Title:

Effect of hangekobokuto for amelioration of aggressiveness and social behavior in socially isolated mice

Running title:

Effect of hangekobokuto for behavior

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● Abstract

Aim: Hangekobokuto, a kampo medicine, is considered effective against anxiety neurosis, amnesia, insomnia, and neurotic gastritis. However, its elucidation of action mechanism and fundamental research of verification of the effect are still not adequate. In this study, we demonstrated the effect of hangekobokuto for amelioration of aggressiveness and social behavior using mice as stress models.

Methods: 4 weeks old male ddY mice were used and socially stressed by isolated rearing for 4 weeks. The control group was reared in a group. Hangekobokuto was then orally administrated in drinking water at a dose of 0.25% and 0.5% for 2 weeks, meanwhile purified water was administrated to the control group. Aggressiveness and social behavior were evaluated. The blood sample was obtained after the test, and corticosterone serum and IL-6 were measured. The prefrontal region of brain and the brain stem region were enucleated, and serotonin, dopamine and IL-6 were measured.

Results: Hangekobokuto dose-dependently controlled the aggressive behavior induced by isolation stress. It was confirmed that the effect disappeared by the administration of $5HT_{1A}$ receptor antagonist. And the blood corticosterone increased by isolation stress decreased by the administration of hangekobokuto. Serotonin and dopamine in the prefrontal region decreased by isolation stress increased by the administration of hangekobokuto. IL-6 in the brain stem increased by isolation stress decreased dose-dependently.

Conclusion: It was confirmed that hangekobokuto has an effect for amelioration of aggressiveness induced by anxiety caused by stress. Furthermore, the test results suggested the mechanism which takes effect through the serotonin system.

●Key words: aggressiveness, dopamine, hangekobokuto, isolation stress, IL-6, serotonin

●Introduction

Stress is involved in clinical conditions of several diseases, especially mental diseases such as adjustment disorder, depression, and chronic fatigue syndrome caused by chronic social stress are recognized as serious social problems. Moreover, mood disorder has been linked to physical diseases for years. For example, endocrine diseases (e.g. hypothyroidism, hyperthyroidism, and Cushing's syndrome), brain diseases (e.g. Parkinson's disease, multiple sclerosis, and cerebrovascular disease), and other diseases including cancer, systemic lupus erythematosus (SLE), cardiac infarction, and chronic pain, can be caused by anxiety and depression resulting from social stress [1)].

Nevertheless, currently, there are no effective treatments available for these stress-related diseases.

At the present time, the condition which threatens to disturb biological homeostasis is defined as stress and the factor which causes stress is called stressor [2)]. Both of the environment factor and the hereditary factor deeply modify the stress reaction.

The sympathetic-adrenal-medullary axis (SAM axis) and the hypothalamic-pituitary-adrenal axis (HPA axis), the stress response, are activated by the outward stimulus (stressor). When the SAM axis is activated, catecholamine is released in blood, the reactions including blood pressure elevation, diaphoresis, blood sugar elevation, arousal, and the condition of preparation for fighting are elicited. Moreover when the HPA axis is activated, glucocorticoid (cortisol) is released in blood [3)], blood sugar elevation, immunosuppression, and inflammatory suppression are caused. This HPA axis has been implicated for years as a pathological mechanism of mood disorder. For example, it has been indicated since 1960's that the level of cortisol in urine and blood and its metabolites are high in the patients of depression [4, 5)].As for the immune region, it has been indicated that stress is involved in aggravation of the infectious diseases and the allergic diseases [6)], however, the relationship between stress and cytokine is regarded as important in recent years [7)]. The patients of depression, for example, it is reported that high sensitive inflammatory cytokine including C-reactive protein (CRP) and Interleukin-6 (IL-6) as an inflammatory marker significantly increases [7)].

Along with the introduction of the concept of chronic stressor [8)], a syndrome called functional somatic syndrome (FSS) is often mentioned. For example, the developmental environment in the juvenile stage is considered as one of the factors to cause pathogenesis of mental diseases, and rodents reared in isolation for a long time in the juvenile stage serve as important model animals [9,10)]. Rearing small laboratory animals such as mice and rats individually for a long time leads to behavioral changes not seen in group-reared normal animals, particularly called "isolation syndrome" [11)] that includes anxiety, depression, increased aggression, and memory disorder [12)]. The isolation syndrome is considered as a model for human mental diseases such as schizophrenia, anxiety disorder, mood disorder, and drug dependence. [13)]

Hangekobokuto, a kampo medicine, is composed of five crude drugs; Pinelliae Tuber, Magnoliae Cortex, Hoelen, Zingiberis Rhizoma and Perilla Herba. It is considered effective against anxiety neurosis, amnesia, insomnia, and neurotic gastritis. However, its elucidation of action mechanism and fundamental research of verification

of the effect are still not adequate. Kaneko and others reported in their research that it acts on the brain monoamine system, however, the ethopharmacological researched have not been conducted.[14)]

In the present study, we performed an experiment for aggressiveness and social behavior using mice as stress models. It is considered that isolation stress enhances aggressiveness and has an effect on the metabolism of the serotonin system in rodents. Therefore, the abnormality of serotonin system is considered one of the factors of augmentation of aggressiveness. The effect against aggressiveness behavior, brain monoamine and IL-6, blood corticosterone, and IL-6 of the group with administration of hangekobokuto was examined.

Materials and methods

Animals

Three weeks male ddY mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). After a week of habituation, the animals of the experimental group were placed individually in a transparent cage (9 × 13 × 20 cm) for 6 weeks. As the control group, 5 mice were reared together in a cage (23 × 31 × 15.5 cm). All animals were reared under the condition of 23±2˚C, relative humidity 55±10 %, 12-hour light and dark cycle from 7:00 to 19:00, free drinking, and normal food consumption (MF, Oriental Yeast Co., Ltd., Tokyo, Japan）through the habituation period to the test period. The animal tests were conducted in accordance with the guideline of Juntendo University Medical School Animal Testing (JACUC) (registration no.1022).

Drugs and reagents

Dry powdered extract manufactured by Tsumura & Co. was used as hangekobokuto. It is composed of Pinelliae Tuber 6.0 g, Magnoliae Cortex 3.0 g, Hoelen 5.0 g, Zingiberis Rhizoma 1.0 g and Perilla Herba 2.0 g, and was dissolved in purified water and administrated.

5-HT_{1A} receptor antagonist WAY-100635 maleate（Sigma, St. Louis, MO, USA）was dissolved in saline.

Social interaction test

Social interaction test is one of the animal tests which evaluates anxious behaviors, and was conducted in accordance with the previously reported method [15)]. A subject mouse and a confronting mouse (non-treated group reared mouse of the same age) were put in an open field equipment (50 x 50 x 50 cm、Neuroscience, Inc., Tokyo,

Japan). The total number of aggressive behaviors (aggressive grooming, tail rattling, chasing, and attacking) as the index of aggressiveness or social behaviors (sniffing, following, and contacting) as the index of sociability of the subject animal toward the control animal for 10 min were counted by a number of people without video monitoring.

Experimental designs

Figure 1 shows the experimental designs for the effects of daily oral administration of hangekobokuto on aggressive and social behaviors in socially isolated mice (Fig.1.).

Hangekobokuto was administrated orally with drinking water at a dose of 0.25 % and 0.5 % (calculated on 0.5 g/kg and 1.0 g/kg) for 14 days to a mouse reared in isolation for 4 weeks. Purified water was administrated to the control group.

On the day of the test, saline or WAY-100635 (0.1 mg/kg) was injected intraperitoneally 30 minutes before the social interaction test, and their aggressiveness and social behaviors were observed. To confirm the effect of the single-agent administration of WAY-100635, the evaluation when intraperitoneal injection of WAY-100635 or saline to the group of socially isolated mice without administration was conducted as well.

The measurement of blood serum corticosterone and IL-6

After 6 weeks of isolated rearing, blood was collected from an artery at the back of the eye under isoflurane anesthesia, and the blood serum was obtained after centrifugation (15000 rpm x 20 minutes, 4˚C). The blood serum was frozen and preserved at −20˚C until the measurement. Serum corticosterone and IL-6 were measured by ELISA Kit. Mouse Corticosterone was measured by Mouse Corticosterone Kit (Assay Designs, Michigan, USA), and IL-6 was measured by IL-6 Kit (mouse IL-6 Immunoassay R & D Systems USA)

The measurement of brain monoamines and IL-6

After the ethopharmacological test, the frontal cortex and the brainstem were collected, frozen and preserved at −80˚C until the measurement. The tissue was homogenized in the buffer solution (0.01 N hydrochloric acid (wako), 1 mM EDTA (sigma), 4 mM sodium disulfite (sigma)), and the supernatant fluid was obtained after centrifugation (15,000 rpm x 30 minutes, 4˚C). Dopamine (Dopamine Research RIA, Labor Diagnostika Nord, Nordhorn, Germany), serotonin (Serotonin Research RIA,

Labor Diagnostika Nord, Nordhorn, Germany), and IL-6（mouse IL-6 Immunoassay, R&D Systems, USA） were measured by each ELIZA Kit. The total amount of protein was then measured (BCA Protein Assay kit Pierce, Rockford, IL, USA).

Statistical analysis

All data were presented as mean ± S.E.M. The data were analyzed with Bonferoni's test to evaluate differences between the administration group and the control group. P values of <0.05 were considered as statistically significant on all the tests.

Results

Effects of hangekobokuto on increased aggressive and decreased social behaviors in socially isolated mice

Isolation stress increased aggressive behaviors and decreased social behaviors. Hangekobokuto dose-dependently controlled aggressive behaviors induced by isolation stress. Especially in the administered group of 0.5 % of hangekobokuto, significant improvements of the increased aggressiveness and the decreased social behaviors were observed (Fig2). This improvement effect was disappeared with a single administration of WAY-100635. Moreover, WAY-100635 did not have an effect on the behavior independently (Fig3). Therefore, it was confirmed that the effect of hangekobokuto acts on $5HT_{1A}$ receptor.

Effects of hangekobokuto on serum corticosterone of socially isolated mice

Isolation stress increased blood corticosterone. Increased corticosterone significantly and dose-dependently decreased by the administration of hangekobokuto for 2 weeks (Fig.4.A).

The blood serum IL-6 increased by isolation stress decreased by the 2-week administration of hangekobokuto, however, it was not a significant difference (Fig.4.B).

Effects of hangekobokuto on serotonin and dopamine in the frontal cortex of social isolated mice

Serotonin in the frontal cortex decreased by isolation stress increased by the administration of hangekobokuto but dose effect was not shown (Fig.5.A). Meanwhile, dopamine increased by the administration of hangekobokuto dose dependently, and significantly in the administered group of 0.5 % hangekobokuto (Fig.5.B).

Effects of hangekobokuto on IL-6 in the brain stem of social isolated mice

IL-6 in the brain stem increased by isolation stress. Hangekobokuto significantly and dose-dependently decreased the increased IL-6 (Fig.6.).

●Discussion

In this study, we discussed the effect of hangekobokuto for amelioration of aggressive behavior induced by social isolation stress. Hangekobokuto was administrated at the dose of 0.25 % and 0.5 % for 14 days. Hangekobokuto dose-dependently controlled the aggressive behavior induced by isolation stress, and significant ameliorations of the increase of aggressiveness and the decrease of social behavior were observed in the administration group of 0.5 % of hangekobokuto. These ameliorations were disappeared by the administration of WAY-100635, $5HT_{1A}$ receptor antagonist. In behavioral pharmacological evaluation, hangekobokuto dose-dependently controlled aggressive behavior induced by isolation stress, and it was confirmed that its effect acted on $5HT_{1A}$ receptor. Furthermore, blood corticosterone significantly increased by social isolation stress, and the increase significantly decreased by hangekobokuto. It was confirmed that the amount of serotonin in the frontal cortex and dopamine significantly decreased by social isolation stress, and the decrease significantly increased by hangekobokuto. Finally, it was confirmed that IL-6 in the brain stem significantly increased by social isolation stress, and the increase significantly decreased by hangekobokuto.

Chronic social stress is known to exert its impact on the body through the neuroendocrine system, autonomic nervous system, chronic inflammation, and oxidant stress [16]. Stimulation by stress is thought to be transmitted to the hypothalamic area through the cortex and the limbic system, and promotes the production of corticotrophin releasing hormone (CRH) in the paraventricular nucleus of hypothalamus; as a result, adrenocorticotropic hormone (ACTH) is released and promotes a production and release of glucocorticoid (the main component of which is cortisol in humans and corticosterone in rodents) from the adrenal cortex [13]. In the present study, we found that serum corticosterone was indeed significantly increased by isolated rearing compared with the group-rearing control without stress load.

The physiological responses including the autonomic nervous system and the HPA axis and the behavioral reactions such as avoidance and escape, both caused by the exposure to stress, are essentially protective responses to the stress [17,18]. Besides the peripheral effects such as increased blood sugar level, the release of glucocorticoid is known to release dopamine in the prefrontal cortex [19], another example for the adaptive

response. However, it is also indicated that the brain system involved in stress reactions is formed dynamically depending on the developing process [20,21], where stress given in the juvenile stage decreases hippocampal volume [22] and has impacts on emotions and cognitive functions after maturation [23]. Furthermore, a prolonged stress chronically decreases the release of dopamine in the prefrontal cortex and impairs working memory [24]. The results of the present study confirmed that a prolonged isolation stress from the adolescence stage, i.e. 28 days after birth, increased anxiety and declined the amount of serotonin in the frontal cortex. Social isolation stress has been previously shown to decrease dopamine in the prefrontal cortex, as well as serotonin and glutamate [25].

It is unknown at present how the prolonged social stress induces decreases of the amount of brain monoamine. However it is possible that the promotion of the production and release of glucocorticoid by stress stimulation is involved. As mentioned above, glucocorticoid receptor stimulation in the prefrontal cortex increases the release of stress-related neuromodulator dopamine [19] in the prefrontal cortex, through the positive-feedback system to midbrain dopamine neurons [8]. The present results demonstrated that serum glucocorticoid was significantly increased 4 weeks after the isolated rearing. Such chronic increases of glucocorticoid may rather induce adapted decreases of dopamine release in the prefrontal cortex. It is also reported that the serotonin release in the prefrontal cortex is decreased by isolation stress [25], thus consistent with the present results, although the underlying mechanisms are still unclear.

Whatever the mechanisms, brain monoamines are involved in controlling emotions and cognitive behaviors, and a decrease of these neuromodulators would result in the abnormality of emotions and cognitive behaviors. As is well known, dopamine neurons project to the limbic and the cortical areas from the ventral tegmental area (VTA), and controls cognition, reward-related learning, and emotional behavior [26,27,28]. Especially, decreased dopamine in the prefrontal cortex results in the dysfunction of this cortex, and this may in turn lead to a decreased inhibition on the amygdala [29], resulting in the aggressive behavior from anxiety. Amygdala is a key region of the brain for anxiety, but the brain of the mouse was very small and difficult to collect amygdala only. As mentioned above, we considered the frontal cortex also related to anxiety. So we investigated the brainstem and the frontal cortex.

On the other hand, serotonin, synthesized in the regions including dorsal raphe nucleus, is projected to various areas in the brain including the cortex and the hippocampus [30, 31]. It is thought that a decrease of serotonin is the most important biological factor for causing depression [32], and serotonin is indeed involved in controlling of emotions [33]. It was shown in rodents that the decrease of serotonin

synthesis in the brain caused declines of cognitive functions, depression, and anxiety [34]. Moreover, the changes of the aggressive behaviors and the serotonin system induced by stress were indicated on many animal tests [15]. In the present study, hangekobokuto recovered the amount of serotonin decreased significantly by stress. It is thought that $5HT_{1A}$ receptor is involved in controlling anxiety, and $5HT_{2A}$ receptor is involved in inducing anxiety, thus the abnormality of the serotonin system can lead anxiety and increase aggressive behaviors. It was confirmed that the effect of hangekobokuto on aggressive behaviors induced by isolation stress was an act to the serotonin nervous system through $5HT_{1A}$ receptor.

Hangekobokuto is composed of Pinelliae Tuber, Magnoliae Cortex, Hoelen, Zingiberis Rhizoma and Perilla Herba, and it is considered effective against anxiety neurosis, amnesia, insomnia, and neurotic gastritis. In kampo medicine, Magnoliae Cortex and Hoelen are considered effective to mental symptoms, however, Kuribara and others reported that honikiol, the component of Magnoliae Cortex, has strong anti-anxiety effect as pharmacological effect of crude drugs [35] .

Gamo and others indicated the possibility that GABAA receptor which is analogous to diazepam, benzodiazepine anxiolytic, is involved as an effect of Hangekobokuto on anxiety[36].

Kaneko and others found out that hangekobokuto acts on the brain monoamine system and reported that the metabolism of 5-HIAA was promoted in the hypothalamic area with 28 days of administration, and serotonin increased. They also reported that as a result of decrease of intrastriatal dopamine turnover, dopamine increased as well [14].

In the present study, hangekobokuto decreased inflammatory cytokine, IL-6 in the brain especially. The relationship between stress and cytokine is regarded as important in recent years[7], and IL-6 is known as an inflammatory marker cytokine for schizophrenia, depression, anxiety disorder, and mood disorders caused by chronic stress in the brain[37]. There is a possibility that hangekobokuto may contribute to improve such mental diseases, so further data needs to be accumulated and evaluated in the future.

●Conclusion

It was confirmed that hangekobokuto has amelioration effect of aggressiveness by stress. The present study also demonstrated the mechanism which takes effect through the serotonin system.

●Conflict of Interests

Authors declare no conflict of interests for this artcle.

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Figure Legends

Fig.1

The experimental designs show the effects of daily oral administration of hangekobokuto on aggressive and social behaviors in socially isolated mice.

Fig.2.

Effects of hangekobokuto on aggressive behavior (A) and social behavior (B) of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). Animals were housed either in social groups (5 mice per cage) or in social isolation (1 mouse per cage) for 4 weeks. Then, isolated rearing with oral administration of hangekobokuto (0, 0.25 and 0.5 %) for 2 weeks. Each value are expressed as mean ± S.E.M (n=10). **p<0.01 vs Control group, and †p<0.05 vs water.

Fig.3.

Effects of hangekobokuto with WAY-100635 (0.1 mg/kg) on aggressive behavior (A) and social behavior (B) of socially isolated mice with hangekobokuto (0 and 0.5 %), and effect of single-agent administration of WAY-100635. Animals were housed either in social groups (5 mice per cage) or in social isolation (1 mouse per cage) for 4 weeks. Then, isolated rearing with oral administration of hangekobokuto (0 and 0.5 %) for 2 weeks. WAY-100635（0.1 mg/kg） or saline was injected intraperitoneally 30 minutes before the social interaction test, and their aggressiveness and social behaviors were observed. Each value are expressed as mean ± S.E.M (n=10). **p<0.01 vs Control group, †p<0.05 vs water and #p<0.05 vs. WAY-100635.

Fig.4.

Effects of hangekobokuto on serum corticostrone (A) and IL-6 (B) of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). Animals were housed either in social groups (5 mice per cage) or in social isolation (1 mouse per cage) for 4 weeks. Then, isolated rearing with oral administration of hangekobokuto (0, 0.25 and 0.5 %) for 2 weeks, blood was collected from an artery at the back of the eye under isoflurane anesthesia, and the blood serum was obtained after centrifugation (15000 rpm x 20 minutes, 4˚C), and frozen and preserved at −20˚C until the measurement.. Each value are expressed as mean ± S.E.M (n=10). *p<0.05, **p<0.01 vs Control group, and †p<0.05 vs water.

Fig.5.

Effects of hangekobokuto on serotonin (A) and dopamine (B) content in the frontal cortex of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). After the ethopharmacological test, the frontal cortex and the brainstem were collected, frozen and preserved at −80˚C until the measurement. The tissue was homogenized in the buffer solution, and the supernatant fluid was obtained after centrifugation (15,000 rpm x 30 minutes, 4˚C). Each value are expressed as mean ± S.E.M (n=10). *$p<0.05$ vs Control group, and †$p<0.05$ vs water.

Fig.6.

Effects of hangekobokuto on IL-6 content in the brain stem of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). After the ethopharmacological test, the frontal cortex and the brainstem were collected, frozen and preserved at −80˚C until the measurement. The tissue was homogenized in the buffer solution, and the supernatant fluid was obtained after centrifugation (15,000 rpm x 30 minutes, 4˚C). Each value are expressed as mean ± S.E.M (n=10). **$p<0.01$ vs Control group, and ††$p<0.05$ vs water.

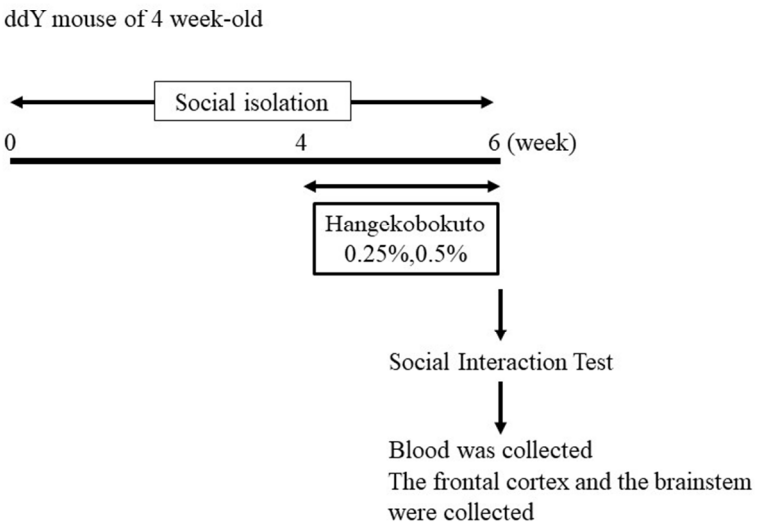

Fig.1:The experimental designs show the effects of daily oral administration of hangekobokuto on aggressive and social behaviors in socially isolated mice.

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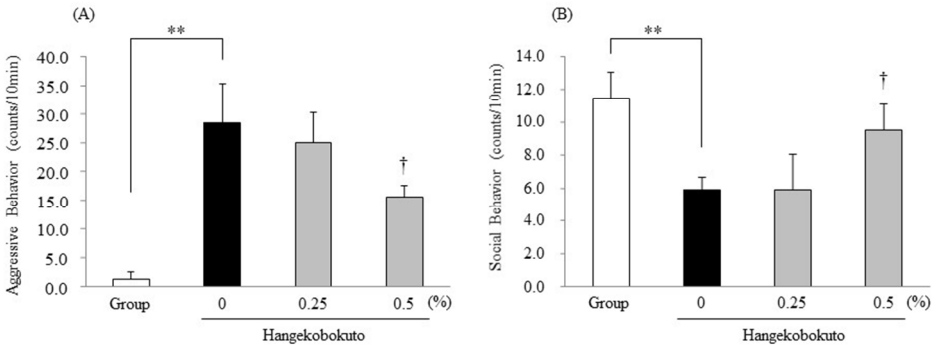

Fig.2:Effects of hangekobokuto on aggressive behavior (A) and social behavior (B) of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). Animals were housed either in social groups (5 mice per cage) or in social isolation (1 mouse per cage) for 4 weeks. Then, isolated rearing with oral administration of hangekobokuto (0, 0.25 and 0.5 %) for 2 weeks. Each value are expressed as mean ± S.E.M (n=10). **p<0.01 vs Control group, and †p<0.05 vs water.

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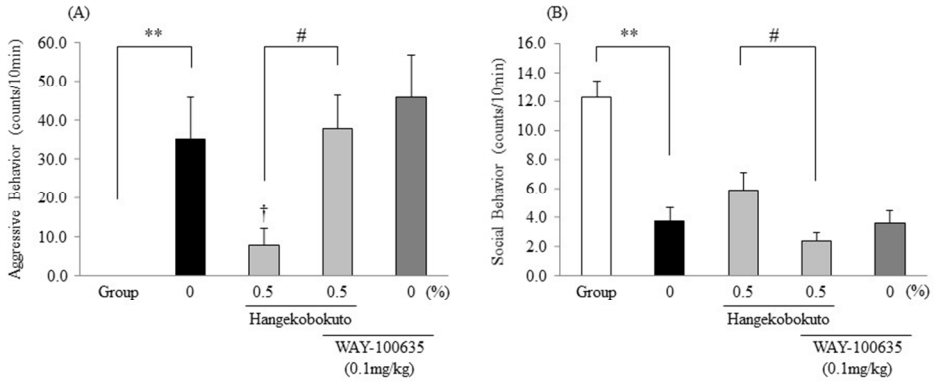

Fig.3:Effects of hangekobokuto with WAY-100635 (0.1 mg/kg) on aggressive behavior (A) and social behavior (B) of socially isolated mice with hangekobokuto (0 and 0.5 %), and effect of single-agent administration of WAY-100635. Animals were housed either in social groups (5 mice per cage) or in social isolation (1 mouse per cage) for 4 weeks. Then, isolated rearing with oral administration of hangekobokuto (0 and 0.5 %) for 2 weeks. WAY-100635（0.1 mg/kg）or saline was injected intraperitoneally 30 minutes before the social interaction test, and their aggressiveness and social behaviors were observed. Each value are expressed as mean ± S.E.M (n=10). **p<0.01 vs Control group, †p<0.05 vs water and #p<0.05 vs. WAY-100635.

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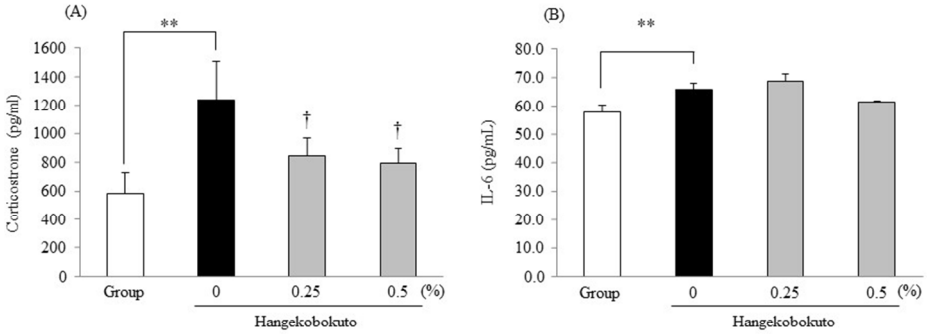

Fig.4:Effects of hangekobokuto on serum corticostrone (A) and IL-6 (B) of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). Animals were housed either in social groups (5 mice per cage) or in social isolation (1 mouse per cage) for 4 weeks. Then, isolated rearing with oral administration of hangekobokuto (0, 0.25 and 0.5 %) for 2 weeks, blood was collected from an artery at the back of the eye under isoflurane anesthesia, and the blood serum was obtained after centrifugation (15000 rpm x 20 minutes, 4°C), and frozen and preserved at −20°C until the measurement.. Each value are expressed as mean ± S.E.M (n=10). *p<0.05, **p<0.01 vs Control group, and †p<0.05 vs water.

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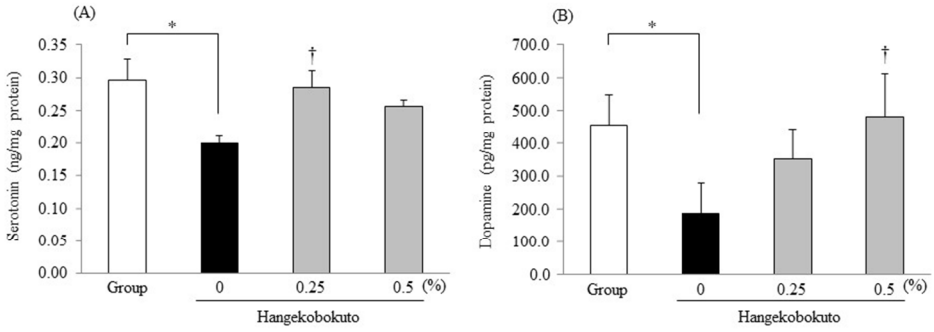

Fig.5:Effects of hangekobokuto on serotonin (A) and dopamine (B) content in the frontal cortex of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). After the ethopharmacological test, the frontal cortex and the brainstem were collected, frozen and preserved at −80˚C until the measurement. The tissue was homogenized in the buffer solution, and the supernatant fluid was obtained after centrifugation (15,000 rpm x 30 minutes, 4˚C). Each value are expressed as mean ± S.E.M (n=10). *p<0.05 vs Control group, and †p<0.05 vs water.

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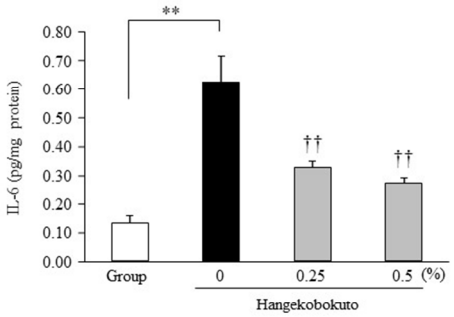

Fig.6:Effects of hangekobokuto on IL-6 content in the brain stem of socially isolated mice with hangekobokuto (0, 0.25 and 0.5 %). After the ethopharmacological test, the frontal cortex and the brainstem were collected, frozen and preserved at −80˚C until the measurement. The tissue was homogenized in the buffer solution, and the supernatant fluid was obtained after centrifugation (15,000 rpm x 30 minutes, 4˚C). Each value are expressed as mean ± S.E.M (n=10). **p<0.01 vs Control group, and ††p<0.05 vs water.

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