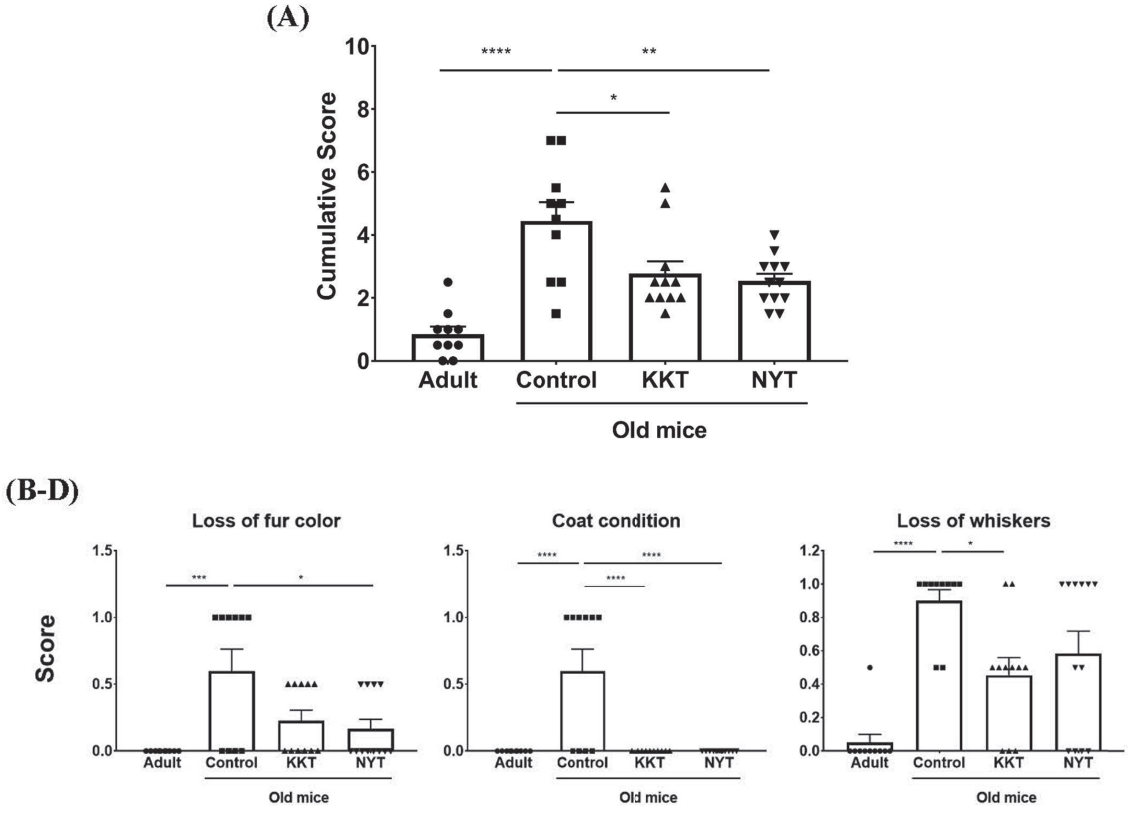

Fig. 4. Effect of Ninjin'yoeito (NYT) and Kamikihito (KKT) on Frailty Assessment Scores in Old Mice

(A) The cumulative scores of frailty-like symptoms in each group at week 4 (B) Fading of coat color, (C) coat condition and (D) loss of whiskers. Data are presented as the mean ± SEM. N = 10–12/group. One-way ANOVA + Bonferroni's s multiple comparison test was used to calculate significance. *; P < 0.05, **; P < 0.01, ***; P < 0.001, ****; P < 0.0001 vs. Old-control.

condition (Fig. 4-C, $p < 0.001$ for both drug groups), and loss of whiskers (Fig. 4-D, $p < 0.05$ for the Old KKT group) were lower than those of the Old-control group.

Motivation-Related Behavior The results of the sucrose splash test at week 6 of treatment are indicated in Fig. 5. The grooming number of the Old-control mice group was significantly lower than that of the adult mice group ($p < 0.0001$), but the grooming number of the Old-KKT and NYT groups was significantly higher than that of the Old-control ($p < 0.05$ for both drug groups).

Prosocial Behavior Figure 6 indicates the results of the rescue-like behavior test of the mice at week 7 of treatment. Compared to the adult mice group, the response latency of the Old-control group that broke the paper lid of the tube containing the restrained mouse tended to increase. The latency of rescue-like behavior in the Old-KKT group was significantly shorter than that in the Old-control group ($p < 0.05$). The reaction latency of the Old-KKT group were comparable to those of the adult mice group. The reaction latency of the Old-NYT group did not differ from those of the Old-control group.

Forelimb Grip Strength Test Figure 7 indicates the results of the forelimb grip strength test of each group at week 12 of treatment. The forelimb grip strength of the Old-control group was significantly weaker than that of the adult mice group ($p < 0.01$) and that of the Old-NYT group was significantly stronger than that of the Old-control group ($p < 0.05$). The grip strength of the Old-KKT group was not different from that of the Old-control group.

DISCUSSION

This study investigated the potential ameliorative effects of NYT and KKT, two Kampo medicines, on physical, psychological, and social aspects of frailty in naturally aged animals. To the best of our knowledge, this is the first time that comparison with the effects of NYT and KKT in general frailty-like symptoms on old mice. NYT improved the declines of muscle strength and motivation; on the other hand, KKT enhanced motivation and rescue-like behavior. We also observed decrease the locomotor activity of old mice; however, neither KKT nor NYT improved those (Supplementary Fig. 1A). Interestingly, NYT, not KKT, significantly prolonged the survival of old mice in this study. Nobiletin, one of the NYT-derived components that migrates into the blood, might be involved in the pharmacological effects of NYT since it was reported to demonstrate the life-prolonging effects in old mice.[22,23]

In this study, frailty-like symptoms in old mice were assessed using the Mouse Frailty Assessment Score. The score of 23-month-old aged control mice was significantly higher than that of adult mice and was close to the previously reported value.[8] Therefore, the aged animals used in this study were verified to exhibit frailty-like symptoms and serve as a useful tool for assessing the effects of drugs on those. The frailty scores of the old mice after four weeks of treatment with NYT and KKT were significantly lower than the scores of the old control mice. This suggests that both drugs may improve or prevent progression of overall frailty-like symptoms in old

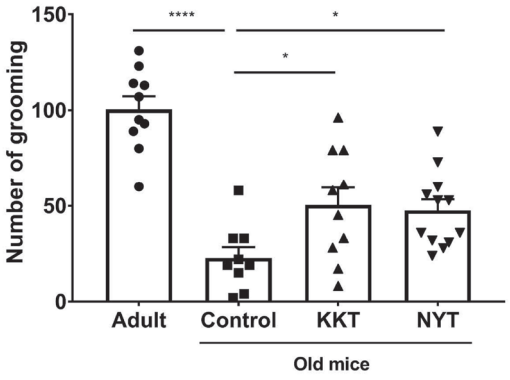

Fig. 5. Effect of Ninjin'yoeito (NYT) and Kamikihito (KKT) on Self-Care Motivation in Old Mice

The frequency of grooming behaviors over 5 min after spraying a 10% sucrose solution in each group at week 6. Data are presented as the mean ± SEM. N = 9–12/group. One-way analysis of variance + Bonferroni's multiple comparison test was used to calculate significance. *; $P < 0.05$, ***; $P < 0.001$ vs. Old-control.

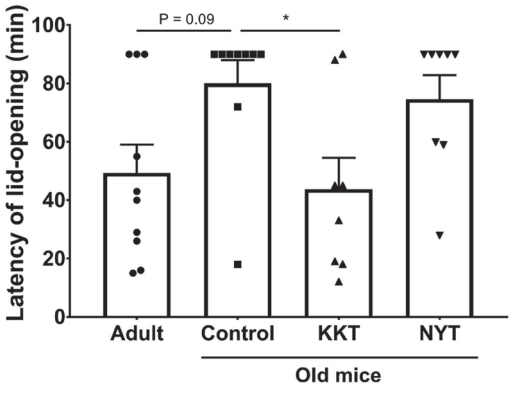

Fig. 6. Effect of Ninjin'yoeito (NYT) and Kamikihito (KKT) on Rescue-Like Behavior in Old Mice

The latency of opening the paper lid of the tube from the outside within 90 min in each group at week 7. Data are presented as the mean ± SEM. N = 8–10/group. Kruskal Wallis test + Dunn's multiple comparison test was used to calculate significance. *; $P < 0.05$ vs. Old-control.

mice. These effects were primarily due to the improvement of skin-level symptoms (fading of fur color and loss of hairiness and whiskers). Although the mechanism of age-related changes in skin-level symptoms is unclear, oxidative stress is a factor that may explain these alterations in systemic conditions. Oxidative stress has been implicated in the pathophysiology of frailty.[24] Furthermore, oxidative stress has been shown to increase in old mice due to reduced mitochondrial function.[25] In our previous study, liquiritin and gallic acid contained in Kampo medicines, as well as their metabolites, exhibited antioxidant activity and absorbed into the body.[26,27] Recently, liquiritin and gallic acid have been identified as NYT components that are transferred into the plasma. Although the pharmacokinetics of KKT-derived components are unclear, it is possible that liquiritin, which has been shown to migrate into the bloodstream, and its metabolites exert antioxidant effects *in vivo*.

Additionally, a sucrose splash test was conducted to measure motivational decline, which is a sign of psychological frailty in old mice. In this test, the frequency of self-grooming in old mice (23 months old) was significantly lower than that in adult mice, indicating a reduction in self-care behavior. Although we did not measure the duration of self-grooming in this study, the grooming duration in old mice decreased with the frequency according to the previous report.[9] This reduction in grooming frequency was increased significantly when old mice were treated with KKT and NYT for six weeks. The act of eliminating the unpleasant viscous sucrose fluid from the fur is an innate self-care behavior for hygienic care of the body surface in mice. The reduction in such behavior is considered to a result of reduction in motivation or affective disorder in terms of reduced self-reward activity, loss of pleasant sensations, or abandonment of the behavior entirely.[9,28–30] In this study, KKT and NYT increased the frequency of grooming in old mice, suggesting the recovery of daily self-care behavior (activity for self-reward). On the other hand, Hirata *et al*. previously reported that NYT (1000 mg/kg/day), but not KKT (1000 mg/kg/day) increased the grooming time of SAMP8 mice in the sucrose splash test.[31] We considered that differences in dosage and animal models might be reflected the differences in the efficacy of improving self-care motivation between KKT and NYT. The splash test is also used as a

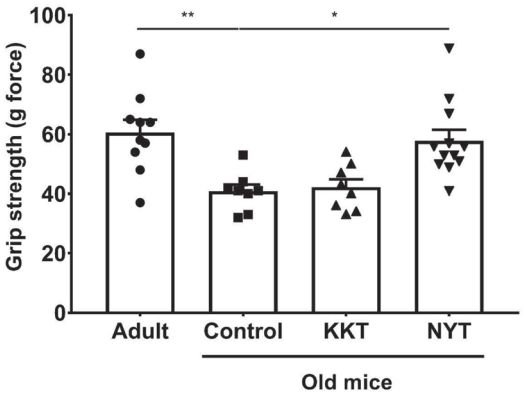

Fig. 7. Effect of Ninjin'yoeito (NYT) and Kamikihito (KKT) on Forelimb Grip Strength in Old Mice

Forelimb grip strength in each group at week 12. Data are presented as the mean ± SEM. One-way analysis of variance + Bonferroni's multiple comparison test was used to calculate significance. *; $P < 0.05$, **; $P < 0.01$ vs. Old-control.

method to detect depression-like and anxiety-like symptoms in mice, and it has been reported that the self-grooming behavior is reduced in old mice[9] and depression model mice[32] which recovered by the administration of antidepressants (e.g., imipramine). Although we did not detect the anxiety-like behavior in old mice in this study (Supplementary Fig. 1B), the results of the sucrose splash test might include effects of KKT and NYT on the response of old mice to the novel environment. Therefore, it is expected that KKT and NYT alleviate depression-like symptoms in aged animals, but further verification in other behavioral tests (including forced swimming test and tail suspension test) may be necessary.

Rescue-like behavior is an altruistic behavior without reward in rodents and primates and is considered a form of prosocial behavior.[33] It has been reported that rescue-like behavior of young mice (10 weeks old) toward restrained mice is based on at least social interest and increased interest in the restraining tube.[20,34] In this study, most of the old mice tore the paper lid of the tube containing the restrained mice later than the adult mice, suggesting that old mice have less interest in the restrained mice and the restraint tube. Interestingly, NYT

did not affect the decline of prosocial behavior in old mice, and only administration of KKT for seven weeks reduced the paper-breaking latency of old mice to the same level as that of adult mice. This suggests that KKT may restore social interest (prosociality) in old mice. Recently, much attention has been paid to KKT effects on the oxytocin system. Shimomura *et al.*[35] have reported that KKT activates oxytocin neurons in the hypothalamus. Additionally, it has been reported that KKT has anti-stress and anti-anxiety effects by releasing oxytocin into the brain in acute stress model rats.[36] Oxytocin is a peptide hormone synthesized and secreted in the paraventricular and suprachiasmatic nuclei of the hypothalamus. Recently, it has been reported to be an essential molecule that regulates advanced prosocial behavior in rodents and humans, such as the formation of relationships of trust and human-human bonds, altruism and empathy toward unidentified others.[37,38] Thus, oxytocin may be involved in KKT's action mechanism for the restoration of prosocial behavior. KKT contains three crude drugs, Zizyphi Fructus, Angelicae Radix and Zingiberis Rhizoma, that activate oxytocin receptor, while NYT contains only Angelicae Radix.[35] It is possible that differences in components between KKT and NYT affected in the efficacy of drugs on prosocial behavior test in this study.

The effects of KKT on oxytocin expression and neural functions in old mice are yet to be investigated. Additionally, it has been reported that KKT enhances reward-related behaviors in old mice[16] and ameliorates fatigue, depression, and anxiety caused by a sympathetic-parasympathetic balance in patients with cancer.[14] At the molecular level, administration of KKT affects the binding of radioligands to acetylcholine, dopamine, 5-HT, and GABA receptors, suggesting that KKT affects the central nervous system function.[39–41] In the future, it is necessary to investigate the mechanism of central KKT effects, including oxytocin, in old mice.

To investigate the effects of KKT and NYT on motor skill decline, which is a sign of physical frailty in old mice, a forelimb grip strength test was conducted. The test data indicated that the forelimb grip strength of 25-month-old mice was significantly weaker than that of 7-month-old mice. This finding was similar with the report that the grip strength of old mice C57BL/6J (≥ 22 months) reduced compared to 4-month-old mice.[42] The reduced grip strength of the old mice was restored to the same level as that of the adult mice after 12 weeks of treatment with NYT. No such effect was observed with KKT. In old mice, skeletal muscle function deteriorates with age, which is thought to be related to a reduction in mitochondrial function in skeletal muscle that correlates with mitochondrial oxidative stress.[43] NYT-derived catalpol has anti-inflammatory and antioxidant effects and effectively relieves Duchenne muscle dystrophy in model animals by promoting myogenesis.[44,45] Similarly, NYT-derived gallic acid activates the AMPK/Sirt1/PGC1α signaling pathway throughout the body, including in skeletal muscle. Its effects in the C2C12 cell culture system include the enhancement of mitochondrial ATP synthesis through Sirt1.[46,47] The possibility that certain NYT components, including catalpol and gallic acid, migrate into the blood and then relieve the decline in skeletal muscle function in old mice is a topic for further investigation.

In this exploratory study, the medicinal effects of NYT and KKT on the phenotypes that occur in aging mice were evaluated. Despite our results, the mechanisms of the pharmacotherapeutic effects of NYT and KKT remain unclear. Further behavioral and biochemical studies will be necessary to elucidate the mechanism of each drug effect.

In conclusion, NYT showed efficacy against physical and psychological frailty-like symptoms, including general frailty-like symptoms, motor skills, and reduced motivation in aged animals and further increased the survival rate of aged animals. In addition to improving general frailty-like symptoms in aged animals, KKT showed efficacy against psychological and social frailty-like symptoms, such as reduced motivation and prosocial behavior. These findings present the usefulness of Kampo medicines for multifaceted frailty symptoms and provide helpful information on the use of different drugs for frailty symptoms.

Acknowledgments We sincerely thank Dr. Kazushige Mizoguchi for invaluable advice. We would like to thank Crimson Interactive Pvt. Ltd. (Ulatus) – www.ulatus.jp for their assistance in English language editing.

Conflict of interest This study was supported by Tsumura & Co. All authors are employees of Tsumura & Co. There is no other conflict of interests regarding the publication of this paper.

REFERENCES

1) Khezrian M, Myint PK, McNeil C, Murray AD. A Review of Frailty Syndrome and Its Physical, Cognitive and Emotional Domains in the Elderly. *Geriatrics (Basel)*, **2**, (2017).
2) Dent E, Martin FC, Bergman H, Woo J, Romero-Ortuno R, Walston JD. Management of frailty: opportunities, challenges, and future directions. *Lancet*, **394**, 1376–1386 (2019).
3) Hoogendijk EO, Afilalo J, Ensrud KE, Kowal P, Onder G, Fried LP. Frailty: implications for clinical practice and public health. *Lancet*, **394**, 1365–1375 (2019).
4) de Labra C, Guimaraes-Pinheiro C, Maseda A, Lorenzo T, Millan-Calenti JC. Effects of physical exercise interventions in frail older adults: a systematic review of randomized controlled trials. *BMC Geriatr.*, **15**, 154 (2015).
5) Liu P, Li Y, Ma L. Caloric Restriction May Help Delay the Onset of Frailty and Support Frailty Management. *Front. Nutr.*, **8**, 731356 (2021).
6) Palliyaguru DL, Moats JM, Di Germanio C, Bernier M, de Cabo R. Frailty index as a biomarker of lifespan and healthspan: focus on pharmacological interventions. *Mech. Ageing Dev.*, **180**, 42–48 (2019).
7) Whitehead JC, Hildebrand BA, Sun M, Rockwood MR, Rose RA, Rockwood K, Howlett SE. A clinical frailty index in aging mice: comparisons with frailty index data in humans. *J. Gerontol. A Biol. Sci. Med. Sci.*, **69**, 621–632 (2014).
8) Sukoff Rizzo SJ, Anderson LC, Green TL, McGarr T, Wells G, Winter SS. Assessing Healthspan and Lifespan Measures in Aging Mice: Optimization of Testing Protocols, Replicability, and Rater Reliability. *Curr. Protoc. Mouse Biol.*, **8**, e45 (2018).
9) Malatynska E, Steinbusch HW, Redkozubova O, Bolkunov A, Kubatiev A, Yeritsyan NB, Vignisse J, Bachurin S, Strekalova T. Anhedonic-like traits and lack of affective deficits in 18-month-old C57BL/6 mice: implications for modeling elderly depression. *Exp. Gerontol.*, **47**, 552–564 (2012).
10) Arnold WD, Taylor RS, Li J, Nagy JA, Sanchez B, Rutkove SB. Electrical impedance myography detects age-related muscle change in mice. *PLoS One*, **12**, e0185614 (2017).
11) Ohsawa M, Tanaka Y, Ehara Y, Makita S, Onaka K. A Possibility of Simultaneous Treatment with the Multicomponent Drug, Ninjin'yoeito, for Anorexia, Apathy, and Cognitive Dysfunction in Frail Alzheimer's Disease Patients: An Open-Label Pilot Study. *J. Alzheimers Dis. Rep.*, **1**, 229–235 (2017).
12) Morinaga A, Nakamura H, Hattanmaru K, Rokot NT, Kimura Y, Ito T. Good Rehabilitation Outcomes and Improved Nutritional Status After Treatment With the Japanese Herbal Medicine Ninjin'yoeito in

an Elderly Patient With Hip Fracture and Sarcopenia: A Case Report. *Front. Nutr.*, **7**, 85 (2020).
13) Yamada C, Mogami S, Kanno H, Nishi A, Shimobori C, Hattori T. Ninjinyoeito Ameliorates Anorexia and Decrease in the Nest Building via a Dopamine D2Receptor-dependent Manner in a Novel Murine Apathy Model. *Jpn. Pharmacol. Ther.*, **46**, 207–216 (2018).
14) Tamada S, Ebisu K, Yasuda S, Kato M, Ninomiya N, Yamasaki T, Iguchi T, Nakatani T, Watanabe Y. Kamikihito improves cancer-related fatigue by restoring balance between the sympathetic and parasympathetic nervous systems. *Prostate Int.*, **6**, 55–60 (2018).
15) Kobayashi A, Nagashima K, Hu A, Harada Y, Kobayashi H. Effectiveness and safety of kamikihito, a traditional Japanese medicine, in managing anxiety among female patients with intractable chronic constipation. *Complement. Ther. Clin. Pract.*, **46**, 101526 (2022).
16) Oizumi H, Miyazaki S, Tabuchi M, Endo T, Omiya Y, Mizoguchi K. Kamikihito Enhances Cognitive Functions and Reward-Related Behaviors of Aged C57BL/6J Mice in an Automated Behavioral Assay System. *Front. Pharmacol.*, **11**, 1037 (2020).
17) Adachi N, Sakhri FZ, Ikemoto H, Ohashi Y, Kato M, Inoue T, Hisamitsu T, Sunagawa M. Kamikihito rescued depressive-like behaviors and hippocampus neurogenesis in chronic restraint stress rats. *J. Tradit. Complement. Med.* (2021).
18) Horie H, Fujii Y, Katabami K, Iwama M, Yamada A. Suppressive effects of traditional herbal medicines on reversion of attenuated polio vaccine viruses to neurovirulent genotype. *J. Tradit. Medicines*, **24**, 156–163 (2007).
19) Rosa JM, Pazini FL, Cunha MP, Colla ARS, Manosso LM, Mancini G, Souza ACG, de Bem AF, Prediger RD, Rodrigues ALS. Antidepressant effects of creatine on amyloid beta1-40-treated mice: the role of GSK-3beta/Nrf2 pathway. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, **86**, 270–278 (2018).
20) Ueno H, Suemitsu S, Murakami S, Kitamura N, Wani K, Takahashi Y, Matsumoto Y, Okamoto M, Ishihara T. Rescue-like Behaviour in Mice is Mediated by Their Interest in the Restraint Tool. *Sci. Rep.*, **9**, 10648 (2019).
21) Aartsma-Rus A, van Putten M. Assessing functional performance in the mdx mouse model. *J. Vis. Exp.* (2014).
22) Matsumoto T, Takiyama M, Sakamoto T, Kaifuchi N, Watanabe J, Takahashi Y, Setou M. Pharmacokinetic study of Ninjin'yoeito: absorption and brain distribution of Ninjin'yoeito ingredients in mice. *J. Ethnopharmacol.*, **279**, 114332 (2021).
23) Nohara K, Mallampalli V, Nemkov T, Wirianto M, Yang J, Ye Y, Sun Y, Han L, Esser KA, Mileykovskaya E, D'Alessandro A, Green CB, Takahashi JS, Dowhan W, Yoo SH, Chen Z. Nobiletin fortifies mitochondrial respiration in skeletal muscle to promote healthy aging against metabolic challenge. *Nat. Commun.*, **10**, 3923 (2019).
24) Alvarez-Satta M, Berna-Erro A, Carrasco-Garcia E, Alberro A, Saenz-Antonanzas A, Vergara I, Otaegui D, Matheu A. Relevance of oxidative stress and inflammation in frailty based on human studies and mouse models. *Aging (Albany NY)*, **12**, 9982–9999 (2020).
25) Kadoguchi T, Shimada K, Miyazaki T, Kitamura K, Kunimoto M, Aikawa T, Sugita Y, Ouchi S, Shiozawa T, Yokoyama-Nishitani M, Fukao K, Miyosawa K, Isoda K, Daida H. Promotion of oxidative stress is associated with mitochondrial dysfunction and muscle atrophy in aging mice. *Geriatr. Gerontol. Int.*, **20**, 78–84 (2020).
26) Matsumoto T, Matsubara Y, Mizuhara Y, Sekiguchi K, Koseki J, Tsuchiya K, Nishimura H, Watanabe J, Kaneko A, Maemura K, Hattori T, Kase Y. Plasma Pharmacokinetics of Polyphenols in a Traditional Japanese Medicine, Jumihaidokuto, Which Suppresses Propionibacterium acnes-Induced Dermatitis in Rats. *Molecules*, **20**, 18031–18046 (2015).
27) Matsubara Y, Matsumoto T, Sekiguchi K, Koseki J, Kaneko A, Yamaguchi T, Kurihara Y, Kobayashi H. Oral Administration of the Japanese Traditional Medicine Keishibukuryogan-ka-yokuinin Decreases Reactive Oxygen Metabolites in Rat Plasma: Identification of Chemical Constituents Contributing to Antioxidant Activity. *Molecules*, **22**, (2017).
28) Spruijt BM, van Hooff JA, Gispen WH. Ethology and neurobiology of grooming behavior. *Physiol. Rev.*, **72**, 825–852 (1992).
29) Haj-Mirzaian A, Nikbakhsh R, Ramezanzadeh K, Rezaee M, Amini-Khoei H, Haj-Mirzaian A, Ghesmati M, Afshari K, Haddadi NS, Dehpour AR. Involvement of opioid system in behavioral despair induced by social isolation stress in mice. *Biomed. Pharmacother.*, **109**, 938–944 (2019).
30) Kosel F, Torres Munoz P, Yang JR, Wong AA, Franklin TB. Age-related changes in social behaviours in the 5xFAD mouse model of Alzheimer's disease. *Behav. Brain Res.*, **362**, 160–172 (2019).
31) Hirata M, Taniguchi C, Watanabe T, Horikawa T, Akizuki Y, Kubota K, Katsurabayashi S, Iwasaki K. Effects of two kinds of Kampo-hozai, ninjinyoeito and kamikihito, on mental disorder-like behaviors in senescence-accelerated mouse-prone 8 mice. *Tradit. Kampo Med.*, **8**, 176–180 (2021).
32) Detanico BC, Piato AL, Freitas JJ, Lhullier FL, Hidalgo MP, Caumo W, Elisabetsky E. Antidepressant-like effects of melatonin in the mouse chronic mild stress model. *Eur. J. Pharmacol.*, **607**, 121–125 (2009).
33) Cronin KA. Prosocial behaviour in animals: the infuence of social relationships,communication and rewards. *Anim. Behav.*, **84**, 1085–1093 (2012).
34) Ueno H, Suemitsu S, Murakami S, Kitamura N, Wani K, Matsumoto Y, Okamoto M, Ishihara T. Helping-Like Behaviour in Mice Towards Conspecifics Constrained Inside Tubes. *Sci. Rep.*, **9**, 5817 (2019).
35) Yuko Maejima SH. Shoko Yokota, Tomoyuki Ono, Peter Proks, Hiromi Yoshida-Komiya, Yoichi Ueta, Katsuhiko Nishimori, Shingen Misaka, Kenju Shimomura: identification of oxytocin receptor activating chemical components from traditional Japanese medicines. *Yao Wu Shi Pin Fen Xi*, **29**, 653–675 (2021).
36) Tsukada M, Ikemoto H, Lee XP, Takaki T, Tsuchiya N, Mizuno K, Inoue T, Tsunokawa Y, Okumo T, Matsuyama T, Sunagawa M. Kamikihito, a traditional Japanese Kampo medicine, increases the secretion of oxytocin in rats with acute stress. *J. Ethnopharmacol.*, **276**, 114218 (2021).
37) Kosfeld M, Heinrichs M, Zak PJ, Fischbacher U, Fehr E. Oxytocin increases trust in humans. *Nature*, **435**, 673–676 (2005).
38) Rilling JK, Young LJ. The biology of mammalian parenting and its effect on offspring social development. *Science*, **345**, 771–776 (2014).
39) Ishihara S, Yamada K, Hayashi T, Hasegawa T, Kameyama T, Morimasa T, Kaneyuki T, Shohmori T, Nabeshima T. Effects of kamikihito, a traditional Chinese medicine, on neurotransmitter receptor binding in the aged rat brain determined by *in vitro* autoradiography: changes in dopamine D1 and serotonin 5-HT2A receptor binding. *Biol. Pharm. Bull.*, **17**, 1132–1134 (1994).
40) Yamada K, Hayashi T, Hasegawa T, Ishihara S, Kameyama T, Morimasa T, Kaneyuki T, Shohmori T, Nabeshima T. Effects of Kamikihito, a traditional Chinese medicine, on neurotransmitter receptor binding in the aged rat brain determined by *in vitro* autoradiography (2): changes in GABAA and benzodiazepine receptor binding. *Jpn. J. Pharmacol.*, **66**, 53–58 (1994).
41) Hayashi T, Yamada K, Hasegawa T, Ishihara S, Kameyama T, Morimasa T, Kaneyuki T, Shohmori T, Nabeshima T. Effects of kamikihito, a traditional Chinese medicine, on neurotransmitter receptor binding in the aged rat brain determined by *in vitro* autoradiography (1): changes in the [3H]QNB binding. *Jpn. J. Pharmacol.*, **64**, 303–306 (1994).
42) Fahlstrom A, Zeberg H, Ulfhake B. Changes in behaviors of male C57BL/6J mice across adult life span and effects of dietary restriction. *Age (Dordr.)*, **34**, 1435–1452 (2012).
43) Figueiredo PA, Powers SK, Ferreira RM, Appell HJ, Duarte JA. Aging impairs skeletal muscle mitochondrial bioenergetic function. *J. Gerontol. A Biol. Sci. Med. Sci.*, **64**, 21–33 (2009).
44) Hu H, Wang C, Jin Y, Meng Q, Liu Q, Liu Z, Liu K, Liu X, Sun H. Catalpol Inhibits Homocysteine-induced Oxidation and Inflammation via Inhibiting Nox4/NF-kappaB and GRP78/PERK Pathways in Human Aorta Endothelial Cells. *Inflammation*, **42**, 64–80 (2019).
45) Xu D, Zhao L, Jiang J, Li S, Sun Z, Huang X, Li C, Wang T, Sun L, Li X, Jiang Z, Zhang L. A potential therapeutic effect of catalpol in Duchenne muscular dystrophy revealed by binding with TAK1. *J. Cachexia Sarcopenia Muscle*, **11**, 1306–1320 (2020).
46) Doan KV, Ko CM, Kinyua AW, Yang DJ, Choi YH, Oh IY, Nguyen NM, Ko A, Choi JW, Jeong Y, Jung MH, Cho WG, Xu S, Park KS, Park WJ, Choi SY, Kim HS, Moh SH, Kim KW. Gallic acid regulates body weight and glucose homeostasis through AMPK activation. *Endocrinology*, **156**, 157–168 (2015).
47) Chang WT, Huang SC, Cheng HL, Chen SC, Hsu CL. Rutin and Gallic Acid Regulates Mitochondrial Functions via the SIRT1 Pathway in C2C12 Myotubes. *Antioxidants*, **10**, (2021).