Effect of Yokukansan and Yokukansankachimpihange on Aggressive Behavior, 5-HT Receptors and Arginine Vasopressin Expression in Social Isolation-Reared Mice

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INTRODUCTION

Yokukansan (YKS: Yi-gan san in Chinese) is a traditional herbal medicine comprising seven types of medicinal herbs. It has been approved by the Ministry of Health, Labour and Welfare of Japan as a remedy for neurosis, insomnia or night crying and irritability in children. Yokukansankachimpihange (YKSCH), which was developed in Japan, includes YKS combined with two medicinal herbs, Citrus unshiu peel (chimpi) and Pinellia tuber (hange). Although YKSCH is similar to YKS, it is more commonly prescribed for patients whose symptoms include digestive function deficiencies. However, the differences between the effects of YKS and YKSCH on brain function are unclear. The present study examined the effects of YKS and YKSCH on aggressive behavior in mice reared under a social isolation (SI) condition. Mice were housed individually for 6 weeks. YKS and YKSCH were administered orally for 2 weeks before aggression tests. SI increased aggressive behavior against naïve mice, and YKS, but not YKSCH, significantly attenuated this aggressive behavior. Because serotonin (5-HT)2A and 5-HT3A receptor antagonists are reported to have anti-aggressive effects, the mRNA levels of these receptors were examined. YKS attenuated the SI-induced increase in 5-HT2A and 5-HT3A receptor mRNA in the amygdala. On the other hand, YKSCH attenuated the SI-induced increase in 5-HT1A receptor mRNA. YKS and YKSCH did not affect 5-HT and its metabolite 5-hydroxyindoleacetic acid content in the amygdala. However, YKSCH increased the mRNA level of arginine vasopressin (AVP), which is a neuropeptide that has been implicated in aggression, in the amygdala.

Key words yokukansan; yokukansankachimpihange; aggressive behavior; arginine vasopressin; serotonin (5-HT)1A receptor

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MATERIALS AND METHODS

Animals Male ddY mice (4 weeks old) were supplied by Japan SLC, Inc. (Shizuoka, Japan). After habituation for 1 week, mice were housed individually in cages (136 × 208 × 115 mm) for 6 weeks prior to testing. Group-reared controls were housed with 3–5 mice per cage (182 × 260 × 128 mm). Naïve male mice (5 weeks old) that were used for the aggression test were also supplied by Japan SLC, Inc. and were group-housed for one week prior to the test. All mice were housed at a temperature of 23 ± 2°C with a relative humidity of 60 ± 10% under a 12 h light–dark cycle (lights on 07:00–19:00). Food and water were available ad libitum. All procedures regarding animal care and use were carried out in keeping with the regulations dictated by the Experimental Animal Care and Use Committee of Fukuoka University (#1507852, #1704040).

Drugs Dry powdered extracts of YKS (Lot. No. 331039200) and YKSCH (Lot. No. 331036600) were provided by Tsumura & Co. (Tokyo, Japan). YKS comprises seven dried extracts as follows: Poria Sclerotium (4.0 g, sclerotium of Wolfiporia cocos Ryvarden et Gilbertson), Atractylodes Lancea Rhizome (4.0 g, rhizome of Atractylodes lancea De Candolle), Uncaria Hook (3.0 g, hook of Uncaria rhynchophylla Miquel), Cnidium Rhizome (3.0 g, rhizome of Cnidium officinale Makino), Japanese Angelica Root (3.0 g, root of Angelica acutiloba Kitagawa), Bupleurum Root (2.0 g, root of Bupleurum falcatum Linné) and Glycyrrhiza (1.5 g, root and stolon of Glycyrrhiza uralensis Fisher). YKSCH consists of YKS and two additional herbs, Pinellia tuber (5.0 g, tuber of Pinellia ternata Breitenbach, hange) and Glycyrrhiza (1.5 g, root and stolon of Glycyrrhiza uralensis Fisher). YKSCH is a blend of YKS and a relative of YKSCH with the most effective dose found to be 1000 mg/kg.

Quantitative (q)RT-PCR After the aggression test, mice were rapidly decapitated, and the amygdala was quickly dissected. The total RNA was extracted using the TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.). First-strand cDNA was reverse-transcribed from total RNA using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd., Osaka, Japan). Real-time PCR was conducted on a LightCycler® 96 System (Roche, Basel, Switzerland) using THUNDERBIRD SYBR qPCR Mix (TOYOBO Co., Ltd.) and primers as per the manufacturer’s protocol. The following PCR conditions were employed: 95°C for 15 s, 59°C (for 5-HT receptor), 63°C (for 5-HT receptor) and 5-HT receptor, 5-HT receptor, and 5-HT receptor receptors), 65°C (for 5-HT receptor) or 66°C (for AVP) for 30 s and 72°C for 45 s. The sequences of the primers were as follows: 5'-GGATTTTCTCCCGTCTGTT-3' and 5'-CACAGGTCCTTTCAAGAC-3' for 5-HT receptor (NM_008380); 5'-TCATCTGCGATTGTTGAGAAT-3' and 5'-CAGTGTGTGGAAGCCTGTTT-3' for 5-HT receptor (NM_014082); 5'-AGACCCATCTCACCATACTG-3' and 5'-AACCAATCTGCTTCAAT-3' for 5-HT receptor (NM_013561); 5'-GCCAGGATGTCATAACACTACG-3' and 5'-TCTCAGTCATGCTGAGATG-3' for AVP (NM_009732.2); and 5'-GGCAGTATTCCCTCCTCG-3' and 5'-CCAGTGTGTGGAAGCCTGTTT-3' for 5-HT receptor (NC_008312.4); 5'-CAGGTTTCTGATGCTGAGATG-3' and 5'-CACACTCGCCCCTGATT-3' for 5-HT receptor (NM_013561).

5-HT and 5-HIAA Content The content of 5-HT and 5-HIAA were measured using an HPLC-electrochemical detector (ECD) system (Eicom Co., Ltd., Kyoto, Japan). Mice were sacrificed immediately after the aggression test. Brain regions were quickly dissected, weighed and homogenized in 150 µL of ice-cold 0.2 M perchloric acid containing 0.1 M ethylenediaminetetraacetic acid (EDTA) and 10 ng/mL iso- protoren (internal standard). Following centrifugation at 2000 g × 15 min, 50 µL of the supernatant was mixed with 20 µL of 1 M sodium acetate and filtered through a membrane filter (0.45 µm; Millex, Merck, Darmstadt, Germany). Then, 10 µL of the sample was injected into the HPLC-ECD system, which used an Eicompak SC-5ODS column (3.0 mm i.d. × 150 mm, Eicom Co., Ltd.) and was set at a potential of +750 mV against an Ag/AgCl reference electrode with a graphite carbon working electrode (WE-3G, Eicom Co., Ltd.). The mobile phase consisted of 0.1 M acetate-citrate buffer (pH 3.5), 200 mg/L sodium 1-octanesulfonate, 5 mg/L EDTA and 17% methanol. The flow rate was maintained at 0.5 µL/min. The monoamine levels were calculated on the basis of standard values using PowerChrom (version 2.2.4; Eicom Co., Ltd.).

Experimental Procedure Mice were isolated for 6 weeks, and daily YKS and YKSCH administration (1000 mg/kg/d, for 2 weeks, per os (p.o.)) was started at 4 weeks after the initiation of SI (Fig. 1). The aggression test was performed at 1 h after the final drug administration. Immediately after the...
aggression test, mice were sacrificed to analyze monoamine content and mRNA expression.

**Statistical Analysis** Data were evaluated for statistical significance using one-way ANOVA followed by Tukey’s multiple comparisons test. An outlier was removed from each PCR data set (5-HT_2A, 5-HT_3A and AVP mRNA) using the ROUT method (Prism 6.04 software, GraphPad, La Jolla, CA, U.S.A.). The criterion for statistical significance was \( p < 0.05 \). Data are shown as the mean ± standard error of the mean (S.E.M.).

**RESULTS**

**Effect of YKS and YKSCH on Aggressive Behavior in SI-Reared Mice** SI-reared mice showed an increased number of attacks compared with group-reared mice \( F(3,52) = 4.206, p < 0.05 \) by Tukey’s multiple comparisons test, Fig. 2. YKS administration to SI-reared mice significantly attenuated this increase in the number of attacks \( F(3,53) = 3.201, p < 0.05 \) by one-way ANOVA, \( p < 0.05 \) by Tukey’s multiple comparisons test, Fig. 2, while YKSCH administration did not produce a significant attenuation. However, YKSCH administration also did not increase the number of attacks compared with group-reared mice. These results suggest that the anti-aggressive effect of YKS is weaker than that of YKS.

**Effect of YKS and YKSCH on 5-HT Receptor Expression in the Amygdala of SI-Reared Mice** 5-HT_1A receptor agonists, 5-HT_2A receptor antagonists and serotonin reuptake inhibitors reduce aggressive behavior in SI-reared mice,\(^4,8,9\) which suggests that aggressive behavior is mediated by 5-HT receptors. Chronic administration of YKS decreases 5-HT_2A receptor levels in the mouse frontal cortex.\(^8\) Therefore, the effect of YKS and YKSCH on 5-HT receptor expression was examined. The mRNA levels of several 5-HT receptors (5-HT_1A, 5-HT_2A and 5-HT_3A) were measured in SI-reared mice compared with group-reared mice \( F(3,53) = 3.322, p < 0.05 \) by one-way ANOVA, \( p < 0.05 \) by Tukey’s multiple comparisons test; 5-HT_2A receptor, \( F(3,53) = 5.354, p < 0.01 \) by one-way ANOVA, \( p < 0.05 \) by Tukey’s multiple comparisons test; 5-HT_3A receptor, \( F(3,52) = 4.206, p < 0.01 \) by one-way ANOVA, \( p < 0.05 \) by Tukey’s multiple comparisons test; however, the 5-HT_3C receptor mRNA level was not increased (Fig. 3). YKSCH administration significantly decreased the enhanced 5-HT_1A mRNA level \( F(3,53) = 4.060, p < 0.05 \) by one-way ANOVA, \( p < 0.05 \) by Tukey’s multiple comparisons test, whereas, YKS administration did not (Fig. 3A). Neither YKS administration nor YKSCH administration decreased the enhanced 5-HT_1B mRNA level (Fig. 3B). On the other hand, YKS administration significantly decreased the enhanced 5-HT_2A and 5-HT_3A receptor mRNA levels \( F(3,52) = 5.354, p < 0.01 \) by one-way ANOVA, \( p < 0.05 \) by Tukey’s multiple comparisons test; 5-HT_3A receptor, \( F(3,52) = 4.206, p < 0.01 \) by one-way ANOVA, \( p < 0.05 \) by Tukey’s multiple comparisons test, whereas, YKSCH administration did not (Figs. 3C, E). These results suggest that a decrease in 5-HT_2A and 5-HT_3A receptor levels contributes to the anti-aggressive effect of YKS.

**Effect of YKS and YKSCH on the Content of 5-HT and Its Metabolite 5-HIAA in the Amygdala of SI-Reared Mice** Since the mRNA levels of 5-HT receptors were altered in the amygdala of SI-reared mice treated with YKS, the content of 5-HT and its metabolite 5-HIAA were then examined. The contents of 5-HT and 5-HIAA were unchanged in SI-reared mice compared with group-reared mice. Likewise, YKS and YKSCH did not affect the 5-HT and 5-HIAA content (Figs. 4A, B). These data suggest that YKS and YKSCH are not responsible for the underlying mechanisms of 5-HT metabolism.

**Effect of YKS and YKSCH on AVP Expression in the Amygdala** AVP plays a key role in aggressive behavior.\(^11\) Therefore, AVP mRNA levels were examined. Although SI-reared mice did not show increased AVP mRNA levels, YKSCH administration significantly increased the AVP mRNA level compared with group-reared mice and YKS-administered mice \( F(3,52) = 4.972, p < 0.01 \) by one-way ANOVA, \( p < 0.01 \) by Tukey’s multiple comparisons test (Fig. 5).
Fig. 3. Effect of YKS and YKSCH on 5-HT Receptor Expression in the Amygdala of Social Isolation-Reared Mice

5-HT₁A (A), 5-HT₁B (B), 5-HT₂A (C), 5-HT₂C (D) and 5-HT₃A (E) mRNA levels in the amygdala were examined after the aggression test. Data are expressed as a percentage of control values (group-reared mice). Values indicate the means ± S.E.M. (n = 12–15/group). *p < 0.05 and **p < 0.01, significantly different.

Fig. 4. Effect of YKS and YKSCH on the Content of 5-HT and Its Metabolite 5-HIAA in the Amygdala of Social Isolation-Reared Mice

5-HT (A) and 5-HIAA (B) levels in the amygdala were examined using HPLC after the aggression test. Values indicate the means ± S.E.M. (n = 16–19/group).
suprachiasmatic nucleus [SCN]) and the extended amygdala (bed nucleus of the stria terminals [BNST] and medial amygdala). AVP projections from the amygdala are suggested to facilitate aggressive behavior. However, SI did not significantly increase AVP mRNA in the amygdala (Fig. 5). Another study also demonstrated no alteration of plasma AVP levels in prairie voles subjected to chronic SI-rearing. However, SI increased the density of AVP immunoreactive cells in the hypothalamus of prairie voles. We also observed increased AVP mRNA levels in the hypothalamus of SI-reared mice (data not shown). SI may induce aggressive behavior via hypothalamic AVP expression. Previous studies have reported that AVP mRNA expression in the amygdala is decreased by adrenalectomy, abolished by a combination of adrenalectomy and gonadectomy, and restored by androgen replacement. In addition, adrenalectomy and gonadectomy have been found to cause a reduction in aggressiveness of SI-reared mice. These reports imply that AVP expression in the amygdala is mediated by androgen and enhances aggressiveness. Given that citrus peel extract has been found to increase serum androgen levels in mice, chimps might enhance aggressiveness via androgen. YKSCH administration increased AVP mRNA levels in the amygdala compared with YKS administration (Fig. 5), implying that chimps disrupts the anti-aggressive effects of YKS via AVP expression. Taken together, SI and YKSCH may induce aggressive behavior via AVP expression in the hypothalamus and amygdala, respectively. However, the mechanism by which YKSCH increases AVP mRNA is unknown.

The 5-HT and 5-HIAA content in amygdala were unchanged by SI although SI affected the 5-HT receptors expression (Figs. 3, 4). Other study also demonstrates the unchanged 5-HT and 5-HIAA content in hippocampus of SI-reared mice. Thus, SI may not affect the synthesis and metabolism of 5-HT. The present study also showed YKS and YKSCH do not alter the 5-HT and 5-HIAA content (Fig. 4). YKS attenuates SI-induced aggressive behavior in zinc-deficient mice while YKS does not affect the 5-HT content in brain tissue. These results suggest that the 5-HT and 5-HIAA content are not implicated in anti-aggressive effect of YKS.

**DISCUSSION**

YKS and YKSCH are prescribed for neurosis, insomnia or night crying and irritability in children. Prescription of these medicines is dependent on the digestive condition. However, the differences between the effects of YKS and YKSCH on brain function are unclear. Therefore, the present study examined the difference between the effects of YKS and YKSCH on aggressive behavior in SI-reared mice. YKS, but not YKSCH, significantly ameliorated SI-increased aggressive behavior. The RT-PCR results suggested that the anti-aggressive effect of YKS may be mediated by a decrease in the expression levels of 5-HT2A and 5-HT3A receptors. In addition, the YKSCH-induced increase in the AVP level may disrupt the anti-aggressive effect because AVP is implicated in aggression.

The 5-HT and 5-HIAA content in amygdala were identical to that of YKS (1000 mg/kg) and 719 mg/kg in YKSCH. However, the decreased effect of YKSCH on 5-HT and 5-HIAA content was not observed in this study.
aggression and 5-HT receptor levels may be, in part, due to the decreased YKS content in YKSCH. However, YKSCH produced a significant increase in AVP mRNA compared with YKS, while the vehicle did not induce a significant increase. These results suggest that increased AVP expression is not due to the decreased YKS content in YKSCH.

In conclusion, the present study indicates that YKS ameliorates SI-induced aggressive behavior, likely by inhibiting the increase in 5-HT2A and 5-HT3A receptors. In addition, the YKSCH-induced increase in the AVP level may contribute to the weaker anti-aggressive effect of YKSCH. YKS may be more effective for the treatment of irritability than YKSCH if digestive function deficiencies are not considered.

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