近　畿　大　学

東 洋 医 学 研 究 所　紀 要

（ 2024 年度 ）

Journal of Research Institute of Traditional Asian Medicine

KINDAI UNIVERSITY 2024

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（ 2024年度 ）

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２０２４年度 東洋医学研究所紀要発刊によせて

東洋医学研究所　所長・教授
武田　卓

東洋医学研究所は、近畿大学病院の開設と同時に大学直属の漢方専門研究所として発足し、日本で最初の漢方の臨床・基礎を研究する研究所として、多くの実績をあげてまいりました。私は、平成 24 年 4 月 1 日より、新所長・新教授として東北大学医学部先進漢方治療医学講座より赴任いたしました。

漢方診療は、西洋医学だけでは改善しにくいような難治性疾患や西洋医学では病気として相手にされないような不定愁訴に対して有効な場合がしばしば認められます。また、最近ではがん治療に伴う様々な副作用対策にも漢方が用いられるようになってきました。このように、従来からの東洋医学だけではなく、西洋医学の確かな専門性を持ったうえでの漢方治療も必要です。そこで、私の専門性を生かした女性漢方外来、冷え症外来を開設しています。さらに、最近のフェムテックへの感心の高まりを背景に、月経前症候群・月経前不快気分障害への新規治療薬開発や、アプリを用いた認知行動療法開発といった、東洋医学の枠組みを超えた女性ヘルスケア領域全般を対象とした臨床研究も実施中です。これらにも関連して、最近では AMED 研究や厚生労働省研究斑において女性特有疾患に関する指針策定にも関与しております。

また、大阪大学医学部より、脳神経科学研究をリードする遠山正彌名誉教授を客員教授にお迎えし、宮田信吾教授を中心とした基礎研究部門も同時にスタートしました。漢方薬の作用メカニズムを最先端の分子生物学的手法によって科学的に解明します。現在、「抑肝散」という漢方薬を中心に、漢方薬の神経機能に対する効果についての科学的解析を行っており、有効成分の同定、作用機序の解明から新規創薬への展開を目指しています。

古代中国からの長い歴史をもつ漢方治療ですが、これからは 21 世紀の現代医療にマッチした診療・研究、さらにはトランスレーショナルリサーチを展開していきたいと考えております。

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Kampo Prescriptions for Premenstrual Syndrome and Premenstrual Dysphoric Disorder: A Secondary Analysis of Nationwide Survey by JSOG Women's Health Care Committee

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Original Article

Kampo Prescriptions for Premenstrual Syndrome and Premenstrual Dysphoric Disorder: A Secondary Analysis of Nationwide Survey by JSOG Women's Health Care Committee

Running title: Kampo Prescriptions for PMS/PMDD

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Abstract

Premenstrual syndrome (PMS) and Premenstrual Dysphoric Disorder (PMDD) significantly impair women's quality of life. A survey of obstetricians and gynecologists showed that 19.5% of the doctors preferred Kampo medicine, including *Tokishakuyakusan* (TSS), *Kamishoyosan* (KSS), *Keishibukuryogan* (KBG), and *Yokukansan* (YKS), as the first-choice treatment for these conditions. We aimed to analyze the characteristics of each Kampo prescription. A secondary analysis was conducted on survey results from members of the Japan Society of Obstetrics and Gynecology collected from September to November 2021. Data from 1,259 respondents treating PMS/PMDD were analyzed. Our correspondence analysis plotted relationships among treatments, showing that Kampo prescriptions of TSS, KSS, KBG, and YKS were distributed similarly to oral contraceptives (OCPs), but different from selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), and gonadotropin-releasing hormone analogs. Among the Kampo formulas, prescription YKS was the closest to SSRIs/SNRIs. Logistic regression analysis revealed that shorter physician experience (< 10 years, ≥ 10 and < 20 years) was associated with selecting prescriptions for TSS and KBG, whereas being a private practitioner was linked to selecting prescriptions for KSS and YKS (clinic vs. hospital OR: 1.57, 1.77; clinic vs. university OR: 1.53, 1.71). The prescription of YKS was also associated with choosing SSRIs/SNRIs (OR: 1.81), chasteberry (OR: 6.24), and other medications or supplements (OR: 2.41). Kampo prescriptions were strongly correlated with OCPs. Prescription TSS and KBG were likely chosen by less experienced practitioners, whereas prescription YKS was used by those more familiar with PMS/PMDD treatments, including SSRIs/SNRIs.

Keywords: correspondence analysis; PMS; PMDD; Traditional Japanese herbal medicine; treatment selection

Introduction

Premenstrual symptoms are characterized by unpleasant psychosomatic symptoms that impair the quality of life in many women (Takeda 2023, Yonkers and Simoni 2018). Symptoms appear in the luteal phase before menstruation, and diminish and disappear with the onset thereof. Premenstrual syndrome (PMS) is a pathological condition characterized by premenstrual symptoms, and is considered a premenstrual dysphoric disorder (PMDD), especially when psychological symptoms are severe. Recently, the concept of premenstrual disorders (PMDs) has also been proposed, which takes both into account on a continuous basis (O'Brien et al. 2011).

Selective serotonin reuptake inhibitors (SSRIs) and oral contraceptive pills (OCPs) are standard treatments for PMS/PMDD (2023, 2017), Kampo, a traditional herbal medicine, is widely used in Japan. The Japan Society of Obstetrics and Gynecology (JSOG) guidelines for PMS/PMDD recommend OCPs, such as drospirenone-ethinylestradiol, Kampo medicines, and SSRIs, especially for psychiatric symptoms (Kawaguchi et al. 2019). However, OCPs and SSRIs are not covered by the national health insurance. Additionally, these medications have a negative image in Japan, which complicates treatment. Against this background, in a survey conducted by the Women's Health Care Committee of the JSOG on the diagnosis and treatment of PMS/PMDD, OCPs (76.8%) were the first choice of treatment, followed by Kampo medicine (19.5%) (Yoshimi et al. 2023).

Kampo treatment is derived from traditional medicine that originated in ancient China and was uniquely developed and applied in Japan. Kampo medicines are complex mixtures of natural-product-derived ingredients in specific proportions. It is available as an industrial product in the form of an extract under strict quality control and is easy to use in Western medicine. In addition, these medicine can be used for medical treatments covered by national insurance, which is one of the characteristics of Japanese medical care. According to the results of the JSOG survey on the actual status of diagnosis and treatment of PMS/PMDD, the most commonly used Kampo medicines were *Kamishoyosan* (KSS), *Tokishakuyakusan* (TSS), *Yokukansan* (YKS), and *Keishibukuryogan* (KBG), in that order.

In traditional Kampo medicine, drugs are selected based on a unique diagnostic method known as "Sho," but in modern Kampo medicine, drugs are selected mostly based on the name of the disease (Watanabe et al. 2001). TSS, KSS, and KBG are well-known generic prescriptions for menstruation-

related disorders such as dysmenorrhea, irregular menstruation, PMS, and menopausal disorders. YKS is a drug used universally in the palliation of behavioral and psychological symptoms of dementia and has been the subject of many clinical trials (Ikarashi and Mizoguchi 2016). YKS has been shown to be effective in stabilizing emotions.

Although these drugs are widely used in the treatment of PMS/PMDD, few clinical trials have examined symptom improvement, and only an open-label study of the KSS has reported its efficacy in psychiatrist-diagnosed PMDD (Yamada and Kanba 2007). There are no fixed criteria for drug selection; therefore, the characteristics of drug selection in actual clinical practice should be examined. We aimed to reanalyze previous data and identify the characteristics of selected Kampo formulas in the treatment of PMS/PMDD.

Materials and Methods

Ethics

We reanalyzed data from physicians in the JSOG from a survey conducted by the Academic Committee on Women's Health Care. All the data have been previously published(Yoshimi et al. 2023). This study was conducted in accordance with the principles of the Declaration of Helsinki. The survey was anonymous and recorded no personal information. Participants read a description of the study's purpose and consented to participate online before completing the survey.

Participants

In the original study, an email was sent to all 16,732 JSOG members inviting them to participate in a web survey via Google Forms from late September to November 2021. A total of 1,312 members completed the questionnaire, of which 1,265 were involved in PMS/PMDD treatment (Figure 1). Of these, those who reported using Kampo medicine for treatment (1,263 respondents) and those who reported their sex (1,259 respondents) were selected for analysis.

Questionnaire

The survey items have been detailed in a previous report (Yoshimi et al. 2023). This study focused on specific items, including the following basic attributes: sex (male or female), years of experience as a physician, specialist qualifications, and workplace type (university hospital, general hospital, clinic, others). The second item of investigation concerned generic therapeutic modalities: OCPs,

SSRIs/serotonin and norepinephrine reuptake inhibitors (SNRIs) (luteal phase only and continuous administration), Kampo medicines (TSS, KSS, KBG, YKS, and others), gonadotropin-releasing hormone (GnRH)-analogs, anti-anxiety agents, hypnotics, chasteberries, vitamin B6, and other medications or supplements. In the case of Kampo medicines, even preparations with the same name may contain different types and amounts of crude drugs, depending on the pharmaceutical company that manufactures them. For example, formulations of TSS from five different companies are available (see STORK for details, http://mpdb.nibiohn.go.jp/stork/). In general clinical practice in obstetrics and gynecology, only a few cases exist where Kampo medicines are selected with consideration of the differences between manufacturers. Therefore, we did not request detailed information on these aspects in this survey. SNRIs such as venlafaxine are also effective for treating PMDD and are considered the second-line treatment after SSRIs, according to the American Academy of Family Physicians (Cohen et al. 2004, Hofmeister and Bodden 2016). Therefore, in this survey, SSRIs and SNRIs are grouped together as therapeutic agents.

Statistical analysis

Proportions were calculated for the categorical variables. A correspondence analysis was used to analyze the characteristics of drug selection for TSS, KSS, KBG, and YKS. To compare these Kampo preparations with the standard treatments for PMS/PMDD, OCPs and SSRIs/SNRIs (luteal phase only and continuous administration) were also included in the analysis, as well as the GnRH analog, which is not a standard treatment, but has been shown to have therapeutic effects (Mezrow et al. 1994). The original treatment methods survey form asked for multiple responses regarding the methods used to treat PMS/PMDD; however, in the correspondence analysis, each method was dichotomized with or without the choice of method.

Logistic regression analysis was used to determine the factors significantly associated with the selection of each Kampo formulation (TSS, KSS, KBG, and YKS). Fourteen items consisting of the post-licensure period for medical practitioners, sex, board-certified OBs/GYNs, working hospital, OCPs, GnRH analog, SSRIs/SNRIs continuous, SSRIs/SNRIs cyclic, anti-anxiety agent, hypnotics, other Kampo formulas, chasteberry, vitamin B6, and other medications or supplements were included in the model. Chasteberries and vitamin B6 were included in the analysis because of a large number

of efficacy reports (Robinson et al. 2024, Verkaik et al. 2017). Statistical analyses were performed using JMP 17.0.0 (SAS, Cary, NC). Statistical significance was set at $p < 0.05$.

Results

Basic patient characteristics are listed in Table 1. Almost all participants in the analysis chose OCPs as their treatment of choice for PMS/PMDD. KSS was the second most common, followed by TSS and YKS. Continuous administration of SSRIs/SNRIs was the next most common treatment, indicating that Kampo treatment is more commonly used than SSRIs/SNRIs.

Next, a correspondence analysis was conducted to analyze the selection characteristics of generic Kampo medicines such as TSS, KSS, KBG, and YKS (Figure 2A). Correspondence analysis is a statistical technique that reduces the dimensionality of categorical data to reveal and visualize the patterns and relationships between variables in a contingency table. Kampo preparations (TSS, KSS, KBG, and YKS) were placed in a group similar to the OCPs, with SSRIs/SNRIs (continuous and cyclic) and GnRH analogs as separate groups. The groups of OCPs and Kampo preparations were densely distributed and difficult to distinguish; therefore, this section was enlarged and displayed. Figure 2B shows an expanded group of OCPs and Kampo formulations (TSS, KSS, KBG, and YKS). TSS, KSS, and KBG were found in one group and in a different position from YKS. Taken together, Figures 2A and 2B indicate that YKS was the closest to the SSRIs/SNRIs position among the Kampo formulations.

In addition, the factors significantly associated with the selection of each Kampo formulation (TSS, KSS, KBG, and YKS) were analyzed (Table 2). Table 2 presents the data showing significant associations with each Kampo formulation. Shorter physician experience was associated with selecting KSS and KBG prescriptions (< 10 vs. ≥ 20 and < 30 OR: 2.16, 2.52; vs. ≥ 30 and < 40 OR: 2.02, 2.56; vs. ≥ 40 and < 50 OR: 2.60, 2.32; vs. ≥ 50 OR: 7.00, 15.0; ≥ 10 and < 20 vs. ≥ 20 and < 30 OR: 2.02, 1.88; vs. ≥ 30 and < 40 OR: 1.88, 1.92; vs. ≥ 40 and < 50 OR: 2.43, 1.73; vs. ≥ 50 OR: 6.53, 11.2), whereas being a clinic practitioner was associated with selecting KSS and YKS prescriptions (private vs. hospital OR: 1.57, 1.77; private vs. university OR: 1.53, 1.71). The prescription of YKS was also associated with choosing SSRI/SNRI (OR: 1.81), chasteberry (OR: 6.24), and other

medications or supplements (OR: 2.41). KSS, KBG, and YKS were significantly associated with the choice of other Kampo formulations (OR: 1.59, 1.56, and 2.49, respectively), whereas TKS was not.

Discussion

Our results indicate the same position for OCPs and Kampo prescriptions (TSS, KSS, KBG, and YKS), which may support the fact that both are universally selected in the treatment of PMS/PMDD. Interestingly, SSRIs/SNRIs and GnRH analogs showed very different positions in the correspondence analysis, which seems to indicate that they are drugs with completely different mechanisms of action. That is, SSRIs/SNRIs increase the brain transmitter serotonin and GnRH analogs suppress ovulation. Japanese obstetricians and gynecologists (OBs/GYNs) may be aware of significant differences in their prescription choices.

Despite its origins in traditional medicine, Kampo therapy plays a major role in medical care in Japan, where it is universally covered by national insurance (Suzuki et al. 2009). Although Kampo treatments are used to treat a variety of diseases, to date, no study has examined the characteristics of the choice of various Kampo medicines for specific diseases.

A detailed examination of the Kampo formulations showed separate positions for the TSS, KSS, and KBG groups, as well as for the YKS group. TSS, KSS, and KBG are the three major formulations often used in women, suggesting that YKS is used differently. Of these four formulations, YKS was the closest to the SSRIs/SNRIs, and the results of logistic regression analysis were consistent, with only YKS having a significant association with SSRIs/SNRIs selection. With respect to YKS, a mechanism of action analysis based on several basic studies suggested that it acts on multiple serotonin receptors to control emotions (Mizoguchi and Ikarashi 2017). Although specific data are unavailable from this secondary analysis of the JSOG survey results, conducting a similar survey to investigate medication choices for the neuropsychiatric symptoms of PMS/PMDD and comprehensively explore Kampo treatments would be a valuable avenue for future research.

For TSS and KBG, we found a significant association with lower years of medical licensure (< 10 years, ≥ 10 years, and < 20 years), and they were perhaps more likely to be used by first-time students of Kampo treatment. However, with respect to the KSS and YKS, being a practitioner was associated

with a greater choice of each drug than being a working physician. Given that PMS/PMDD is more likely to be treated by primary care physicians than by university or general hospitals, it is likely that physicians who are more familiar with PMS/PMDD treatment have the characteristics to select these medications. For YKS, in particular, there was also a significant association with the choice of SSRIs/SNRIs, chasteberry, and other medications and supplements. This suggests that YKS may be selected by physicians who are familiar with PMS/PMDD treatment.

This study is important because it is a unique survey of the characteristics of Kampo medicine selection among OBs/GYNs engaged in clinical practice. However, this study has several limitations. First, the cross-sectional study design precluded a causal explanation. Second, the response rate was low. As the survey was conducted using a web-based system, responses were not mandatory and were inevitably biased toward those interested in the survey content. Therefore, our results indicate the characteristics of OBs/GYNs committed to PMS/PMDD treatment. Third, the participants in this study were limited to OBs/GYNs. In Japan, PMS/PMDD treatment is provided not only by OBs/GYNs, but also by psychiatrists; our survey included both. Psychiatrists were significantly more likely than OBs/GYNs not only to use SSRIs, but also to use Kampo medicine as their first choice, with 42.1% using it as such (Yoshimi et al. 2024). Further studies of the differences in the characteristics of Kampo medicine selection between psychiatrists and OBs/GYNs are warranted.

Although the role of Kampo treatment in Japanese medicine is significant, no systematic educational method has been established, and each physician learns it in their own way. Understanding the characteristics of Kampo medicine selection, as described in this study, is expected to be useful to effectively educate beginners about Kampo medicine treatment. In particular, emphasizing the importance of including YKS and KSS in introductory Kampo medicine education for beginners is crucial.

Data availability statement

All data generated or analyzed in this study were included in a previous article (Yoshimi et al. 2023). Further inquiries can be directed to the corresponding authors.

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Conflict of interest

The other authors declare no conflicts of interest.

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Figure legends

Figure 1. Flow diagram of study participants. Those who reported treating PMS/PMDD and using Kampo medicines in their treatment and who reported their sex as male or female were selected. JSOG, Japanese Society of Obstetrics and Gynecology; PMS, premenstrual syndrome; PMDD, premenstrual dysphoric disorder

Figure 2. Correspondence analysis results. A. Selection characteristics of generic Kampo medicines such as TSS, KSS, KBG, and YKS. B. Enlarged portions of OCPs, TSS, KSS, KBG, and YKS. TSS, *Tokishakuyakusan*; KSS, *Kamishoyosan*; KBG, *Keishibukuryogan*; YKS, *Yokukansan*; OCPs, oral contraceptives; GnRH analog, gonadotropin-releasing hormone analog; SSRIs, selective serotonin reuptake inhibitors; SNRIs, serotonin and norepinephrine reuptake inhibitors

Table 1. Characteristics of study participants (n = 1259)

	Number (%)
Post-licensure period (years) for medical practitioners	
< 10	117 (9.3)
≥ 10 and < 20	321 (25.5)
≥ 20 and < 30	373 (29.6)
≥ 30 and < 40	308 (24.5)
≥ 40 and < 50	127 (10.1)
≥ 50	13 (1.0)
Gender (female)	639 (50.8)
Board-certified OBs/GYNs	1203 (95.6)
Working hospital	
University hospital	272 (21.6)
General hospital	521 (41.4)
Clinic	404 (32.1)
Others	62 (4.9)
Generic therapeutic medication for PMS/PMDD (multiple answers allowed)	
OCPs	1235 (98.1)
GnRH-analog	64 (5.1)
SSRIs/SNRIs (continuous dosing)	496 (39.4)
SSRIs/SNRIs (luteal phase dosing)	214 (17.0)
Anti-anxiety agent	357 (28.4)
Hypnotics	250 (19.9)
Kampo medicine, *Tokishakuyakusan*	670 (53.2)
Kampo medicine, *Kamishoyosan*	926 (73.6)
Kampo medicine, *Keishibukuryogan*	460 (36.5)

Kampo medicine, *Yokukansan*	648 (51.5)
Kampo medicine, others	321 (25.5)
Chasteberry	18 (1.4)
Vitamin B6	34 (2.7)
Other medications or supplements	64 (5.1)

OBs/GYNs, obstetricians and gynecologists; PMS/PMDD, premenstrual syndrome and premenstrual dysphoric disorder; GnRH analog, gonadotropin-releasing hormone analog; OCPs, oral contraceptives; SSRIs, selective serotonin reuptake inhibitors; SNRIs, serotonin and norepinephrine reuptake inhibitors.

Table 2. Factors associated with Kampo formulation selection

	TSS	KSS	KBG	YKS
	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
Post-licensure period for medical practitioners				
< 10 vs. ≥ 20 and < 30	2.16 (1.30–3.58)		2.52 (1.52–4.17)	
≥ 30 and < 40	2.02 (1.18–3.44)		2.56 (1.50–4.37)	
≥ 40 and < 50	2.60 (1.40–4.82)		2.32 (1.24–4.33)	
≥ 50	7.00 (1.72–28.5)		15.0 (1.82–123.20)	
≥ 10 and < 20 vs. ≥ 20 and < 30	2.02 (1.47–2.77)		1.88 (1.36–2.61)	
≥ 30 and < 40	1.88 (1.33–2.67)		1.92 (1.34–2.74)	
≥ 40 and < 50	2.43 (1.53–3.87)		1.73 (1.06–2.82)	
≥ 50	6.53 (1.70–25.1)		11.2 (1.41–88.8)	
Gender (female)		1.82 (1.36–2.43)		2.41 (1.84–3.15)
Board-certified OBs/GYNs		2.00 (1.01–3.94)	2.06 (1.05–4.04)	
Working hospital				
Clinic vs. general hospital		1.57 (1.13–2.19)		1.77 (1.31–2.40)
Clinic vs. university hospital		1.53 (1.02–2.28)		1.71 (1.18–2.47)
OCPs				
GnRH-analog				
SSRIs/SNRIs (continuous dosing)				1.81 (1.32–2.47)
SSRIs/SNRIs (luteal phase dosing)			0.61 (0.40–0.91)	
Anti-anxiety agent				
Hypnotics	1.76 (1.28–2.44)	2.18 (1.45–3.29)	2.43 (1.77–3.35)	1.60 (1.15–2.24)
Other Kampo		1.59 (1.13–2.23)	1.56 (1.17–2.08)	2.49 (1.85–3.36)
Chasteberry				6.24 (1.31–29.7)
Vitamin B6				
Other medications or supplements				2.41 (1.20–4.84)

Only results showing significant associations are presented. R2 = 0.06 (TSS), 0.06 (KSS), 0.06 (KBG), 0.12 (YKS).

TSS, *Tokishakuyakusan*; KSS, *Kamishoyosan*; KBG, *Keishibukuryogan*; YKS, *Yokukansan*; OR, odds ratio; CI, confidence interval; OBs/GYNs, obstetricians and gynecologists; OCPs, oral contraceptives; GnRH analog, gonadotropin-releasing hormone analog; SSRIs, selective serotonin reuptake inhibitor; SNRIs, serotonin and norepinephrine reuptake inhibitors.

Fig. 1

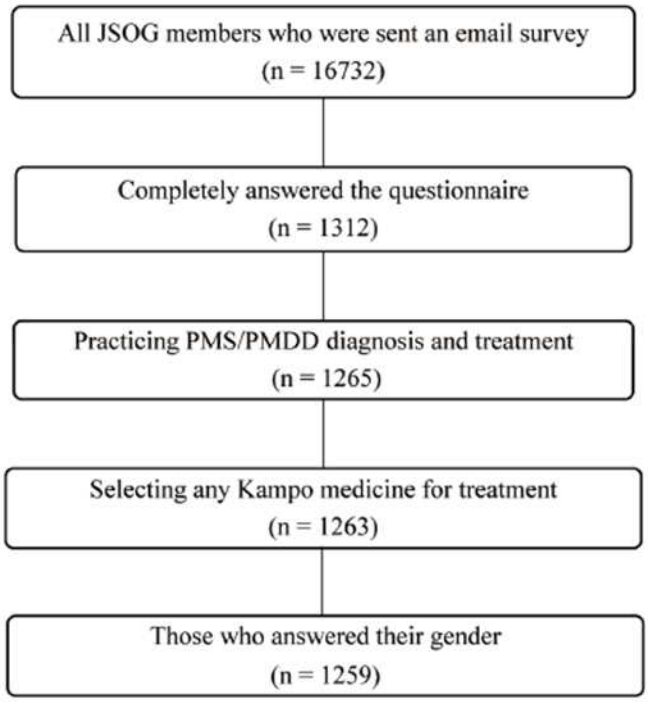
All JSOG members who were sent an email survey
(n = 16732)
Completely answered the questionnaire
(n = 1312)
Practicing PMS/PMDD diagnosis and treatment
(n = 1265)
Selecting any Kampo medicine for treatment
(n = 1263)
Those who answered their gender
(n = 1259)

Fig. 2A

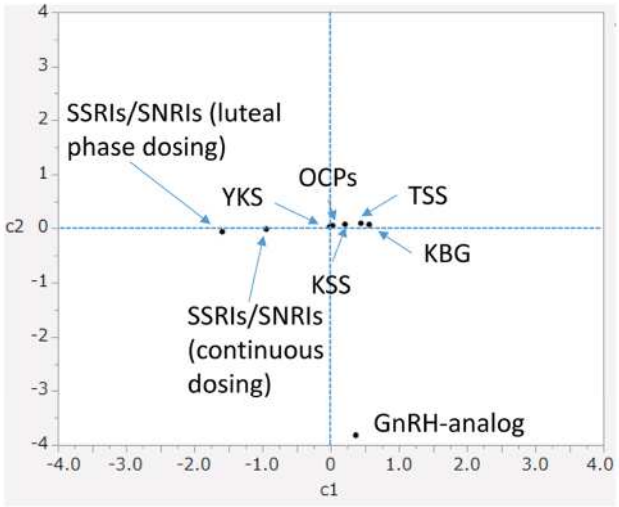
SSRIs/SNRIs (luteal phase dosing)
YKS
OCPs
TSS
KBG
KSS
SSRIs/SNRIs (continuous dosing)
GnRH-analog
c1
c2
4
3
2
1
0
-1
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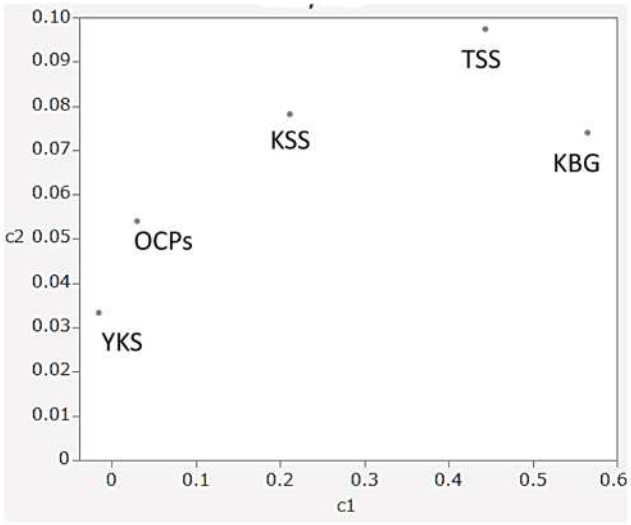
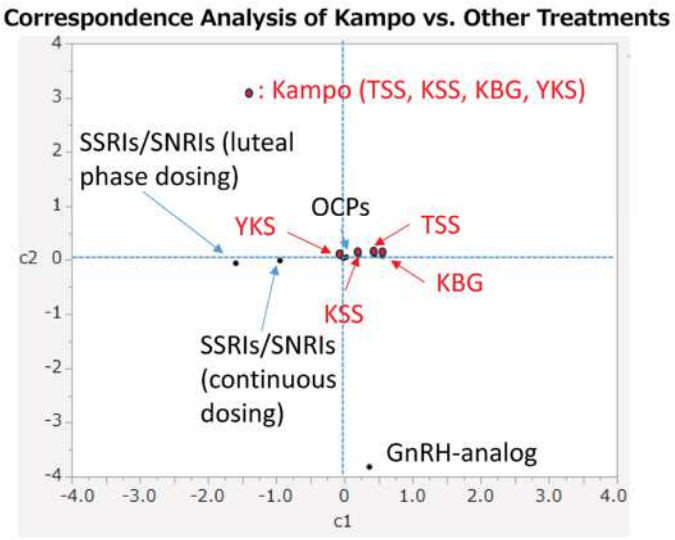
Fig. 2B



Graphical Abstract



BMC Pregnancy and Childbirth

RESEARCH

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Mental health risks in pregnancy and early parenthood among male and female parents following unintended pregnancy or fertility treatment: a cross-sectional observational study

Naoki Mizunuma[1,2,3*], Keiko Yamada[4], Takashi Kimura[5], Yutaka Ueda[6], Takashi Takeda[7], Takahiro Tabuchi[8,9] and Kunihiko Kurosaki[10]

Abstract

Background Unintended pregnancy at higher risk of perinatal mood disorders; however, concurrent factors such as socioeconomic conditions may be more critical to mental health than pregnancy intention. Mental health risks among individuals undergoing fertility treatment are inconsistent. We investigated mental health risks during pregnancy and parenthood in parents who conceived unintentionally or through fertility treatment compared to those who conceived naturally and intentionally.

Methods We conducted a web-based study with 10,000 adults ≥ 18 years old, either pregnant or with a child aged < 2 years. Male and female respondents weren't couples. We analyzed 1711 men and 7265 women, after filtering out invalid responses. We used a questionnaire including conception methods (e.g., naturally conceived intended/unintended pregnancies, fertility treatment such as scheduled intercourse or ovulation inducers [SI/OI], intrauterine insemination [IUI], and in-vitro fertilization or intracytoplasmic sperm injection [IVF/ICSI]) and mental health risks (e.g., psychological distress, chronic pain, death fantasies). Using a modified Poisson regression, we estimated relative risks (RR [CI]) for mental health risks compared to those with intended pregnancies.

Results Unintended pregnancy showed higher mental health risks during pregnancy in both genders, with women having significantly higher psychological distress, chronic pain, and death fantasies (RR 1.63 [1.05–2.54], RR 1.63 [1.14–2.33], and RR 2.18 [1.50–3.18], respectively). Women's death fantasies risk remained high in parenthood: RR 1.40 (1.17–1.67). In relation to fertility treatments, men using SI/OI during their partner's pregnancy showed higher mental health risks, especially for chronic pain (RR 1.75 [1.01–3.05]). Men who underwent IUI showed higher mental health risks during parenthood, notably death fantasies (RR 2.41 [1.13–5.17]). Pregnant women using SI/OI experienced higher mental health risks, with a significant risk of chronic pain (RR 1.63 [1.14–2.33]). Pregnant women using IVF/ICSI

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had a significantly lower risk of chronic pain (RR 0.44 [0.22–0.87]), but women who used IVF/ICSI had a significantly higher risk of death fantasies during parenthood (RR 1.40 [1.04–1.88]).

Conclusions Mental health risks vary by parenting stage (pregnancy or early parenthood) and gender, especially for those who conceived unintentionally or through fertility treatment. Both stages require adaptable mental health support for all parents.

Trial registration N/A (non-interventional study).

Keywords Mental health, Pregnancy, Parenthood, Unintended pregnancy, Fertility treatment

Background

There are several methods of intentional and assisted conception and all have unique perinatal and postnatal mental health risks. Pregnancy intentions often influence parental psychological factors during pregnancy and post-delivery (e.g., unintended pregnancies can cause unhappiness) [1]. Since 1978, in vitro fertilization (IVF) has provided an option for infertility problems. However, couples undergoing fertility treatment may experience substantial physical, psychological, and financial burdens, including clinic visits, treatment costs, and planning intimacy around menstrual cycles [2, 3]. Infertile couples may have a higher risk of psychological disorders [4]. Individuals undergoing fertility treatment may experience psychological distress, including depression [5], anxiety [6], anger, frustration, isolation, and death fantasies [7].

Such negative psychosocial factors can trigger somatic symptoms like chronic pain, which can be affected by physiological, psychological, and social factors [8]. Chronic pain during pregnancy and postpartum is a common issue, with studies showing that up to 70% of pregnant women experience low back pain and 45% report pelvic pain [9]. Male partners may also experience chronic pain, such as back pain or headaches [10]. Furthermore, there is a bidirectional relationship between chronic pain and mental health issues such as depression and anxiety [11]. For instance, parents experiencing chronic pain during pregnancy are at a higher risk of developing postpartum depression [12]. Conversely, psychological distress such as anxiety and depression during pregnancy is associated with an increased risk of developing chronic pain after childbirth [13]. Given these interrelationships, it is important to consider chronic pain alongside mental health outcomes when examining the effects of different conception methods.

Couples must manage these burdens until the birth of their child, and some cease fertility treatment owing to psychological stress [6]. Parents also face post-pregnancy challenges. Mental health problems (e.g., depression or anxiety, post-traumatic stress disorder) may arise during pregnancy and post-delivery [14]. Psychological stress in either parent during pregnancy may engender bonding disorders [15]. Furthermore, poor perinatal and postnatal mental health in both men and women are risk factors for child abuse or maltreatment [16].

We chose to investigate both unintended pregnancies and pregnancies resulting from fertility treatments in this study because, despite their differences, they represent opposite ends of the "pregnancy intentionality spectrum" and both have the potential to significantly impact mental health. By examining both types of pregnancies, we aim to explore how different circumstances surrounding pregnancy intention can lead to varying mental health outcomes. Comparing these two types of pregnancies allows us to identify different mechanisms that might influence mental health in these distinct contexts.

Several studies have reported associations between conception methods and female perinatal mental health. Women who have experienced naturally conceived unintended pregnancies have a higher risk of psychological distress [17] and maternal depression [18], particularly during postpartum [19, 20], than women with naturally conceived intended pregnancy. In contrast, the postpartum mental health risks for women who have undergone fertility treatment, compared with those who have conceived naturally, vary depending on individual circumstances and the type of treatment received. While some studies suggest that women who have conceived through fertility treatment are not at higher risk of postpartum depression or anxiety [21], other research suggests a higher prevalence of mental health problems, such as depression, obsessive-compulsive symptoms, and somatization, in these women [22, 23]. In addition, unintended pregnancy has been associated with an increased risk of mental health problems in men, particularly depression. A systematic review and meta-analysis found that men who became unintended fathers were more than twice as likely to report mental health problems, including depression, compared with men who had planned pregnancies [24]. Similarly, men who experience fertility problems may report lower sexual and personal quality of life, leading to social distancing and increased psychological distress [25–27]. In contrast, few studies have assessed the specific mental health outcomes of women facing fertility problems, although some evidence suggests that these women may also face increased emotional distress during the perinatal period [23]. Thus,

both unintended pregnancy and fertility problems may have significant psychological effects on both mothers and fathers, although the exact nature and extent of these effects may vary.

We hypothesized that understanding the distinct mental health risks associated with naturally conceived unintended pregnancies and those resulting from fertility treatments could provide a more comprehensive understanding of how pregnancy intention affects mental health. To test this, we investigated the association between conception methods (i.e., naturally conceived intended pregnancy, naturally conceived unintended pregnancy, pregnancy through fertility treatment) and mental health risks (psychological distress, prevalence of chronic pain, death fantasies) among male and female parents who were either pregnant or had children aged < 2 years.

Methods

Study design and population

We used data from a cross-sectional web-based special survey conducted in 2021 as part of Japan Coronavirus Disease (COVID)-19 and Society Internet Survey (JACSIS) related to perinatal and early parenthood. This survey specifically targeted pregnant women and parents with children under two years old. It is important to note that the participants in this survey are independent and do not overlap with those in the main JACSIS survey, which covers a broader demographic. The data for this study were exclusively drawn from this non-overlapping subset of the JACSIS panel. Figure 1 shows the participant enrollment process. Random sampling using a computer algorithm was used to recruit participants. All participants electronically provided informed consent before responding to the questionnaire. A total of 440,323 panelists registered with a Japanese internet survey agency, Rakuten Insight, Inc. (based in Tokyo, Japan). A screening survey to identify eligible participants, defined as those aged ≥ 18 years who were either expecting a child or

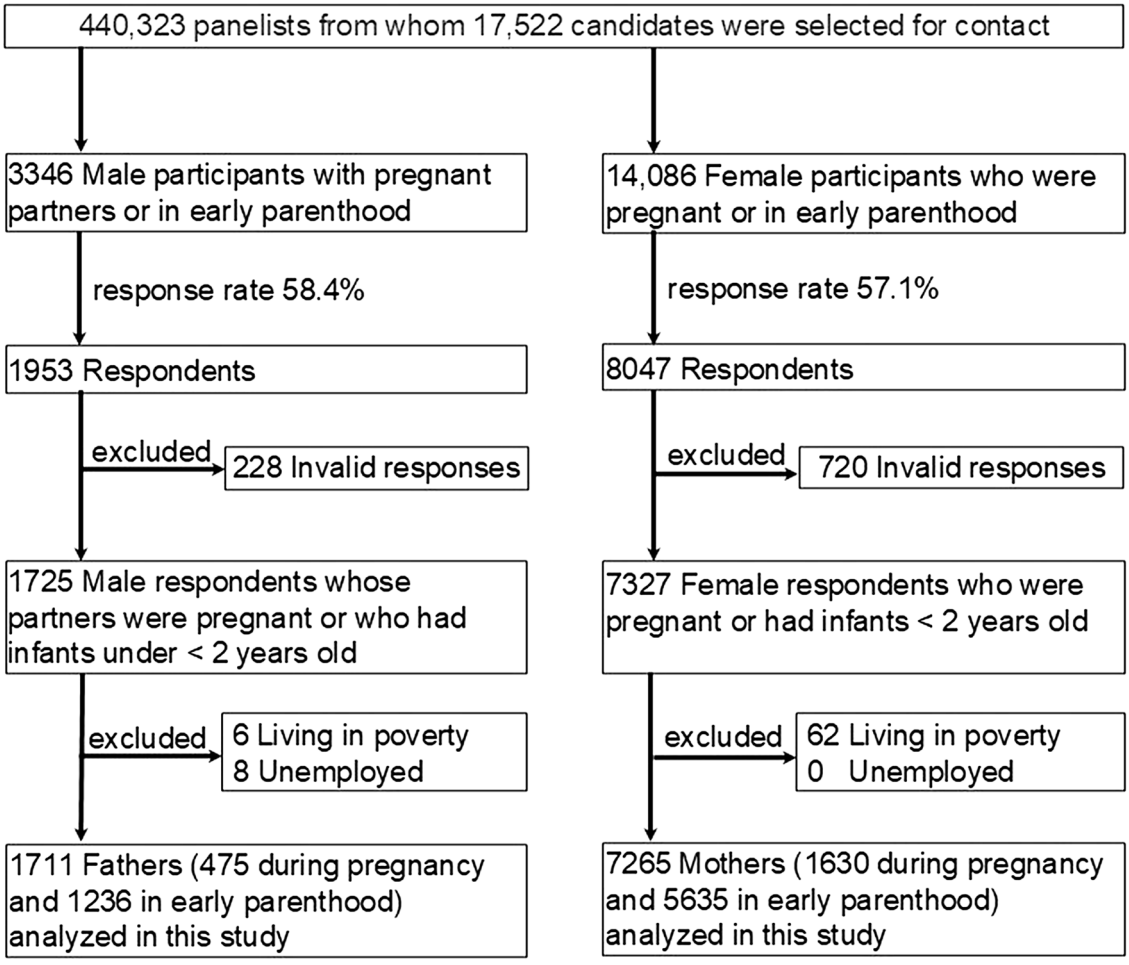

Fig. 1 Enrollment process. Parents aged ≥ 18 years, either expecting a child or with a child aged < 2 years, were recruited from 28 July to 30 August 2021

had a child aged < 2 years, was distributed to a subset of these panelists based on a computer algorithm designed to optimize the final sample size to 10,000 participants. The exact number of panelists who received the screening survey was not disclosed. Subsequently, we sent email invitations and questionnaires to 3436 male and 14,086 female eligible participants. Of those invited to participate, 10,000 (1953 men and 8047 women) responded between 28 July and 30 August 2021 (response rate: 58.4% for men; 57.1% for women). Of the 1953 male and 8047 female respondents, we excluded 228 men and 720 women with invalid responses, defined as respondents who did not select one of the five possible options in response to a dummy question; those who reported abusing all seven substances on the questionnaire (alcohol, sleeping medications, opioids, sniffing paint thinner, legal high drugs, marijuana, and cocaine/heroin); or those who selected all past medical history listed. These criteria aim to exclude respondents who reported abusing all seven substances listed in the questionnaire or who selected all past medical history options. This exclusion is intended to enhance the reliability and consistency of the data by minimizing the influence of extreme cases on the analysis results. These criteria are standardized and consistently used across the JACSIS study. We also excluded 6 men and 60 women living in poverty and 8 unemployed men (there were no unemployed women) as these factors were considered confounders with a small sample size. We defined poverty as an annual equivalized income of < 1.24 million JPY (8857 USD at 140 JPY/USD), the poverty line in 2018 as defined by the Organisation for Economic Co-operation and Development [28]. In total, we analyzed data from 1711 men (475 expectant fathers and 1236 early-stage fathers) and 7265 women (1630 expectant mothers and 5635 early-stage mothers). Characteristics of these participants are shown in Table 1 and Supplementary Table 1 [see Additional file 1].

Notably, these expectant mothers may have included women who were considering abortion or even planning to have an abortion at the time they completed their surveys.

Measures

Conceptional methods: exploratory variable

We used a single question to identify conception methods: "Which method was used to achieve your most recent pregnancy?" Respondents chose from six options: (i) naturally conceived intended pregnancy (desired or intended pregnancy planned in terms of timing), (ii) naturally conceived unintended pregnancy (unexpected or undesirable pregnancy), (iii) fertility treatment (scheduled intercourse [SI] or ovulation inducer [OI], combined owing to the relatively low incidence of these among men), (iv) fertility treatment (intrauterine insemination [IUI]), (v) fertility treatment (IVF and intracytoplasmic sperm injection [ICSI]).

Psychological distress: outcome measure 1

Psychological distress was defined as a score of ≥ 13 on the Kessler Psychological Distress Scale (K6) [29–31]. Participants were asked "During the past 30 days, about how often did you feel i) nervous, ii) hopeless, iii) restless or fidgety, iv) so depressed that nothing could cheer you up, v) that everything was an effort, and vi) worthless?" Respondents rated the frequency of these feelings as follows: 0 (none of the time), 1 (a little of the time), 2 (some of the time), 3 (most of the time), or 4 (all of the time). Responses are summed to produce a total scale score (range: 0–24). Binarization of the K6 score using the established cutoff point allows for a consistent analytical approach across our various outcome measures, including the binary measures such as death fantasies. This approach facilitates a uniform assessment of recent psychological distress presence or absence, aligning with our other binary outcome variables.

Chronic pain: outcome measure 2

Chronic pain was defined as pain that persisted or recurred for longer than 3 months, according to the International Classification of Diseases 11th Revision (ICD-11) [32]. We assessed about the presence of pain symptoms in the past month, with a slight variation in wording between male and female participants. For men, we asked about pain symptoms excluding body aches from a cold (yes or no). For women, we asked about pain symptoms excluding labor pain, pain during childbirth, afterpains, and body aches from a cold (yes or no). Participants who answered 'Yes' to this question and indicated that their pain lasted for 3 months or more (≥ 3 and < 6 months, ≥ 6 and < 12 months, or ≥ 1 year) were categorized as having chronic pain. Participants were shown manikins that displayed body regions and asked to identify their dominant pain site.

Death fantasies: outcome measure 3

An original single question was used to assess death fantasies: "Have you ever felt that you wanted to die at any time since January 2021?" (yes or no). It should be noted that this single question does not constitute a validated measure of suicide risk, such as the Columbia-Suicide Severity Rating Scale (C-SSRS) [33]. The C-SSRS is a psychometrically validated tool with demonstrated reliability and validity in assessing the full spectrum of suicidal ideation and behaviors, including its ability to differentiate between passive and active ideation, and to predict future suicide attempts [33]. Our single-question measure was designed to assess death fantasies rather than suicidal ideation and should be interpreted with caution, as it

Table 1 Mean values and proportions for participant characteristics ($n = 8976$)

	Men				Women			
	During pregnancy		Within 2 years after delivery		During pregnancy		Within 2 years after delivery	
	n = 475		*n* = 1236		*n* = 1630		*n* = 5635	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age, years	35.2	5.1	35.5	5.2	31.5	4.5	32.2	4.4
	n	%	n	%	n	%	n	%
Junior high or high school graduate	56	11.8	134	10.8	249	15.3	885	15.7
Current smoker	97	20.4	266	21.5	34	2.1	244	4.3
Current drinker	261	54.9	737	59.6	63	3.9	1310	23.2
Lives with ≥ 2 children	77	16.2	628	50.8	208	12.8	2576	45.7
Recurrent pregnancy loss	26	5.5	55	4.4	259	15.9	399	7.1
Fetus/infant/child with health problems	19	4.0	40	3.2	27	1.7	198	3.5
History of depression	32	6.7	68	5.5	114	7.0	434	7.7
Paternal leave	-	-	415	33.6	-	-	499	8.9
Maternal leave	-	-	461	37.3	-	-	2420	42.9
Low birth weight infant	-	-	92	7.4	-	-	507	9.0
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Recent K6 score	4.3	4.8	3.9	4.8	4.7	5.5	4.3	5.3
	n	%	n	%	n	%	n	%
Presence of chronic pain	40	8.4	138	11.2	165	10.1	1043	18.5
Presence of death fantasies	54	11.4	98	7.9	142	8.7	598	10.6
Conception method								
NCIP	342	72.0	906	73.3	1017	62.4	3668	65.1
NCUP	37	7.8	126	10.2	221	13.6	1028	34.2
Fertility treatment (SI/OI)	28	5.9	84	6.8	141	8.7	393	7.0
Fertility treatment (IUI)	27	5.7	41	3.3	50	3.1	166	2.9
Fertility treatment (IVF/ICSI)	41	8.6	79	6.4	201	12.3	380	6.7
Dominant site for chronic pain, $n = 1386$	40	(100)	138	(100)	165	(100)	1043	(100)
Head or orofacial	5	(12.5)	11	(8.0)	23	(13.9)	164	(15.7)
Neck or shoulder	17	(42.5)	45	(32.6)	9	(5.5)	197	(18.9)
Chest or abdominal	0	(0)	2	(1.4)	11	(6.7)	87	(8.3)
Back	3	(7.5)	2	(1.4)	6	(3.6)	48	(4.6)
Low back	10	(25.0)	50	(36.2)	66	(40.0)	290	(27.8)
Upper limb, hand, or finger	2	(5.0)	9	(6.5)	2	(1.2)	78	(7.5)
Pelvis	0	(0)	4	(2.9)	26	(15.8)	76	(7.3)
Genitals, urethra, or anus	0	(0)	3	(2.2)	7	(4.2)	21	(2.0)
Hip	0	(0)	0	(0)	10	(6.1)	21	(2.0)
Lower limb	3	(7.5)	12	(8.7)	5	(3.0)	61	(5.8)

ICSI, Intracytoplasmic sperm injection; IUI, Intrauterine insemination; IVF, In vitro fertilization; K6, Kessler psychological distress Scale; NCIP, Naturally conceived intended pregnancy; NCUP, Naturally conceived unintended pregnancy; OI, Ovulation inducer; SD, Standard deviation; SI, Scheduled intercourse

does not capture the full intensity or spectrum of suicidal thoughts.

Covariates

The following factors potentially related to perinatal parental mental health were considered as covariates (i.e., potential confounding factors). Age, education level, and income can influence stress levels and access to social support, both of which are key in managing perinatal mental health [34, 35]. Smoking and drinking habits, often used as coping mechanisms, may negatively affect perinatal mental health [34]. Pregnancy loss, infant health issues, and a history of depression can increase psychological burden and the risk of mental health problems [34, 36–39]. Furthermore, the availability and duration of parental leave are crucial, with extended leave being associated with improved maternal mental health [40, 41]. Finally, experiences such as having a low birthweight baby or caesarean section may contribute to psychological stress due to unexpected medical challenges [42].

For both periods (pregnancy and early parenthood), the following factors were considered

Age (18–24, 25–29, 30–34, 35–39, 40–44, or 45–49 years), educational attainment (junior high or high school graduate, or >high school graduate), smoking status (never-smoker, former smoker, or smoker), drinking status (never-drinker, former drinker, or drinker), equivalized income (quintile), number of children participant lives with (0, 1, or ≤2), recurrent pregnancy loss (yes or no), fetal/infant/child with health problems (yes or no), and history of depression (yes or no). Equivalized household income was calculated by dividing the median value of the multiple-choice response to the annual household income question by the square root of the number of people living in the household. Recurrent pregnancy loss was calculated as two or more pregnancy losses. In our questionnaire, the number of pregnancy losses was derived from the difference between the total number of pregnancies and the number of live births. This approach, while commonly used in Japanese epidemiological studies due to the sensitive nature of direct questions regarding miscarriages and induced abortions, may not distinguish between consecutive and non-consecutive losses. Equivalized household income was calculated by dividing the median value of the multiple-choice response to the annual household income question by the square root of the number of people living in the household.

For early parenthood, the following factors were considered

Paternal leave (yes or no), maternal leave (yes or no), and low birth weight infant, defined as a body weight of <2500 g (yes or no). Experience of a cesarean section (yes or no) was used as a covariate for female participants in early parenthood.

Covariate multicollinearity was examined using the variance inflation factor (VIF). The VIF for all covariates was <3. A VIF of <5 is generally accepted as indicating a lack of problematic multicollinearity [43].

Statistical analysis

We used a modified Poisson regression model to compare the relative risk (RR) with 95% confidence intervals (CIs) of recent psychological distress, prevalence of chronic pain, and death fantasies during pregnancy and early parenthood according to conception method compared with naturally conceived intended pregnancy. We used Statistical Analysis Software (SAS; SAS Institute Inc., Cary, NC, USA) and the SAS PROC GENMOD REPEATED statement to estimate the sandwich error using the modified Poisson regression model. Sandwich error is a robust method for estimating standard errors in regression analysis. It is particularly useful when the data are highly variable. It adjusts for irregularities and dependencies in the data, providing more reliable standard error estimates and increasing the accuracy of statistical inference [44].

Model 1 was adjusted for age. Model 2 was adjusted for age, junior high or high school graduate, smoking, drinking, equivalized income, living with ≥2 children, recurrent pregnancy loss, infant with health problems, and history of depression. To Model 3 (only for participants in early parenthood) we added paternal leave, maternal leave, and low birth weight infant. Model 4 included the variables from Model 3 plus cesarean section experience (only for chronic pain prevalence among women in early parenthood). The reason cesarean section experience was specifically considered only in Model 4 is that it may have a specific impact on the development of chronic pain. Cesarean section was introduced at a different stage than the other covariates because of its potential impact on the physical recovery process, especially as it may be associated with chronic pain in women. P values<0.05 (two-tailed tests) were considered statistically significant. All statistical analyses were performed using SAS, Version 9.4.

Results

Participant characteristics

Table 1 and Supplemental Table 1 in the Additional file 1 show the participant characteristics. Men whose partners were pregnant were 22–49 years old, men within two years after delivery were 21–49 years old, women during pregnancy were 19–48 years old, and those within two years after delivery were 18–48 years old. The prevalence of recent psychological distress was 10.5% in men expecting a child and 8.7% in men within two years after delivery, whereas it was 7.0% in women during pregnancy and 6.8% in women within two years after delivery. The prevalence of chronic pain was 8.4% in men expecting a child and 11.2% in men within two years after delivery, whereas it was 10.1% in women during pregnancy and 18.5% in women within two years after delivery. The proportion of men experiencing death fantasies was 11.4% among those expecting a child, and 7.9% within two years after delivery. For women, these figures were 8.7% and 10.6%, respectively.

Recent psychological distress

Figure 2 shows the association between conception method and recent psychological distress. When compared with naturally conceived intended pregnancy, the following patterns were observed: In male partners during pregnancy (Fig. 2 [a] upper left), there was a non-significant trend toward a higher risk of recent psychological distress in cases of naturally conceived unintended pregnancy and SI/OI (with wide CIs), whereas lower risks were associated with IUI and IVF/ICSI. For men within two years after delivery (Fig. 2 [b] lower left),

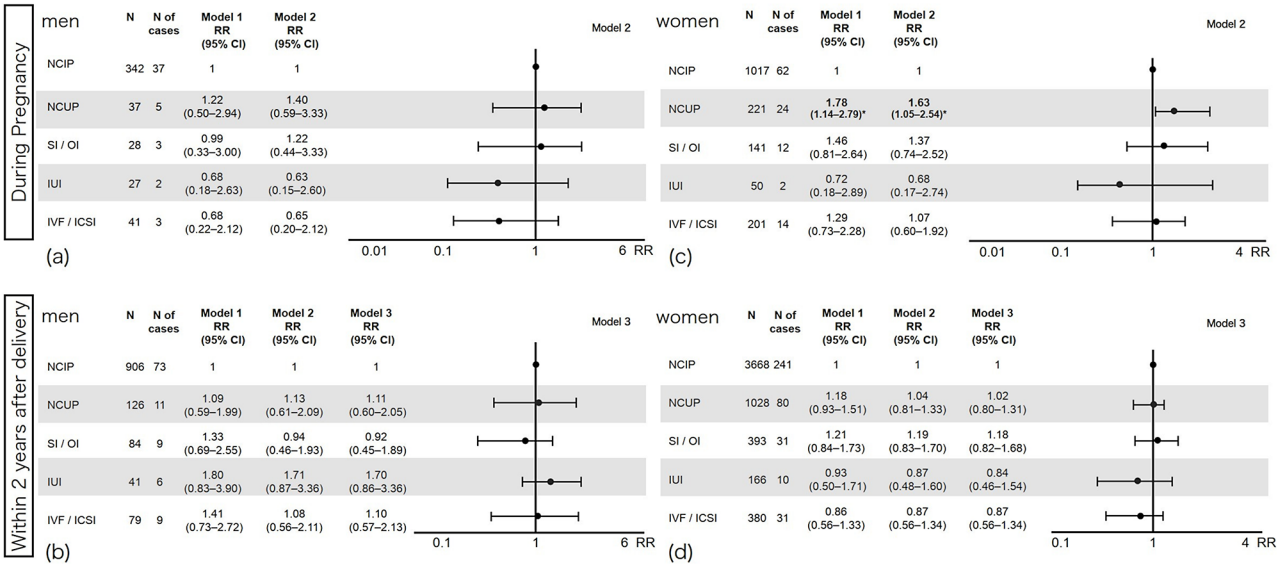

Fig. 2 Conception methods and psychological distress. The figure illustrates the relative risks and 95% confidence intervals of psychological distress by conception methods. The X-axis shows the relative risk of psychological distress. The upper left quadrant (**a**) shows male parents whose partners were pregnant; the lower left (**b**) shows male parents in early parenthood; the upper right (**d**) shows female parents during pregnancy; and the lower right (**d**) shows female parents in early parenthood. CI, Confidence interval; ICSI, Intracytoplasmic sperm injection; IUI, Intrauterine insemination; IVF, In vitro fertilization; N, Number; NCIP, naturally conceived intended pregnancy; NCUP, Naturally conceived unintended pregnancy; OI, Ovulation inducer; RR, Relative risk; SI, Scheduled intercourse

naturally conceived unintended pregnancy, IUI, and IVF/ICSI were associated with a non-significant trend toward higher risk of recent psychological distress, whereas the risk was lower for SI/OI. However, none of these observed trends reached statistical significance.

Figure 2 (c) shows the results for women during pregnancy. Naturally conceived unintended pregnancy was associated with a significantly increased the risk of recent psychological distress within 30 days (Model 2: RR 1.63, 95% CI 1.05–2.54, $p<0.05$) among women who were still pregnant. However, this higher risk of recent psychological distress was not observed among women who had already given birth to a naturally conceived unintended pregnancy (Fig. 2 [d] lower right). During pregnancy, there was a non-significant trend towards a higher risk associated with SI/OI, and IUI was linked with a lower risk of recent psychological distress (Fig. 2 [c] upper right). Conversely, within two years after delivery (Fig. 2 [d] lower right), SI/OI exhibited a non-significant trend toward a higher risk of recent psychological distress (with wide CIs), whereas IUI and IVF/ICSI were associated with a trend toward a lower risk of recent psychological distress. However, none of these trends reached statistical significance.

Chronic pain

Figure 3 shows the association between conception method and prevalence of chronic pain. Compared with naturally conceived intended pregnancy, the following patterns were observed.

For male partners during pregnancy (Fig. 3 [a] upper left), the risk of prevalence of chronic pain in cases of naturally conceived unintended pregnancy tended to be twice as high (with wide CIs), and the risk was also higher for SI/OI. However, the risks for chronic pain prevalence with IUI and IVF/ICSI showed a non-significant trend towards being lower (with wide CIs). These results did not reach statistical significance. Conversely, for males during early parenthood (Fig. 3 [b] lower left), the risk of chronic pain prevalence with IVF/ICSI was similar to that of naturally conceived intended pregnancy, whereas naturally conceived unintended pregnancy and IUI were associated with a higher risk. For SI/OI, the risk of chronic pain prevalence was significantly higher (Model 3: RR 1.75, 95% CI 1.01–3.05, $p<0.05$).

For women during pregnancy (Fig. 3 [c] upper right), the risk of chronic pain prevalence for naturally conceived unintended pregnancy was significantly higher (Model 2: RR 1.63, 95% CI 1.14–2.33, $p<0.01$). Although not statistically significant, there was a slight, non-significant trend indicating a higher risk of chronic pain prevalence with SI/OI, and a lower risk with IUI. The risk of chronic pain prevalence with IVF/ICSI was significantly lower (Model 2: RR 0.44, 95% CI 0.22–0.87, $p<0.05$). After delivery (Fig. 3 [d] lower right), the higher risk of chronic pain prevalence observed during pregnancy for naturally conceived unintended pregnancies decreased, indicating that the association was limited to the pregnancy period. While there was a slight tendency for a higher risk of chronic pain prevalence with IVF/ICSI

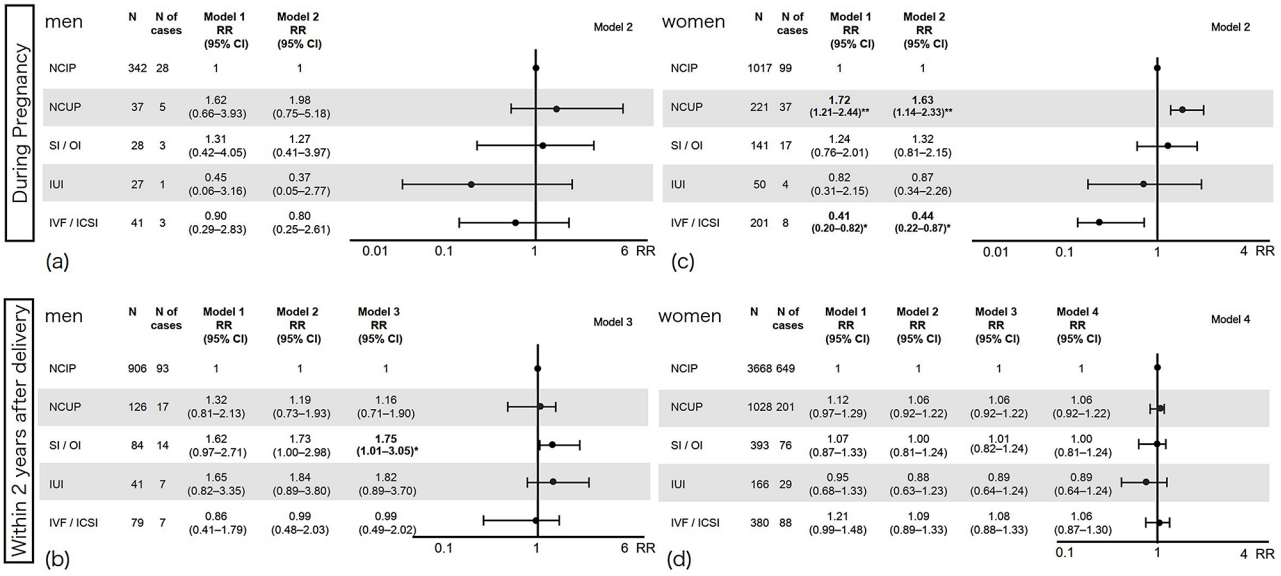

Fig. 3 Conception methods and chronic pain. The figure illustrates the relative risks and 95% confidence intervals of prevalence of chronic pain by conception methods. The X-axis shows the relative risk of the prevalence of chronic pain. The upper left quadrant (**a**) shows male parents whose partners were pregnant; the lower left (**b**) shows male parents in early parenthood; the upper right (**c**) shows female parents during pregnancy; and the lower right (**d**) shows female parents in early parenthood. CI, Confidence interval; ICSI, Intracytoplasmic sperm injection; IUI, Intrauterine insemination; IVF, In vitro fertilization; N, Number; NCIP, naturally conceived intended pregnancy; NCUP, Naturally conceived unintended pregnancy; OI, Ovulation inducer; RR, Relative risk; SI, Scheduled intercourse

(with wide CIs) and a lower risk with IUI. none of these results reached statistical significance.

Death fantasies

Figure 4 shows the association between conception method and death fantasies. When compared with naturally conceived intended pregnancies, the following patterns were observed.

For men whose partners were pregnant (Fig. 4 [a] upper left), the risk of death fantasies for naturally conceived unintended pregnancy and IVF/ICSI showed a non-significant trend towards being higher (with wide CIs), whereas the risk for SI/OI and IUI showed a non-significant trend towards being lower (with wide CIs). None of these findings were statistically significant. In contrast, men within two years after delivery (Fig. 4 [b] lower left) tended to have a higher risk of death fantasies in cases of naturally conceived unintended pregnancy and IVF/ICSI. The risk of death fantasies also tended to be higher for SI/OI. Men who had undergone IUI had a significantly higher risk of death fantasies (Model 3: RR 2.41, 95% CI 1.13–5.17, $p<0.05$).

For women during pregnancy (Fig. 4 [c] upper right), the risk of death fantasies was significantly higher for naturally conceived unintended pregnancy (Model 2: RR 2.18, 95% CI 1.50–3.18, $p<0.001$) and for SI/OI (Model 2: RR 1.76, 95% CI 1.05–2.96, $p<0.05$). Although not statistically significant, the risk of death fantasies in cases of IUI tended to be lower, whereas the risk for IVF tended to be higher. Conversely, women within two years after delivery (Fig. 4 [d] lower right) had a significantly higher risk of death fantasies for naturally conceived unintended pregnancy (Model 3: RR 1.40, 95% CI 1.17–1.67, $p<0.001$). The risk of death fantasies for IVF/ICSI was also significantly higher (Model 3: RR 1.40, 95% CI 1.04–1.88, $p<0.01$). The risk of death fantasies for SI/OI and IUI tended to be roughly equivalent or slightly lower compared with naturally conceived intended pregnancy. None of these findings were statistically significant.

Covariates

The covariate risks associated with mental health outcomes are shown in Supplemental Tables 2A, 2B, 3A, 3B, 4A and B in Additional File 2. Among the key covariates, history of depression and health problem of fetus/infant/child were strongly associated with mental health risks during pregnancy and up to two years postpartum. Increasing age was linked to higher levels of chronic pain and lower levels of death fantasies in postpartum women. Regarding socioeconomic status, low education was associated with recent psychological distress in both pregnant women and men. Low income showed significant associations with recent psychological distress, chronic pain, and death fantasies in postpartum women. In terms of substance use, smoking was linked to recent psychological distress in postpartum women, as well as to chronic pain and increased death fantasies in men. Alcohol consumption was associated with lower recent

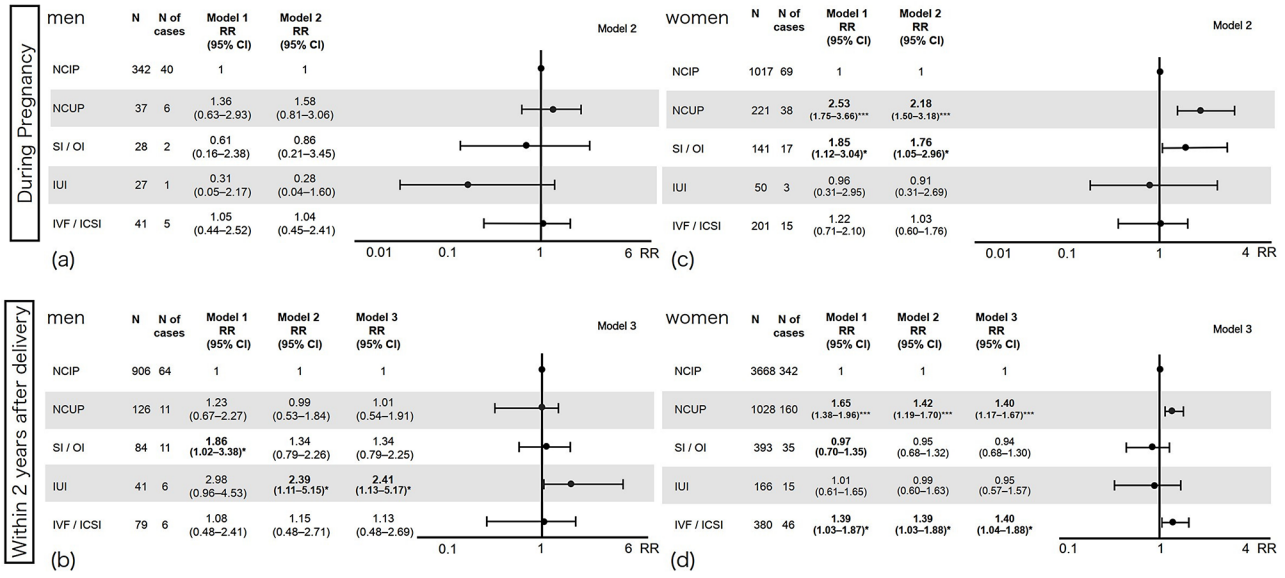

Fig. 4 Conception methods and death fantasies. The figure illustrates the relative risks and 95% confidence intervals of death fantasies by conception methods. The X-axis shows the relative risk of death fantasies. The upper left quadrant (**a**) shows male parents whose partners were pregnant; the lower left (**b**) shows male parents in early parenthood; the upper right (**c**) shows female parents during pregnancy; and the lower right (**d**) shows female parents in early parenthood. CI, Confidence interval; ICSI, Intracytoplasmic sperm injection; IUI, Intrauterine insemination; IVF, In vitro fertilization; N, Number; NCIP, Naturally conceived intended pregnancy; NCUP, Naturally conceived unintended pregnancy; OI, Ovulation inducer; RR, Relative risk; SI, Scheduled intercourse

psychological distress and death fantasies in men, but in postpartum women, it was linked to increased chronic pain and death fantasies during pregnancy. Notably, several covariates showed stronger associations with mental health outcomes than conception method variables, including unintended pregnancy itself.

Discussion

We used three markers to assess mental health risks: recent levels of psychological distress, chronic pain as a somatic symptom, and death fantasies. The results of this study suggest that mental health risks associated with unintended natural pregnancies or fertility treatments may vary depending on the time before and after childbirth, as well as the parent's gender. These findings are consistent with previous meta-analyses showing an increased risk of depression and stress, although variations were observed according to gender and conception method [45, 46]. Notably, women with naturally conceived unintended pregnancies showed a higher tendency for psychological distress during pregnancy, but this distress tended to decrease after childbirth. Moreover, several covariates, such as history of depression, fetal/infant/child health problems, and socioeconomic status, showed stronger associations with mental health outcomes than the conception method variable itself. However, caution is needed in interpreting these results, considering the study's limitations. These findings highlight the need for flexible and comprehensive mental health support for parents who experience unintended pregnancies or undergo fertility treatments, both during pregnancy and after childbirth. The limitations of this study, including those discussed below, must be taken into account when interpreting these results.

Naturally conceived unintended pregnancy significantly was associated with the risk of recent psychological distress, chronic pain, and death fantasies in women. Notably, recent psychological distress within the past 30 days was higher during pregnancy for women with naturally conceived unintended pregnancies, but lower after delivery, suggesting that the distress may be related to the pregnancy period rather than being prolonged. In men, non-significant trends toward increased risks were observed, but these did not reach statistical significance. Given the confidence intervals, these trends may be clinically important. This finding is consistent with previous studies showing that unintended pregnancy increases the risk of maternal depression and stress, although these risks may diminish as parents adjust after childbirth [47]. These findings support meta-analyses showing an association between naturally conceived unintended pregnancy and increased risk of depression, although this risk may decrease over time [48]. Although this study did not examine whether or not the women were planning to have an abortion during their pregnancy, it is possible that the strong psychological stress of considering whether or not to have an abortion or of having an abortion planned for the near future may have affected

the women with naturally conceived unintended pregnancies. They may be associated with feelings of pressure from the male partner, family, or others to have an abortion that is contrary to the woman's own values and preferences. The mental health risks related to pregnancy loss including abortion on mental health are well known [35–37, 39, 49]. Therefore, it is important to consider the negative impact that unintended pregnancies can have on mental health, especially during pregnancy.

Our findings from this study support recent critiques of the unintended pregnancy framework, as highlighted by Auerbach et al. (2023) [50], suggesting that underlying factors may play a greater role in mental health outcomes than pregnancy intention itself. The strong association between mental health risks during pregnancy and postpartum supports the argument that pre-existing mental health conditions are more predictive of postpartum mental health than pregnancy intention [50]. In addition, our findings add to the evidence that the relationship between unintended pregnancy and mental health is complex and multifaceted [50]. Pre-existing mental health problems may influence both the perception of a pregnancy as unintended and the subsequent postpartum challenges [51]. This perspective shifts the focus from the unintended pregnancy itself to the broader context of pre-pregnancy mental health conditions. This interpretation is consistent with the findings of comprehensive reviews, as highlighted in a previous report, which concluded that abortion is not a direct cause of mental health problems [49]. Instead, associations often reflect pre-existing or concurrent mental health conditions associated with unintended pregnancy or abortion. These findings underscore the importance of addressing the broader psychosocial factors that shape both pregnancy intentions and mental health outcomes. In light of these findings, future research should take a more nuanced approach that includes the broader social and structural context, including pre-existing mental health conditions, socioeconomic factors, and other potential confounders. This comprehensive approach will better capture the interplay between pregnancy intentions and mental health outcomes.

We found that during pregnancy, SI/OI use was generally associated with higher mental health risks for both men and women, compared with individuals who had children through naturally conceived intended pregnancies. Notably, men who used SI/OI during early parenthood also demonstrated a significantly higher risk of chronic pain and death fantasies than men who experienced a naturally conceived intended pregnancy. Conversely, women who used SI/OI during early parenthood did not show an increased mental health risk. The reasons for the observed differences in mental health risks, with men showing an increased risk of chronic pain and death fantasies while women did not, are unclear, but the stress associated with timed intercourse may place additional strain on relationships. Women may be less affected during early parenthood, when the pressure to time intercourse has subsided.

This study is the first to categorize assisted reproductive technology (ART) into IUI and IVF/ICSI to examine their differential effects on mental health risks in male and female parents in early parenthood. During pregnancy, participants who had children using ART demonstrated lower or the same mental health risks as those who had children through naturally conceived intended pregnancy. It is reasonable to assume that individuals who have had a child after enduring the difficulties of infertility and the physical and financial burdens of ART have lower mental health risk than those who conceived easily without such challenges. Men who used IUI during early parenthood showed an unexpected increase in risk of death fantasies, whereas IVF had no effect on mental health outcomes at any stage. For women, ART did not generally increase mental health risks, with the exception of postpartum death fantasies among IVF/ICSI users. The reasons why IUI had a greater effect on men during early parenthood remain unclear.

Differences in stress experienced by men undergoing IUI and IVF/ICSI may reflect factors such as the timing of semen sample collection, which member of the couple is infertile, and differences in men's perception of their role in fertility treatment. IUI semen sample collection is typically timed to coincide with ovulation, which is predicted by monitoring ovarian follicle growth. In contrast, IVF tends to be more predictable, with semen collection typically timed to a controlled cycle. Additionally, when IUI is used, there is often no cause of female infertility (e.g., tubal occlusion), which may increase pressure on the male partner. Furthermore, in Japan, fertility treatments typically progress from SI/OI to IUI (up to six attempts) and finally to IVF [52], which may increase psychological relief for men after successful IVF attempts. A previous study also showed that women who used IVF or ICSI experienced less recent psychological distress than those who used OI or male artificial insemination [53], which is consistent with our findings.

This study's approach of investigating both naturally conceived unintended pregnancies and those resulting from fertility treatments offers a more comprehensive understanding of how pregnancy intention affects mental health. While these scenarios represent opposite ends of the pregnancy intentionality spectrum, they share certain mental health impacts. Women with unintended pregnancies experienced higher risks of psychological distress, chronic pain, and death fantasies during pregnancy, likely due to unexpected stressors and lack of preparation associated with unplanned pregnancies. In contrast,

women who underwent IVF/ICSI showed a significantly lower risk of chronic pain during pregnancy, possibly reflecting the extensive medical support and psychological preparation involved in fertility treatments.

However, despite these differences, both groups also share some common mental health challenges. For example, while the IVF/ICSI group exhibited a lower risk of chronic pain during pregnancy, they showed a higher risk of suicidal ideation and death fantasies during early parenthood. This might reflect a mismatch between the expectations formed during long-term fertility treatment and the realities of parenting, or a lack of post-treatment support. Similarly, unintended pregnancies, while associated with heightened stress during pregnancy, may lead to ongoing relationship tensions and adjustment difficulties in the postpartum period.

Both unintended pregnancy and fertility treatment can contribute to mental health risks through direct stressors, such as the emotional and financial burdens of treatment or the strain of an unplanned pregnancy. Relationship deterioration of fertility treatment is a common reason for discontinuation [6]. These stressors may also lead to indirect impacts, such as relationship strain and disruptions to work or social life. While the immediate stress from fertility treatments after childbirth, relationship tensions and difficulties in accepting an unintended pregnancy could persist, potentially contributing to mental health risks in the postpartum period. Further research is needed to explore these shared and distinct pathways.

This is the first study to indicate that men who use IVF are not associated with increased psychological risk during pregnancy and early parenthood. These findings provide psychological reassurance to both individuals and health care providers. However, men undergoing SI/OI and IUI may face increased mental health risks during early parenthood. Targeted mental health support is critical for male parents using these treatments, as well as for female parents coping with unintended pregnancy.

There are several limitations that should be considered when interpreting these findings. First, the data do not necessarily represent all residents in Japan, and respondents to web-based surveys may differ in important ways from the general population. However, the data were obtained by sending random invitations to many registrants across Japan, and therefore have some validity regarding representation. Second, this was a cross-sectional study, so temporal causality cannot be assumed. For instance, individuals at psychological risk may be more likely to experience infertility and to use ART [54]. However, this seems unlikely because mental health risk among participants who used IVF did not increase during pregnancy and early parenthood. Moreover, the results remained unchanged after the model was adjusted for depression history. Third, this study was conducted during the COVID-19 pandemic in 2021. The pandemic may have exacerbated negative emotions and thus increased the risk of mental health problems. Fourth, we did not consider the duration of fertility treatment. Generally, even with the same conception method, patient burden increases with longer duration, so length of treatment may have affected the findings. Fifth, while we accounted for pregnancy loss by calculating the difference between the number of pregnancies and live births, this approach does not allow us to distinguish between induced abortions, miscarriages, and stillbirths. In addition, our definition of recurrent pregnancy loss does not take into account whether losses were consecutive, which is the standard criterion. This limitation may affect the precision of our findings regarding the impact of pregnancy loss on psychological outcomes. Sixth, the potential overlap of two different time periods—during pregnancy and postpartum—for women who gave birth or had an abortion in 2021, which may affect the interpretation of the results. Additionally, we did not collect data on whether pregnant women were considering abortion, planning to abort, or facing pressure to abort from others, which may have contributed to psychological distress across all three outcome measures. Furthermore, the measure used to assess death fantasies was not a validated scale for suicidal risk, which may limit the interpretation of the results in this area. Seventh, the study used a binary measure of pregnancy intention rather than a specific scale, which may have missed the more nuanced aspects of participants' attitudes. Respondents were asked directly whether the pregnancy was considered "intended" or "unintended." This binary approach may not fully capture the complexity of pregnancy intentions. As Santelli et al. (2009) suggest [55], pregnancy intentions are multidimensional and include aspects such as the timing of pregnancy, emotional reactions, and partner-specific factors. The lack of a more detailed measure in our study limits the depth of insight into participants' pregnancy intentions and may obscure important variations that could affect the observed outcomes.

Conclusions

Markers for mental health risks associated with unintended naturally conceived pregnancy or fertility treatment varied before and after live births, as well as according to the parent's gender. Notably, levels of recent psychological distress within the past 30 days were higher during pregnancy for women with naturally conceived unintended pregnancies, but declined after delivery. Flexible and comprehensive mental health support is needed both during pregnancy and within two years after delivery for individuals who conceived unintentionally or through fertility treatment.

Abbreviations

IVF	In vitro fertilization
COVID	Coronavirus Disease
JACSIS	The 2021 Japan Coronavirus Disease-19 and Society Internet Survey
SI	Scheduled intercourse
OI	Ovulation inducer
IUI	Intrauterine insemination
ICSI	Intracytoplasmic sperm injection
K6	Kessler Psychological Distress Scale
ICD-11	International Classification of Diseases 11th Revision
VIF	Variance inflation factor
RR	Relative risk
CI	Confidence interval
SAS	Statistical Analysis Software
ART	Assisted reproductive technology

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12884-024-07082-x.

Supplementary Material 1

Supplementary Material 2

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Author contributions

Conceptualization, N.M. and K.Y.; Data curation, T.T.2; Formal analysis, N.M. and K.Y.; Funding acquisition, T.T.2; Investigation, N.M.; Methodology, N.M., K.Y., and T.K.; Project administration, N.M. and K.K.; Resources, K.Y. and T.T.2; Software, N.M. and K.Y.; Supervision, K.K.; Validation, N.M., K.Y., and T.K.; Visualization, N.M.; Roles/Writing - original draft N.M. and K.Y.; and Writing - review and editing, T.K., Y.U., T.T.1, T.T.2, and K.K.

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Data availability

The data used in this study are not publicly available because they contain personally identifiable or potentially sensitive patient information. Determining whether data are truly non-identifying can be challenging, especially when combining multiple low-frequency responses, which could lead to participant identification. For this reason, we obtained approval from the Research Ethics Committee of the Osaka International Cancer Institute under the condition that data would not be redistributed without explicit participant consent. However, we are open to sharing de-identified data, pending consultation with the Ethics Committee to ensure compliance with ethical guidelines. Researchers interested in accessing the data should contact Dr. Takahiro Tabuchi at tabuchitak@gmail.com. Requests will be reviewed on a case-by-case basis.

Declarations

Ethics approval and informed consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Electronic informed consent was obtained from all patients for being included in the study. Participants were provided with credit points known as 'Rakuten Points,' which can be used for internet shopping or converted into cash, as an incentive for completing the survey. The study was approved by the Institutional Review Board of the Osaka International Cancer Institute (approval number 20084-8) and the Institutional Review Board of Toho University (approval number A23057_A23001).

Consent to publish

Not applicable.

Competing interests

The authors declare no competing interests.

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Publisher's note

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Calcium, Vitamin D, and Dairy Intake and Premenstrual Syndrome: A Cross-Sectional Study

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Summary Calcium and vitamin D have been suggested to be associated with the amelioration of symptoms of premenstrual syndrome (PMS). However, evidence for an association between the dietary intake of these nutrients and PMS is limited and inconsistent. We examined the cross-sectional association of calcium and vitamin D intake with PMS. Participants were 390 women aged 22–49 y who responded to a mail survey in 2022–2023. Dietary intake was assessed using a validated self-administered diet history questionnaire. PMS were assessed using the Premenstrual Symptoms Questionnaire. Logistic regression analysis was used to estimate odds ratios of PMS according to tertiles of calcium and vitamin D intake with adjustment for potential confounding variables. The prevalence of moderate to severe PMS was 10% (39 women). Neither calcium nor vitamin D intake was significantly associated with PMS. However, calcium intake was associated with a decreased prevalence of PMS, albeit without statistical significance, with multivariable-adjusted odds ratios (95% CI) for PMS in the lowest through highest tertiles of calcium intake of 1.00 (reference), 0.47 (0.18–1.25), and 0.27 (0.07–1.08), respectively (p for trend=0.06). The odds ratio of PMS was low in the highest tertile of vitamin D intake compared with the lowest, but without statistical significance (odds ratio 0.56, 95% CI 0.19–1.66). Our findings suggest that calcium and vitamin D intake was not appreciably associated with PMS. The suggestive inverse association between calcium intake and PMS requires further investigation.

Key Words calcium, dairy, Japanese, premenstrual syndrome, vitamin D

Premenstrual syndrome (PMS) is identified by mood, behavioral, and physical symptoms during the luteal phase of the menstrual cycle, which often resolve by the end of menstruation (*1*, *2*). Although the symptoms are typically mild, 5–8% of women experience moderate to severe symptoms which interfere with personal or social relationships or work (*2*). In Japan, Takeda et al. (*3*) reported a prevalence of moderate to severe PMS among 1,187 women aged 20–49 y of 5.3%, and estimated that 1.8 million women currently suffer from untreated premenstrual disorders. These numbers indicate the importance of identifying the determinants of PMS in preventing or reducing its symptoms and improving quality of life in women. While the etiology of PMS remains unclear, suggested factors include not only fluctuations in hormone level and neurotransmitters but also lifestyle, including diet, alcohol consumption, smoking, and physical activity (*4*–*9*).

Circulating levels of calcium and vitamin D decrease during the luteal phase of the menstrual cycle when PMS symptoms appear, which has been suggested to cause the symptoms of PMS (*10*). Randomized trials have found that calcium supplementation has significant positive effects on the PMS symptoms (*11*). Interventional studies have also reported that vitamin D supplementation is effective in ameliorating PMS symptoms (*12*). Dietary intake of calcium and vitamin D are therefore suggested to be associated with reductions in the symptoms and risk of PMS. However, observational studies on this issue are limited. In a case-control study nested within the Nurses' Health Study (NHS) II (*4*), dietary intake of calcium and vitamin D was significantly associated with decreased risk of PMS among 3,025 women. In contrast, a US cross-sectional study observed no association between calcium intake and premenstrual symptoms among 3,013 women (*13*). Other cross-sectional studies have examined the association between them, but findings have been inconsistent, with small sample sizes (30–<200 participants) and a lack of adjustment for factors associated with PMS (*14*–*20*). Here, we examined the cross-sectional association of calcium, vitamin D, and dairy intake

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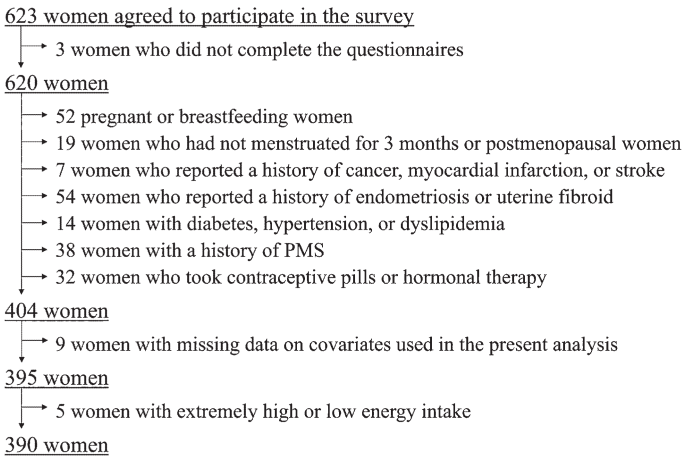

Fig. 1. Flowchart of study participants.

with PMS in Japanese women.

MATERIALS AND METHODS

Study procedure. The study was conducted under a cross-sectional design. A mail survey was conducted among graduates of Fukuoka Women's University, Fukuoka, Japan. Of 5,456 graduates from all faculties and departments, including nutrition, liberal arts, and science departments from fiscal years 1995 to 2021, 3,567 graduates were invited to participate, after excluding those whose address was unknown. We mailed two types of survey questionnaire, one specifically designed for diet and the second for overall health-related lifestyle, including PMS, in March and November 2022, and asked participants to fill in the questionnaires and then return them. We re-sent the questionnaires to non-respondents in July 2023 and again asked them to participate in the study. This study was approved by the Ethics Committee of Fukuoka Women's University (2021-29). Written informed consent was obtained from all participants.

Participants. Of 3,567 participants, surveys sent to 311 were returned without a response due to address change, etc. Among the remaining 3,256 participants, 623 participated in the survey (response rate 19%). Of these, we excluded 3 participants who did not complete the questionnaires (Fig. 1). We then excluded 52 participants who were pregnant or breastfeeding; 19 who had not menstruated for 3 mo or were postmenopausal; 7 who reported a history of cancer, myocardial infarction, or stroke; 54 who reported a history of endometriosis or uterine fibroids; 14 with diabetes, hypertension, or dyslipidemia; 38 with a history of PMS; and 32 who took the contraceptive pill or hormonal therapy. Of the remaining 404 women, we further excluded 9 participants with missing data on covariates used in the present analysis, and 5 with extremely high or low energy intake (exceeding mean±3 standard deviations). Finally, 390 women aged 22–49 y were included in the analysis.

Dietary assessment. Dietary habits during the preceding month were assessed using a validated brief self-administered diet history questionnaire (BDHQ) (*21*, *22*). Participants were asked about the frequency of intake of 46 foods and non-alcoholic beverages, daily frequency of rice and miso soup intake, frequency of alcohol consumption and amount consumed per typical drinking occasion for five alcoholic beverages, usual cooking methods, and dietary behaviors. Intake of energy and selected nutrients was estimated based on the intake of food items obtained with the BDHQ and the corresponding food composition list in the Standard Tables of Food Composition in Japan (*23*). A validation study of the BDHQ using 16-d weighed dietary records as gold standard in 92 women aged 31–69 y reported a Spearman's correlation coefficient for energy-adjusted intake of dairy products of 0.54 (*22*), and Pearson correlation coefficients for energy-adjusted intake of 0.51 for calcium, 0.35 for vitamin B1, 0.52 for vitamin B2, 0.49 for vitamin B6, 0.56 for potassium, and 0.53 for iron (*21*).

We obtained information on the use of calcium supplements, but supplemental calcium intake was not incorporated into the analysis due to the small number of participants who used calcium supplements ($n=7$). In addition, we did not measure the use of vitamin D supplements.

Premenstrual syndrome. PMS were assessed using the Premenstrual Symptoms Questionnaire (PSQ) developed by Takeda et al. (*3*). The PSQ consists of 11 symptoms, including depressed mood, anxiety or tension, tearfulness, anger or irritability; decreased interest in work, home, or social activities; difficulty concentrating; fatigue or lack of energy; overeating or food cravings; insomnia or hypersomnia; feeling of being overwhelmed; and physical symptoms such as tender breasts, feeling of bloating, headache, joint or muscle pain, or weight gain experienced 1–2 wk before menses. In addition, the PSQ asked whether the premenstrual symptoms interfered with work efficiency or productivity, or home responsibilities; social activities; and relationships with coworkers or family. Participants were asked to denote the severity of premenstrual symptoms and their interference with activities from four options

(not at all, mild, moderate, or severe). We divided participants with premenstrual symptoms into three groups: "no/mild PMS," "moderate to severe PMS," and "premenstrual dysphoric disorder (PMDD)" according to criteria reported previously (*3*). Since few participants were identified as having PMDD (n=9), we combined them together with those having moderate to severe PMS. The PSQ is useful, and its reliability and validity have been fully evaluated (*3*, *24*, *25*).

Other variables. Information on age at menarche, body height, body weight, pregnancy or childbirth experience, employment status, overtime work, leisure-time physical activity, physical activity during work and housework or while commuting, alcohol consumption, and supplement use was elicited in the questionnaire. Body mass index was calculated using self-reported body height and body weight.

Statistical analysis. Participants were divided into tertiles of energy-adjusted intake (by the density method) of calcium, vitamin D, and dairy. Characteristics of participants according to tertile of calcium, vitamin D, and dairy intake were expressed as means (standard deviation) for continuous variables and percentage for categorical variables. Trend associations between confounding factors and calcium, vitamin D, and dairy intake were tested using linear regression analysis for continuous variables, treating the median intake in each tertile of calcium, vitamin D, and dairy as a continuous variable, and the Mantel-Haenszel chi-square test for categorical variables.

We used multiple logistic regression analysis to estimate odds ratios and 95% confidence intervals (CIs) of moderate to severe PMS for tertiles of calcium, vitamin D, and dairy intake, taking the lowest tertile category as reference. The first model was adjusted for age (y) and survey period (March in 2022, November in 2022, or July in 2023), and the second model was further adjusted for age at menarche (<15 or ≥15 y old), body mass index (kg/m^2), pregnancy or childbirth experience (yes or no), employment status and overtime work (unemployed or employed and overtime work <11 or ≥11 h/mo), moderate to vigorous physical activity in leisure time (min/d, tertile), sedentary time during work and housework or while commuting (tertile; <5, 5–<9, or ≥9 h/d), alcohol consumption (non-drinker or drinker ≥1 d/mo), supplement use (regardless of the type of supplement) (yes or no), and total energy intake (kcal/d). Since the number of current smokers was two, we did not adjust for smoking status. In the analysis of calcium and vitamin D, as the third model, intake (/1,000 kcal) of vitamin B6 (mg), vitamin B1 (mg), vitamin B2 (mg), potassium (mg), and iron (mg) was further adjusted for, and intake of calcium (mg) and vitamin D (μg) was mutually adjusted for. Trend associations were assessed by treating the median intake in each tertile of calcium, vitamin D, and dairy intake as a continuous variable.

Vitamin D enhances intestinal absorption of calcium and the renal conservation of absorbed calcium (*26*). We therefore analyzed the association between calcium and PMS by vitamin D intake (<median or ≥median) to examine the joint association of calcium and vitamin D with PMS. An interaction term, created by multiplying calcium intake (tertile) and vitamin D intake (dichotomous), was added to the model to assess statistical interactions. Two-sided p values <0.05 were considered to indicate statistical significance. All analyses were conducted using Statistical Analysis System (SAS) software version 9.4 (SAS Institute, Cary, NC, USA).

Table 1. Characteristics of participants.

	Mean±SD (range) or n (%)
No. of participants	390
Age (y)	31.4±8.0 (22–49)
BMI (kg/m^2)	20.2±2.3 (16.0–32.4)
Age at menarche (<15 y old)	355 (91.0)
Pregnancy or childbirth experience (yes)	95 (24.4)
Current drinker (≥1 d/mo)	216 (55.4)
Current smoker (yes)	2 (0.51)
Employment status (yes)	347 (89.0)
Overtime work (≥11 h/mo)[1]	143 (41.2)
Leisure-time physical activity (high)[2]	98 (25.1)
Sedentary time during work (high)[3]	106 (27.2)
Supplement use (yes)	122 (31.3)
Total energy intake (kcal/d)	1,480±398 (241–2,860)
Nutrient intake (/d)	
Vitamin B6 (mg/1,000 kcal)	0.71±0.18 (0.22–2.03)
Vitamin B1 (mg/1,000 kcal)	0.44±0.09 (0.18–0.71)
Vitamin B2 (mg/1,000 kcal)	0.74±0.18 (0.27–2.03)
Potassium (mg/1,000 kcal)	1,401±379 (545–3,790)
Iron (mg/1,000 kcal)	4.45±1.14 (1.81–14.28)
Calcium (mg/1,000 kcal)	281±90 (85–831)
Vitamin D (μg/1,000 kcal)	5.92±4.12 (0.26–56.46)

[1] The denominator was the number of participants who reported yes to their employment status.
[2] Moderate to vigorous physical activity in leisure time of ≥10.5 min/d.
[3] Sedentary time during work and housework or while commuting ≥9 h/d.

RESULTS

In the study participants, the mean (standard deviation) of age and BMI was 31.4 (8.0) y and 20.2 (2.3) kg/m^2, respectively (Table 1). The characteristics of the study participants by tertiles of calcium, vitamin D, and dairy intake are shown in Table 2. Participants with higher intake of calcium and vitamin D were more likely to be older and to have higher consumption of vitamin B6, vitamin B1, vitamin B2, potassium, and iron, and less likely to work compared with those with lower intake. In addition, participants with higher intake of calcium were less likely to be current drinkers. Participants with higher dairy intake were less likely to be current drinkers and to work. They also consumed more vitamin B1, vitamin B2, potassium, and calcium.

Table 2. Characteristics of participants according to tertile (T) of calcium, vitamin D, and dairy intake.

	Calcium intake			Vitamin D intake			Dairy intake		
	T1 (low)	T3 (high)	*p* for trend[1]	T1 (low)	T3 (high)	*p* for trend[1]	T1 (low)	T3 (high)	*p* for trend[1]
No. of participants	130	130		130	130		130	130	
Age (mean±SD, y)	30.0±6.8	32.6±8.9	<0.01	30.2±7.3	33.1±8.9	<0.01	31.2±7.8	31.9±8.3	0.47
BMI (mean±SD, kg/m^2)	20.4±2.6	20.2±1.9	0.43	20.5±2.5	20.2±2.3	0.27	20.0±2.3	20.5±2.1	0.11
Age at menarche (<15 y old, %)	91.5	90.0	0.66	93.1	91.5	0.74	91.5	89.2	0.51
Pregnancy or childbirth experience (%)	19.2	25.4	0.26	22.3	25.4	0.58	23.8	25.4	0.77
Current drinker (≥1 d/mo, %)	62.3	46.2	<0.01	55.4	53.1	0.67	60.8	46.2	0.017
Current smoker (%)	1.5	0.0	0.086	0.8	0.8	0.93	1.5	0.0	0.09
Employment status (yes, %)	95.4	84.6	<0.01	91.5	83.8	0.039	94.6	84.6	0.011
Overtime work (≥11 h/mo, %)[2]	45.2	40.9	0.50	42.0	44.0	0.72	39.0	37.3	0.80
Leisure-time physical activity (high, %)[3]	22.3	32.3	0.061	23.1	26.2	0.59	28.5	30.8	0.62
Sedentary time during work (high, %)[4]	27.7	27.7	1.00	27.7	25.4	0.65	26.9	27.7	0.89
Supplement use (yes, %)	26.9	36.2	0.11	26.9	32.3	0.40	29.2	33.8	0.42
Total energy intake (kcal/d)	1,479±422	1,420±389	0.22	1,459±420	1,487±441	0.61	1,464±433	1,448±371	0.72
Nutrient intake (mean±SD,/d)									
Vitamin B6 (mg/1,000 kcal)	0.60±0.13	0.82±0.21	<0.01	0.60±0.14	0.82±0.20	<0.01	0.71±0.21	0.73±0.16	0.26
Vitamin B1 (mg/1,000 kcal)	0.38±0.07	0.50±0.08	<0.01	0.40±0.09	0.48±0.08	<0.01	0.43±0.10	0.47±0.08	<0.01
Vitamin B2 (mg/1,000 kcal)	0.60±0.13	0.89±0.18	<0.01	0.65±0.17	0.82±0.19	<0.01	0.67±0.20	0.84±0.17	<0.01
Potassium (mg/1,000 kcal)	1,100±221	1,707±392	<0.01	1,215±329	1,596±409	<0.01	1,335±420	1,518±356	<0.01
Iron (mg/1,000 kcal)	3.71±0.70	5.20±1.30	<0.01	3.84±0.92	5.02±1.25	<0.01	4.52±1.40	4.50±1.01	0.93
Calcium (mg/1,000 kcal)	191±33	378±72	<0.01	235±79	334±93	<0.01	228±89	348±75	<0.01
Vitamin D (μg/1,000 kcal)	4.07±2.01	8.02±5.87	<0.01	2.97±0.96	9.56±5.23	<0.01	5.86±5.83	6.19±2.92	0.51

[1] Based on the Mantel-Haenszel chi-square test for categorical variables and linear regression analysis for continuous variables, assigning median intake to tertile of calcium, vitamin D, or dairy intake.

[2] The denominator was the number of participants who reported yes to their employment status.

[3] Moderate to vigorous physical activity in leisure time of ≥10.5 min/d.

[4] Sedentary time during work and housework or while commuting ≥9 h/d.

Table 3. Odds ratio (95% confidence interval) of moderate to severe premenstrual syndrome according to tertile (T) of calcium, vitamin D, and dairy intake.

	T1 (low)	T2	T3 (high)	*p* for trend[1]
Calcium intake				
Median (mg/1,000 kcal)	192.4	274.3	360.6	
No. of participants	130	130	130	
No. of cases	17	11	11	
Model 1[2]	1.00 (reference)	0.70 (0.31–1.59)	0.77 (0.34–1.75)	0.50
Model 2[3]	1.00 (reference)	0.77 (0.33–1.79)	0.82 (0.34–1.96)	0.63
Model 3[4]	1.00 (reference)	0.47 (0.18–1.25)	0.27 (0.07–1.08)	0.060
Vitamin D intake				
Median (μg/1,000 kcal)	3.1	5.1	8.0	
No. of participants	130	130	130	
No. of cases	14	15	10	
Model 1[2]	1.00 (reference)	1.20 (0.54–2.64)	0.83 (0.35–1.99)	0.67
Model 2[3]	1.00 (reference)	1.26 (0.56–2.84)	0.83 (0.34–2.04)	0.68
Model 3[4]	1.00 (reference)	1.00 (0.43–2.37)	0.56 (0.19–1.66)	0.29
Milk and dairy product intake				
Median (g/1,000 kcal)	17.8	61.3	108.0	
No. of participants	130	130	130	
No. of cases	19	10	10	
Model 1[2]	1.00 (reference)	0.45 (0.19–1.03)	0.52 (0.23–1.18)	0.094
Model 2[3]	1.00 (reference)	0.44 (0.19–1.04)	0.51 (0.21–1.21)	0.099

CI, confidence interval; OR, odds ratio.

[1] Based on multiple logistic regression analysis, assigning median intake to tertile of calcium, vitamin D, or dairy intake.

[2] Adjusted for age (y) and survey month (March in 2022, November in 2022, and July in 2023).

[3] Additionally adjusted for age at menarche (<15 or ≥15 y old), body mass index (kg/m^2), pregnancy or childbirth experience (yes or no), employment status and overtime work (unemployed or employed and overtime work <11 or ≥11 h/mo), moderate to vigorous physical activity in leisure time (min/d, tertile), sedentary time during work and housework or while commuting (<5, 5–<9, or ≥9 h/d), alcohol consumption (non-drinker or drinker ≥1 d/mo), supplement use (yes or no), and total energy intake (kcal/d).

[4] Additionally adjusted for intake (/1,000 kcal) of vitamin B6 (mg), vitamin B1 (mg), vitamin B2 (mg), potassium (mg), and iron (mg). Models for calcium (mg) and vitamin D (μg) were mutually adjusted.

In total, 39 women (10%) were identified as having moderate to severe PMS. The odds ratios of moderate to severe PMS according to tertiles of calcium, vitamin D, and dairy intake are shown in Table 3. Calcium intake was not associated with PMS in model 1, which was adjusted for age and survey month, or in model 2, which was adjusted for covariates other than dietary factors. In model 3, which additionally adjusted for dietary factors, calcium intake was suggestively associated with decreased odds of PMS (p for trend=0.06), with multivariable-adjusted odds ratio (95% CI) of PMS for the lowest through highest tertile of calcium intake of 1.00 (reference), 0.47 (0.18–1.25), and 0.27 (0.07–1.08), respectively. The odds ratio of PMS in the highest tertile of vitamin D intake was lower than in the lowest tertile after adjustment for covariates, including dietary factors (0.56, 95% CI 0.19–1.66). In addition, the odds ratios of PMS in the middle and highest tertile of dairy intake tended to be approximately 50% lower than in the lowest tertile. However, these associations were not statistically significant (p for trend=0.29 for vitamin D intake and 0.099 for dairy intake).

In stratified analyses by vitamin D intake, the interaction was not statistically significant (p for interaction=0.87). The multivariable-adjusted odds ratio (95% CI) of PMS for the lowest through highest tertile of calcium intake was 1.00 (reference), 0.21 (0.04–1.03), and 0.14 (0.01–1.43) among participants with lower intake of vitamin D; and 1.00 (reference), 1.24 (0.17–8.81), and 0.86 (0.06–12.28) among those with higher intake.

DISCUSSION

In this cross-sectional study of Japanese women, calcium, vitamin D, and dairy intake were not significantly associated with PMS. However, we found a suggestive decrease in prevalence of moderate to severe PMS associated with calcium intake after adjustment for covariates. To our knowledge, this study is one of only a few that have examined the association between these by adjusting for factors associated with PMS, and is the first to investigate the association of vitamin D and dairy intake with PMS in Asia.

We observed a suggestive inverse association between calcium intake and PMS. This finding is consistent with those from some but not all previous studies. A case-control study nested within the NHS II observed that intake of calcium from food sources was associated with decreased risk of PMS among 1,057 women who developed PMS and 1,968 women with no diagnosis of

PMS aged 27–44 y (*4*). A cross-sectional study found that lower calcium intake was associated with PMS among 108 Italian women aged 19–51 y (*15*). Another cross-sectional study reported a lower intake of calcium in 31 Iranian students with PMS than in 31 without (*19*). In contrast, some cross-sectional studies have reported no association between calcium intake and PMS (*13*, *16*, *18*, *20*). Some of these studies did not adjust or insufficiently adjusted for covariates, including dietary factors (*16*, *18*, *20*), and had a small sample size (30–<200 participants) (*16*, *18*, *20*). In addition, the age of study participants (42–52 y old) in one previous study (*13*) was older than in the others (from 19–30 to 25–51 y old) (*4*, *15*, *16*, *18–20*). These limitations may partly explain the lack of association in the previous studies. In our study, we found a suggestive association between high intake of calcium and low prevalence of PMS after adjustment for covariates including dietary factors such as vitamin B6, vitamin B1, vitamin B12, potassium, iron and vitamin D among nearly 400 women aged 22–49 y.

Although the prevalence of PMS tended to decrease with increasing dairy intake, we observed that dairy intake was not clearly associated with PMS. Findings differed among previous studies: milk (or dairy) intake was associated with decreased risk of PMS in one nested case-control study (US) (*4*); no association was observed in two cross-sectional studies (US and UAE) (*17*, *27*); and a positive association was seen in another study (Italy) (*15*). Although the reason for this inconsistency among findings is unclear, these studies differed in their study design, sample size, method of assessing PMS, and use or lack of adjustment for covariates. In the case-control study nested within the NHS II among nearly 3,000 women—which did adjust for covariates (*4*)—the inverse association between milk intake and PMS risk was stronger in overweight than in normal-weight women, although the interaction was not statistically significant. In addition, in an analysis by type of milk, skim or low-fat milk intake was significantly associated with lower risk of PMS whereas whole milk intake was not (*4*). Most of our present participants were not obese (BMI <25 kg/m^2) (95% of participants) and did not consume low-fat milk (82% of participants), which might accordingly explain why we did not observe a significant association between dairy intake and PMS.

In the present study, vitamin D intake was not associated with PMS. Previous US studies did observe a decreased risk or prevalence of PMS associated with higher intake of vitamin D (*4*, *14*, *17*). In contrast, an Italian study and Iranian studies reported no association (*18–20*). In addition to issues with small sample size and lack of adjustment for covariates, studies which reported no association were conducted in regions (Europe and Asia) which have a higher prevalence of vitamin D deficiency than North America (*28*). Vitamin D status is largely due to exposure to sunlight, which induces vitamin D production in the skin (*26*). Accordingly, the association between vitamin D intake and PMS might depend on vitamin D levels. This is further supported by the fact that the only reported efficacy among intervention trials administering vitamin D is a report in vitamin D-deficient subjects (*29*). Clarification of this issue will require the measurement of vitamin D concentrations in blood.

Calcium and vitamin D levels fluctuate across the menstrual cycle in response to changes in estradiol at ovulation and during the luteal phase (*10*). As estrogen increases immediately before ovulation and during the luteal phase, calcium levels are lower at ovulation than during the early follicular phase and return to their follicular levels by the end of the luteal phase (*10*). The symptoms of PMS appear during the luteal phase, when calcium levels are low, and are similar to those of hypocalcemia, including depression, anxiety, and fatigue (*30*). Calcium has been reported to be protectively associated with depression, one of the symptoms of PMS, through the synthesis of serotonin and dopamine and the influence of the excitability of neuromuscular tissues involved in emotional regulation (*31–33*). Thus, calcium intake and the maintenance of calcium levels may alleviate symptoms of PMS.

Among the major strengths of this study are its use of validated questionnaires for PMS and diet and adjustment for known and suspected risk factors of PMS. Several limitations also warrant mention. First, an association derived from a cross-sectional study does not necessarily indicate causality. Second, since we assessed dietary intake using a self-administered questionnaire and at a single time point only, misclassification due to measurement error is possible. Third, we could not consider calcium or vitamin D intake from supplements. Fourth, the low participation rate (19%) raises the possibility of selection bias. Fifth, our sample size was not large enough to allow detection of an association between PMS and calcium with statistical significance. Sixth, we used BMI, calculated using self-reported weight and height, which may be subject to under- or over-reporting, as an adjustment factor. However, self-reported weight and height was confirmed to be highly correlated with the measured one (*34*). Seventh, we cannot rule out the possibility of bias resulting from unrecognized confounders or residual confounding. Finally, as the study participants were graduates of a university, the findings may not be applicable to populations with a different background.

In conclusion, we observed no significant association between calcium, vitamin D, and dairy intake and PMS. However, we did find a suggestive inverse association between calcium intake and PMS. As the present study was cross-sectional, the observed association of calcium intake with PMS requires confirmation in prospective studies.

Authorship

Research conception and design: AN; investigation: AN, MS, NY, MN, and MO; the data curation: AN, NY, and MN; statistical analysis of the data: AN, HM, AH, and MF; interpretation of the data: AN, MS, TT, and

MO; manuscript drafting: AN. All authors have read and approved the final manuscript.

Disclosure of state of COI

No conflicts of interest to be declared.

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ORIGINAL ARTICLE

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Practical diagnosis and treatment of premenstrual syndrome and premenstrual dysphoric disorder by psychiatrists and obstetricians/gynecologists in Japan

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Abstract

Aim: To investigate and compare the diagnoses and treatment of premenstrual syndrome (PMS) and premenstrual dysphoric disorder (PMDD) from the perspectives of psychiatrists and obstetricians/gynecologists (OB/GYNs) in Japan.

Methods: Between December 2021 and February 2022, a web-based survey was conducted among the members of the Japanese Association of Neuro-Psychiatric Clinics. Data from 262 psychiatrists who responded to the aforementioned survey were compared with data from 409 OB/GYNs from a survey conducted in 2021 among members of the Japanese Society of Obstetrics and Gynecology.

Results: Overall, 79.8% of psychiatrists and 97.3% of OB/GYNs were involved in practicing PMS/PMDD diagnosis and treatment. Most psychiatrists believed that PMS should be treated by OB/GYNs (74.4%) and PMDD by psychiatrists (75.6%). Only vague medical interviews were conducted by 86.6% of psychiatrists, and only 9.7% maintained a two-cycle symptom diary. Psychiatrists mostly prescribed selective serotonin/serotonin and noradrenaline reuptake inhibitor (SSRI/SNRI) continuous dosing (91.1%), followed by Kampo medicines, especially *Kamishoyosan* (73.3%); only 2.8% chose oral contraceptive pills, unlike OB/GYNs, while SSRI continuous (32.8%) and luteal phase dosing (20.6%) and Kampo medicine (42.1%) were the most common first-line treatments. Lifestyle guidance was prescribed by 63.6% of psychiatrists, followed by cognitive behavioral therapy (13.8%) and the symptom diary observation method (11.1%), which were similar to OB/GYNs' choices.

Conclusions: Many Japanese psychiatrists and OB/GYNs do not base PMS/PMDD diagnoses on prospective monitoring methods using specific diagnostic criteria and therefore do not provide evidence-based treatment. Moreover, a tendency of being biased toward treatments in which the department specialized was observed.

KEYWORDS
diagnostic techniques and procedures, Kampo, oral contraceptive pills, prospective daily charting, selective serotonin reuptake inhibitors

INTRODUCTION

Premenstrual syndrome (PMS) and premenstrual dysphoric disorder (PMDD) are characterized by psychiatric or physical/behavioral symptoms that manifest during the luteal phase of the menstrual cycle, affect patients' activities of daily living, and resolve shortly after the onset of menstruation. Affective symptoms include affective lability, irritability, depressed mood, and anxiety. Physical/behavioral symptoms include decreased interest, difficulty in concentration, anergia, cravings or changes in appetite, insomnia or hypersomnia, a sense of being overwhelmed or out of control, and physical symptoms such as breast tenderness or swelling, joint or muscle pain, bloating or weight gain.[1–3]

Epidemiological surveys have estimated that the frequency of premenstrual symptoms is relatively high (80%–90%) and that the prevalence of PMS in menstruating women is approximately 20%–30%. Furthermore, 1.2%–6.4% of women of reproductive age exhibit severe psychotic premenstrual symptoms associated with PMDD that interfere with daily life.[1,4–6]

PMS and PMDD are classified as premenstrual conditions from the perspective of gynecology and psychiatry, respectively, and although the established diagnostic methods are different, there is some overlap between them. The American Psychiatric Association has defined and published specific criteria for a severe clinical syndrome of PMDD in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5-TR).[3] Similarly, the American Congress of Obstetricians and Gynecologists (ACOG) has defined guidelines for PMS.[7] Nevertheless, there has been considerable controversy and unevenness in guidelines across disciplines about the appropriate diagnostic criteria for syndromes with clinically significant premenstrual symptoms. Notably, PMS and PMDD have recently been viewed as a part of the continuous disease concept. The International Society for the Study of Premenstrual Disorders (ISPMD) describes a spectrum of premenstrual disorders (PMDs), including core and variant PMDs, and both PMS and PMDD are considered core PMDs.[8] The DSM-5-TR,[3] ISPMD, and ACOG guidelines[7,8] state that symptoms must occur reproducibly during two cycles of prospective recording for a diagnosis of PMS and PMDD. As a prospective symptom diary, the Daily Record of Problem Severity (DRSP) is considered a valid and reliable tool to diagnose PMS or PMDD and is frequently used for research purposes, including in clinical trials worldwide.[9,10] However, while screening tools and structured clinical interviews are available and are clinically used, such retrospective assessments are often considered to be limited because of their subjectivity and concerns regarding recall bias. This is because patients can overestimate the cyclical nature of their symptoms, which are irregular and often worse during the luteal phase.[1,8] Moreover, 2 months of prospective daily symptom monitoring requires a considerable amount of time and effort from patients, therefore its adoption in routine clinical practice remains questionable. In 2021, we conducted a survey to investigate the current status and problems associated with diagnosing and treating PMS/PMDD among Japanese obstetricians and gynecologists (OB/GYNs). The results showed that only 8.4% of the 1267 OB/GYN respondents engaged in routine PMS/PMDD treatment used a two-cycle symptom diary.[11] This was similar to the percentage (11.5%) reported for a 2012 study that included gynecologists and family physicians in the United States,[12] indicating a dissociation between research and actual clinical practice.

Standard pharmacological treatments for PMS/PMDD include the use of selective serotonin reuptake inhibitors (SSRIs), which affect the levels of serotonin and other neurotransmitters in the brain, and oral contraceptive pills (OCPs), which influence hormonal activity by suppressing ovulation. SSRI therapy, dosed continuously or only in the luteal phase of the menstrual cycle, is the gold standard treatment for PMDD as per expert guidelines.[13,14] Serotonin and norepinephrine reuptake inhibitors (SNRIs) such as venlafaxine have shown to be therapeutically effective for PMDD.[15] For treating women with PMS, drospirenone-containing OCPs may represent an effective treatment and should be considered as the first-line pharmaceutical intervention OCPs.[10,16] The guidelines of the Japanese Society of Obstetrics and Gynecology (JSOG) state that treatment can also include counseling, lifestyle guidance, exercise therapy, and administration of Kampo medicines (traditional Japanese herbal medicines) and diuretics.[17]

Notwithstanding these recommendations, insurance does not cover SSRIs and OCPs for patients with PMS/PMDD in Japan. Moreover, in addition to the medical complications, both these drug types are associated with several other complications. OCPs have side effects such as the increased risk of venous thromboembolism, stroke, and breast cancer, and SSRIs can increase suicide-related behavior in patients younger than 24 years, activation, and post-discontinuation symptoms.[18–20] In this context, according to the results of the survey of OB/GYNs in 2021, OCPs were the most common first-line drugs for the treatment of PMS/PMDD (76.8%), following Kampo medicine (19.5%) and SSRIs, which were significantly less frequently used (2.6%).[11]

PMS/PMDD is often treated by OB/GYNs and psychiatrists in Japan, and the definition of the disease concept has not been fully established. Due to the variety of disease concepts and diagnosis methods for PMS/PMDD, deciding which department a patient should be referred to remains a significant problem. It is assumed that psychiatrists and OB/GYNs also face various challenges in diagnosing and treating PMS/PMDD; however, the actual situation is unclear. The present study therefore aimed to clarify the current status and problems associated with the diagnosis and treatment of PMS/

PMDD in Japan by psychiatrists and to compare them with those by OB/GYNs.

METHODS

Ethical considerations

This study was performed as a Women's Health Care Academic Committee survey of the JSOG and targeted physicians who belong to the JSOG and the Japanese Association of Neuro-Psychiatric Clinics (JAPC). This study was conducted in accordance with the principles of the Declaration of Helsinki. The survey was conducted anonymously and contained no personal information. A description of the study's objectives was provided to all participants and they agreed to participate by submitting online consent.

Participants

A survey questionnaire was mailed to all JSOG members (n = 16,732) between the end of September and the end of November 2021. The details of the survey have been reported previously.[11] A survey questionnaire was also emailed to all JAPC members (n = 1670) between December 1, 2021 and February 18, 2022, and a web-based survey was administered using Google Forms. Of these, 262 JAPC members responded (15.7%) with the completed questionnaires. Among them, 247 psychiatrists who routinely engaged in PMS/PMDD treatment and provided answers to questions about their PMS/PMDD diagnosis and treatment were selected (Figure 1). The results of this survey have already been outlined in JSOG's annual report.[21] Of the 1312 members of the JSOG who responded to the survey, 409 OB/GYNs (2.4% of all JSOG members [16,732]) whose place of work was a clinic were selected because all JAPC members work in clinics. Among them, 407 who engaged in routine PMS/PMDD treatment and answered the questions regarding routine PMS/PMDD diagnosis and treatment were included in this study.

Questionnaire

With regard to general characteristics, participants were questioned about the number of years since licensure as doctors and their gender. We also surveyed their knowledge about disease names, diagnosis, and treatment, which department (OB/GYN, psychiatry, internal medicine, or others) should diagnose and treat PMS and PMDD according to them, and the frequency of involvement. Only those engaged in PMS/PMDD practice were questioned about their routine diagnostic procedure for PMS/PMDD, which first-line drugs and pharmacotherapies they used most commonly in the treatment of PMS/PMDD, and which non-pharmacotherapy treatments they used most frequently. Multiple answers were allowed for the question regarding which department should diagnose and treat PMS and PMDD, and regarding treatment and diagnosis in accordance with

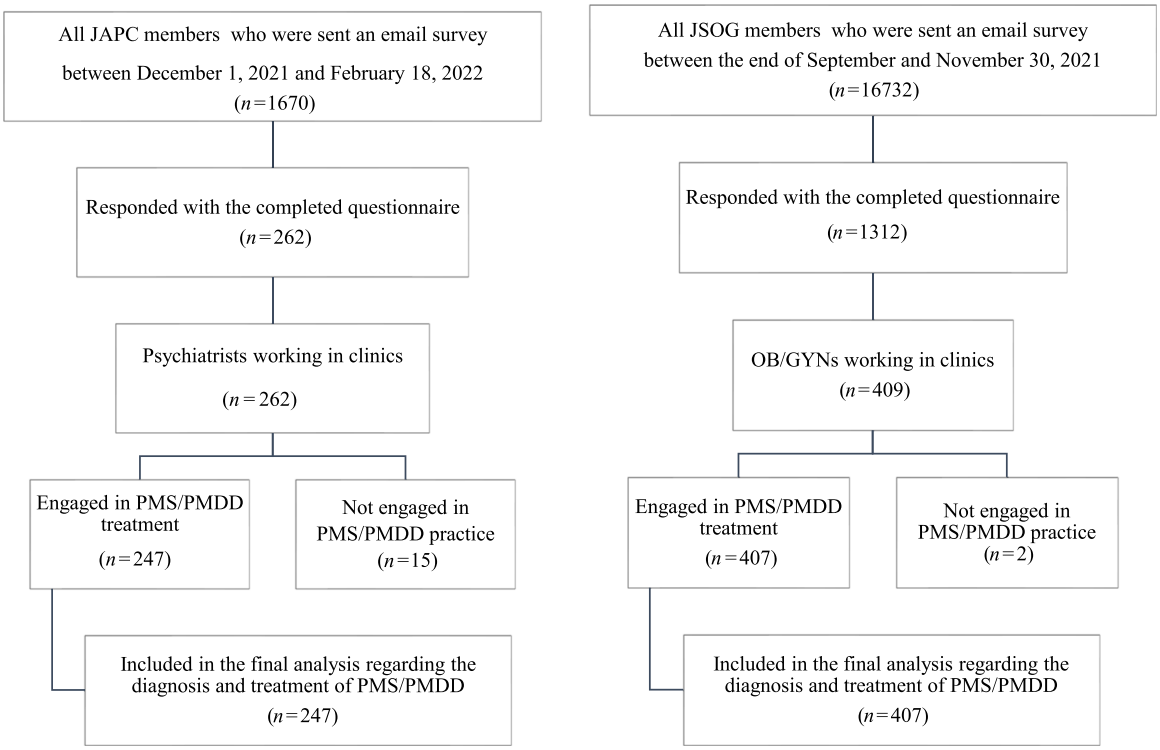

FIGURE 1 Participant selection flowchart. JAPC, Japanese Association of Neuro-Psychiatric Clinics; JSOG, Japanese Society of Obstetrics and Gynecology; OB/GYNs, obstetricians/gynecologists; PMS, premenstrual syndrome; PMDD, premenstrual dysphoric disorder.

the previous survey for OB/GYNs in 2021.[11] "Interview based on the DSM-5" was included in the answers to the question on "General diagnostic procedure for PMS/PMDD."

The DSM-5-TR published in 2022 maintained the same diagnostic criteria for PMDD without specific changes, compared to the DSM-5.[3,22]

Statistical analysis

Cross-tabulations were performed, and Pearson's χ^2 tests were conducted between psychiatrists and OB/GYNs. Statistical significance was set at $P < 0.05$. The effect size was measured using Cramer's V calculated with BellCurve for Excel (Social Survey Research Information Co., Ltd). The effect sizes of 0.10, 0.30, and 0.50 were judged as small, medium, and large, respectively.[23]

RESULTS

The background characteristics of participants are listed in Table 1. The gender ratio (female vs. male) was 19.5 versus 80.5 in psychiatrists, and 41.8 versus 57.5 in OB/GYNs. The number of male psychiatrists was significantly higher than that of female psychiatrists. The postlicensure period was significantly longer for the psychiatrists than for the OB/GYNs.

Table 2 presents data regarding the involved degree of engagement in and awareness of PMS/PMDD care. Notably, 79.8% of psychiatrists ($n = 262$) answered "practice on diagnosis and treatment of PMS/PMDD." Significantly more psychiatrists (60.3%) chose psychiatry as the preferred treatment approach for PMS, whereas only 32.0% of OB/GYNs shared this perspective. Conversely, for PMDD, 77.8% of OB/GYNs indicated a preference for treatment within the OB/GYN specialty, a significantly higher proportion compared to the 63.4% of psychiatrists who expressed the same opinion. In total, 247 (94.3%) psychiatrists and 407 (99.5%) OB/GYNs answered that they were engaged in routine PMS/PMDD treatment; OB/GYNs treated PMS/PMDD more frequently than psychiatrists.

TABLE 1 Characteristics of study participants.

	Psychiatrists ($n = 262$) n (%)	OB/GYNs ($n = 409$) n (%)	P value (effect size)
Postlicensure period (years) for medical practitioners			0.014* (0.15)
<10	2 (0.8)	4 (1.0)	
≥10 and <20	19 (7.3)	58 (14.2)	
≥20 and <30	70 (26.7)	128 (31.3)	
≥30 and <40	95 (36.3)	139 (34.0)	
≥40 and <50	70 (26.7)	73 (17.8)	
≥50	6 (2.3)	7 (1.7)	
Gender			<0.01** (0.24)
Female	51 (19.5)	171 (41.8)	
Male	211 (80.5)	235 (57.5)	
No response	0 (0)	3 (0.7)	

Abbreviation: OB/GYNs, obstetricians/gynecologists.
*$P < 0.05$; **$P < 0.01$.

The results of the questions concerning the diagnosis and treatment of PMS/PMDD are presented in Table 3. Regarding the generic diagnostic procedures, 86.6% of psychiatrists answered "Interview only vague premenstrual health problems," similar to the result for OB/GYNs (83.5%). Only 9.7% of psychiatry respondents maintained a symptom diary rating for two cycles, as described by the ACOG and DSM-5-TR diagnostic criteria.[3,7] These findings did not differ significantly from those of the OB/GYNs. Some OB/GYNs choose an ACOG-based interview (17.0%), while psychiatrists chose a DSM-5-based interview (28.3%). Additionally, psychiatrists (3.2%) used a screening questionnaire like the Premenstrual Symptoms Screening Tool or Premenstrual Symptoms Questionnaire[24–27] significantly less often than OB/GYNs (11.3%).

SSRI/SNRI continuous dosing was the most commonly prescribed drug therapy by psychiatrists (91.1%). In contrast, the percentage for SSRI/SNRI–luteal phase dosing was 47.8%. Kampo medicine (especially *Kamishoyosan* [73.3%], *Tokishakuyakusan* [61.1%], and *Yokukansan* [46.2%]) was the next most common treatment. Psychiatrists more frequently prescribed psychotropic drugs, for example anti-anxiety agents (58.7%), sleep-inducing drugs (44.9%), and atypical/typical antipsychotics (31.2%). In contrast, hormone therapy was much less commonly chosen by psychiatrists compared to OB/GYNs.

Regarding the first-line drugs for PMS/PMDD treatment, psychiatrists chose Kampo medicine (42.1%), followed by SSRI/SNRI continuous dosing (32.8%) and SSRI/SNRI luteal phase dosing (20.6%); only 0.8% chose OCPs. Significantly more psychiatrists chose Kampo medicine than OB/GYNs.

Lifestyle guidance was chosen by 63.6% of psychiatrists as the most common non-pharmacological treatment, following counseling (52.6%), exercise guidance (21.9%), cognitive behavioral therapy (CBT) (17.4%), and the symptom diary observation method (13.0%).

DISCUSSION

This study aimed to identify the current PMS/PMDD diagnosis and treatment practices among psychiatrists and compare them with previously published results for OB/GYNs in Japan. PMS/PMDD and other menstruation-related disorders are often treated by psychiatrists and OB/GYNs in Japan, and according to this survey, many practicing psychiatrists and OB/GYNs were treating PMS/PMDD.

Both groups of practitioners had overwhelmingly vague opinions regarding diagnostic methods (86.6% of psychiatrists and 83.5% of OB/GYNs), with only 28% of psychiatrists using the DSM-5 and only 17% of OB/GYNs using the ACOG diagnostic criteria. Very few (9.7% of

TABLE 2 Knowledge about and involvement in PMS/PMDD diagnosis and treatment.

	Psychiatrists (n = 262) n (%)	OB/GYNs (n = 409) n (%)	P value (effect size)
Knowledge about PMS/PMDD diagnosis and treatment			<0.01* (0.32)
No knowledge	2 (0.8)	5 (1.2)	
Knowledge of name only	36 (13.7)	3 (0.7)	
Knowledge of diagnosis and treatment	15 (5.7)	3 (0.7)	
Practicing diagnosis and treatment	209 (79.8)	398 (97.3)	
Departments that should diagnose and treat PMS (multiple answers allowed)			
Psychiatry	158 (60.3)	131 (32.0)	<0.01* (0.28)
Gynecology	195 (74.4)	382 (93.3)	<0.01* (0.26)
Internal medicine	17 (6.5)	20 (4.9)	0.48 (0.034)
Either	67 (25.6)	34 (8.3)	<0.01* (0.24)
Departments that should diagnose and treat PMDD (multiple answers allowed)			
Psychiatry	198 (75.6)	295 (72.1)	0.37 (0.038)
Gynecology	166 (63.4)	318 (77.8)	<0.01* (0.16)
Internal medicine	8 (3.1)	12 (2.9)	0.92 (0.0034)
Either	48 (18.3)	40 (9.8)	<0.01* (0.12)
Engaged in PMS/PMDD treatment			<0.01* (0.47)
No	15 (5.7)	2 (0.5)	
Rarely (a few patients per year)	66 (25.2)	18 (4.4)	
Occasionally (a few patients per month)	113 (43.1)	99 (24.2)	
Daily (several patients per week)	68 (26.0)	290 (70.9)	

Abbreviations: OB/GYNs, obstetricians/gynecologists; PMS/PMDD, premenstrual syndrome and premenstrual dysphoric disorder.
*P < 0.01.

psychiatrists and 6.6% of OB/GYNs) had an assessment of prospective two consecutive menstrual cycles based on the diagnostic criteria for PMS/PMDD. This indicates that there is a discrepancy between the various diagnostic criteria and actual clinical practice. However, the findings of our study closely align with those of a 2012 US study that reported a similar prevalence rate of 11.5%,[12] although it is important to note that the previous study focused specifically on OB/GYN and family medicine physicians, while our research encompassed all OB/GYNs, including those working in hospital settings, in Japan.[28] According to the DSM-5-TR, women with psychiatric disorders other than PMS/PMDD may experience chronic or intermittent symptoms that are not related to the menstrual cycle; however, the onset of menstruation is likely to be a memorable event, leading to complaints that symptoms worsen or only appear during the premenstrual period. The differential diagnosis is further complicated by the overlap of symptoms between PMDD and several other psychiatric diagnoses. This challenge is particularly pronounced when clinicians depend solely on recalled symptoms, therefore it is crucial to confirm PMDD through prospective rating scales.[3]

Although diagnoses based on prospective assessments such as the DRSP are necessary for research objectives, the diagnostic methods proposed by these guidelines are inconsistent with clinical practice.[9] The proportion of OB/GYNs using the screening tool was low (11.3%) and for psychiatrists was even lower (3.2%). The reason for the significant difference between them is unclear, but it could be the fact that this tool is not well known in both departments. Therefore, it is questionable whether many psychiatrists are making accurate diagnoses, and further education of psychiatrists regarding PMS/PMDD diagnosis appears to be needed. Future studies are required to establish clinically appropriate and convenient diagnostic procedures and biomarkers that can be used to measure the severity of the disease quantitatively.

Regarding treatment drugs when multiple responses were allowed, SSRI/SNRI (continuous dosing) was 91.1%, SSRI/SNRI (luteal phase dosing) was 47.8%, followed by Kampo medicines (Kamishoyosan: 73.3%), whereas only 2.8% administered OCPs among psychiatrists. These results were in the reverse order for OB/GYNs. Furthermore, as for first-line treatment, most chose SSRIs (53.2% [continuous dosing 32.8%, luteal phase dosing 20.6%]), followed by Kampo medicines (42.1%). Psychiatrists and OB/GYNs also had contrasting choices for this question. Regarding pharmacotherapy, there was a tendency for treatment to be biased toward the speciality of each department. Regarding the high proportion of psychiatrists

TABLE 3 Responses regarding the diagnosis and treatment of PMS/PMDD.

	Psychiatrists (*n* = 247) *n* (%)	OB/GYNs (*n* = 407) *n* (%)	*P* value (effect size)
General diagnosis procedure for PMS/PMDD (multiple answers allowed)			
Interview only vague premenstrual health problems	214 (86.6)	340 (83.5)	0.34 (0.04)
Interview based on the ACOG diagnostic criteria	6 (2.4)	69 (17.0)	<0.01** (0.22)
Interview based on the DSM-5	70 (28.3)	28 (6.9)	<0.01** (0.29)
Rating for one cycle	7 (2.8)	24 (5.9)	0.11 (0.29)
Rating for two cycles	24 (9.7)	27 (6.6)	0.20 (0.06)
Screening measure/questionnaire (e.g., PSST or PSQ)	8 (3.2)	46 (11.3)	<0.01** (0.14)
Measurement of basal body temperature	13 (5.3)	46 (11.3)	0.01* (0.10)
Other measurements	5 (2.0)	3 (0.7)	0.16 (0.057)
Treatment generally prescribed for PMS/PMDD (multiple answers allowed)			
OCPs	7 (2.8)	399 (98.0)	<0.01** (0.95)
HRT	4 (1.6)	69 (17.0)	<0.01** (0.24)
Dienogest	4 (1.6)	148 (36.4)	<0.01** (0.40)
LNG-IUS	1 (0.4)	54 (13.3)	<0.01** (0.22)
GnRH analogues	2 (0.8)	18 (4.4)	0.018* (0.10)
SSRI/SNRIs (continuous dosing)	225 (91.1)	164 (40.3)	<0.01** (0.50)
SSRI/SNRIs (luteal phase dosing)	118 (47.8)	89 (21.9)	<0.01** (0.27)
Other antidepressant	71 (28.7)	31 (7.6)	<0.01** (0.28)
Anxiolytic	145 (58.7)	114 (28.0)	<0.01** (0.30)
Sleep-inducing drugs	111 (44.9)	83 (20.4)	<0.01** (0.26)
Atypical/typical antipsychotics	77 (31.2)	5 (1.2)	<0.01** (0.44)
Tokishakuyakusan	151 (61.1)	195 (47.9)	<0.01** (0.13)
Kamishoyosan	181 (73.3)	320 (78.6)	0.14 (0.06)
Keishibukuryogan	102 (41.3)	132 (32.4)	0.027* (0.090)
Yokukansan	114 (46.2)	247 (60.7)	<0.01** (0.14)
Other Kampo medicine	50 (20.2)	150 (36.9)	<0.01** (0.17)
Chasteberry	2 (0.8)	10 (2.5)	0.22 (0.060)
Vitamin B_6	4 (1.6)	16 (3.9)	0.15 (0.065)
Other drugs or supplements	7 (2.8)	32 (7.9)	0.014* (0.10)
First-line medication			<0.01** (0.79)
OCPs	2 (0.8)	315 (77.4)	
SSRIs (luteal phase dosing)	51 (20.6)	4 (1.0)	
SSRIs (continuous dosing)	81 (32.8)	5 (1.2)	
Kampo medicine	104 (42.1)	77 (18.9)	
Depends on the case	9 (3.6)	6 (1.5)	
Nonpharmacological treatment generally prescribed for PMS/PMDD (multiple answers allowed)			
Lifestyle guidance	157 (63.6)	303 (74.4)	<0.01** (0.12)
Cognitive behavioral therapy	43 (17.4)	56 (13.8)	0.25 (0.049)
Symptom diary observation method	32 (13.0)	45 (11.1)	0.54 (0.029)
Counseling	130 (52.6)	116 (28.5)	<0.01** (0.24)

TABLE 3 (Continued)

	Psychiatrists (*n* = 247) *n* (%)	OB/GYNs (*n* = 407) *n* (%)	*P* value (effect size)
Exercise	54 (21.9)	72 (17.7)	0.23 (0.05)
Acupuncture and moxibustion	5 (2.0)	8 (2.0)	0.96 (0.0020)
Others	8 (3.2)	2 (0.2)	<0.01** (0.12)
None	15 (6.1)	14 (3.4)	0.16 (0.06)

Abbreviations: ACOG, the American College of Obstetricians and Gynecologists; DSM-5, the Diagnostic and Statistical Manual of Mental Disorders-5; GnRH-analogue, gonadotropin-releasing hormone agonists and antagonists; HRT, hormone replacement therapy; LNG-IUS, levonorgestrel intrauterine system; OB/GYNs, obstetricians/gynecologists; OCPs, oral contraceptives; PMS/PMDD, premenstrual syndrome and premenstrual dysphoric disorder; PSQ, the Premenstrual Symptoms Questionnaire; PSST, the Premenstrual Symptoms Screening Tool, SSRI/SNRI, selective serotonin reuptake inhibitors/serotonin noradrenaline reuptake inhibitors.

*$P < 0.05$; **$P < 0.01$.

administering SSRIs, it is assumed that a high proportion of them treat patients with severe psychiatric symptoms, which makes this the correct treatment method, as indicated in the treatment guidelines for PMDD.[10,13] As for SSRI administration methods, luteal phase administration alone is as effective as continuous administration.[29] The present results suggest that psychiatrists may be unaware of this fact, as many of them use continuous dosing as their first treatment choice.

This study also showed that although the frequency of SSRI use was relatively high (53.2%) in psychiatry, Kampo medicine was also preferred (42.1%) as a first-line drug. This may be due to concerns regarding the side effects of SSRIs.[20] Kampo medicines are more common as first-line treatment, which means that many practitioners are making treatment choices contrary to the guidelines for PMS/PMDD. Both departments chose a variety of Kampo medications that are universally used in Japan and are well accepted by patients.[30,31] Kampo medicines are probably chosen in Japan because OCPs and SSRIs are not easily accepted by the general public.

There have been some studies on the therapeutic efficacy of drospirenone-containing OCPs for PMDD; however, based on the results of this study, some OB/GYNs opt for this drug, but most psychiatrists do not use it.[16] SSRIs are considered the first choice for treatment, but OCPs are also effective, and prescribing OCPs would expand psychiatrists' treatment toolkit.[10,32] In Japan, there is a prevailing tendency for OB/GYNs to address PMS due to its predominantly physical nature, whereas patients with PMDD cases are usually considered to be managed by psychiatrists. However, as seen in Table 2, a considerable portion of psychiatrists (60%) advocated for the involvement of psychiatry in addressing PMS, while the majority of OB/GYNs (77.8%) believed PMDD management should fall within their domain. These findings underscore the necessity for both departments to encourage the involvement of a multidisciplinary team; this situation could be managed by both departments working closely together and leveraging their combined expertise with their familiarity of treatment procedures. Specifically, psychiatrists should be able to confidently prescribe OC/LEPs, while OB/GYNs should be equipped to administer SSRIs.

Many psychiatrists also selected anti-anxiety agents (58.7%), and despite benzodiazepines having a history of being one among the clinically used agents for the treatment of PMS/PMDD, there are very few reliable studies evaluating their efficacy, and they should be considered only for adjunctive therapeutic option in intractable cases.[33,34]

Regarding non-pharmacological therapies, lifestyle guidance was the most common, followed by counseling, whereas CBT, said to be as effective as SSRIs, was less common in both departments.[35] The effectiveness of CBT for PMS/PMDD is supported by existing evidence, and the CBT implementation rate for depression and anxiety in psychiatric clinics in Japan is relatively high at 37.9%.[36] However, the adoption of a CBT approach for PMS/PMDD is not widespread in Japan; the establishment of a standardized protocol could likely promote its wider adoption as a treatment modality.

The survey revealed that many doctors use methods unique to Japan that are not included in the global guidelines for diagnosis and treatment. The existing evidence supports the use of psychological therapies and non-pharmacological complementary and alternative approaches for patients with mild symptoms who refuse pharmacotherapy. When the symptoms are more severe and the patient consents, SSRIs and OCPs should be preferred, as they represent the most reliable evidence-based treatments.

Differences were found in the proportion of male and female psychiatrists who responded to the survey. In our previous study, we showed that gender differences among Japanese OB/GYNs influence the choice of diagnosis and treatment of PMS/PMDD.[37] Originally, there was a larger gender difference in the number of Japanese psychiatrists compared to OB/GYNs, which influenced the composition of the participants in this study, therefore even if the gender difference observed in psychiatrists affects diagnosis and treatment, it cannot be simply compared with that amongst the OB/GYNs. As the purpose of this study was to investigate the current state of diagnosis and treatment between psychiatrists and OB/GYNs, the gender difference amongst psychiatrists was not investigated.

This is the first study in Japan to present the current status of psychiatry in diagnosing and treating PMS/PMDD. However, it should be mentioned that this study has some limitations. First, the psychiatrists who responded to this survey were members of JAPC, an organization mainly composed of physicians working in clinics and

excluding those working in hospitals, therefore these data might be biased, even after considering that treatment for PMS/PMDD is mainly provided in outpatient clinics. Additionally, even though 15.7% of JAPC members responded to the survey, it is deduced that the survey responses should be considered as data from those highly concerned about PMS/PMDD who are active in those treatments. Second, regarding the assessment methods, only self-reported data were used. Moreover, it was not possible to confirm the frequency with which the departments in this study would be encountering patients with PMS/PMDD, and it is possible that doctors under- or over-reported certain information. Because this study relied on retrospective reporting of typical practices, the accuracy of answers concerning diagnosing and treating PMS/PMDD could be improved if these practices were assessed prospectively. Finally, other types of health professionals who might be related to the assessment of PMS/PMDD, such as internists and family physicians, were excluded. It is also important to investigate the clinical practices of PMS/PMDD in other countries to assess the practices in other regions.

CONCLUSIONS

Most psychiatrists as well as OB/GYNs in Japan based their diagnosis of PMS/PMDD on vague medical interviews rather than on a prospective monitoring method in accordance with the relevant diagnostic criteria. Moreover, our results also indicate that they do not treat patients based on appropriate evidence. With regard to treatment, there was a tendency for bias toward treatments in which their department specializes, which differs from treatments in accordance with global standard guidelines, and many treatments are unique to Japan, such as Kampo medicine. Collectively, our findings indicate that it is necessary to further educate psychiatrists and OB/GYNs about PMS/PMDD. Tailor-made treatment regimens based on evidence-based medicine need to be developed by assessing the needs of individual patients and appropriate use of a multidisciplinary approach that involves both psychiatrists and OB/GYNs as needed.

AUTHOR CONTRIBUTION

All authors contributed to the study's conception, study design, and manuscript revision and approved the final manuscript. Kana Yoshimi analyzed the data and wrote the manuscript. Takashi Takeda was the main contributor to the study design and conception.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL STATEMENT

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. The survey was anonymous and did not include any personal information.

PATIENT CONSENT STATEMENT

Before completing the survey, all participants read the description of the study's purpose and agreed to participate in the study by providing online consent.

CLINICAL TRIAL REGISTRATION

N/A.

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Kamishoyosan Normalizes Dendritic Spine Morphology in the Medial Prefrontal Cortex by Regulating microRNA-18 and Glucocorticoid Receptor Expressions in Postmenopausal Chronic Stress-Exposed Mice

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Abstract

Objective: Kamishoyosan (KSS), a traditional Japanese Kampo medicine, is widely used to treat neuropsychiatric symptoms in perimenopausal and postmenopausal women. We aimed to elucidate the functional mechanisms underlying KSS-mediated reduction of stress response behaviors and neuropsychological symptoms in perimenopausal and postmenopausal women.

Methods: Female mice were bilaterally ovariectomized (OVX) at the age of 12 weeks and exposed to chronic water immersion and restraint stress for three weeks. Among them, mice in the OVX+stress+KSS group were fed chow containing KSS from one week before exposure to chronic stress until the end of the experiment. Firstly, we performed a marble burying test and measured serum corticosterone levels to assess irritability and stress conditions. Next, we examined whether KSS affects microRNA-18 (miR-18) and glucocorticoid receptor (GR) protein expression, as well as the basal dendritic spine morphology of pyramidal neurons in the medial prefrontal cortex (mPFC) of postmenopausal chronic stress-exposed mice. Analyzed data were expressed as mean ± standard deviation. Tukey's post hoc test, followed by analysis of variance (ANOVA), was used for among-group comparisons.

Results: KSS administration normalized chronic stress-induced unstable emotion-like behavior and upregulated plasma corticosterone levels. Furthermore, KSS ameliorated GR protein expression by downregulating miR-18 expression in the mPFC and recovered the immature morphological changes in spine formation of pyramidal neurons in the mPFC of OVX mice following chronic stress exposure.

Conclusions: KSS administration in postmenopausal chronic stress-exposed mice exerted anti-stress effects and improved the basal dendritic spine morphology of pyramidal neurons by regulating miR-18 and glucocorticoid receptor expression in the mPFC.

Categories: Psychiatry, Anatomy, Obstetrics/Gynecology
Keywords: medial prefrontal cortex, spine morphology, glucocorticoid receptor, microrna, hypothalamic-pituitary-adrenal axis, menopause, kamishoyosan

Introduction

Menopause-related neuropsychological symptoms, including irritation, depression, and anxiety, are characterized by cognitive, autonomic, emotional, and endocrine function disturbances [1]. One of the traditional Japanese Kampo medicines, Kamishoyosan (KSS), is composed of 10 crude compounds containing a specified mixture derived from plant sources and is widely prescribed to improve various neuropsychiatric symptoms in perimenopausal and postmenopausal women [2,3]. A previous KSS clinical study in postmenopausal women and a premenstrual rat model reported that KSS administration caused significant improvements in excitability and irritability scores [4,5]. Apart from these studies, we previously demonstrated that continuous KSS administration in postmenopausal chronic stress-exposed mice attenuated stress-related depressive behavior and normalized hypothalamic-pituitary-adrenal (HPA) axis activity [6]. However, the molecular mechanisms underlying the beneficial effects of KSS-mediated regulation of the HPA axis remain unclear.

The prefrontal cortex (PFC), particularly the medial prefrontal cortex (mPFC) in humans, is critical to higher-order executive functions, memory, decision-making, cognition, and emotional control [7]. The mPFC is vulnerable to stress, which can decrease its volume and synaptic density by changing spine

morphology in patients with depression [8]. Decreased PFC activity is closely related to the onset of stress-related psychiatric diseases such as depression [9]. Therefore, prefrontal hypofunction induced by stress exposure is strongly implicated in the onset of psychiatric symptoms. However, the molecular mechanisms underlying menopause-related neuropsychological symptoms in mPFC functions remain unclear.

Glucocorticoid receptors (GRs) are also distributed in the PFC, and prefrontal GRs have been recently implicated in HPA axis regulation and mood regulation [10,11]. These studies suggest a functional association between defective prefrontal GR signaling and stress-related psychiatric diseases. Furthermore, a previous study indicated that elevated microRNA-18 (miR-18) expression and reduced GR protein expression were reported in the paraventricular hypothalamic nucleus of stress-vulnerability model rats [12]. However, the functions of miR-18 in the mPFC of postmenopausal environmental stress-exposed mice remain unclear.

Accordingly, as a continuation of our previous KSS research, we aimed to evaluate the effects of KSS on miRNA-mediated regulation of GR expression in the mPFC and the basal dendritic spine morphology of pyramidal neurons in the mPFC of postmenopausal environmental stress-exposed mice in this study.

Materials And Methods

Ethics statement

All animal experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals, the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal care and handling procedures approved by the International Animal Care and Use Committee of Kindai University (No. KAME-25-009).

Animals

Ten-week-old C57BL/6N female mice were purchased from SLC (Japan SLC, Inc., Hamamatsu, Japan). Three mice per cage were kept at room temperature (22 ± 2°C; humidity, 55 ± 10%) in a 12-h light/dark cycle (lights on at 07:00 a.m. and off at 07:00 p.m.). The animals had free access to water and food for breeding (CE-2; CLEA Japan Inc., Tokyo, Japan).

KSS administration and stress exposure

KSS is composed of the extractions of 10 medicinal herbs [6] (Table *1*) and was supplied by Tsumura & Co. (Tokyo, Japan). These ingredient content percentages were calculated from the KSS product label (Tsumura & Co.). Dry powdered extracts of KSS were mixed with CE-2 chow at a final concentration of 3% (w/w) and used as previously reported [6].

Ingredient	Content (%)
Bupleuri Radix (*Bupleurum falcatum*)	13.3
Paeoniae Radix (*Paeonia lactiflora*)	13.3
Atractylodis Rhizoma (*Atractylodes ovate*)	13.3
Angelicae Radix (*Angelica acutiloba*)	13.3
Hoelen (*Poria cocos*)	13.3
Gardeniae Fructus (*Gardenia jasminoides*)	8.9
Moutan Cortex (*Paeonia suffruticosa*)	8.9
Glycyrrhizae Radix (*Glycyrrhizae uralensis*)	6.7
Zingiberis Rhizoma (*Zingiber officinale*)	4.4
Menthae Herba (*Menthae arvensis*)	4.4

TABLE 1: Composition of Kamishoyosan

All female mice were bilaterally ovariectomized (OVX) at age 12 weeks. After two weeks of postoperative recovery, the 36 mice were randomly allocated into three groups (n=12) after the ovariectomy: the control group (non-stressed OVX mice), the chronically stressed group (OVX+Stress mice), and the chronically stressed group administered with KSS (OVX+Stress+KSS mice). There was no significant difference in body

weight and average daily consumption of chow between groups before stress exposure. Chronic stress was induced as previously described [6]. Briefly, OVX mice were exposed to chronic Water Immersion and Restraint Stress (WIRS) for three weeks (at 14-17 weeks of age). Specifically, they were restrained in a 50-mL conical polypropylene centrifuge tube and vertically immersed to the level of the xiphoid process in a water bath maintained at 23°C for two hours once a day for three weeks. For KSS administration, mice in the OVX+Stress+KSS group were fed CE-2 chow containing 3% KSS one week before chronic stress exposure (13 weeks old) until the end of the experiment as previously reported [6]. The mice were confirmed to be free of gastric and duodenal ulcers by visual observation for bleeding and sores on the surface of the stomach and duodenal mucosa.

Enzyme-linked immunosorbent assay (ELISA)

Serum corticosterone levels were measured using a Corticosterone Enzyme Immunoassay Kit (Arbor Assays, K014, Ann Arbor, Michigan), following the manufacturer's instructions. Briefly, one day after the chronic stress exposure period, the mice were deeply anesthetized, and their blood samples were collected into tubes containing heparin. The tubes were immediately placed on ice, followed by centrifugation at 1000 g for 15 minutes at 4°C. Plasma samples were stored at -80°C prior to assays. Absorbance at 450 nm was measured using a plate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, Massachusetts), and the corticosterone concentration in each sample was calculated using the SkanIt™ microplate reader software (Thermo Fisher Scientific Inc.).

Marble burying test

To assess behaviors representing unstable emotions, anxiety, and irritability, a marble burying test was performed two days after the end of the chronic stress exposure period [13]. Briefly, 20 glass marbles were evenly distributed on 5-cm-deep sawdust bedding in 4 × 5 grids in standard cages (25 × 25 × 31 cm). Each mouse was placed in a cage for 15 minutes. Subsequently, the mice were removed from the cage, and the number of marbles buried by the mice was counted at the end of the test. Marbles buried to at least 2/3 of their depth were considered buried [13]. The light intensity in both the breeding and test rooms was ~150 lux at 40 cm from the floor.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the PFC of mice using Isogen II (NipponGene, Toyama, Japan), following the manufacturer's instructions. To analyze mRNA expression, reverse transcription of the total RNA was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). To evaluate GR and GAPDH expression, quantitative real-time PCR (qRT-PCR) was conducted using KOD SYBR qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan) with the following forward/reverse primers: GR, 5'-ACCTGGATGACCAAATGACCC-3'/5'-GCATAGCAGGTTTCCACTTGC-3' and GAPDH, 5'-GTGTTCCTACCCCCAATGTG-3'/5'-AGGAGACAACCTGGTCCTCA-3'. The housekeeping gene GAPDH was used as the internal control [6]. Specific ratio comparisons (gene of interest/GAPDH) were used to assess between-group differences in transcript expression. To analyze miRNA expression, reverse transcription of total RNA was performed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. To detect mature miR-18, qRT-PCR was performed using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific Inc.). TaqMan assays specific for miR-18 (Thermo Fisher Scientific Inc.) were performed with an ABI7900HT PCR System according to the manufacturer's instructions (Thermo Fisher Scientific Inc.). The relative levels of miR-18 in the mPFC were calculated with the 2−ΔΔCT method, with U6 as an internal control.

Golgi staining

Golgi staining was performed using an FD Rapid Golgi Stain Kit (FD NeuroTechnologies Inc., Columbia, Maryland), as previously described [14]. Briefly, the brains were removed from anesthetized mice and immersed in an equal mixture of solutions A and B for three weeks at 22±2°C in the dark. Next, the brains were transferred into solution C for seven days at 22±2°C. After freezing on dry ice, 200-µm serial coronal sections of the brain samples were prepared using a cryostat at -24°C and mounted on a 0.5% gelatin-coated glass slide, incubated overnight at 22±2°C, and soaked in solution C for five minutes. Subsequently, the slides were stained with a mixture of solution D, solution E, and deionized water (1:1:2) for 10 minutes, then rinsed in distilled water twice for four minutes. Coronal sections were dehydrated in an ascending ethanol series, cleared with xylene, and sealed with Entellan (Merck KGaA, Darmstadt, Germany). All images of the pyramidal neurons in the mPFC were obtained using a Keyence microscope (Keyence Corp., Osaka, Japan).

Immunohistochemistry

Immunohistochemical staining of the mouse brain was performed as previously described [6]. Briefly, mice were perfused transcranially with 4% paraformaldehyde three days after the three-week exposure to chronic stress. Next, their brains were collected and immersion-fixed in 4% paraformaldehyde at 4°C overnight. After post-fixing, the brain tissues were stored in a 30% (w/v) sucrose solution in 0.1 M phosphate-buffered saline (PBS) for 48 hours at 4°C. Free-floating, 30-µm-thick sections were rinsed with PBS and incubated in blocking buffer (5% bovine serum albumin and 0.3% Triton X-100 in PBS) for one hour at room temperature.

Subsequently, the sections were incubated with primary antibodies overnight at 4°C (Table 2). Next, the sections were washed in PBS and incubated with Alexa 488 anti-rabbit IgG secondary antibody (1:1000, Thermo Fisher Scientific Inc.; A-11008, RRID: AB_143165) for two hours at room temperature. All images were acquired using a laser scanning confocal microscope (C2; Nikon Corp., Tokyo, Japan). Immunohistochemical staining intensities were determined using ImageJ (National Institutes of Health). To quantify the GR expression level, the images were analyzed with pixel values of fluorescence intensity using the ImageJ software relative to a predetermined threshold intensity (the background intensity of the images set to zero). The same threshold setting was applied to all the images in each comparison group.

Immune blotting analyses

Immune blotting analysis was performed as previously described [6] using the antibodies in Table 2. Immunodetection of target proteins was performed using horseradish peroxidase-conjugated secondary antibodies (1:5000; Cell Signaling Technology Inc.) and an ECL Prime Western Blotting Detection System (GE Healthcare Systems Inc., Chicago, Illinois). Densitometric quantification was performed using ImageJ (National Institutes of Health), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. PSD95, postsynaptic density protein 95.

Antibody	Code No,	Manufacturer	Dilution
GR	ab183127	Abcam Plc, Cambridge, England	1:500 (IHC)
GR	ab183127	Abcam Plc, Cambridge, England	1:1000 (WB)
PSD95	3450	Cell Signaling Technology Inc., Danvers, Massachusetts	1:1000 (WB)
GAPDH	sc-32233	Santa Cruz Biotechnology Inc., Dallas, Texas	1:1000 (WB)

TABLE 2: The information about antibodies

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; WB; western blotting

Statistical analyses

Statistical analyses were performed using GraphPad Prism 10, a statistical software commonly used for data analysis in basic medical research (GraphPad Software, Boston, Massachusetts). Data are expressed as mean ± standard deviation (SD). Analysis of variance (one-way ANOVA) was used for 3 among-group statistical differences, followed by Tukey's post hoc test. Tukey's post-hoc test is a widely used statistical method with robust power to identify significant differences between groups and handle unequal sample sizes and variances. Statistical significance was set at $P < 0.05$.

Results

KSS normalized stress-upregulated plasma corticosterone level and irritability behavior

Compared with control OVX mice, OVX+Stress mice showed upregulated plasma corticosterone levels (Figure 1a, b), whereas the levels in OVX+Stress+KSS mice resembled those of the control (Figure 1a, b). Moreover, OVX+Stress mice showed an increased number of buried marbles, which was lower in OVX+Stress+KSS mice (Figure 1c). These results suggest that KSS administration ameliorated chronic stress-induced continuous hyperactivity of the HPA axis and unstable emotional behavior in OVX+Stress+KSS mice.

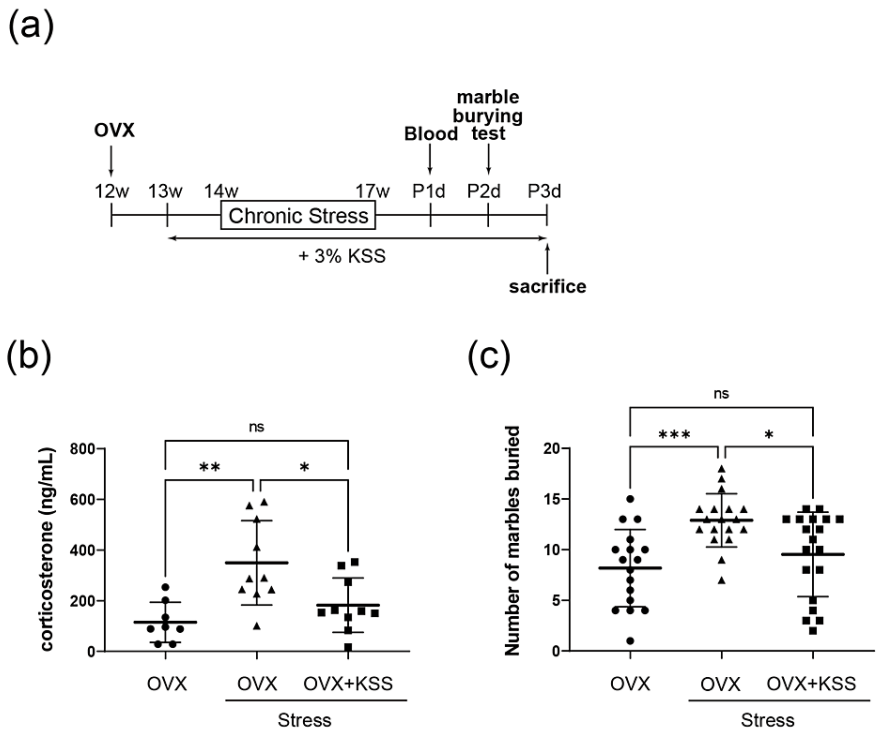

FIGURE 1: KSS treatment effects on plasma corticosterone levels and irritability-like behavior in OVX+Stress mice.

(a) Experimental timeline. The mice were used from 12 w (weeks) to 17w. Several analyses were performed from P1d (post 1 day) to P3d. (b) Levels of plasma corticosterone were measured by ELISA using blood samples. Results are shown as means ±SD (n = 8-10). (c) Marble Burying Test. Chronic stress exposure increased the number of buried marbles, which was normalized by the KSS administration. Results are shown as means ± SD (n = 17-19). *P < 0.05, **P < 0.01 one-way ANOVA followed by Tukey's post-test.

KSS, Kamishoyosan; OVX, ovariectomized mice; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ANOVA, analysis of variance; ns, no statistically significant difference.

Dendritic spine maturation and synaptic function in the mPFC were ameliorated from the effects of chronic stress by KSS

Chronic stress significantly decreased the spine area and spine head width in the mPFC of OVX mice, while these changes were absent in chronic stress-exposed OVX mice administered KSS (Figures *2a*, b, d). Furthermore, OVX+Stress mice showed increased spine length compared with stress-exposed OVX mice that received KSS (Figures *2a*, c). To evaluate synaptic function in the mPFC, we assessed the expression level of the postsynaptic density protein 95 (PSD95). Western blot analysis revealed decreased PSD95 expression in the mPFC of OVX+Stress mice, whereas expression in OVX+Stress+KSS mice was equivalent to that of the control (Figures *2e*, f). These results suggest that KSS administration ameliorated defects induced by chronic stress in spine maturation and synaptic function in the mPFC of OVX+Stress mice.

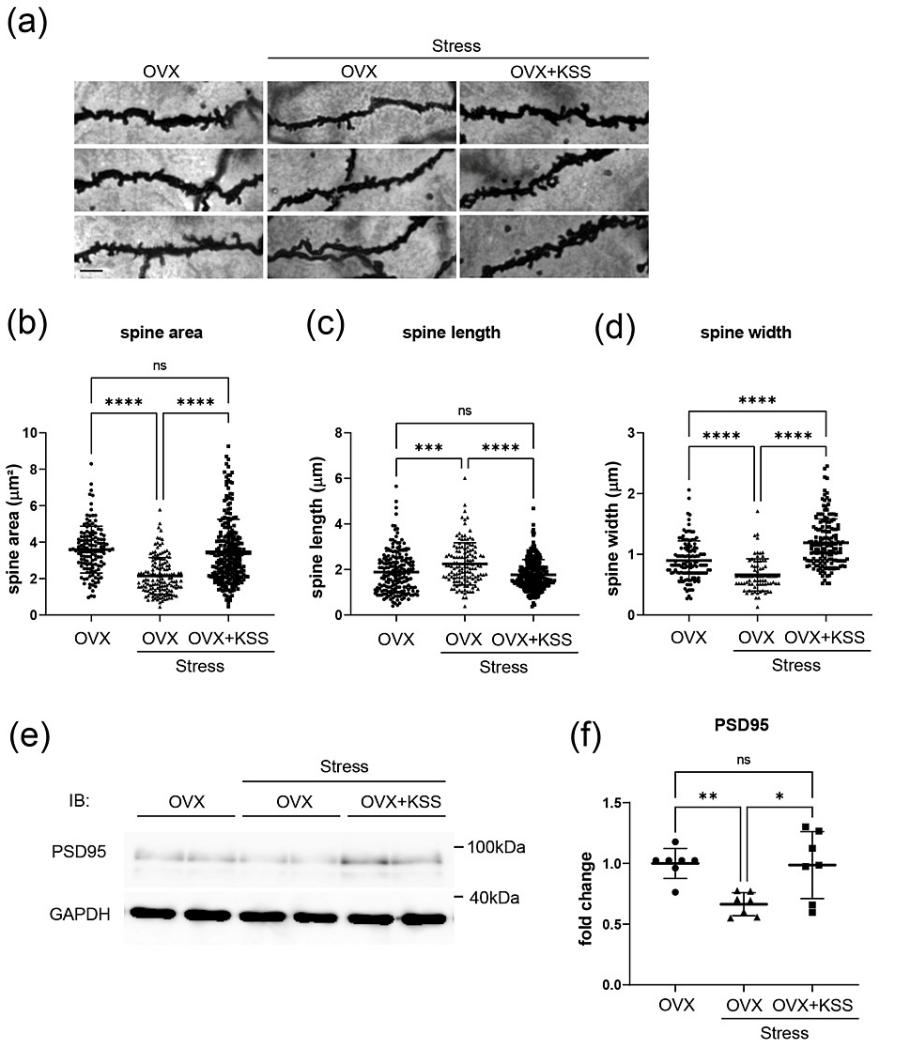

FIGURE 2: KSS treatment effects on basal dendritic spines of pyramidal neurons in the mPFC.

(a) High-magnification image of the representative Golgi-stained dendritic segments of pyramidal neurons in the mPFC. Scale bar, 200 nm. (b-d) Spine area, length, and width of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Data are presented as means ± SD. Two hundred spines from four slices from three animals per group were analyzed. Compared with OVX and OVX+Stress+KSS mice, OVX+Stress mice showed reduced spine area, length, and width. Data are presented as means ± SD. (e) PSD95 expression was evaluated through western blot analysis. (f) Densitometric quantification of PSD95 expression. The results are shown as means ± SD (n = 6). One-way ANOVA, Tukey's multiple comparisons test, *p < 0.05, **P < 0.01, ***P < 0.001, ****P□0.0001. Scale bar: 200 nm.

KSS, Kamishoyosan; OVX, ovariectomized mice; mPFC, medial prefrontal cortex; IB, immune blotting; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo Dalton; ns, no statistically significant difference.

KSS recovered GR protein expression changes through miR-18 upregulation in the mPFC

Next, we evaluated the relationship between GR protein and miR-18 expression levels in the mPFC region of the OVX+Stress mice with or without KSS administration. OVX+Stress mice showed significantly increased miR-18 expression in the mPFC, which was normalized by KSS administration in OVX+Stress+KSS mice (Figure *3a*). Moreover, western blot analysis revealed decreased GR protein expression in the mPFC of OVX+Stress mice, whereas it resembled the control level in the OVX+Stress+KSS mice (Figures *3b*, c). Chronic stress exposure also decreased the number of GR-immunoreactive cells in the mPFC, which was prevented by KSS administration (Figures *3d*, e). Taken together, these findings suggest that KSS reduced miR-18 expression and increased GR protein expression levels by reversing the inhibitory effects of chronic stress exposure on GR protein translation in the mPFC of OVX+Stress mice.

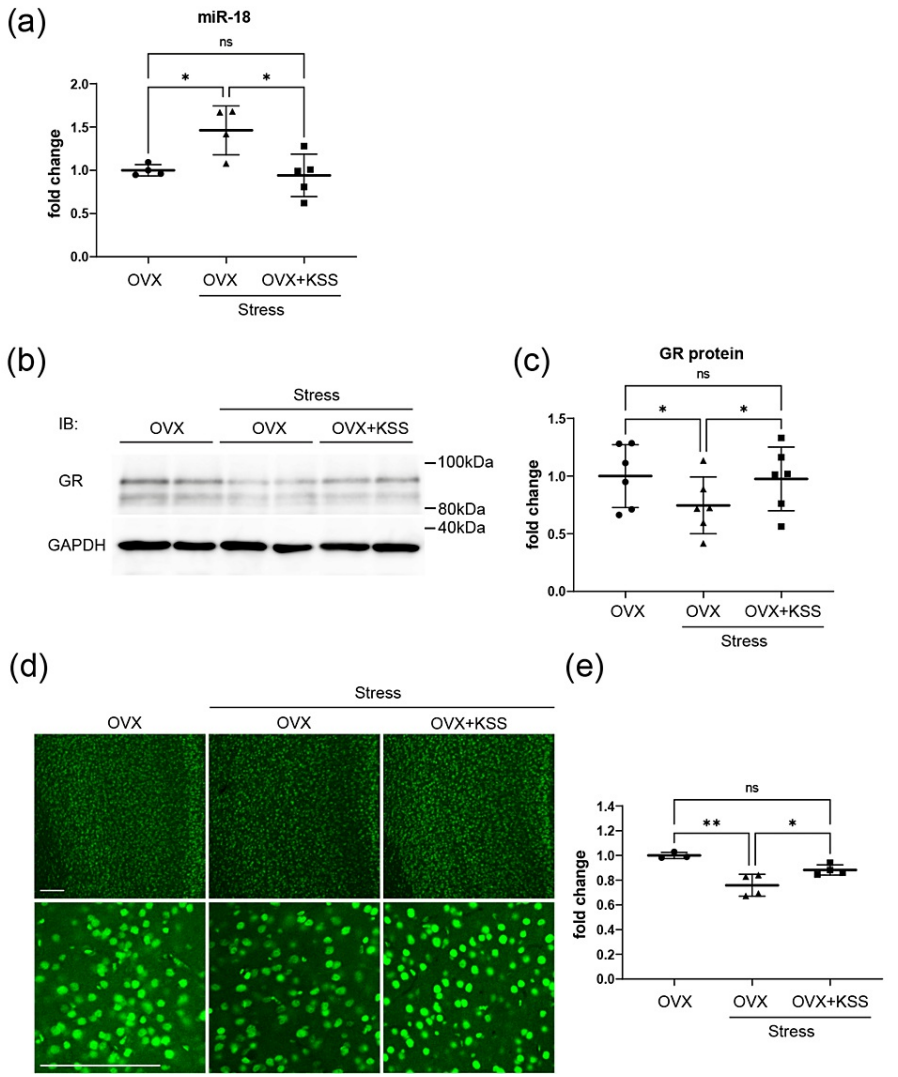

FIGURE 3: KSS treatment effects on miR-18 level and GR protein expression in the mPFC in OVX+Stress mice.

(a) Expression of miR-18 in the PFC was quantified through quantitative RT-PCR. (b) GR expression was assessed through western blot analysis. (c) Densitometric quantification of GR expression. These results are shown as means ± SD (n = 6). (d) Representative staining (upper panels) and high magnification (lower panels) images of GRs (green) in the mPFC. (e) The relative fluorescence intensity of GR signals in the mPFC. These results are shown as means ± SD (n = 4). Scale bar: 100 μm.

PFC, prefrontal cortex; RT-PCR, reverse transcription-polymerase chain reaction; GR, glucocorticoid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-18, microRNA-18; ns, no statistically significant difference.

Discussion

The present study demonstrated that KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which in turn improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figures *1*-*3*). The mPFC is involved in numerous important cognitive functions, including decision-making, working memory, attention, and emotional control [7]. Several clinical studies have demonstrated the effects of menopause on the mPFC [15]. Menopause is associated with decreased gray matter volume in the mPFC, which may contribute to cognitive changes such as memory problems and difficulties in decision-making [16]. During menopause, there are changes in the levels of hormones such as estrogen, which is involved in the formation and maintenance of synapses and dendritic spines [17]. Furthermore, estrogen replacement therapy increased the density and size of hippocampal dendritic spines in a rat menopause model [18]. These findings suggest that the menopause-related decrease in estrogen levels may be associated with changes in the synaptic function and spine morphology of mPFC neurons. Menopause-related changes in hormone levels may exacerbate the effects of chronic stress. Thus, future studies should focus on understanding the

anti-stress mechanisms involved in regulating neuronal functions by the effective chemical components of KSS.

We previously found that Yokukansan, a Japanese herbal medicine, downregulated miR-18 expression and normalized HPA axis activity by regulating GR protein expression in the hypothalamus and corpus callosum of stress-exposed mice. Similarly, our present findings indicated that KSS ameliorated chronic stress-induced unstable emotional behavior and upregulated plasma corticosterone levels (Figure *1*). A previous study reported that postmenopausal women had higher levels of cortisol and perceived stress than premenopausal women [19]. However, the molecular mechanisms underlying the changes in cortisol levels and HPA axis activity in postmenopausal women remain unclear. The present study demonstrated that the effects of KSS on the HPA axis involve miR-18 and GR protein expression (Figure *3*). Specifically, KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figure *2*).

The limitation of this study is that we did not identify the constituent herbal medicines included in KSS and did not show the function of estrogen receptors (ERs). Among the constituent herbal medicines in KSS, Bupleuri radix is well-known as the main component that may be effective for psychiatric symptoms and could serve as a possible alternative to current antidepressant medicines. Thus, a single administration of Bupleuri radix might show practical antidepressant-like and anti-stress effects in rodents. The next step in our research is to determine the effective chemical components of KSS. Previous studies have reported that miR-18 prevents ERα expression; furthermore, postmenopausal women have shown decreased ERα expression [20]. Therefore, brain miR-18 expression might be crucially involved in GR and/or ERα expression during the onset of menopausal symptoms. However, further studies linking the effective chemical components of KSS to its anti-stress function related to microRNAs in the brain are warranted to clarify the functional implications of these novel findings in the mPFC of chronic stress-exposed postmenopausal model mice.

From a clinical point of view, one previous KSS clinical trial study for postmenopausal women indicated that it was not able to show significant improvement effects in the main survey values [4]. One of the reasons why no significant improvements were found in the main survey values is that this clinical study might include several problems with the study design of participant selection. However, this clinical study also showed that KSS administration for post-menopause women showed significant improvements in excitability and irritability scores [4]. Furthermore, in this study, we indicated that KSS administration showed improvement effects of irritability behaviors for stress-exposed-OVX mice (Figure *1*). Furthermore, we found that the manufacturer did not perform the clinical survey of adverse reactions, and the KSS clinical trial study for postmenopausal women for 12 weeks indicated that no serious adverse events were reported [4]. From the results of these clinical trials and our basic research, it is assumed that KSS has a possibility of an effect on improving neuropsychiatric symptoms during menopause, especially irritability.

Conclusions

In conclusion, KSS administration in chronic stress-exposed postmenopausal mice exerted anti-stress effects and facilitated recovery from immature spine morphologies of basal dendritic pyramidal neurons, at least partly by regulating miR-18 and GR expression in the mPFC.

Appendices

□	OVX (n=8)	OVX+stress (n=10)	OVX+stress+KSS (n=10)
Mean	114.8	349.5*	182.2†
SD	79.1	166.6	107.6

TABLE 3: KSS treatment effects on plasma corticosterone levels (Figure 1b)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0017 between OVX and OVX+stress, P = 0.0174 between OVX+stress and OVX+stress+KSS groups).

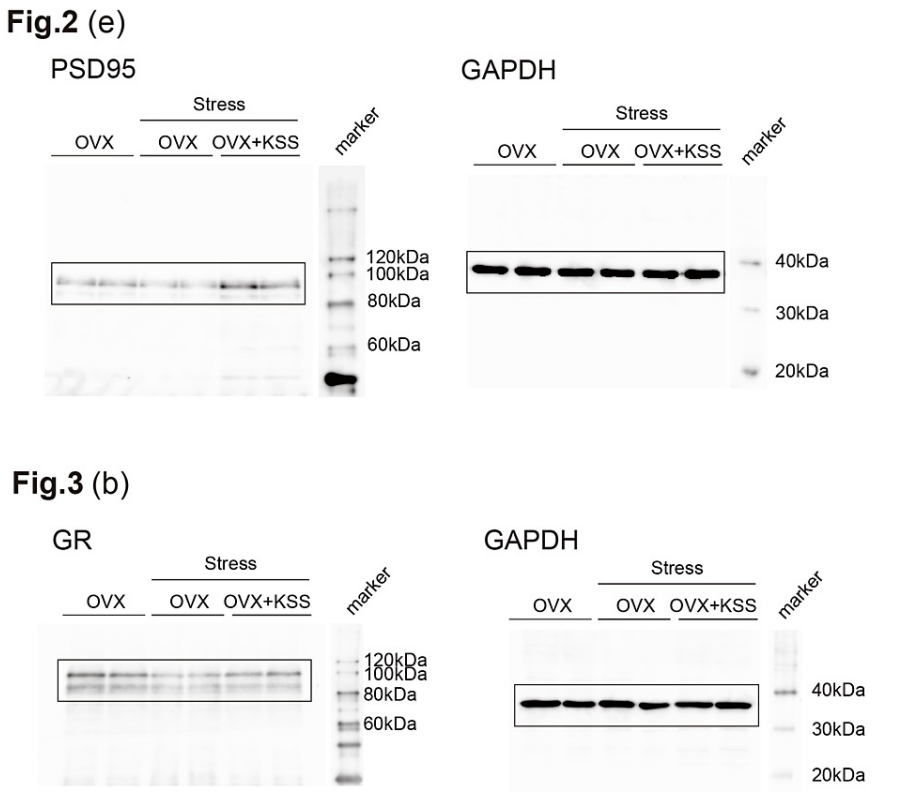

FIGURE 4: Full-size gels for immunoblots and molecular weight markers in Figures 2, 3.

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo-Dalton.

	OVX (n=17)	OVX+stress (n=18)	OVX+stress+KSS (n=19)
Mean	8.18	12.89*	9.53†
SD	3.81	2.63	4.17

TABLE 4: Number of marbles buried in the marbles burying test (Figure 1c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0009 between OVX and OVX+stress, P = 0.0177 between OVX+stress and OVX+stress+KSS groups).

□(a) □	OVX (n=135)	OVX+stress (n=146)	OVX+stress+KSS (n=260)
Mean	3.59	2.15**	3.47†
SD	1.28	1.01	1.78
□(b) □	**OVX (n=192)**	**OVX+stress (n=131)**	**OVX+stress+KSS (n=261)**
Mean	1.88	2.24*	1.76†
SD	0.92	0.93	0.67
□(c) □	**OVX (n=127)**	**OVX+stress (n=79)**	**OVX+stress+KSS (n=157)**
Mean	0.90	0.66**	1.19†
SD	0.32	0.26	0.39

TABLE 5: Morphological analysis of spines of pyramidal neurons in mPFC using Golgi staining (Figure 2b-d)

Spine area (a), length (b), and width (c) of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (*P <0.001, **P <0.0001 between OVX and OVX+stress, †P <0.0001 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=7)	OVX+stress (n=7)	OVX+stress+KSS (n=7)
Mean	1.00	0.66*	0.99†
SD	0.12	0.10	0.28

TABLE 6: Densitometric quantification of PSD95 expression in mPFC (Figure 2f)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0079 between OVX and OVX+stress, P = 0.0107 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=4)	OVX+stress (n=4)	OVX+stress+KSS (n=5)
Mean	1.00	1.46*	0.94†
SD	0.07	0.28	0.25

TABLE 7: Expression levels of miR-18 in mPFC (Figure 3a)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0362 between OVX and OVX+stress, P = 0.0146 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=6)	OVX+stress (n=6)	OVX+stress+KSS (n=6)
Mean	1.00	0.75*	0.97†
SD	0.27	0.25	0.28

TABLE 8: Densitometric quantification of GR expression (Figure 3c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0123 between OVX and OVX+stress, P = 0.0212 between OVX+stress and OVX+stress+KSS groups).

GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=3)	OVX+stress (n=4)	OVX+stress+KSS (n=4)
Mean	1.00	0.76*	0.88†
SD	0.03	0.09	0.04

TABLE 9: The relative fluorescence intensity of GR signals in the mPFC (Figure 3e)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0022 between OVX and OVX+stress, P = 0.0492 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Drafting of the manuscript: Shingo Miyata

Supervision: Shingo Miyata

Acquisition, analysis, or interpretation of data: Shoko Shimizu, Yoshihisa Koyama, Yugo Ishino

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Disclosures

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原著論文

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和文総説

1. 漢方薬の作用機序からみたトランスレーショナルリサーチ
   宮田信吾、清水尚子、石野雄吾、遠山正彌
   自律神経、62(1)：2-4, 2025.

ＷＥＢセミナー

1. MDPI's Journal Cluster of Neurosciences Webinar
   Glial-Neuronal Interactions in Nervous System
   2024.12.25 (Zoom)
   Miyata S.
   Oligodendrocyte development and function
   ---from signal transductions to stress responses

学会発表

1. NEURO2024 第 47 回日本神経科学大会 第 67 回日本神経化学会大会 第 46 回日本生物学的精神医学会年会 第 8 回アジアオセアニア神経科学連合コングレス 合同大会　2024.7.24-27, 福岡コンベンションセンター
   *石野雄吾、清水尚子、遠山正彌、**宮田信吾**
   神経発生におけるアルギニンメチル化酵素 PRMT7 の機能解析
2. NEURO2024 第 47 回日本神経科学大会 第 67 回日本神経化学会大会 第 46 回日本生物学的精神医学会年会 第 8 回アジアオセアニア神経科学連合コングレス 合同大会　2024.7.24-27, 福岡コンベンションセンター
   *清水尚子、石野雄吾、遠山正彌、**宮田信吾**
   生後発達期におけるオリゴデンドロサイト分化の分子機構解明
3. 第 100 回日本解剖学会近畿支部学術集会　2024.11.16, 大阪大学 吹田キャンパス 銀杏会館
   *清水尚子、石野雄吾、**宮田信吾**
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1. うつ病発症機構におけるタンパクメチル化酵素によるリン酸化シグナル制御の重要性
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2. 転写因子 NFATc2 の新規活性制御機構による髄鞘機能調節
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   石野雄吾, **宮田信吾**(分担研究者)

学会活動、等

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Kamishoyosan Normalizes Dendritic Spine Morphology in the Medial Prefrontal Cortex by Regulating microRNA-18 and Glucocorticoid Receptor Expressions in Postmenopausal Chronic Stress-Exposed Mice

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Abstract

Objective: Kamishoyosan (KSS), a traditional Japanese Kampo medicine, is widely used to treat neuropsychiatric symptoms in perimenopausal and postmenopausal women. We aimed to elucidate the functional mechanisms underlying KSS-mediated reduction of stress response behaviors and neuropsychological symptoms in perimenopausal and postmenopausal women.

Methods: Female mice were bilaterally ovariectomized (OVX) at the age of 12 weeks and exposed to chronic water immersion and restraint stress for three weeks. Among them, mice in the OVX+stress+KSS group were fed chow containing KSS from one week before exposure to chronic stress until the end of the experiment. Firstly, we performed a marble burying test and measured serum corticosterone levels to assess irritability and stress conditions. Next, we examined whether KSS affects microRNA-18 (miR-18) and glucocorticoid receptor (GR) protein expression, as well as the basal dendritic spine morphology of pyramidal neurons in the medial prefrontal cortex (mPFC) of postmenopausal chronic stress-exposed mice. Analyzed data were expressed as mean ± standard deviation. Tukey's post hoc test, followed by analysis of variance (ANOVA), was used for among-group comparisons.

Results: KSS administration normalized chronic stress-induced unstable emotion-like behavior and upregulated plasma corticosterone levels. Furthermore, KSS ameliorated GR protein expression by downregulating miR-18 expression in the mPFC and recovered the immature morphological changes in spine formation of pyramidal neurons in the mPFC of OVX mice following chronic stress exposure.

Conclusions: KSS administration in postmenopausal chronic stress-exposed mice exerted anti-stress effects and improved the basal dendritic spine morphology of pyramidal neurons by regulating miR-18 and glucocorticoid receptor expression in the mPFC.

Categories: Psychiatry, Anatomy, Obstetrics/Gynecology
Keywords: medial prefrontal cortex, spine morphology, glucocorticoid receptor, microrna, hypothalamic-pituitary-adrenal axis, menopause, kamishoyosan

Introduction

Menopause-related neuropsychological symptoms, including irritation, depression, and anxiety, are characterized by cognitive, autonomic, emotional, and endocrine function disturbances [1]. One of the traditional Japanese Kampo medicines, Kamishoyosan (KSS), is composed of 10 crude compounds containing a specified mixture derived from plant sources and is widely prescribed to improve various neuropsychiatric symptoms in perimenopausal and postmenopausal women [2,3]. A previous KSS clinical study in postmenopausal women and a premenstrual rat model reported that KSS administration caused significant improvements in excitability and irritability scores [4,5]. Apart from these studies, we previously demonstrated that continuous KSS administration in postmenopausal chronic stress-exposed mice attenuated stress-related depressive behavior and normalized hypothalamic-pituitary-adrenal (HPA) axis activity [6]. However, the molecular mechanisms underlying the beneficial effects of KSS-mediated regulation of the HPA axis remain unclear.

The prefrontal cortex (PFC), particularly the medial prefrontal cortex (mPFC) in humans, is critical to higher-order executive functions, memory, decision-making, cognition, and emotional control [7]. The mPFC is vulnerable to stress, which can decrease its volume and synaptic density by changing spine

morphology in patients with depression [8]. Decreased PFC activity is closely related to the onset of stress-related psychiatric diseases such as depression [9]. Therefore, prefrontal hypofunction induced by stress exposure is strongly implicated in the onset of psychiatric symptoms. However, the molecular mechanisms underlying menopause-related neuropsychological symptoms in mPFC functions remain unclear.

Glucocorticoid receptors (GRs) are also distributed in the PFC, and prefrontal GRs have been recently implicated in HPA axis regulation and mood regulation [10,11]. These studies suggest a functional association between defective prefrontal GR signaling and stress-related psychiatric diseases. Furthermore, a previous study indicated that elevated microRNA-18 (miR-18) expression and reduced GR protein expression were reported in the paraventricular hypothalamic nucleus of stress-vulnerability model rats [12]. However, the functions of miR-18 in the mPFC of postmenopausal environmental stress-exposed mice remain unclear.

Accordingly, as a continuation of our previous KSS research, we aimed to evaluate the effects of KSS on miRNA-mediated regulation of GR expression in the mPFC and the basal dendritic spine morphology of pyramidal neurons in the mPFC of postmenopausal environmental stress-exposed mice in this study.

Materials And Methods

Ethics statement

All animal experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals, the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal care and handling procedures approved by the International Animal Care and Use Committee of Kindai University (No. KAME-25-009).

Animals

Ten-week-old C57BL/6N female mice were purchased from SLC (Japan SLC, Inc., Hamamatsu, Japan). Three mice per cage were kept at room temperature (22 ± 2°C; humidity, 55 ± 10%) in a 12-h light/dark cycle (lights on at 07:00 a.m. and off at 07:00 p.m.). The animals had free access to water and food for breeding (CE-2; CLEA Japan Inc., Tokyo, Japan).

KSS administration and stress exposure

KSS is composed of the extractions of 10 medicinal herbs [6] (Table *1*) and was supplied by Tsumura & Co. (Tokyo, Japan). These ingredient content percentages were calculated from the KSS product label (Tsumura & Co.). Dry powdered extracts of KSS were mixed with CE-2 chow at a final concentration of 3% (w/w) and used as previously reported [6].

Ingredient	Content (%)
Bupleuri Radix (*Bupleurum falcatum*)	13.3
Paeoniae Radix (*Paeonia lactiflora*)	13.3
Atractylodis Rhizoma (*Atractylodes ovate*)	13.3
Angelicae Radix (*Angelica acutiloba*)	13.3
Hoelen (*Poria cocos*)	13.3
Gardeniae Fructus (*Gardenia jasminoides*)	8.9
Moutan Cortex (*Paeonia suffruticosa*)	8.9
Glycyrrhizae Radix (*Glycyrrhizae uralensis*)	6.7
Zingiberis Rhizoma (*Zingiber officinale*)	4.4
Menthae Herba (*Menthae arvensis*)	4.4

TABLE 1: Composition of Kamishoyosan

All female mice were bilaterally ovariectomized (OVX) at age 12 weeks. After two weeks of postoperative recovery, the 36 mice were randomly allocated into three groups (n=12) after the ovariectomy: the control group (non-stressed OVX mice), the chronically stressed group (OVX+Stress mice), and the chronically stressed group administered with KSS (OVX+Stress+KSS mice). There was no significant difference in body

weight and average daily consumption of chow between groups before stress exposure. Chronic stress was induced as previously described [6]. Briefly, OVX mice were exposed to chronic Water Immersion and Restraint Stress (WIRS) for three weeks (at 14-17 weeks of age). Specifically, they were restrained in a 50-mL conical polypropylene centrifuge tube and vertically immersed to the level of the xiphoid process in a water bath maintained at 23°C for two hours once a day for three weeks. For KSS administration, mice in the OVX+Stress+KSS group were fed CE-2 chow containing 3% KSS one week before chronic stress exposure (13 weeks old) until the end of the experiment as previously reported [6]. The mice were confirmed to be free of gastric and duodenal ulcers by visual observation for bleeding and sores on the surface of the stomach and duodenal mucosa.

Enzyme-linked immunosorbent assay (ELISA)

Serum corticosterone levels were measured using a Corticosterone Enzyme Immunoassay Kit (Arbor Assays, K014, Ann Arbor, Michigan), following the manufacturer's instructions. Briefly, one day after the chronic stress exposure period, the mice were deeply anesthetized, and their blood samples were collected into tubes containing heparin. The tubes were immediately placed on ice, followed by centrifugation at 1000 g for 15 minutes at 4°C. Plasma samples were stored at -80°C prior to assays. Absorbance at 450 nm was measured using a plate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, Massachusetts), and the corticosterone concentration in each sample was calculated using the SkanIt™ microplate reader software (Thermo Fisher Scientific Inc.).

Marble burying test

To assess behaviors representing unstable emotions, anxiety, and irritability, a marble burying test was performed two days after the end of the chronic stress exposure period [13]. Briefly, 20 glass marbles were evenly distributed on 5-cm-deep sawdust bedding in 4 × 5 grids in standard cages (25 × 25 × 31 cm). Each mouse was placed in a cage for 15 minutes. Subsequently, the mice were removed from the cage, and the number of marbles buried by the mice was counted at the end of the test. Marbles buried to at least 2/3 of their depth were considered buried [13]. The light intensity in both the breeding and test rooms was ~150 lux at 40 cm from the floor.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the PFC of mice using Isogen II (NipponGene, Toyama, Japan), following the manufacturer's instructions. To analyze mRNA expression, reverse transcription of the total RNA was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). To evaluate GR and GAPDH expression, quantitative real-time PCR (qRT-PCR) was conducted using KOD SYBR qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan) with the following forward/reverse primers: GR, 5'-ACCTGGATGACCAAATGACCC-3'/5'-GCATAGCAGGTTTCCACTTGC-3' and GAPDH, 5'-GTGTTCCTACCCCCAATGTG-3'/5'-AGGAGACAACCTGGTCCTCA-3'. The housekeeping gene GAPDH was used as the internal control [6]. Specific ratio comparisons (gene of interest/GAPDH) were used to assess between-group differences in transcript expression. To analyze miRNA expression, reverse transcription of total RNA was performed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. To detect mature miR-18, qRT-PCR was performed using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific Inc.). TaqMan assays specific for miR-18 (Thermo Fisher Scientific Inc.) were performed with an ABI7900HT PCR System according to the manufacturer's instructions (Thermo Fisher Scientific Inc.). The relative levels of miR-18 in the mPFC were calculated with the 2−ΔΔCT method, with U6 as an internal control.

Golgi staining

Golgi staining was performed using an FD Rapid Golgi Stain Kit (FD NeuroTechnologies Inc., Columbia, Maryland), as previously described [14]. Briefly, the brains were removed from anesthetized mice and immersed in an equal mixture of solutions A and B for three weeks at 22±2°C in the dark. Next, the brains were transferred into solution C for seven days at 22±2°C. After freezing on dry ice, 200-µm serial coronal sections of the brain samples were prepared using a cryostat at -24°C and mounted on a 0.5% gelatin-coated glass slide, incubated overnight at 22±2°C, and soaked in solution C for five minutes. Subsequently, the slides were stained with a mixture of solution D, solution E, and deionized water (1:1:2) for 10 minutes, then rinsed in distilled water twice for four minutes. Coronal sections were dehydrated in an ascending ethanol series, cleared with xylene, and sealed with Entellan (Merck KGaA, Darmstadt, Germany). All images of the pyramidal neurons in the mPFC were obtained using a Keyence microscope (Keyence Corp., Osaka, Japan).

Immunohistochemistry

Immunohistochemical staining of the mouse brain was performed as previously described [6]. Briefly, mice were perfused transcranially with 4% paraformaldehyde three days after the three-week exposure to chronic stress. Next, their brains were collected and immersion-fixed in 4% paraformaldehyde at 4°C overnight. After post-fixing, the brain tissues were stored in a 30% (w/v) sucrose solution in 0.1 M phosphate-buffered saline (PBS) for 48 hours at 4°C. Free-floating, 30-µm-thick sections were rinsed with PBS and incubated in blocking buffer (5% bovine serum albumin and 0.3% Triton X-100 in PBS) for one hour at room temperature.

Subsequently, the sections were incubated with primary antibodies overnight at 4°C (Table 2). Next, the sections were washed in PBS and incubated with Alexa 488 anti-rabbit IgG secondary antibody (1:1000, Thermo Fisher Scientific Inc.; A-11008, RRID: AB_143165) for two hours at room temperature. All images were acquired using a laser scanning confocal microscope (C2; Nikon Corp., Tokyo, Japan). Immunohistochemical staining intensities were determined using ImageJ (National Institutes of Health). To quantify the GR expression level, the images were analyzed with pixel values of fluorescence intensity using the ImageJ software relative to a predetermined threshold intensity (the background intensity of the images set to zero). The same threshold setting was applied to all the images in each comparison group.

Immune blotting analyses

Immune blotting analysis was performed as previously described [6] using the antibodies in Table 2. Immunodetection of target proteins was performed using horseradish peroxidase-conjugated secondary antibodies (1:5000; Cell Signaling Technology Inc.) and an ECL Prime Western Blotting Detection System (GE Healthcare Systems Inc., Chicago, Illinois). Densitometric quantification was performed using ImageJ (National Institutes of Health), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. PSD95, postsynaptic density protein 95.

Antibody	Code No,	Manufacturer	Dilution
GR	ab183127	Abcam Plc, Cambridge, England	1:500 (IHC)
GR	ab183127	Abcam Plc, Cambridge, England	1:1000 (WB)
PSD95	3450	Cell Signaling Technology Inc., Danvers, Massachusetts	1:1000 (WB)
GAPDH	sc-32233	Santa Cruz Biotechnology Inc., Dallas, Texas	1:1000 (WB)

TABLE 2: The information about antibodies

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; WB; western blotting

Statistical analyses

Statistical analyses were performed using GraphPad Prism 10, a statistical software commonly used for data analysis in basic medical research (GraphPad Software, Boston, Massachusetts). Data are expressed as mean ± standard deviation (SD). Analysis of variance (one-way ANOVA) was used for 3 among-group statistical differences, followed by Tukey's post hoc test. Tukey's post-hoc test is a widely used statistical method with robust power to identify significant differences between groups and handle unequal sample sizes and variances. Statistical significance was set at $P < 0.05$.

Results

KSS normalized stress-upregulated plasma corticosterone level and irritability behavior

Compared with control OVX mice, OVX+Stress mice showed upregulated plasma corticosterone levels (Figure 1a, b), whereas the levels in OVX+Stress+KSS mice resembled those of the control (Figure 1a, b). Moreover, OVX+Stress mice showed an increased number of buried marbles, which was lower in OVX+Stress+KSS mice (Figure 1c). These results suggest that KSS administration ameliorated chronic stress-induced continuous hyperactivity of the HPA axis and unstable emotional behavior in OVX+Stress+KSS mice.

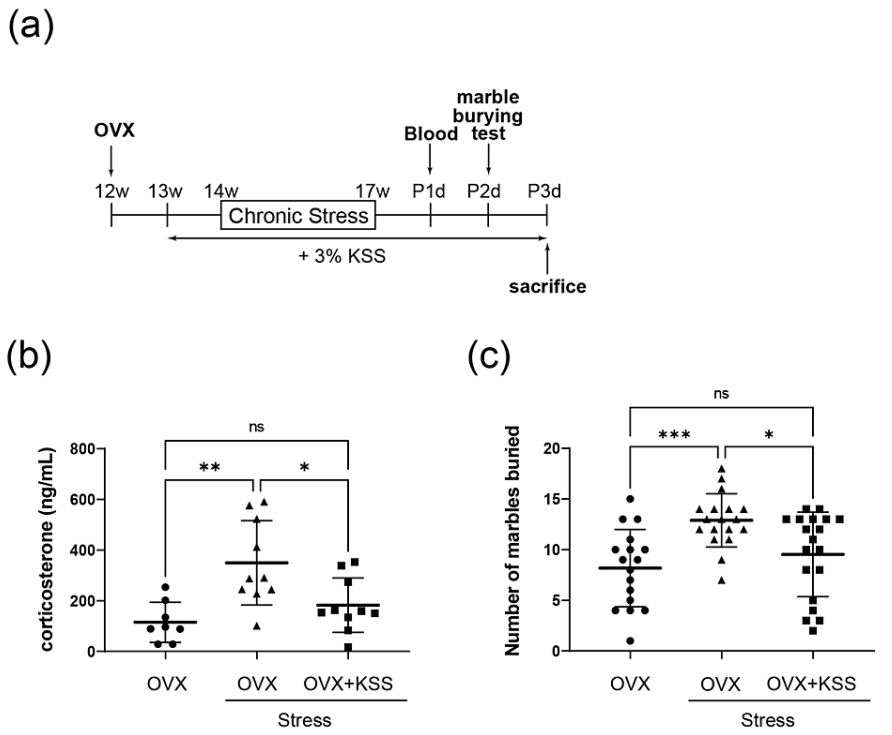

FIGURE 1: KSS treatment effects on plasma corticosterone levels and irritability-like behavior in OVX+Stress mice.

(a) Experimental timeline. The mice were used from 12 w (weeks) to 17w. Several analyses were performed from P1d (post 1 day) to P3d. (b) Levels of plasma corticosterone were measured by ELISA using blood samples. Results are shown as means ±SD (n = 8-10). (c) Marble Burying Test. Chronic stress exposure increased the number of buried marbles, which was normalized by the KSS administration. Results are shown as means ± SD (n = 17-19). *P < 0.05, **P < 0.01 one-way ANOVA followed by Tukey's post-test.

KSS, Kamishoyosan; OVX, ovariectomized mice; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ANOVA, analysis of variance; ns, no statistically significant difference.

Dendritic spine maturation and synaptic function in the mPFC were ameliorated from the effects of chronic stress by KSS

Chronic stress significantly decreased the spine area and spine head width in the mPFC of OVX mice, while these changes were absent in chronic stress-exposed OVX mice administered KSS (Figures *2a*, b, d). Furthermore, OVX+Stress mice showed increased spine length compared with stress-exposed OVX mice that received KSS (Figures *2a*, c). To evaluate synaptic function in the mPFC, we assessed the expression level of the postsynaptic density protein 95 (PSD95). Western blot analysis revealed decreased PSD95 expression in the mPFC of OVX+Stress mice, whereas expression in OVX+Stress+KSS mice was equivalent to that of the control (Figures *2e*, f). These results suggest that KSS administration ameliorated defects induced by chronic stress in spine maturation and synaptic function in the mPFC of OVX+Stress mice.

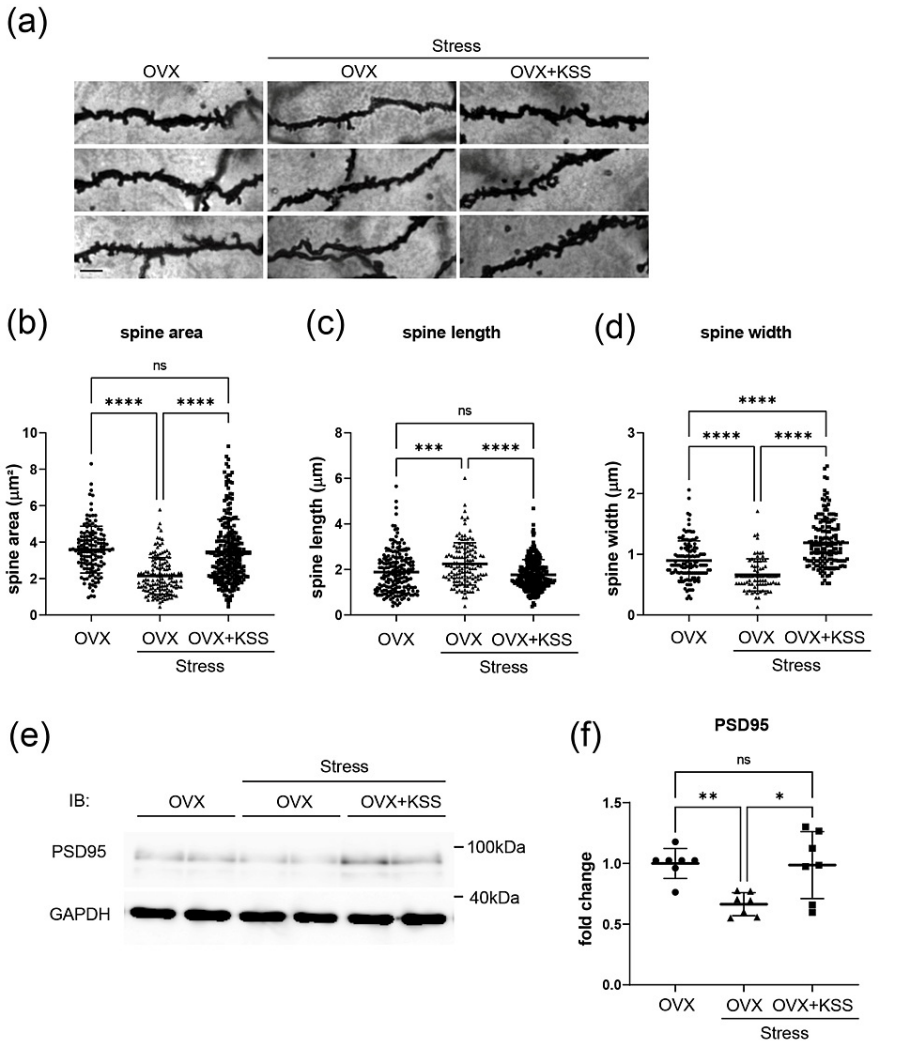

FIGURE 2: KSS treatment effects on basal dendritic spines of pyramidal neurons in the mPFC.

(a) High-magnification image of the representative Golgi-stained dendritic segments of pyramidal neurons in the mPFC. Scale bar, 200 nm. (b-d) Spine area, length, and width of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Data are presented as means ± SD. Two hundred spines from four slices from three animals per group were analyzed. Compared with OVX and OVX+Stress+KSS mice, OVX+Stress mice showed reduced spine area, length, and width. Data are presented as means ± SD. (e) PSD95 expression was evaluated through western blot analysis. (f) Densitometric quantification of PSD95 expression. The results are shown as means ± SD (n = 6). One-way ANOVA, Tukey's multiple comparisons test, *p < 0.05, **P < 0.01, ***P < 0.001, ****P□0.0001. Scale bar: 200 nm.

KSS, Kamishoyosan; OVX, ovariectomized mice; mPFC, medial prefrontal cortex; IB, immune blotting; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo Dalton; ns, no statistically significant difference.

KSS recovered GR protein expression changes through miR-18 upregulation in the mPFC

Next, we evaluated the relationship between GR protein and miR-18 expression levels in the mPFC region of the OVX+Stress mice with or without KSS administration. OVX+Stress mice showed significantly increased miR-18 expression in the mPFC, which was normalized by KSS administration in OVX+Stress+KSS mice (Figure *3a*). Moreover, western blot analysis revealed decreased GR protein expression in the mPFC of OVX+Stress mice, whereas it resembled the control level in the OVX+Stress+KSS mice (Figures *3b*, c). Chronic stress exposure also decreased the number of GR-immunoreactive cells in the mPFC, which was prevented by KSS administration (Figures *3d*, e). Taken together, these findings suggest that KSS reduced miR-18 expression and increased GR protein expression levels by reversing the inhibitory effects of chronic stress exposure on GR protein translation in the mPFC of OVX+Stress mice.

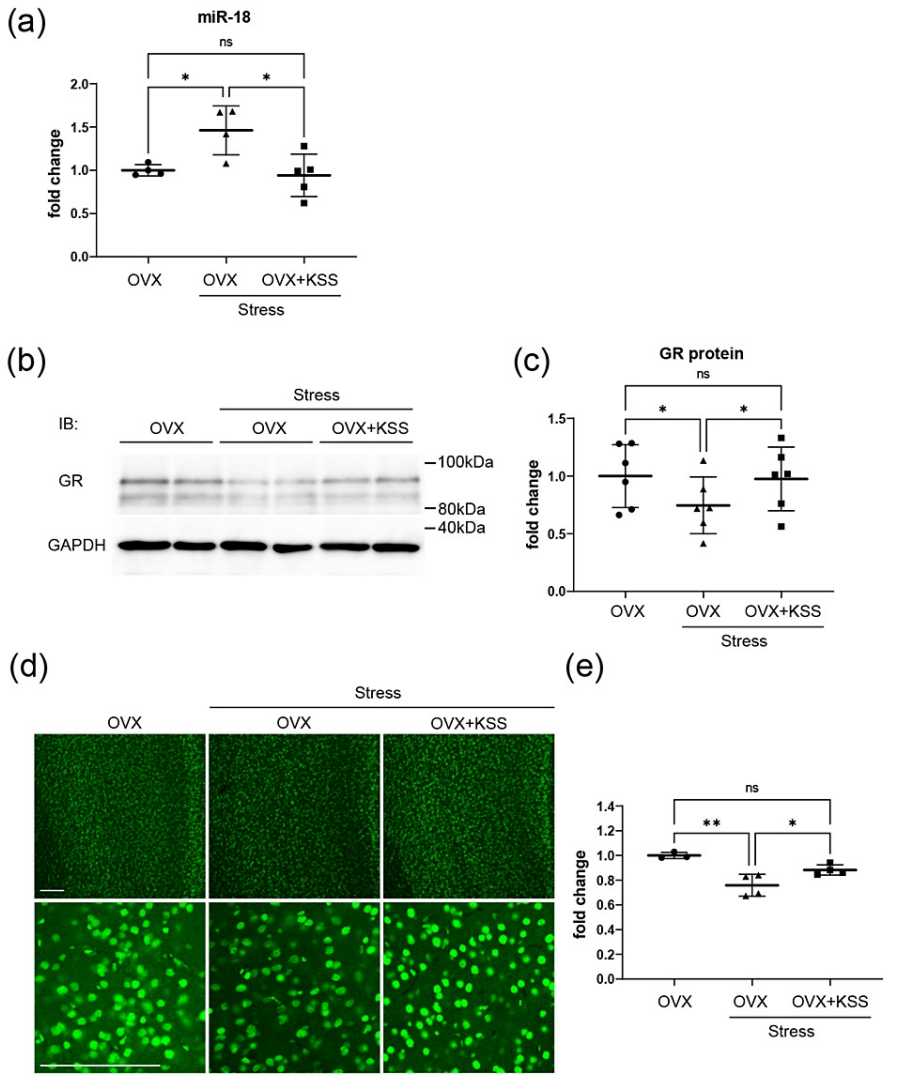

FIGURE 3: KSS treatment effects on miR-18 level and GR protein expression in the mPFC in OVX+Stress mice.

(a) Expression of miR-18 in the PFC was quantified through quantitative RT-PCR. (b) GR expression was assessed through western blot analysis. (c) Densitometric quantification of GR expression. These results are shown as means ± SD (n = 6). (d) Representative staining (upper panels) and high magnification (lower panels) images of GRs (green) in the mPFC. (e) The relative fluorescence intensity of GR signals in the mPFC. These results are shown as means ± SD (n = 4). Scale bar: 100 μm.

PFC, prefrontal cortex; RT-PCR, reverse transcription-polymerase chain reaction; GR, glucocorticoid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-18, microRNA-18; ns, no statistically significant difference.

Discussion

The present study demonstrated that KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which in turn improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figures *1*-*3*). The mPFC is involved in numerous important cognitive functions, including decision-making, working memory, attention, and emotional control [7]. Several clinical studies have demonstrated the effects of menopause on the mPFC [15]. Menopause is associated with decreased gray matter volume in the mPFC, which may contribute to cognitive changes such as memory problems and difficulties in decision-making [16]. During menopause, there are changes in the levels of hormones such as estrogen, which is involved in the formation and maintenance of synapses and dendritic spines [17]. Furthermore, estrogen replacement therapy increased the density and size of hippocampal dendritic spines in a rat menopause model [18]. These findings suggest that the menopause-related decrease in estrogen levels may be associated with changes in the synaptic function and spine morphology of mPFC neurons. Menopause-related changes in hormone levels may exacerbate the effects of chronic stress. Thus, future studies should focus on understanding the

anti-stress mechanisms involved in regulating neuronal functions by the effective chemical components of KSS.

We previously found that Yokukansan, a Japanese herbal medicine, downregulated miR-18 expression and normalized HPA axis activity by regulating GR protein expression in the hypothalamus and corpus callosum of stress-exposed mice. Similarly, our present findings indicated that KSS ameliorated chronic stress-induced unstable emotional behavior and upregulated plasma corticosterone levels (Figure *1*). A previous study reported that postmenopausal women had higher levels of cortisol and perceived stress than premenopausal women [19]. However, the molecular mechanisms underlying the changes in cortisol levels and HPA axis activity in postmenopausal women remain unclear. The present study demonstrated that the effects of KSS on the HPA axis involve miR-18 and GR protein expression (Figure *3*). Specifically, KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figure *2*).

The limitation of this study is that we did not identify the constituent herbal medicines included in KSS and did not show the function of estrogen receptors (ERs). Among the constituent herbal medicines in KSS, Bupleuri radix is well-known as the main component that may be effective for psychiatric symptoms and could serve as a possible alternative to current antidepressant medicines. Thus, a single administration of Bupleuri radix might show practical antidepressant-like and anti-stress effects in rodents. The next step in our research is to determine the effective chemical components of KSS. Previous studies have reported that miR-18 prevents ERα expression; furthermore, postmenopausal women have shown decreased ERα expression [20]. Therefore, brain miR-18 expression might be crucially involved in GR and/or ERα expression during the onset of menopausal symptoms. However, further studies linking the effective chemical components of KSS to its anti-stress function related to microRNAs in the brain are warranted to clarify the functional implications of these novel findings in the mPFC of chronic stress-exposed postmenopausal model mice.

From a clinical point of view, one previous KSS clinical trial study for postmenopausal women indicated that it was not able to show significant improvement effects in the main survey values [4]. One of the reasons why no significant improvements were found in the main survey values is that this clinical study might include several problems with the study design of participant selection. However, this clinical study also showed that KSS administration for post-menopause women showed significant improvements in excitability and irritability scores [4]. Furthermore, in this study, we indicated that KSS administration showed improvement effects of irritability behaviors for stress-exposed-OVX mice (Figure *1*). Furthermore, we found that the manufacturer did not perform the clinical survey of adverse reactions, and the KSS clinical trial study for postmenopausal women for 12 weeks indicated that no serious adverse events were reported [4]. From the results of these clinical trials and our basic research, it is assumed that KSS has a possibility of an effect on improving neuropsychiatric symptoms during menopause, especially irritability.

Conclusions

In conclusion, KSS administration in chronic stress-exposed postmenopausal mice exerted anti-stress effects and facilitated recovery from immature spine morphologies of basal dendritic pyramidal neurons, at least partly by regulating miR-18 and GR expression in the mPFC.

Appendices

□	OVX (n=8)	OVX+stress (n=10)	OVX+stress+KSS (n=10)
Mean	114.8	349.5*	182.2†
SD	79.1	166.6	107.6

TABLE 3: KSS treatment effects on plasma corticosterone levels (Figure 1b)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0017 between OVX and OVX+stress, P = 0.0174 between OVX+stress and OVX+stress+KSS groups).

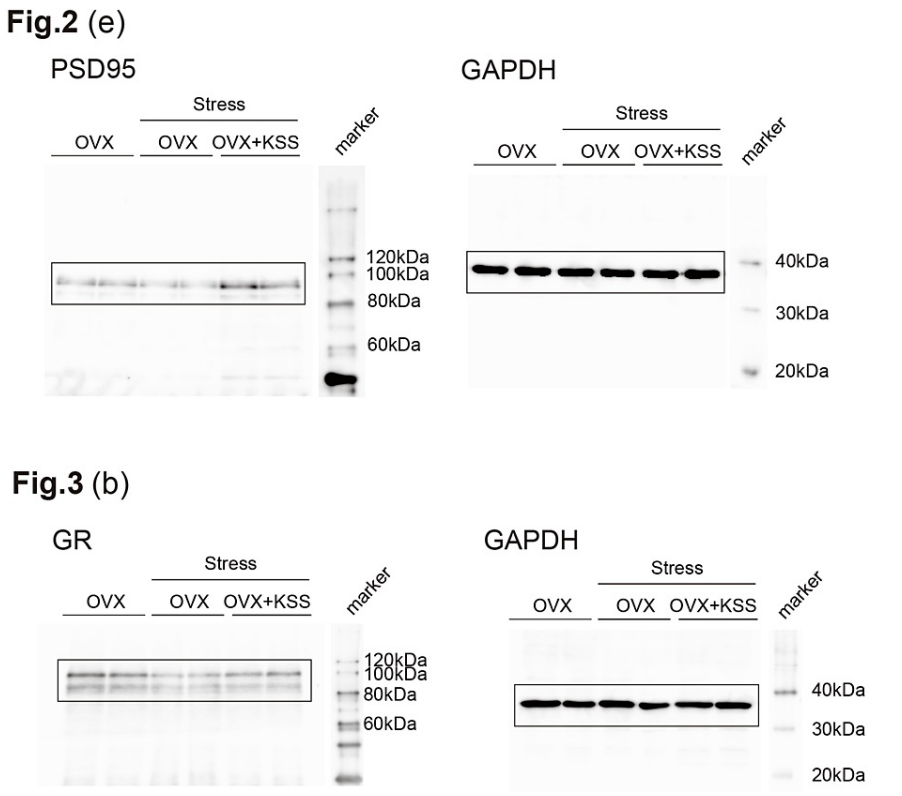

FIGURE 4: Full-size gels for immunoblots and molecular weight markers in Figures 2, 3.

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo-Dalton.

	OVX (n=17)	OVX+stress (n=18)	OVX+stress+KSS (n=19)
Mean	8.18	12.89*	9.53†
SD	3.81	2.63	4.17

TABLE 4: Number of marbles buried in the marbles burying test (Figure 1c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0009 between OVX and OVX+stress, P = 0.0177 between OVX+stress and OVX+stress+KSS groups).

□(a) □	OVX (n=135)	OVX+stress (n=146)	OVX+stress+KSS (n=260)
Mean	3.59	2.15**	3.47†
SD	1.28	1.01	1.78
□(b) □	**OVX (n=192)**	**OVX+stress (n=131)**	**OVX+stress+KSS (n=261)**
Mean	1.88	2.24*	1.76†
SD	0.92	0.93	0.67
□(c) □	**OVX (n=127)**	**OVX+stress (n=79)**	**OVX+stress+KSS (n=157)**
Mean	0.90	0.66**	1.19†
SD	0.32	0.26	0.39

TABLE 5: Morphological analysis of spines of pyramidal neurons in mPFC using Golgi staining (Figure 2b-d)

Spine area (a), length (b), and width (c) of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (*P <0.001, **P <0.0001 between OVX and OVX+stress, †P <0.0001 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=7)	OVX+stress (n=7)	OVX+stress+KSS (n=7)
Mean	1.00	0.66*	0.99†
SD	0.12	0.10	0.28

TABLE 6: Densitometric quantification of PSD95 expression in mPFC (Figure 2f)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0079 between OVX and OVX+stress, P = 0.0107 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=4)	OVX+stress (n=4)	OVX+stress+KSS (n=5)
Mean	1.00	1.46*	0.94†
SD	0.07	0.28	0.25

TABLE 7: Expression levels of miR-18 in mPFC (Figure 3a)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0362 between OVX and OVX+stress, P = 0.0146 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=6)	OVX+stress (n=6)	OVX+stress+KSS (n=6)
Mean	1.00	0.75*	0.97†
SD	0.27	0.25	0.28

TABLE 8: Densitometric quantification of GR expression (Figure 3c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0123 between OVX and OVX+stress, P = 0.0212 between OVX+stress and OVX+stress+KSS groups).

GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=3)	OVX+stress (n=4)	OVX+stress+KSS (n=4)
Mean	1.00	0.76*	0.88†
SD	0.03	0.09	0.04

TABLE 9: The relative fluorescence intensity of GR signals in the mPFC (Figure 3e)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0022 between OVX and OVX+stress, P = 0.0492 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Drafting of the manuscript: Shingo Miyata

Supervision: Shingo Miyata

Acquisition, analysis, or interpretation of data: Shoko Shimizu, Yoshihisa Koyama, Yugo Ishino

Critical review of the manuscript for important intellectual content: Shoko Shimizu, Yoshihisa Koyama, Yugo Ishino, Takashi Takeda, Shoichi Shimada, Masaya Tohyama

Disclosures

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司会：上園保仁

漢方薬の作用機序解明からみたトランスレーショナルリサーチ

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キーワード：漢方薬，ストレス応答経路，マイクロRNA，セロトニン受容体，ジェンダーインクルージブ社会
Kampo medicine, stress-response pathway, microRNA, serotonin receptor, gender inclusive society

抄録：うつ病などの精神疾患の発症には様々な環境ストレス要因の関与が大きいことが知られているものの，これら神経精神症状表出に関与する分子機序や脳の機能的・構造的変化等については，いまだ十分に明らかにされていないのが現状である．我々は認知症に伴う行動心理学的症状（BPSD）の治療に対して臨床的に有効であることが知られている抑肝散および女性の更年期障害に対して処方される三大漢方薬のひとつであり，主にイライラなどの精神神経症状に有効である加味逍遥散の抗ストレス効果に関する作用機序の解明を実施し，それぞれの漢方薬の特異的応答経路を見出した．今後は有効成分の同定が必須であり，更なる詳細な解析が期待される．
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はじめに

近年，特にこの20年程度の間に基礎研究領域における漢方薬の作用機序の解明が盛んに行われると共に臨床研究における漢方薬の有効性の検討も多く実施されてきた．しかし，未だに国際的な評価に耐えうる漢方薬への発展にまでには至っていない．これは基礎研究領域での漢方薬の作用機序の解明がいまだ十分ではなく，Evidence-based medicine（EBM）に基づいた漢方薬による臨床適用にまで至っていない状況によるものと考えることができる．本稿では，近年の漢方薬の作用機序解明の中心にいた抑肝散と共に，近年のジェンダーインクルージブな社会に対応するためには極めて重要であると考えられる月経前症候群や月経困難症，更には更年期障害症状などの女性特有の症状克服に有効な漢方薬の一つである加味逍遥散について，我々の基礎研究で得られた研究成果を概説したい．

抑肝散の抗ストレス効果について

抑肝散は，柴胡，釣藤鈎，蒼朮，茯苓，当帰，川芎，甘草の7種類の構成生薬からなる多成分合剤であり，古くから神経症や不眠症，更には小児の夜泣きや疳症に使用されてきた．近年，認知症に伴う周辺症状の行動心理学的症状（behavioral and psychological symptoms of dementia; BPSD）への有効性が示されるなど，非常に多くの研究論文の存在する漢方薬の代表格の一つである[2]．著者らのグループでは認知症中核症状にも抑肝散が有効性を示すのではないかとの仮説から検討を実施し，抑肝散が確かに神経細胞死を抑制するという事実だけでなく，構成生薬の中で神経細胞死を抑制する成分は川芎である事を同定した．さらに，川芎に含まれるフェルラ酸に神経細胞死抑制効果がある事や家族性アルツハイマー型認知症の原因遺伝子の一つプレセニリン1の変異体（deltaE9）発現神経細胞において，抑肝散および川芎が小胞体ストレス応答経路を介して細胞死を抑制するという分子機序も見出している[1]．

また，認知症BPSDの治療にはリスペリドンなどの非定型抗精神病薬だけでなく，抗うつ薬も効果があるとの報告があることから，抗うつ薬と類似した成分が抑肝散の中にも含有されている可能性がある，すなわち抑肝散の構成成分の中にうつ病態の一つである不安や気分の落ち込みなどの症状にも有効な成分が含まれている可能性がるのではと著者らは考えた．そこで著者らは，環境ストレス曝露によるうつ病モデルマウスを作成し，抑肝散の効果の有無について検討を行った．各種環境ストレス暴露動物は不安や攻撃性など様々なストレス症状を呈する．これらのストレス暴露に対する生体反応系として視床下部－下垂体－副腎軸（Hypothalamic-Pituitary-Adrenal axis; HPA axis）が知られており，通常はこのHPA axisは負のフィードバック機能により過剰な刺激が持続しない．しかし，うつ病をはじめとする一部のストレス性の精神疾患では，慢性的な繰り返しのストレス暴露により，HPA axisが過剰に反応し続け，負のフィードバックシステムが機能不全に至るこ

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とが大きく関連すると考えられている．著者らは環境ストレス曝露によるうつ病モデルマウスのHPA axis応答経路を中心に検討を重ねた．その結果，抑肝散投与群ではストレスに上昇した血中コルチコステロン（グルココルチコイド）値が非ストレス群と同程度にまで低下すること．HPA axis制御に深く関わるGlucocorticoid Receptor（GR）タンパク量は視床下部のparaventricular nucleus（室傍核；PVN）および脳梁オリゴデンドロサイトにおいて有意に増加することを見出した．次にこの分子機序について検討したところ，タンパク発現量を転写後に調節するnon-coding RNAのひとつであるmicroRNA（miR）としてmiR-18又はmiR-124aによるGRタンパク質の翻訳レベルの制御機構を明らかにした．すなわち，抑肝散投与により，ストレス負荷マウスの視床下部神経細胞ではmiR-18，脳梁オリゴデンドロサイトではmiR-124aの発現が低下し，GR翻訳抑制が解除され，その結果GRタンパク量を正常量に増加させることでHPA axisを正常化するという抑肝散の新たな抗ストレス効果の分子機序を明らかにすることが出来た[4)5)]．更に，抑肝散の構成生薬の中で柴胡を含む数個に抗ストレス効果がある可能性まで見出している．

加味逍遥散の抗ストレス効果について

女性の月経困難症や月経前症候群は特定の性周期に表出することで日常生活に支障をきたす事があり，近年のジェンダーインクルージブな社会環境の安定的構築にとって，

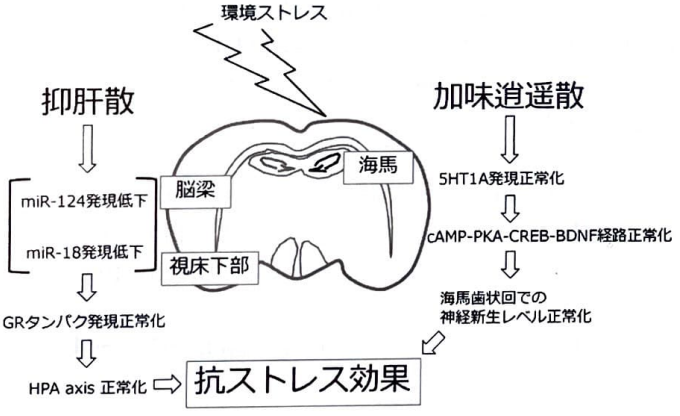

図1 著者らが見出した抑肝散と加味逍遥散の抗ストレス効果の分子機序．（左）抑肝散によるmiR-18制御で視床下部PVN neuronの，miR-124a制御で脳梁オリゴデンドロサイトのGR protein levelをそれぞれ正常化することで抗ストレス効果を発揮する．（右）加味逍遙散による海馬神経細胞でのセロトニン5HT1A受容体発現量正常化による細胞内シグナル伝達を活性化しストレスにより低下した神経新生レベルを正常化する．PVN: paraventricular nucleus, GR: glucocorticoid receptor, HPA axis: hypothalamic-pituitary-adrenal axis, PKA: protein kinase A, CREB: cAMP response element binding protein, BDNF: brain-derived neurotrophic factor.

それら症状の治療・予防は大きな課題となっている．更に，更年期障害では卵巣機能低下によるエストロゲン分泌減少という生物学的要因に加えて，社会心理的ストレスなどの環境要因が複雑に関与する事で，のぼせ・冷えなどの身体的症状や不安，イライラなどの精神的症状を呈することから，この症状の治療・予防も女性活躍社会構築における課題の一つである．これらの症状に対しては，女性三大漢方薬が処方されることが多く，特に精神的症状が強い場合には加味逍遥散が臨床的に使用される事が多い．

加味逍遥散は柴胡，当帰，芍薬，茯苓，白朮，甘草，生姜，薄荷，牡丹皮，山梔子の10種類の生薬からなる多成分合剤であり，抑肝散と一部構成生薬が重なっている．そこで著者らは抑肝散研究と同様に精神症状に効果を示す加味逍遥散には不安や気分の落ち込みなどの症状にも有効な成分が含まれている可能性があるものと考え，更年期障害モデルマウスへの環境ストレス曝露により不安，イライラなどの精神的症状を呈する更年期障害モデルマウスを作成し，加味逍遥散の効果の有無について検討を行った．その結果，加味逍遥散投与群ではストレスに上昇した血中コルチコステロン（グルココルチコイド）値が非ストレス群と同程度にまで低下すること，海馬歯状回おける神経幹細胞からの神経新生レベルが正常化することを見出した．次にこの分子機序について検討したところ，海馬歯状回の神経細胞におけるセロトニン受容体の一つ5-HT1A受容体の発現量が正常化することが明らかになった．うつ病患者における死後脳解析から海馬や扁桃体などのうつ病と関連する領域で5-HT1A受容体の密度低下が報告されており，更年期障害時でも海馬歯状回の神経細胞で5-HT1A受容体が発現低下することによりうつ病の症状に関連する様な精神症状が表出する可能性が考えられた．今回の著者らの検討により，加味逍遥散はこの海馬歯状回の5-HT1A受容体発現レベルを正常化することによりHPA axisを正常化し，神経症状を改善するという新規分子機序を見出した[3)]．更に著者らは詳細な解析を実施し，加味逍遥散が5-HT1A受容体の下流シグナルであるcAMP-PKA-CREB-BDNF経路を正常化するという事実も見出している[3)]．この加味逍遥散は，抑肝散と同様に構成生薬の中に柴胡を含んでおり，加味逍遥散の抗ストレス効果への柴胡の関与の可能性に今後の興味がもたれた．

おわりに

主に精神症状に有効であり臨床適用されている抑肝散と加味逍遥散の抗ストレス効果についての著者らの基礎研究は現在も進行中で，更なる分子機序の解明を実施しており，両者の漢方薬の根本的な作用機序に少しでも近づきたい所である（図1）．しかし，どちらの漢方薬も多成分合

剤でありトランスレーショナルリサーチとして探索する必要のある有効成分の同定という点からは，単独の成分なのかくつかの成分が複合的に関与して効果を示しているのか等についてはっきりしないことが多く，古くから議論の余地が大きい課題でもある．著者らだけでなく，全世界的観点から今後の漢方薬の基礎研究およびトランスレーショナルリサーチの進展に期待したい．

利益相反について：すべての著者に開示すべき利益相反はない．

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Abstract

Translational research based on functional mechanisms of Kampo medicine

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Major depressive disorder (MDD) is one of the leading causes of disturbances in emotional, cognitive, autonomic, and endocrine functions, according to the large amount of information on MDD that has been accumulated during recent years. Although dysregulation of the HPA axis by chronic stress is indicative of MDD, the molecular mechanisms and functional changes in the brain underlying depression are largely unknown. Yokukansan (YKS) can affect behavioral and psychological symptoms such as aggression, anxiety, and depression in patients with Alzheimer's disease and other forms of dementia, and Kamishoyosan (KSS) is widely used for the treatment of various neuropsychiatric symptoms in perimenopausal and postmenopausal women. In the previous study, we developed specific animal models and indicated molecular mechanisms of the YKS and KSS functions for these various neuropsychiatric symptoms. These results suggest that one or more active ingredients in YKS and KSS could be used as a possible alternative to current antidepressant drugs.

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原著論文

1. Kamishoyosan Normalizes Dendritic Spine Morphology in the Medial Prefrontal Cortex by Regulating microRNA-18 and Glucocorticoid Receptor Expressions in Postmenopausal Chronic Stress-Exposed Mice.
   Shimizu S, Koyama Y, **Ishino Y,** Takeda T, Shimada S, Tohyama M, Miyata S*.
   Cureus. 2024 Jun 30;16(6):e63526.

和文総説

1. 漢方薬の作用機序からみたトランスレーショナルリサーチ
   宮田信吾、清水尚子、**石野雄吾**、遠山正彌
   自律神経、62(1)：2-4, 2025.

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   神経発生におけるアルギニンメチル化酵素 PRMT7 の機能解析
2. NEURO2024 第 47 回日本神経科学大会 第 67 回日本神経化学会大会 第 46 回日本生物学的精神医学会年会 第 8 回アジアオセアニア神経科学連合コングレス 合同大会　2024.7.24-27, 福岡コンベンションセンター
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1. 転写因子 NFATc2 の新規活性制御機構による髄鞘機能調節
   日本学術振興会 科学研究費助成事業 基盤研究(C) 2024 年 4 月- 2027 年 3 月
   石野雄吾(代表研究者), 宮田信吾

2. うつ病発症機構におけるタンパクメチル化酵素によるリン酸化シグナル制御の重要性
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宮田信吾, 遠山正彌, 清水尚子, **石野雄吾**(分担研究者)

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Kamishoyosan Normalizes Dendritic Spine Morphology in the Medial Prefrontal Cortex by Regulating microRNA-18 and Glucocorticoid Receptor Expressions in Postmenopausal Chronic Stress-Exposed Mice

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Abstract

Objective: Kamishoyosan (KSS), a traditional Japanese Kampo medicine, is widely used to treat neuropsychiatric symptoms in perimenopausal and postmenopausal women. We aimed to elucidate the functional mechanisms underlying KSS-mediated reduction of stress response behaviors and neuropsychological symptoms in perimenopausal and postmenopausal women.

Methods: Female mice were bilaterally ovariectomized (OVX) at the age of 12 weeks and exposed to chronic water immersion and restraint stress for three weeks. Among them, mice in the OVX+stress+KSS group were fed chow containing KSS from one week before exposure to chronic stress until the end of the experiment. Firstly, we performed a marble burying test and measured serum corticosterone levels to assess irritability and stress conditions. Next, we examined whether KSS affects microRNA-18 (miR-18) and glucocorticoid receptor (GR) protein expression, as well as the basal dendritic spine morphology of pyramidal neurons in the medial prefrontal cortex (mPFC) of postmenopausal chronic stress-exposed mice. Analyzed data were expressed as mean ± standard deviation. Tukey's post hoc test, followed by analysis of variance (ANOVA), was used for among-group comparisons.

Results: KSS administration normalized chronic stress-induced unstable emotion-like behavior and upregulated plasma corticosterone levels. Furthermore, KSS ameliorated GR protein expression by downregulating miR-18 expression in the mPFC and recovered the immature morphological changes in spine formation of pyramidal neurons in the mPFC of OVX mice following chronic stress exposure.

Conclusions: KSS administration in postmenopausal chronic stress-exposed mice exerted anti-stress effects and improved the basal dendritic spine morphology of pyramidal neurons by regulating miR-18 and glucocorticoid receptor expression in the mPFC.

Categories: Psychiatry, Anatomy, Obstetrics/Gynecology
Keywords: medial prefrontal cortex, spine morphology, glucocorticoid receptor, microrna, hypothalamic-pituitary-adrenal axis, menopause, kamishoyosan

Introduction

Menopause-related neuropsychological symptoms, including irritation, depression, and anxiety, are characterized by cognitive, autonomic, emotional, and endocrine function disturbances [1]. One of the traditional Japanese Kampo medicines, Kamishoyosan (KSS), is composed of 10 crude compounds containing a specified mixture derived from plant sources and is widely prescribed to improve various neuropsychiatric symptoms in perimenopausal and postmenopausal women [2,3]. A previous KSS clinical study in postmenopausal women and a premenstrual rat model reported that KSS administration caused significant improvements in excitability and irritability scores [4,5]. Apart from these studies, we previously demonstrated that continuous KSS administration in postmenopausal chronic stress-exposed mice attenuated stress-related depressive behavior and normalized hypothalamic-pituitary-adrenal (HPA) axis activity [6]. However, the molecular mechanisms underlying the beneficial effects of KSS-mediated regulation of the HPA axis remain unclear.

The prefrontal cortex (PFC), particularly the medial prefrontal cortex (mPFC) in humans, is critical to higher-order executive functions, memory, decision-making, cognition, and emotional control [7]. The mPFC is vulnerable to stress, which can decrease its volume and synaptic density by changing spine

morphology in patients with depression [8]. Decreased PFC activity is closely related to the onset of stress-related psychiatric diseases such as depression [9]. Therefore, prefrontal hypofunction induced by stress exposure is strongly implicated in the onset of psychiatric symptoms. However, the molecular mechanisms underlying menopause-related neuropsychological symptoms in mPFC functions remain unclear.

Glucocorticoid receptors (GRs) are also distributed in the PFC, and prefrontal GRs have been recently implicated in HPA axis regulation and mood regulation [10,11]. These studies suggest a functional association between defective prefrontal GR signaling and stress-related psychiatric diseases. Furthermore, a previous study indicated that elevated microRNA-18 (miR-18) expression and reduced GR protein expression were reported in the paraventricular hypothalamic nucleus of stress-vulnerability model rats [12]. However, the functions of miR-18 in the mPFC of postmenopausal environmental stress-exposed mice remain unclear.

Accordingly, as a continuation of our previous KSS research, we aimed to evaluate the effects of KSS on miRNA-mediated regulation of GR expression in the mPFC and the basal dendritic spine morphology of pyramidal neurons in the mPFC of postmenopausal environmental stress-exposed mice in this study.

Materials And Methods

Ethics statement

All animal experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals, the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal care and handling procedures approved by the International Animal Care and Use Committee of Kindai University (No. KAME-25-009).

Animals

Ten-week-old C57BL/6N female mice were purchased from SLC (Japan SLC, Inc., Hamamatsu, Japan). Three mice per cage were kept at room temperature (22 ± 2°C; humidity, 55 ± 10%) in a 12-h light/dark cycle (lights on at 07:00 a.m. and off at 07:00 p.m.). The animals had free access to water and food for breeding (CE-2; CLEA Japan Inc., Tokyo, Japan).

KSS administration and stress exposure

KSS is composed of the extractions of 10 medicinal herbs [6] (Table *1*) and was supplied by Tsumura & Co. (Tokyo, Japan). These ingredient content percentages were calculated from the KSS product label (Tsumura & Co.). Dry powdered extracts of KSS were mixed with CE-2 chow at a final concentration of 3% (w/w) and used as previously reported [6].

Ingredient	Content (%)
Bupleuri Radix (*Bupleurum falcatum*)	13.3
Paeoniae Radix (*Paeonia lactiflora*)	13.3
Atractylodis Rhizoma (*Atractylodes ovate*)	13.3
Angelicae Radix (*Angelica acutiloba*)	13.3
Hoelen (*Poria cocos*)	13.3
Gardeniae Fructus (*Gardenia jasminoides*)	8.9
Moutan Cortex (*Paeonia suffruticosa*)	8.9
Glycyrrhizae Radix (*Glycyrrhizae uralensis*)	6.7
Zingiberis Rhizoma (*Zingiber officinale*)	4.4
Menthae Herba (*Menthae arvensis*)	4.4

TABLE 1: Composition of Kamishoyosan

All female mice were bilaterally ovariectomized (OVX) at age 12 weeks. After two weeks of postoperative recovery, the 36 mice were randomly allocated into three groups (n=12) after the ovariectomy: the control group (non-stressed OVX mice), the chronically stressed group (OVX+Stress mice), and the chronically stressed group administered with KSS (OVX+Stress+KSS mice). There was no significant difference in body

weight and average daily consumption of chow between groups before stress exposure. Chronic stress was induced as previously described [6]. Briefly, OVX mice were exposed to chronic Water Immersion and Restraint Stress (WIRS) for three weeks (at 14-17 weeks of age). Specifically, they were restrained in a 50-mL conical polypropylene centrifuge tube and vertically immersed to the level of the xiphoid process in a water bath maintained at 23°C for two hours once a day for three weeks. For KSS administration, mice in the OVX+Stress+KSS group were fed CE-2 chow containing 3% KSS one week before chronic stress exposure (13 weeks old) until the end of the experiment as previously reported [6]. The mice were confirmed to be free of gastric and duodenal ulcers by visual observation for bleeding and sores on the surface of the stomach and duodenal mucosa.

Enzyme-linked immunosorbent assay (ELISA)

Serum corticosterone levels were measured using a Corticosterone Enzyme Immunoassay Kit (Arbor Assays, K014, Ann Arbor, Michigan), following the manufacturer's instructions. Briefly, one day after the chronic stress exposure period, the mice were deeply anesthetized, and their blood samples were collected into tubes containing heparin. The tubes were immediately placed on ice, followed by centrifugation at 1000 g for 15 minutes at 4°C. Plasma samples were stored at -80°C prior to assays. Absorbance at 450 nm was measured using a plate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, Massachusetts), and the corticosterone concentration in each sample was calculated using the SkanIt™ microplate reader software (Thermo Fisher Scientific Inc.).

Marble burying test

To assess behaviors representing unstable emotions, anxiety, and irritability, a marble burying test was performed two days after the end of the chronic stress exposure period [13]. Briefly, 20 glass marbles were evenly distributed on 5-cm-deep sawdust bedding in 4 × 5 grids in standard cages (25 × 25 × 31 cm). Each mouse was placed in a cage for 15 minutes. Subsequently, the mice were removed from the cage, and the number of marbles buried by the mice was counted at the end of the test. Marbles buried to at least 2/3 of their depth were considered buried [13]. The light intensity in both the breeding and test rooms was ~150 lux at 40 cm from the floor.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the PFC of mice using Isogen II (NipponGene, Toyama, Japan), following the manufacturer's instructions. To analyze mRNA expression, reverse transcription of the total RNA was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). To evaluate GR and GAPDH expression, quantitative real-time PCR (qRT-PCR) was conducted using KOD SYBR qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan) with the following forward/reverse primers: GR, 5'-ACCTGGATGACCAAATGACCC-3'/5'-GCATAGCAGGTTTCCACTTGC-3' and GAPDH, 5'-GTGTTCCTACCCCCAATGTG-3'/5'-AGGAGACAACCTGGTCCTCA-3'. The housekeeping gene GAPDH was used as the internal control [6]. Specific ratio comparisons (gene of interest/GAPDH) were used to assess between-group differences in transcript expression. To analyze miRNA expression, reverse transcription of total RNA was performed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. To detect mature miR-18, qRT-PCR was performed using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific Inc.). TaqMan assays specific for miR-18 (Thermo Fisher Scientific Inc.) were performed with an ABI7900HT PCR System according to the manufacturer's instructions (Thermo Fisher Scientific Inc.). The relative levels of miR-18 in the mPFC were calculated with the 2−ΔΔCT method, with U6 as an internal control.

Golgi staining

Golgi staining was performed using an FD Rapid Golgi Stain Kit (FD NeuroTechnologies Inc., Columbia, Maryland), as previously described [14]. Briefly, the brains were removed from anesthetized mice and immersed in an equal mixture of solutions A and B for three weeks at 22±2°C in the dark. Next, the brains were transferred into solution C for seven days at 22±2°C. After freezing on dry ice, 200-µm serial coronal sections of the brain samples were prepared using a cryostat at -24°C and mounted on a 0.5% gelatin-coated glass slide, incubated overnight at 22±2°C, and soaked in solution C for five minutes. Subsequently, the slides were stained with a mixture of solution D, solution E, and deionized water (1:1:2) for 10 minutes, then rinsed in distilled water twice for four minutes. Coronal sections were dehydrated in an ascending ethanol series, cleared with xylene, and sealed with Entellan (Merck KGaA, Darmstadt, Germany). All images of the pyramidal neurons in the mPFC were obtained using a Keyence microscope (Keyence Corp., Osaka, Japan).

Immunohistochemistry

Immunohistochemical staining of the mouse brain was performed as previously described [6]. Briefly, mice were perfused transcranially with 4% paraformaldehyde three days after the three-week exposure to chronic stress. Next, their brains were collected and immersion-fixed in 4% paraformaldehyde at 4°C overnight. After post-fixing, the brain tissues were stored in a 30% (w/v) sucrose solution in 0.1 M phosphate-buffered saline (PBS) for 48 hours at 4°C. Free-floating, 30-µm-thick sections were rinsed with PBS and incubated in blocking buffer (5% bovine serum albumin and 0.3% Triton X-100 in PBS) for one hour at room temperature.

Subsequently, the sections were incubated with primary antibodies overnight at 4°C (Table 2). Next, the sections were washed in PBS and incubated with Alexa 488 anti-rabbit IgG secondary antibody (1:1000, Thermo Fisher Scientific Inc.; A-11008, RRID: AB_143165) for two hours at room temperature. All images were acquired using a laser scanning confocal microscope (C2; Nikon Corp., Tokyo, Japan). Immunohistochemical staining intensities were determined using ImageJ (National Institutes of Health). To quantify the GR expression level, the images were analyzed with pixel values of fluorescence intensity using the ImageJ software relative to a predetermined threshold intensity (the background intensity of the images set to zero). The same threshold setting was applied to all the images in each comparison group.

Immune blotting analyses

Immune blotting analysis was performed as previously described [6] using the antibodies in Table 2. Immunodetection of target proteins was performed using horseradish peroxidase-conjugated secondary antibodies (1:5000; Cell Signaling Technology Inc.) and an ECL Prime Western Blotting Detection System (GE Healthcare Systems Inc., Chicago, Illinois). Densitometric quantification was performed using ImageJ (National Institutes of Health), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. PSD95, postsynaptic density protein 95.

Antibody	Code No,	Manufacturer	Dilution
GR	ab183127	Abcam Plc, Cambridge, England	1:500 (IHC)
GR	ab183127	Abcam Plc, Cambridge, England	1:1000 (WB)
PSD95	3450	Cell Signaling Technology Inc., Danvers, Massachusetts	1:1000 (WB)
GAPDH	sc-32233	Santa Cruz Biotechnology Inc., Dallas, Texas	1:1000 (WB)

TABLE 2: The information about antibodies

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; WB; western blotting

Statistical analyses

Statistical analyses were performed using GraphPad Prism 10, a statistical software commonly used for data analysis in basic medical research (GraphPad Software, Boston, Massachusetts). Data are expressed as mean ± standard deviation (SD). Analysis of variance (one-way ANOVA) was used for 3 among-group statistical differences, followed by Tukey's post hoc test. Tukey's post-hoc test is a widely used statistical method with robust power to identify significant differences between groups and handle unequal sample sizes and variances. Statistical significance was set at $P < 0.05$.

Results

KSS normalized stress-upregulated plasma corticosterone level and irritability behavior

Compared with control OVX mice, OVX+Stress mice showed upregulated plasma corticosterone levels (Figure 1a, b), whereas the levels in OVX+Stress+KSS mice resembled those of the control (Figure 1a, b). Moreover, OVX+Stress mice showed an increased number of buried marbles, which was lower in OVX+Stress+KSS mice (Figure 1c). These results suggest that KSS administration ameliorated chronic stress-induced continuous hyperactivity of the HPA axis and unstable emotional behavior in OVX+Stress+KSS mice.

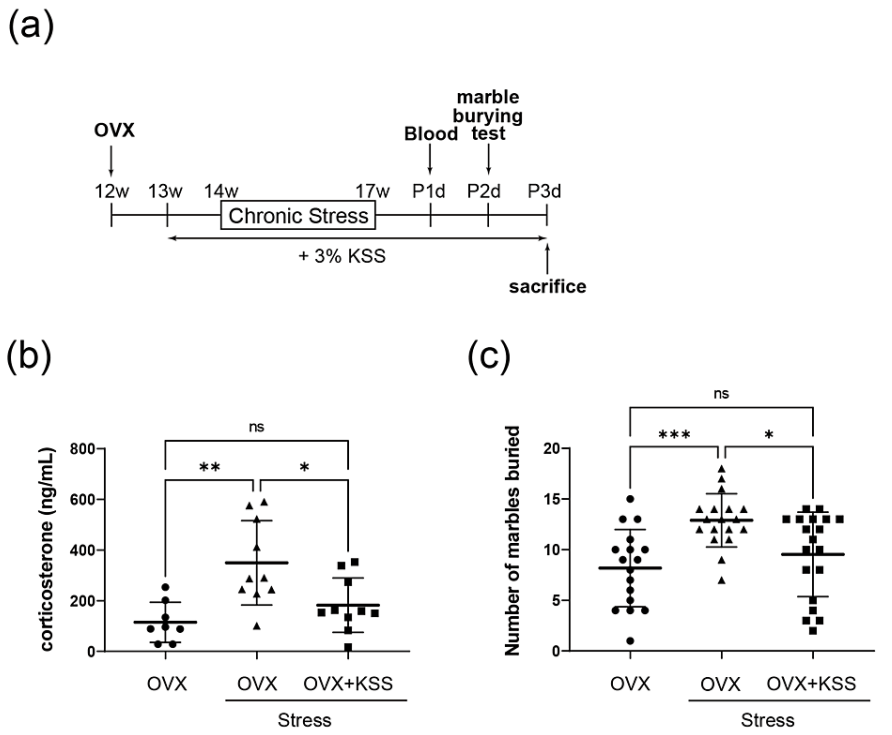

FIGURE 1: KSS treatment effects on plasma corticosterone levels and irritability-like behavior in OVX+Stress mice.

(a) Experimental timeline. The mice were used from 12 w (weeks) to 17w. Several analyses were performed from P1d (post 1 day) to P3d. (b) Levels of plasma corticosterone were measured by ELISA using blood samples. Results are shown as means ±SD (n = 8-10). (c) Marble Burying Test. Chronic stress exposure increased the number of buried marbles, which was normalized by the KSS administration. Results are shown as means ± SD (n = 17-19). *P < 0.05, **P < 0.01 one-way ANOVA followed by Tukey's post-test.

KSS, Kamishoyosan; OVX, ovariectomized mice; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ANOVA, analysis of variance; ns, no statistically significant difference.

Dendritic spine maturation and synaptic function in the mPFC were ameliorated from the effects of chronic stress by KSS

Chronic stress significantly decreased the spine area and spine head width in the mPFC of OVX mice, while these changes were absent in chronic stress-exposed OVX mice administered KSS (Figures *2a*, b, d). Furthermore, OVX+Stress mice showed increased spine length compared with stress-exposed OVX mice that received KSS (Figures *2a*, c). To evaluate synaptic function in the mPFC, we assessed the expression level of the postsynaptic density protein 95 (PSD95). Western blot analysis revealed decreased PSD95 expression in the mPFC of OVX+Stress mice, whereas expression in OVX+Stress+KSS mice was equivalent to that of the control (Figures *2e*, f). These results suggest that KSS administration ameliorated defects induced by chronic stress in spine maturation and synaptic function in the mPFC of OVX+Stress mice.

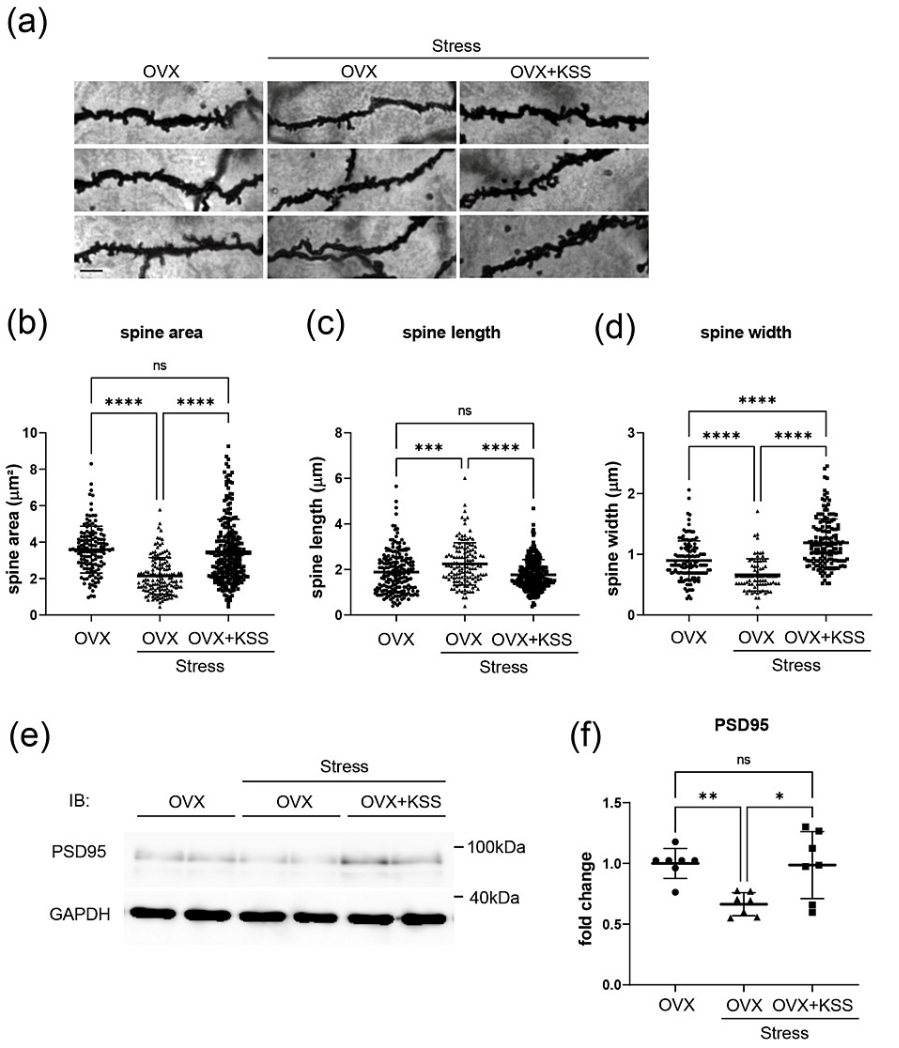

FIGURE 2: KSS treatment effects on basal dendritic spines of pyramidal neurons in the mPFC.

(a) High-magnification image of the representative Golgi-stained dendritic segments of pyramidal neurons in the mPFC. Scale bar, 200 nm. (b-d) Spine area, length, and width of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Data are presented as means ± SD. Two hundred spines from four slices from three animals per group were analyzed. Compared with OVX and OVX+Stress+KSS mice, OVX+Stress mice showed reduced spine area, length, and width. Data are presented as means ± SD. (e) PSD95 expression was evaluated through western blot analysis. (f) Densitometric quantification of PSD95 expression. The results are shown as means ± SD (n = 6). One-way ANOVA, Tukey's multiple comparisons test, *p < 0.05, **P < 0.01, ***P < 0.001, ****P□0.0001. Scale bar: 200 nm.

KSS, Kamishoyosan; OVX, ovariectomized mice; mPFC, medial prefrontal cortex; IB, immune blotting; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo Dalton; ns, no statistically significant difference.

KSS recovered GR protein expression changes through miR-18 upregulation in the mPFC

Next, we evaluated the relationship between GR protein and miR-18 expression levels in the mPFC region of the OVX+Stress mice with or without KSS administration. OVX+Stress mice showed significantly increased miR-18 expression in the mPFC, which was normalized by KSS administration in OVX+Stress+KSS mice (Figure *3a*). Moreover, western blot analysis revealed decreased GR protein expression in the mPFC of OVX+Stress mice, whereas it resembled the control level in the OVX+Stress+KSS mice (Figures *3b*, c). Chronic stress exposure also decreased the number of GR-immunoreactive cells in the mPFC, which was prevented by KSS administration (Figures *3d*, e). Taken together, these findings suggest that KSS reduced miR-18 expression and increased GR protein expression levels by reversing the inhibitory effects of chronic stress exposure on GR protein translation in the mPFC of OVX+Stress mice.

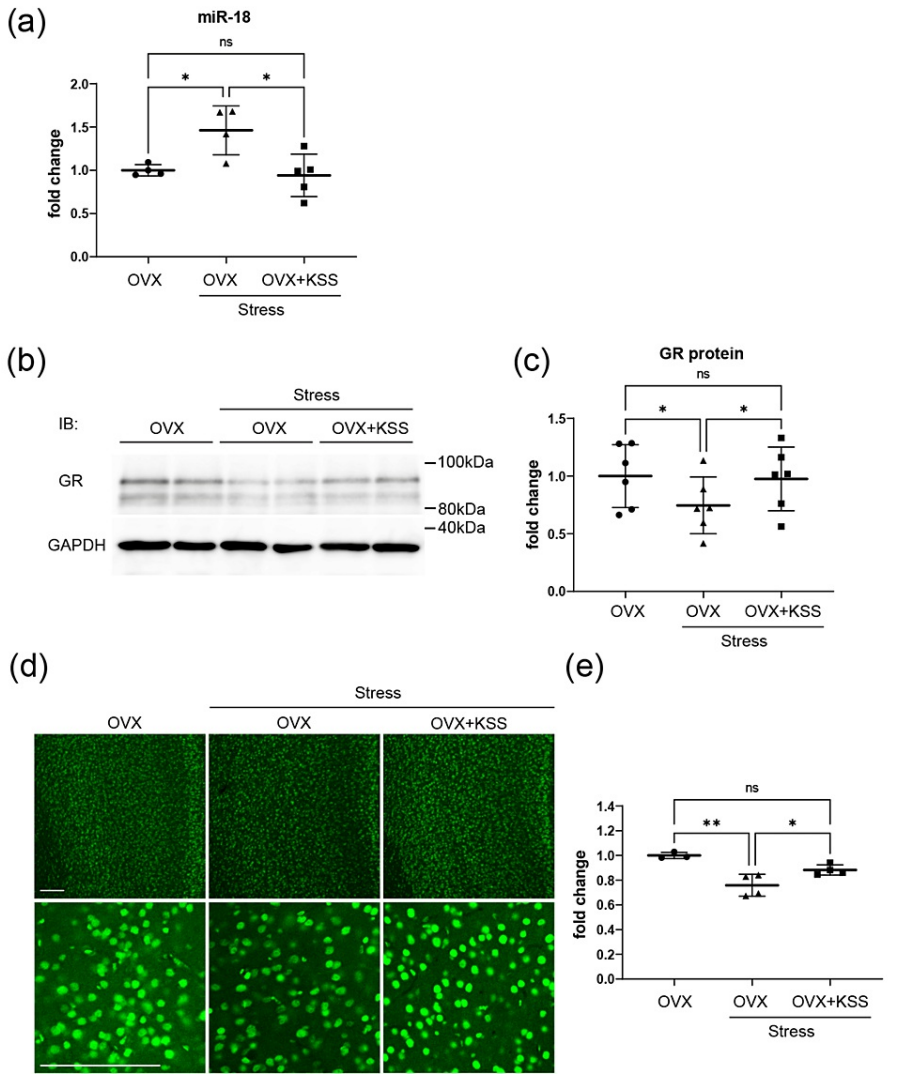

FIGURE 3: KSS treatment effects on miR-18 level and GR protein expression in the mPFC in OVX+Stress mice.

(a) Expression of miR-18 in the PFC was quantified through quantitative RT-PCR. (b) GR expression was assessed through western blot analysis. (c) Densitometric quantification of GR expression. These results are shown as means ± SD (n = 6). (d) Representative staining (upper panels) and high magnification (lower panels) images of GRs (green) in the mPFC. (e) The relative fluorescence intensity of GR signals in the mPFC. These results are shown as means ± SD (n = 4). Scale bar: 100 μm.

PFC, prefrontal cortex; RT-PCR, reverse transcription-polymerase chain reaction; GR, glucocorticoid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-18, microRNA-18; ns, no statistically significant difference.

Discussion

The present study demonstrated that KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which in turn improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figures *1*-*3*). The mPFC is involved in numerous important cognitive functions, including decision-making, working memory, attention, and emotional control [7]. Several clinical studies have demonstrated the effects of menopause on the mPFC [15]. Menopause is associated with decreased gray matter volume in the mPFC, which may contribute to cognitive changes such as memory problems and difficulties in decision-making [16]. During menopause, there are changes in the levels of hormones such as estrogen, which is involved in the formation and maintenance of synapses and dendritic spines [17]. Furthermore, estrogen replacement therapy increased the density and size of hippocampal dendritic spines in a rat menopause model [18]. These findings suggest that the menopause-related decrease in estrogen levels may be associated with changes in the synaptic function and spine morphology of mPFC neurons. Menopause-related changes in hormone levels may exacerbate the effects of chronic stress. Thus, future studies should focus on understanding the

anti-stress mechanisms involved in regulating neuronal functions by the effective chemical components of KSS.

We previously found that Yokukansan, a Japanese herbal medicine, downregulated miR-18 expression and normalized HPA axis activity by regulating GR protein expression in the hypothalamus and corpus callosum of stress-exposed mice. Similarly, our present findings indicated that KSS ameliorated chronic stress-induced unstable emotional behavior and upregulated plasma corticosterone levels (Figure *1*). A previous study reported that postmenopausal women had higher levels of cortisol and perceived stress than premenopausal women [19]. However, the molecular mechanisms underlying the changes in cortisol levels and HPA axis activity in postmenopausal women remain unclear. The present study demonstrated that the effects of KSS on the HPA axis involve miR-18 and GR protein expression (Figure *3*). Specifically, KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figure *2*).

The limitation of this study is that we did not identify the constituent herbal medicines included in KSS and did not show the function of estrogen receptors (ERs). Among the constituent herbal medicines in KSS, Bupleuri radix is well-known as the main component that may be effective for psychiatric symptoms and could serve as a possible alternative to current antidepressant medicines. Thus, a single administration of Bupleuri radix might show practical antidepressant-like and anti-stress effects in rodents. The next step in our research is to determine the effective chemical components of KSS. Previous studies have reported that miR-18 prevents ERα expression; furthermore, postmenopausal women have shown decreased ERα expression [20]. Therefore, brain miR-18 expression might be crucially involved in GR and/or ERα expression during the onset of menopausal symptoms. However, further studies linking the effective chemical components of KSS to its anti-stress function related to microRNAs in the brain are warranted to clarify the functional implications of these novel findings in the mPFC of chronic stress-exposed postmenopausal model mice.

From a clinical point of view, one previous KSS clinical trial study for postmenopausal women indicated that it was not able to show significant improvement effects in the main survey values [4]. One of the reasons why no significant improvements were found in the main survey values is that this clinical study might include several problems with the study design of participant selection. However, this clinical study also showed that KSS administration for post-menopause women showed significant improvements in excitability and irritability scores [4]. Furthermore, in this study, we indicated that KSS administration showed improvement effects of irritability behaviors for stress-exposed-OVX mice (Figure *1*). Furthermore, we found that the manufacturer did not perform the clinical survey of adverse reactions, and the KSS clinical trial study for postmenopausal women for 12 weeks indicated that no serious adverse events were reported [4]. From the results of these clinical trials and our basic research, it is assumed that KSS has a possibility of an effect on improving neuropsychiatric symptoms during menopause, especially irritability.

Conclusions

In conclusion, KSS administration in chronic stress-exposed postmenopausal mice exerted anti-stress effects and facilitated recovery from immature spine morphologies of basal dendritic pyramidal neurons, at least partly by regulating miR-18 and GR expression in the mPFC.

Appendices

□	OVX (n=8)	OVX+stress (n=10)	OVX+stress+KSS (n=10)
Mean	114.8	349.5*	182.2†
SD	79.1	166.6	107.6

TABLE 3: KSS treatment effects on plasma corticosterone levels (Figure 1b)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0017 between OVX and OVX+stress, P = 0.0174 between OVX+stress and OVX+stress+KSS groups).

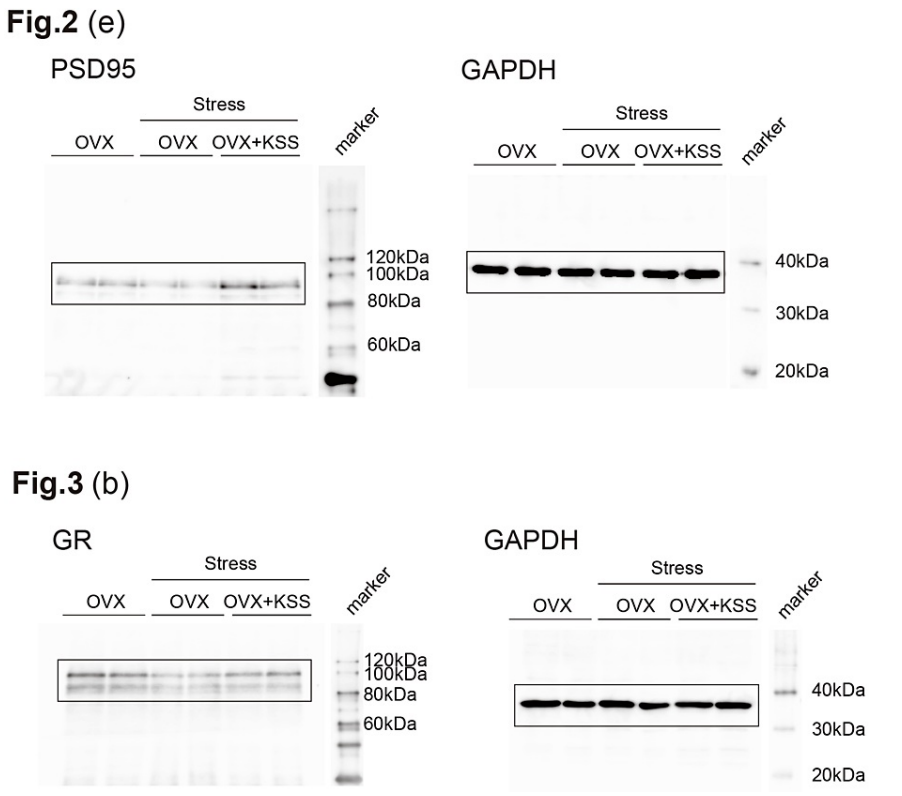

FIGURE 4: Full-size gels for immunoblots and molecular weight markers in Figures 2, 3.

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo-Dalton.

	OVX (n=17)	OVX+stress (n=18)	OVX+stress+KSS (n=19)
Mean	8.18	12.89*	9.53†
SD	3.81	2.63	4.17

TABLE 4: Number of marbles buried in the marbles burying test (Figure 1c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0009 between OVX and OVX+stress, P = 0.0177 between OVX+stress and OVX+stress+KSS groups).

□(a) □	OVX (n=135)	OVX+stress (n=146)	OVX+stress+KSS (n=260)
Mean	3.59	2.15**	3.47†
SD	1.28	1.01	1.78
□(b) □	**OVX (n=192)**	**OVX+stress (n=131)**	**OVX+stress+KSS (n=261)**
Mean	1.88	2.24*	1.76†
SD	0.92	0.93	0.67
□(c) □	**OVX (n=127)**	**OVX+stress (n=79)**	**OVX+stress+KSS (n=157)**
Mean	0.90	0.66**	1.19†
SD	0.32	0.26	0.39

TABLE 5: Morphological analysis of spines of pyramidal neurons in mPFC using Golgi staining (Figure 2b-d)

Spine area (a), length (b), and width (c) of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (*P <0.001, **P <0.0001 between OVX and OVX+stress, †P <0.0001 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=7)	OVX+stress (n=7)	OVX+stress+KSS (n=7)
Mean	1.00	0.66*	0.99†
SD	0.12	0.10	0.28

TABLE 6: Densitometric quantification of PSD95 expression in mPFC (Figure 2f)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0079 between OVX and OVX+stress, P = 0.0107 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=4)	OVX+stress (n=4)	OVX+stress+KSS (n=5)
Mean	1.00	1.46*	0.94†
SD	0.07	0.28	0.25

TABLE 7: Expression levels of miR-18 in mPFC (Figure 3a)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0362 between OVX and OVX+stress, P = 0.0146 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=6)	OVX+stress (n=6)	OVX+stress+KSS (n=6)
Mean	1.00	0.75*	0.97†
SD	0.27	0.25	0.28

TABLE 8: Densitometric quantification of GR expression (Figure 3c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0123 between OVX and OVX+stress, P = 0.0212 between OVX+stress and OVX+stress+KSS groups).

GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=3)	OVX+stress (n=4)	OVX+stress+KSS (n=4)
Mean	1.00	0.76*	0.88†
SD	0.03	0.09	0.04

TABLE 9: The relative fluorescence intensity of GR signals in the mPFC (Figure 3e)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0022 between OVX and OVX+stress, P = 0.0492 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Drafting of the manuscript: Shingo Miyata

Supervision: Shingo Miyata

Acquisition, analysis, or interpretation of data: Shoko Shimizu, Yoshihisa Koyama, Yugo Ishino

Critical review of the manuscript for important intellectual content: Shoko Shimizu, Yoshihisa Koyama, Yugo Ishino, Takashi Takeda, Shoichi Shimada, Masaya Tohyama

Disclosures

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司会：上園保仁

漢方薬の作用機序解明からみたトランスレーショナルリサーチ

宮田信吾　清水尚子　石野雄吾　遠山正彌

キーワード：漢方薬，ストレス応答経路，マイクロRNA，セロトニン受容体，ジェンダーインクルージブ社会
Kampo medicine, stress-response pathway, microRNA, serotonin receptor, gender inclusive society

抄録：うつ病などの精神疾患の発症には様々な環境ストレス要因の関与が大きいことが知られているものの，これら神経精神症状表出に関与する分子機序や脳の機能的・構造的変化等については，いまだ十分に明らかにされていないのが現状である．我々は認知症に伴う行動心理学的症状（BPSD）の治療に対して臨床的に有効であることが知られている抑肝散および女性の更年期障害に対して処方される三大漢方薬のひとつであり，主にイライラなどの精神神経症状に有効である加味逍遥散の抗ストレス効果に関する作用機序の解明を実施し，それぞれの漢方薬の特異的応答経路を見出した．今後は有効成分の同定が必須であり，更なる詳細な解析が期待される．
（自律神経，62：2–4, 2025，doi: 10.32272/ans.62.2_4）

はじめに

近年，特にこの20年程度の間に基礎研究領域における漢方薬の作用機序の解明が盛んに行われると共に臨床研究における漢方薬の有効性の検討も多く実施されてきた．しかし，未だに国際的な評価に耐えうる漢方薬への発展にまでには至っていない．これは基礎研究領域での漢方薬の作用機序の解明がいまだ十分ではなく，Evidence-based medicine（EBM）に基づいた漢方薬による臨床適用にまで至っていない状況によるものと考えることができる．本稿では，近年の漢方薬の作用機序解明の中心にいた抑肝散と共に，近年のジェンダーインクルージブな社会に対応するためには極めて重要であると考えられる月経前症候群や月経困難症，更には更年期障害症状などの女性特有の症状克服に有効な漢方薬の一つである加味逍遥散について，我々の基礎研究で得られた研究成果を概説したい．

抑肝散の抗ストレス効果について

抑肝散は，柴胡，釣藤鈎，蒼朮，茯苓，当帰，川芎，甘草の7種類の構成生薬からなる多成分合剤であり，古くから神経症や不眠症，更には小児の夜泣きや疳症に使用されてきた．近年，認知症に伴う周辺症状の行動心理学的症状（behavioral and psychological symptoms of dementia; BPSD）への有効性が示されるなど，非常に多くの研究論文の存在する漢方薬の代表格の一つである[2]．著者らのグループでは認知症中核症状にも抑肝散が有効性を示すのではないかとの仮説から検討を実施し，抑肝散が確かに神経細胞死を抑制するという事実だけでなく，構成生薬の中で神経細胞死を抑制する成分は川芎である事を同定した．さらに，川芎に含まれるフェルラ酸に神経細胞死抑制効果がある事や家族性アルツハイマー型認知症の原因遺伝子の一つプレセニリン1の変異体（deltaE9）発現神経細胞において，抑肝散および川芎が小胞体ストレス応答経路を介して細胞死を抑制するという分子機序も見出している[1]．

また，認知症BPSDの治療にはリスペリドンなどの非定型抗精神病薬だけでなく，抗うつ薬も効果があるとの報告があることから，抗うつ薬と類似した成分が抑肝散の中にも含有されている可能性がある，すなわち抑肝散の構成成分の中にうつ病態の一つである不安や気分の落ち込みなどの症状にも有効な成分が含まれている可能性がるのではと著者らは考えた．そこで著者らは，環境ストレス曝露によるうつ病モデルマウスを作成し，抑肝散の効果の有無について検討を行った．各種環境ストレス暴露動物は不安や攻撃性など様々なストレス症状を呈する．これらのストレス暴露に対する生体反応系として視床下部－下垂体－副腎軸（Hypothalamic-Pituitary-Adrenal axis; HPA axis）が知られており，通常はこのHPA axisは負のフィードバック機能により過剰な刺激が持続しない．しかし，うつ病をはじめとする一部のストレス性の精神疾患では，慢性的な繰り返しのストレス暴露により，HPA axisが過剰に反応し続け，負のフィードバックシステムが機能不全に至るこ

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とが大きく関連すると考えられている．著者らは環境ストレス曝露によるうつ病モデルマウスのHPA axis応答経路を中心に検討を重ねた．その結果，抑肝散投与群ではストレスに上昇した血中コルチコステロン（グルココルチコイド）値が非ストレス群と同程度にまで低下すること．HPA axis制御に深く関わるGlucocorticoid Receptor（GR）タンパク量は視床下部のparaventricular nucleus（室傍核；PVN）および脳梁オリゴデンドロサイトにおいて有意に増加することを見出した．次にこの分子機序について検討したところ，タンパク発現量を転写後に調節するnon-coding RNAのひとつであるmicroRNA（miR）としてmiR-18又はmiR-124aによるGRタンパク質の翻訳レベルの制御機構を明らかにした．すなわち，抑肝散投与により，ストレス負荷マウスの視床下部神経細胞ではmiR-18，脳梁オリゴデンドロサイトではmiR-124aの発現が低下し，GR翻訳抑制が解除され，その結果GRタンパク量を正常量に増加させることでHPA axisを正常化するという抑肝散の新たな抗ストレス効果の分子機序を明らかにすることが出来た[4)5)]．更に，抑肝散の構成生薬の中で柴胡を含む数個に抗ストレス効果がある可能性まで見出している．

加味逍遥散の抗ストレス効果について

女性の月経困難症や月経前症候群は特定の性周期に表出することで日常生活に支障をきたす事があり，近年のジェンダーインクルージブな社会環境の安定的構築にとって，

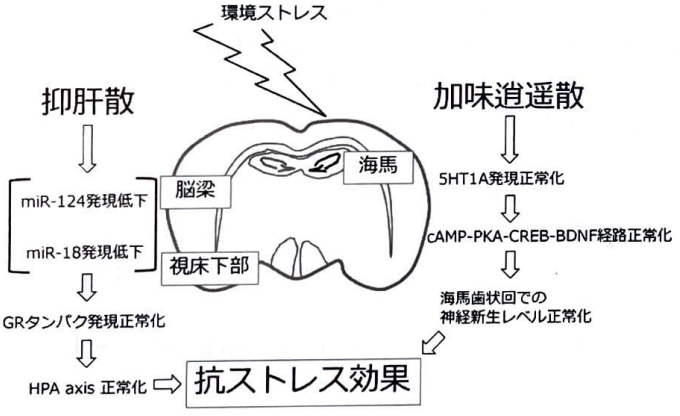

図1 著者らが見出した抑肝散と加味逍遥散の抗ストレス効果の分子機序．（左）抑肝散によるmiR-18制御で視床下部PVN neuronの，miR-124a制御で脳梁オリゴデンドロサイトのGR protein levelをそれぞれ正常化することで抗ストレス効果を発揮する．（右）加味逍遙散による海馬神経細胞でのセロトニン5HT1A受容体発現量正常化による細胞内シグナル伝達を活性化しストレスにより低下した神経新生レベルを正常化する．PVN: paraventricular nucleus, GR: glucocorticoid receptor, HPA axis: hypothalamic-pituitary-adrenal axis, PKA: protein kinase A, CREB: cAMP response element binding protein, BDNF: brain-derived neurotrophic factor.

それら症状の治療・予防は大きな課題となっている．更に，更年期障害では卵巣機能低下によるエストロゲン分泌減少という生物学的要因に加えて，社会心理的ストレスなどの環境要因が複雑に関与する事で，のぼせ・冷えなどの身体的症状や不安，イライラなどの精神的症状を呈することから，この症状の治療・予防も女性活躍社会構築における課題の一つである．これらの症状に対しては，女性三大漢方薬が処方されることが多く，特に精神的症状が強い場合には加味逍遥散が臨床的に使用される事が多い．

加味逍遥散は柴胡，当帰，芍薬，茯苓，白朮，甘草，生姜，薄荷，牡丹皮，山梔子の10種類の生薬からなる多成分合剤であり，抑肝散と一部構成生薬が重なっている．そこで著者らは抑肝散研究と同様に精神症状に効果を示す加味逍遥散には不安や気分の落ち込みなどの症状にも有効な成分が含まれている可能性があるものと考え，更年期障害モデルマウスへの環境ストレス曝露により不安，イライラなどの精神的症状を呈する更年期障害モデルマウスを作成し，加味逍遥散の効果の有無について検討を行った．その結果，加味逍遥散投与群ではストレスに上昇した血中コルチコステロン（グルココルチコイド）値が非ストレス群と同程度にまで低下すること，海馬歯状回おける神経幹細胞からの神経新生レベルが正常化することを見出した．次にこの分子機序について検討したところ，海馬歯状回の神経細胞におけるセロトニン受容体の一つ5-HT1A受容体の発現量が正常化することが明らかになった．うつ病患者における死後脳解析から海馬や扁桃体などのうつ病と関連する領域で5-HT1A受容体の密度低下が報告されており，更年期障害時でも海馬歯状回の神経細胞で5-HT1A受容体が発現低下することによりうつ病の症状に関連する様な精神症状が表出する可能性が考えられた．今回の著者らの検討により，加味逍遥散はこの海馬歯状回の5-HT1A受容体発現レベルを正常化することによりHPA axisを正常化し，神経症状を改善するという新規分子機序を見出した[3)]．更に著者らは詳細な解析を実施し，加味逍遥散が5-HT1A受容体の下流シグナルであるcAMP-PKA-CREB-BDNF経路を正常化するという事実も見出している[3)]．この加味逍遥散は，抑肝散と同様に構成生薬の中に柴胡を含んでおり，加味逍遥散の抗ストレス効果への柴胡の関与の可能性に今後の興味がもたれた．

おわりに

主に精神症状に有効であり臨床適用されている抑肝散と加味逍遥散の抗ストレス効果についての著者らの基礎研究は現在も進行中で，更なる分子機序の解明を実施しており，両者の漢方薬の根本的な作用機序に少しでも近づきたい所である（図1）．しかし，どちらの漢方薬も多成分合

剤でありトランスレーショナルリサーチとして探索する必要のある有効成分の同定という点からは，単独の成分なのかくつかの成分が複合的に関与して効果を示しているのか等についてはっきりしないことが多く，古くから議論の余地が大きい課題でもある．著者らだけでなく，全世界的観点から今後の漢方薬の基礎研究およびトランスレーショナルリサーチの進展に期待したい．

利益相反について：すべての著者に開示すべき利益相反はない．

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Abstract

Translational research based on functional mechanisms of Kampo medicine

Shingo Miyata, Shoko Shimizu, Yugo Ishino, and Masaya Tohyama

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Major depressive disorder (MDD) is one of the leading causes of disturbances in emotional, cognitive, autonomic, and endocrine functions, according to the large amount of information on MDD that has been accumulated during recent years. Although dysregulation of the HPA axis by chronic stress is indicative of MDD, the molecular mechanisms and functional changes in the brain underlying depression are largely unknown. Yokukansan (YKS) can affect behavioral and psychological symptoms such as aggression, anxiety, and depression in patients with Alzheimer's disease and other forms of dementia, and Kamishoyosan (KSS) is widely used for the treatment of various neuropsychiatric symptoms in perimenopausal and postmenopausal women. In the previous study, we developed specific animal models and indicated molecular mechanisms of the YKS and KSS functions for these various neuropsychiatric symptoms. These results suggest that one or more active ingredients in YKS and KSS could be used as a possible alternative to current antidepressant drugs.

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2024 年度 (2024.04-2025.03) 東洋医学研究所 清水 尚子 業績一覧

清水 尚子　助教

原著論文

1. Kamishoyosan Normalizes Dendritic Spine Morphology in the Medial Prefrontal Cortex by Regulating microRNA-18 and Glucocorticoid Receptor Expressions in Postmenopausal Chronic Stress-Exposed Mice.
   Shimizu S (First author), Koyama Y, Ishino Y, Takeda T, Shimada S, Tohyama M, Miyata S*.

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和文総説

1. 漢方薬の作用機序からみたトランスレーショナルリサーチ
   宮田信吾、**清水尚子**、石野雄吾、遠山正彌
   自律神経、62(1)：2-4, 2025.

学会発表

1. NEURO2024 第 47 回日本神経科学大会 第 67 回日本神経化学会大会 第 46 回日本生物学的精神医学会年会 第 8 回アジアオセアニア神経科学連合コングレス 合同大会　2024.7.24-27, 福岡コンベンションセンター
   *石野雄吾、**清水尚子**、遠山正彌、宮田信吾
   神経発生におけるアルギニンメチル化酵素 PRMT7 の機能解析
2. NEURO2024 第 47 回日本神経科学大会 第 67 回日本神経化学会大会 第 46 回日本生物学的精神医学会年会 第 8 回アジアオセアニア神経科学連合コングレス 合同大会　2024.7.24-27, 福岡コンベンションセンター
   ***清水尚子**、石野雄吾、遠山正彌、宮田信吾
   生後発達期におけるオリゴデンドロサイト分化の分子機構解明
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   ***清水尚子**、石野雄吾、宮田信吾
   生後のオリゴデンドロサイト発達における統合失調症関連因子の機能解析

競争的資金等の研究課題（公的資金）

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   日本学術振興会 科学研究費助成事業 基盤研究(C) 2023 年 4 月- 2026 年 3 月

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Kamishoyosan Normalizes Dendritic Spine Morphology in the Medial Prefrontal Cortex by Regulating microRNA-18 and Glucocorticoid Receptor Expressions in Postmenopausal Chronic Stress-Exposed Mice

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Abstract

Objective: Kamishoyosan (KSS), a traditional Japanese Kampo medicine, is widely used to treat neuropsychiatric symptoms in perimenopausal and postmenopausal women. We aimed to elucidate the functional mechanisms underlying KSS-mediated reduction of stress response behaviors and neuropsychological symptoms in perimenopausal and postmenopausal women.

Methods: Female mice were bilaterally ovariectomized (OVX) at the age of 12 weeks and exposed to chronic water immersion and restraint stress for three weeks. Among them, mice in the OVX+stress+KSS group were fed chow containing KSS from one week before exposure to chronic stress until the end of the experiment. Firstly, we performed a marble burying test and measured serum corticosterone levels to assess irritability and stress conditions. Next, we examined whether KSS affects microRNA-18 (miR-18) and glucocorticoid receptor (GR) protein expression, as well as the basal dendritic spine morphology of pyramidal neurons in the medial prefrontal cortex (mPFC) of postmenopausal chronic stress-exposed mice. Analyzed data were expressed as mean ± standard deviation. Tukey's post hoc test, followed by analysis of variance (ANOVA), was used for among-group comparisons.

Results: KSS administration normalized chronic stress-induced unstable emotion-like behavior and upregulated plasma corticosterone levels. Furthermore, KSS ameliorated GR protein expression by downregulating miR-18 expression in the mPFC and recovered the immature morphological changes in spine formation of pyramidal neurons in the mPFC of OVX mice following chronic stress exposure.

Conclusions: KSS administration in postmenopausal chronic stress-exposed mice exerted anti-stress effects and improved the basal dendritic spine morphology of pyramidal neurons by regulating miR-18 and glucocorticoid receptor expression in the mPFC.

Categories: Psychiatry, Anatomy, Obstetrics/Gynecology
Keywords: medial prefrontal cortex, spine morphology, glucocorticoid receptor, microrna, hypothalamic-pituitary-adrenal axis, menopause, kamishoyosan

Introduction

Menopause-related neuropsychological symptoms, including irritation, depression, and anxiety, are characterized by cognitive, autonomic, emotional, and endocrine function disturbances [1]. One of the traditional Japanese Kampo medicines, Kamishoyosan (KSS), is composed of 10 crude compounds containing a specified mixture derived from plant sources and is widely prescribed to improve various neuropsychiatric symptoms in perimenopausal and postmenopausal women [2,3]. A previous KSS clinical study in postmenopausal women and a premenstrual rat model reported that KSS administration caused significant improvements in excitability and irritability scores [4,5]. Apart from these studies, we previously demonstrated that continuous KSS administration in postmenopausal chronic stress-exposed mice attenuated stress-related depressive behavior and normalized hypothalamic-pituitary-adrenal (HPA) axis activity [6]. However, the molecular mechanisms underlying the beneficial effects of KSS-mediated regulation of the HPA axis remain unclear.

The prefrontal cortex (PFC), particularly the medial prefrontal cortex (mPFC) in humans, is critical to higher-order executive functions, memory, decision-making, cognition, and emotional control [7]. The mPFC is vulnerable to stress, which can decrease its volume and synaptic density by changing spine

morphology in patients with depression [8]. Decreased PFC activity is closely related to the onset of stress-related psychiatric diseases such as depression [9]. Therefore, prefrontal hypofunction induced by stress exposure is strongly implicated in the onset of psychiatric symptoms. However, the molecular mechanisms underlying menopause-related neuropsychological symptoms in mPFC functions remain unclear.

Glucocorticoid receptors (GRs) are also distributed in the PFC, and prefrontal GRs have been recently implicated in HPA axis regulation and mood regulation [10,11]. These studies suggest a functional association between defective prefrontal GR signaling and stress-related psychiatric diseases. Furthermore, a previous study indicated that elevated microRNA-18 (miR-18) expression and reduced GR protein expression were reported in the paraventricular hypothalamic nucleus of stress-vulnerability model rats [12]. However, the functions of miR-18 in the mPFC of postmenopausal environmental stress-exposed mice remain unclear.

Accordingly, as a continuation of our previous KSS research, we aimed to evaluate the effects of KSS on miRNA-mediated regulation of GR expression in the mPFC and the basal dendritic spine morphology of pyramidal neurons in the mPFC of postmenopausal environmental stress-exposed mice in this study.

Materials And Methods

Ethics statement

All animal experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals, the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal care and handling procedures approved by the International Animal Care and Use Committee of Kindai University (No. KAME-25-009).

Animals

Ten-week-old C57BL/6N female mice were purchased from SLC (Japan SLC, Inc., Hamamatsu, Japan). Three mice per cage were kept at room temperature (22 ± 2°C; humidity, 55 ± 10%) in a 12-h light/dark cycle (lights on at 07:00 a.m. and off at 07:00 p.m.). The animals had free access to water and food for breeding (CE-2; CLEA Japan Inc., Tokyo, Japan).

KSS administration and stress exposure

KSS is composed of the extractions of 10 medicinal herbs [6] (Table *1*) and was supplied by Tsumura & Co. (Tokyo, Japan). These ingredient content percentages were calculated from the KSS product label (Tsumura & Co.). Dry powdered extracts of KSS were mixed with CE-2 chow at a final concentration of 3% (w/w) and used as previously reported [6].

Ingredient	Content (%)
Bupleuri Radix (*Bupleurum falcatum*)	13.3
Paeoniae Radix (*Paeonia lactiflora*)	13.3
Atractylodis Rhizoma (*Atractylodes ovate*)	13.3
Angelicae Radix (*Angelica acutiloba*)	13.3
Hoelen (*Poria cocos*)	13.3
Gardeniae Fructus (*Gardenia jasminoides*)	8.9
Moutan Cortex (*Paeonia suffruticosa*)	8.9
Glycyrrhizae Radix (*Glycyrrhizae uralensis*)	6.7
Zingiberis Rhizoma (*Zingiber officinale*)	4.4
Menthae Herba (*Menthae arvensis*)	4.4

TABLE 1: Composition of Kamishoyosan

All female mice were bilaterally ovariectomized (OVX) at age 12 weeks. After two weeks of postoperative recovery, the 36 mice were randomly allocated into three groups (n=12) after the ovariectomy: the control group (non-stressed OVX mice), the chronically stressed group (OVX+Stress mice), and the chronically stressed group administered with KSS (OVX+Stress+KSS mice). There was no significant difference in body

weight and average daily consumption of chow between groups before stress exposure. Chronic stress was induced as previously described [6]. Briefly, OVX mice were exposed to chronic Water Immersion and Restraint Stress (WIRS) for three weeks (at 14-17 weeks of age). Specifically, they were restrained in a 50-mL conical polypropylene centrifuge tube and vertically immersed to the level of the xiphoid process in a water bath maintained at 23°C for two hours once a day for three weeks. For KSS administration, mice in the OVX+Stress+KSS group were fed CE-2 chow containing 3% KSS one week before chronic stress exposure (13 weeks old) until the end of the experiment as previously reported [6]. The mice were confirmed to be free of gastric and duodenal ulcers by visual observation for bleeding and sores on the surface of the stomach and duodenal mucosa.

Enzyme-linked immunosorbent assay (ELISA)

Serum corticosterone levels were measured using a Corticosterone Enzyme Immunoassay Kit (Arbor Assays, K014, Ann Arbor, Michigan), following the manufacturer's instructions. Briefly, one day after the chronic stress exposure period, the mice were deeply anesthetized, and their blood samples were collected into tubes containing heparin. The tubes were immediately placed on ice, followed by centrifugation at 1000 g for 15 minutes at 4°C. Plasma samples were stored at -80°C prior to assays. Absorbance at 450 nm was measured using a plate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, Massachusetts), and the corticosterone concentration in each sample was calculated using the SkanIt™ microplate reader software (Thermo Fisher Scientific Inc.).

Marble burying test

To assess behaviors representing unstable emotions, anxiety, and irritability, a marble burying test was performed two days after the end of the chronic stress exposure period [13]. Briefly, 20 glass marbles were evenly distributed on 5-cm-deep sawdust bedding in 4 × 5 grids in standard cages (25 × 25 × 31 cm). Each mouse was placed in a cage for 15 minutes. Subsequently, the mice were removed from the cage, and the number of marbles buried by the mice was counted at the end of the test. Marbles buried to at least 2/3 of their depth were considered buried [13]. The light intensity in both the breeding and test rooms was ~150 lux at 40 cm from the floor.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the PFC of mice using Isogen II (NipponGene, Toyama, Japan), following the manufacturer's instructions. To analyze mRNA expression, reverse transcription of the total RNA was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). To evaluate GR and GAPDH expression, quantitative real-time PCR (qRT-PCR) was conducted using KOD SYBR qPCR Mix (TOYOBO Co., Ltd., Osaka, Japan) with the following forward/reverse primers: GR, 5'-ACCTGGATGACCAAATGACCC-3'/5'-GCATAGCAGGTTTCCACTTGC-3' and GAPDH, 5'-GTGTTCCTACCCCCAATGTG-3'/5'-AGGAGACAACCTGGTCCTCA-3'. The housekeeping gene GAPDH was used as the internal control [6]. Specific ratio comparisons (gene of interest/GAPDH) were used to assess between-group differences in transcript expression. To analyze miRNA expression, reverse transcription of total RNA was performed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. To detect mature miR-18, qRT-PCR was performed using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific Inc.). TaqMan assays specific for miR-18 (Thermo Fisher Scientific Inc.) were performed with an ABI7900HT PCR System according to the manufacturer's instructions (Thermo Fisher Scientific Inc.). The relative levels of miR-18 in the mPFC were calculated with the 2−ΔΔCT method, with U6 as an internal control.

Golgi staining

Golgi staining was performed using an FD Rapid Golgi Stain Kit (FD NeuroTechnologies Inc., Columbia, Maryland), as previously described [14]. Briefly, the brains were removed from anesthetized mice and immersed in an equal mixture of solutions A and B for three weeks at 22±2°C in the dark. Next, the brains were transferred into solution C for seven days at 22±2°C. After freezing on dry ice, 200-µm serial coronal sections of the brain samples were prepared using a cryostat at -24°C and mounted on a 0.5% gelatin-coated glass slide, incubated overnight at 22±2°C, and soaked in solution C for five minutes. Subsequently, the slides were stained with a mixture of solution D, solution E, and deionized water (1:1:2) for 10 minutes, then rinsed in distilled water twice for four minutes. Coronal sections were dehydrated in an ascending ethanol series, cleared with xylene, and sealed with Entellan (Merck KGaA, Darmstadt, Germany). All images of the pyramidal neurons in the mPFC were obtained using a Keyence microscope (Keyence Corp., Osaka, Japan).

Immunohistochemistry

Immunohistochemical staining of the mouse brain was performed as previously described [6]. Briefly, mice were perfused transcranially with 4% paraformaldehyde three days after the three-week exposure to chronic stress. Next, their brains were collected and immersion-fixed in 4% paraformaldehyde at 4°C overnight. After post-fixing, the brain tissues were stored in a 30% (w/v) sucrose solution in 0.1 M phosphate-buffered saline (PBS) for 48 hours at 4°C. Free-floating, 30-µm-thick sections were rinsed with PBS and incubated in blocking buffer (5% bovine serum albumin and 0.3% Triton X-100 in PBS) for one hour at room temperature.

Subsequently, the sections were incubated with primary antibodies overnight at 4°C (Table 2). Next, the sections were washed in PBS and incubated with Alexa 488 anti-rabbit IgG secondary antibody (1:1000, Thermo Fisher Scientific Inc.; A-11008, RRID: AB_143165) for two hours at room temperature. All images were acquired using a laser scanning confocal microscope (C2; Nikon Corp., Tokyo, Japan). Immunohistochemical staining intensities were determined using ImageJ (National Institutes of Health). To quantify the GR expression level, the images were analyzed with pixel values of fluorescence intensity using the ImageJ software relative to a predetermined threshold intensity (the background intensity of the images set to zero). The same threshold setting was applied to all the images in each comparison group.

Immune blotting analyses

Immune blotting analysis was performed as previously described [6] using the antibodies in Table 2. Immunodetection of target proteins was performed using horseradish peroxidase-conjugated secondary antibodies (1:5000; Cell Signaling Technology Inc.) and an ECL Prime Western Blotting Detection System (GE Healthcare Systems Inc., Chicago, Illinois). Densitometric quantification was performed using ImageJ (National Institutes of Health), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. PSD95, postsynaptic density protein 95.

Antibody	Code No,	Manufacturer	Dilution
GR	ab183127	Abcam Plc, Cambridge, England	1:500 (IHC)
GR	ab183127	Abcam Plc, Cambridge, England	1:1000 (WB)
PSD95	3450	Cell Signaling Technology Inc., Danvers, Massachusetts	1:1000 (WB)
GAPDH	sc-32233	Santa Cruz Biotechnology Inc., Dallas, Texas	1:1000 (WB)

TABLE 2: The information about antibodies

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; WB; western blotting

Statistical analyses

Statistical analyses were performed using GraphPad Prism 10, a statistical software commonly used for data analysis in basic medical research (GraphPad Software, Boston, Massachusetts). Data are expressed as mean ± standard deviation (SD). Analysis of variance (one-way ANOVA) was used for 3 among-group statistical differences, followed by Tukey's post hoc test. Tukey's post-hoc test is a widely used statistical method with robust power to identify significant differences between groups and handle unequal sample sizes and variances. Statistical significance was set at $P < 0.05$.

Results

KSS normalized stress-upregulated plasma corticosterone level and irritability behavior

Compared with control OVX mice, OVX+Stress mice showed upregulated plasma corticosterone levels (Figure 1a, b), whereas the levels in OVX+Stress+KSS mice resembled those of the control (Figure 1a, b). Moreover, OVX+Stress mice showed an increased number of buried marbles, which was lower in OVX+Stress+KSS mice (Figure 1c). These results suggest that KSS administration ameliorated chronic stress-induced continuous hyperactivity of the HPA axis and unstable emotional behavior in OVX+Stress+KSS mice.

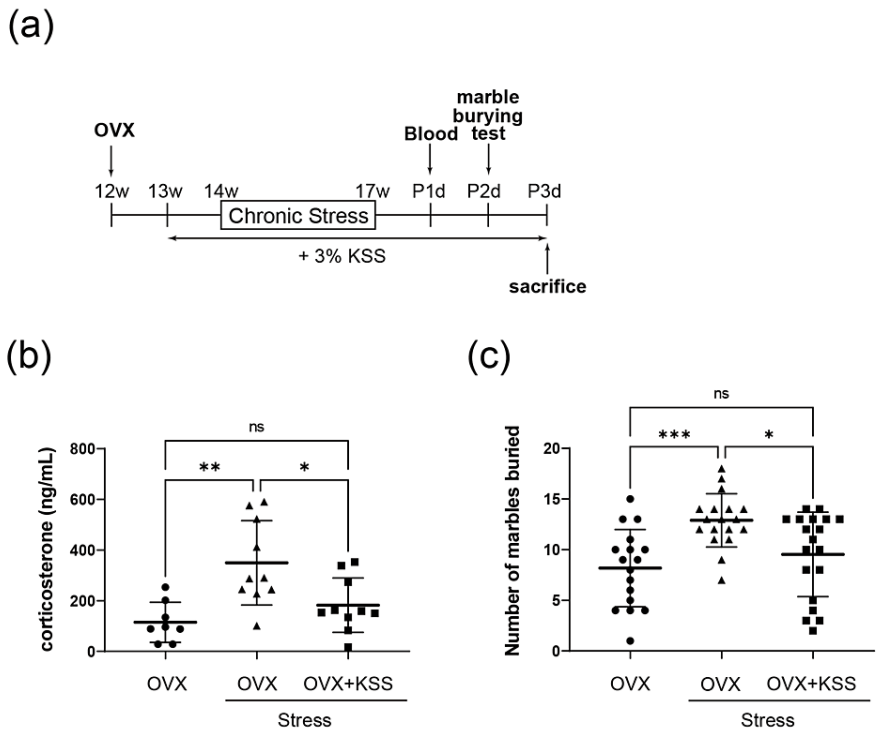

FIGURE 1: KSS treatment effects on plasma corticosterone levels and irritability-like behavior in OVX+Stress mice.

(a) Experimental timeline. The mice were used from 12 w (weeks) to 17w. Several analyses were performed from P1d (post 1 day) to P3d. (b) Levels of plasma corticosterone were measured by ELISA using blood samples. Results are shown as means ±SD (n = 8-10). (c) Marble Burying Test. Chronic stress exposure increased the number of buried marbles, which was normalized by the KSS administration. Results are shown as means ± SD (n = 17-19). *P < 0.05, **P < 0.01 one-way ANOVA followed by Tukey's post-test.

KSS, Kamishoyosan; OVX, ovariectomized mice; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ANOVA, analysis of variance; ns, no statistically significant difference.

Dendritic spine maturation and synaptic function in the mPFC were ameliorated from the effects of chronic stress by KSS

Chronic stress significantly decreased the spine area and spine head width in the mPFC of OVX mice, while these changes were absent in chronic stress-exposed OVX mice administered KSS (Figures *2a*, b, d). Furthermore, OVX+Stress mice showed increased spine length compared with stress-exposed OVX mice that received KSS (Figures *2a*, c). To evaluate synaptic function in the mPFC, we assessed the expression level of the postsynaptic density protein 95 (PSD95). Western blot analysis revealed decreased PSD95 expression in the mPFC of OVX+Stress mice, whereas expression in OVX+Stress+KSS mice was equivalent to that of the control (Figures *2e*, f). These results suggest that KSS administration ameliorated defects induced by chronic stress in spine maturation and synaptic function in the mPFC of OVX+Stress mice.

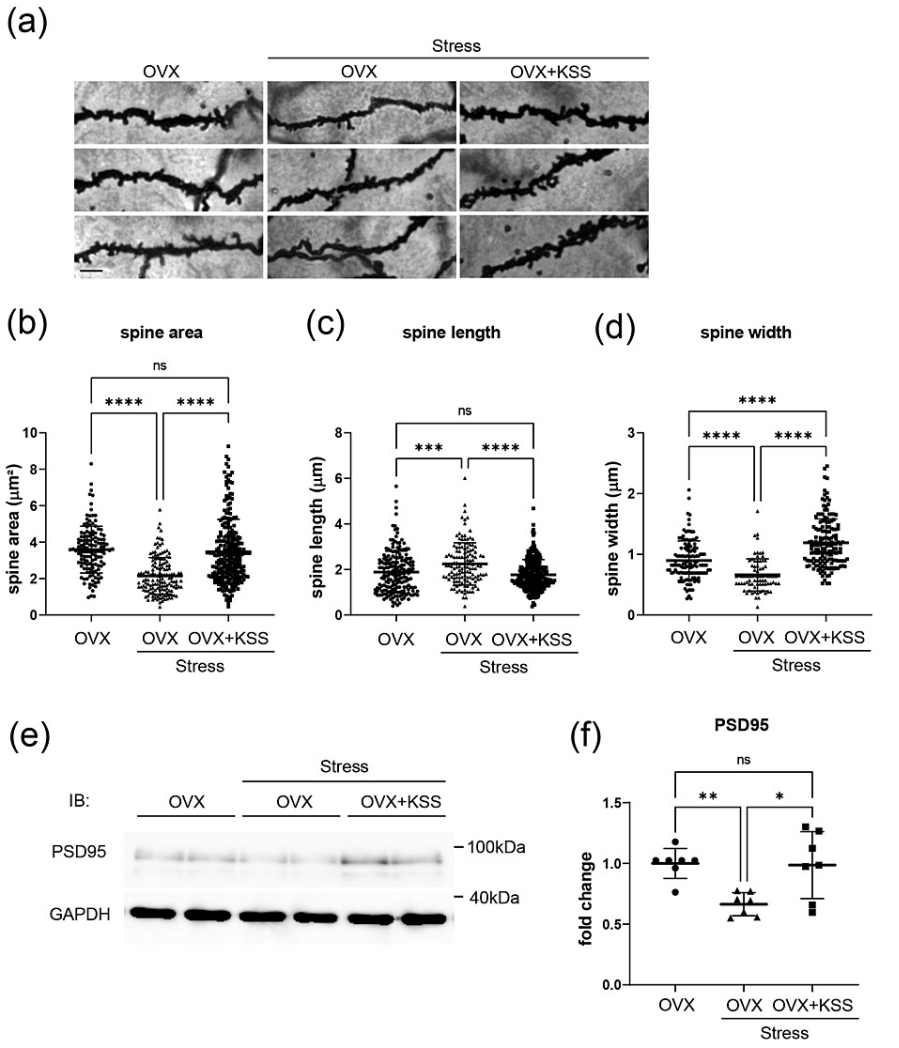

FIGURE 2: KSS treatment effects on basal dendritic spines of pyramidal neurons in the mPFC.

(a) High-magnification image of the representative Golgi-stained dendritic segments of pyramidal neurons in the mPFC. Scale bar, 200 nm. (b-d) Spine area, length, and width of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Data are presented as means ± SD. Two hundred spines from four slices from three animals per group were analyzed. Compared with OVX and OVX+Stress+KSS mice, OVX+Stress mice showed reduced spine area, length, and width. Data are presented as means ± SD. (e) PSD95 expression was evaluated through western blot analysis. (f) Densitometric quantification of PSD95 expression. The results are shown as means ± SD (n = 6). One-way ANOVA, Tukey's multiple comparisons test, *p < 0.05, **P < 0.01, ***P < 0.001, ****P□0.0001. Scale bar: 200 nm.

KSS, Kamishoyosan; OVX, ovariectomized mice; mPFC, medial prefrontal cortex; IB, immune blotting; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo Dalton; ns, no statistically significant difference.

KSS recovered GR protein expression changes through miR-18 upregulation in the mPFC

Next, we evaluated the relationship between GR protein and miR-18 expression levels in the mPFC region of the OVX+Stress mice with or without KSS administration. OVX+Stress mice showed significantly increased miR-18 expression in the mPFC, which was normalized by KSS administration in OVX+Stress+KSS mice (Figure *3a*). Moreover, western blot analysis revealed decreased GR protein expression in the mPFC of OVX+Stress mice, whereas it resembled the control level in the OVX+Stress+KSS mice (Figures *3b*, c). Chronic stress exposure also decreased the number of GR-immunoreactive cells in the mPFC, which was prevented by KSS administration (Figures *3d*, e). Taken together, these findings suggest that KSS reduced miR-18 expression and increased GR protein expression levels by reversing the inhibitory effects of chronic stress exposure on GR protein translation in the mPFC of OVX+Stress mice.

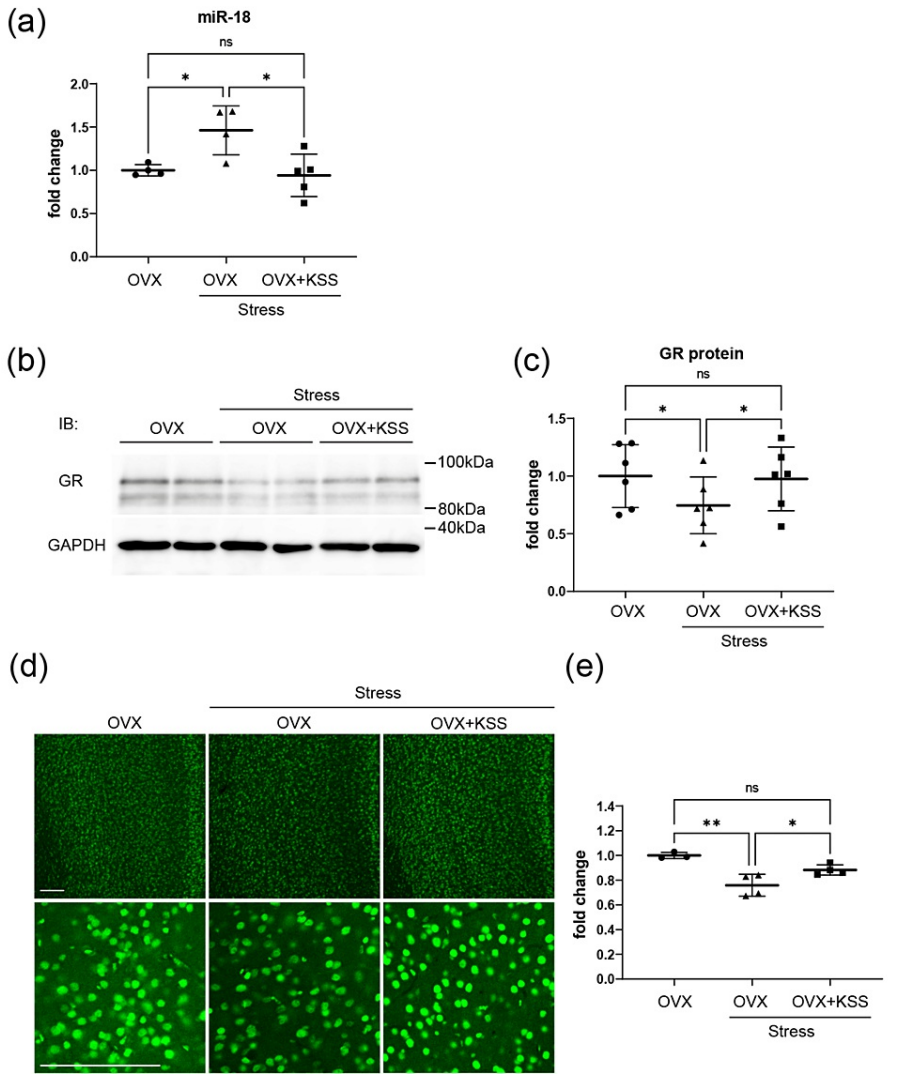

FIGURE 3: KSS treatment effects on miR-18 level and GR protein expression in the mPFC in OVX+Stress mice.

(a) Expression of miR-18 in the PFC was quantified through quantitative RT-PCR. (b) GR expression was assessed through western blot analysis. (c) Densitometric quantification of GR expression. These results are shown as means ± SD (n = 6). (d) Representative staining (upper panels) and high magnification (lower panels) images of GRs (green) in the mPFC. (e) The relative fluorescence intensity of GR signals in the mPFC. These results are shown as means ± SD (n = 4). Scale bar: 100 μm.

PFC, prefrontal cortex; RT-PCR, reverse transcription-polymerase chain reaction; GR, glucocorticoid receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-18, microRNA-18; ns, no statistically significant difference.

Discussion

The present study demonstrated that KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which in turn improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figures *1*-*3*). The mPFC is involved in numerous important cognitive functions, including decision-making, working memory, attention, and emotional control [7]. Several clinical studies have demonstrated the effects of menopause on the mPFC [15]. Menopause is associated with decreased gray matter volume in the mPFC, which may contribute to cognitive changes such as memory problems and difficulties in decision-making [16]. During menopause, there are changes in the levels of hormones such as estrogen, which is involved in the formation and maintenance of synapses and dendritic spines [17]. Furthermore, estrogen replacement therapy increased the density and size of hippocampal dendritic spines in a rat menopause model [18]. These findings suggest that the menopause-related decrease in estrogen levels may be associated with changes in the synaptic function and spine morphology of mPFC neurons. Menopause-related changes in hormone levels may exacerbate the effects of chronic stress. Thus, future studies should focus on understanding the

anti-stress mechanisms involved in regulating neuronal functions by the effective chemical components of KSS.

We previously found that Yokukansan, a Japanese herbal medicine, downregulated miR-18 expression and normalized HPA axis activity by regulating GR protein expression in the hypothalamus and corpus callosum of stress-exposed mice. Similarly, our present findings indicated that KSS ameliorated chronic stress-induced unstable emotional behavior and upregulated plasma corticosterone levels (Figure *1*). A previous study reported that postmenopausal women had higher levels of cortisol and perceived stress than premenopausal women [19]. However, the molecular mechanisms underlying the changes in cortisol levels and HPA axis activity in postmenopausal women remain unclear. The present study demonstrated that the effects of KSS on the HPA axis involve miR-18 and GR protein expression (Figure *3*). Specifically, KSS ameliorated GR protein expression changes by downregulating miR-18 expression in the mPFC, which improved unstable emotion-like behaviors and immature morphological changes in the spine formation of pyramidal neurons in the mPFC (Figure *2*).

The limitation of this study is that we did not identify the constituent herbal medicines included in KSS and did not show the function of estrogen receptors (ERs). Among the constituent herbal medicines in KSS, Bupleuri radix is well-known as the main component that may be effective for psychiatric symptoms and could serve as a possible alternative to current antidepressant medicines. Thus, a single administration of Bupleuri radix might show practical antidepressant-like and anti-stress effects in rodents. The next step in our research is to determine the effective chemical components of KSS. Previous studies have reported that miR-18 prevents ERα expression; furthermore, postmenopausal women have shown decreased ERα expression [20]. Therefore, brain miR-18 expression might be crucially involved in GR and/or ERα expression during the onset of menopausal symptoms. However, further studies linking the effective chemical components of KSS to its anti-stress function related to microRNAs in the brain are warranted to clarify the functional implications of these novel findings in the mPFC of chronic stress-exposed postmenopausal model mice.

From a clinical point of view, one previous KSS clinical trial study for postmenopausal women indicated that it was not able to show significant improvement effects in the main survey values [4]. One of the reasons why no significant improvements were found in the main survey values is that this clinical study might include several problems with the study design of participant selection. However, this clinical study also showed that KSS administration for post-menopause women showed significant improvements in excitability and irritability scores [4]. Furthermore, in this study, we indicated that KSS administration showed improvement effects of irritability behaviors for stress-exposed-OVX mice (Figure *1*). Furthermore, we found that the manufacturer did not perform the clinical survey of adverse reactions, and the KSS clinical trial study for postmenopausal women for 12 weeks indicated that no serious adverse events were reported [4]. From the results of these clinical trials and our basic research, it is assumed that KSS has a possibility of an effect on improving neuropsychiatric symptoms during menopause, especially irritability.

Conclusions

In conclusion, KSS administration in chronic stress-exposed postmenopausal mice exerted anti-stress effects and facilitated recovery from immature spine morphologies of basal dendritic pyramidal neurons, at least partly by regulating miR-18 and GR expression in the mPFC.

Appendices

□	OVX (n=8)	OVX+stress (n=10)	OVX+stress+KSS (n=10)
Mean	114.8	349.5*	182.2†
SD	79.1	166.6	107.6

TABLE 3: KSS treatment effects on plasma corticosterone levels (Figure 1b)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0017 between OVX and OVX+stress, P = 0.0174 between OVX+stress and OVX+stress+KSS groups).

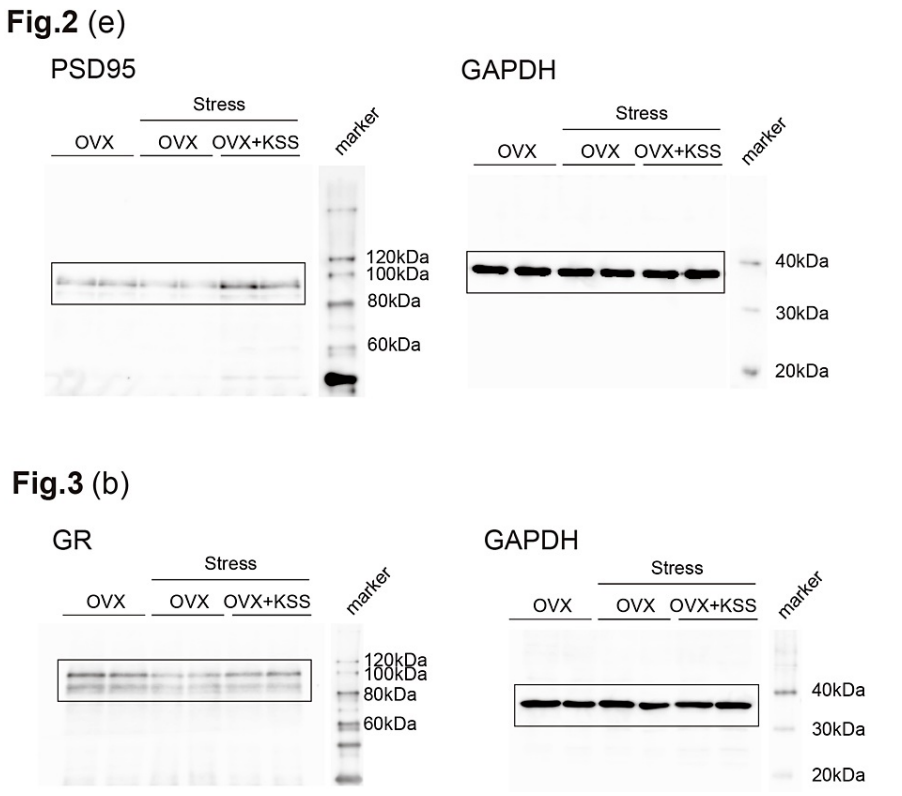

FIGURE 4: Full-size gels for immunoblots and molecular weight markers in Figures 2, 3.

GR, glucocorticoid receptor; PSD95, postsynaptic density protein 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kDa, kilo-Dalton.

	OVX (n=17)	OVX+stress (n=18)	OVX+stress+KSS (n=19)
Mean	8.18	12.89*	9.53†
SD	3.81	2.63	4.17

TABLE 4: Number of marbles buried in the marbles burying test (Figure 1c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0009 between OVX and OVX+stress, P = 0.0177 between OVX+stress and OVX+stress+KSS groups).

□(a) □	OVX (n=135)	OVX+stress (n=146)	OVX+stress+KSS (n=260)
Mean	3.59	2.15**	3.47†
SD	1.28	1.01	1.78
□(b) □	**OVX (n=192)**	**OVX+stress (n=131)**	**OVX+stress+KSS (n=261)**
Mean	1.88	2.24*	1.76†
SD	0.92	0.93	0.67
□(c) □	**OVX (n=127)**	**OVX+stress (n=79)**	**OVX+stress+KSS (n=157)**
Mean	0.90	0.66**	1.19†
SD	0.32	0.26	0.39

TABLE 5: Morphological analysis of spines of pyramidal neurons in mPFC using Golgi staining (Figure 2b-d)

Spine area (a), length (b), and width (c) of basal dendrites from the cell soma of pyramidal neurons in the mPFC. Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (*P <0.001, **P <0.0001 between OVX and OVX+stress, †P <0.0001 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=7)	OVX+stress (n=7)	OVX+stress+KSS (n=7)
Mean	1.00	0.66*	0.99†
SD	0.12	0.10	0.28

TABLE 6: Densitometric quantification of PSD95 expression in mPFC (Figure 2f)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0079 between OVX and OVX+stress, P = 0.0107 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=4)	OVX+stress (n=4)	OVX+stress+KSS (n=5)
Mean	1.00	1.46*	0.94†
SD	0.07	0.28	0.25

TABLE 7: Expression levels of miR-18 in mPFC (Figure 3a)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0362 between OVX and OVX+stress, P = 0.0146 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=6)	OVX+stress (n=6)	OVX+stress+KSS (n=6)
Mean	1.00	0.75*	0.97†
SD	0.27	0.25	0.28

TABLE 8: Densitometric quantification of GR expression (Figure 3c)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0123 between OVX and OVX+stress, P = 0.0212 between OVX+stress and OVX+stress+KSS groups).

GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

□ □	OVX (n=3)	OVX+stress (n=4)	OVX+stress+KSS (n=4)
Mean	1.00	0.76*	0.88†
SD	0.03	0.09	0.04

TABLE 9: The relative fluorescence intensity of GR signals in the mPFC (Figure 3e)

Asterisk (*) and dagger (†) indicate a significant difference between test groups using one-way ANOVA followed by Tukey's post-test (P = 0.0022 between OVX and OVX+stress, P = 0.0492 between OVX+stress and OVX+stress+KSS groups).

mPFC: medial prefrontal cortex, GR: glucocorticoid receptor, OVX: ovariectomized, KSS: Kamishoyosan.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Drafting of the manuscript: Shingo Miyata

Supervision: Shingo Miyata

Acquisition, analysis, or interpretation of data: Shoko Shimizu, Yoshihisa Koyama, Yugo Ishino

Critical review of the manuscript for important intellectual content: Shoko Shimizu, Yoshihisa Koyama, Yugo Ishino, Takashi Takeda, Shoichi Shimada, Masaya Tohyama

Disclosures

Human subjects: All authors have confirmed that this study did not involve human participants or tissue. **Animal subjects:** The International Animal Care and Use Committee of the Kindai University Issued protocol number KAME-25-009. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** This research was funded in part by the Japan Society for a Grant-in-Aid for Scientific Research (C) (grants 19K06916, and 23K06007), the Osaka Medical Research Foundation for Intractable Diseases, the KINDAI COVID-19 Control Support Project. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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司会：上園保仁

漢方薬の作用機序解明からみたトランスレーショナルリサーチ

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キーワード：漢方薬，ストレス応答経路，マイクロRNA，セロトニン受容体，ジェンダーインクルージブ社会
Kampo medicine, stress-response pathway, microRNA, serotonin receptor, gender inclusive society

抄録：うつ病などの精神疾患の発症には様々な環境ストレス要因の関与が大きいことが知られているものの，これら神経精神症状表出に関与する分子機序や脳の機能的・構造的変化等については，いまだ十分に明らかにされていないのが現状である．我々は認知症に伴う行動心理学的症状（BPSD）の治療に対して臨床的に有効であることが知られている抑肝散および女性の更年期障害に対して処方される三大漢方薬のひとつであり，主にイライラなどの精神神経症状に有効である加味逍遥散の抗ストレス効果に関する作用機序の解明を実施し，それぞれの漢方薬の特異的応答経路を見出した．今後は有効成分の同定が必須であり，更なる詳細な解析が期待される．
（自律神経，62：2–4, 2025，doi: 10.32272/ans.62.2_4）

はじめに

近年，特にこの20年程度の間に基礎研究領域における漢方薬の作用機序の解明が盛んに行われると共に臨床研究における漢方薬の有効性の検討も多く実施されてきた．しかし，未だに国際的な評価に耐えうる漢方薬への発展にまでには至っていない．これは基礎研究領域での漢方薬の作用機序の解明がいまだ十分ではなく，Evidence-based medicine（EBM）に基づいた漢方薬による臨床適用にまで至っていない状況によるものと考えることができる．本稿では，近年の漢方薬の作用機序解明の中心にいた抑肝散と共に，近年のジェンダーインクルージブな社会に対応するためには極めて重要であると考えられる月経前症候群や月経困難症，更には更年期障害症状などの女性特有の症状克服に有効な漢方薬の一つである加味逍遥散について，我々の基礎研究で得られた研究成果を概説したい．

抑肝散の抗ストレス効果について

抑肝散は，柴胡，釣藤鈎，蒼朮，茯苓，当帰，川芎，甘草の7種類の構成生薬からなる多成分合剤であり，古くから神経症や不眠症，更には小児の夜泣きや疳症に使用されてきた．近年，認知症に伴う周辺症状の行動心理学的症状（behavioral and psychological symptoms of dementia; BPSD）への有効性が示されるなど，非常に多くの研究論文の存在する漢方薬の代表格の一つである[2]．著者らのグループでは認知症中核症状にも抑肝散が有効性を示すのではないかとの仮説から検討を実施し，抑肝散が確かに神経細胞死を抑制するという事実だけでなく，構成生薬の中で神経細胞死を抑制する成分は川芎である事を同定した．さらに，川芎に含まれるフェルラ酸に神経細胞死抑制効果がある事や家族性アルツハイマー型認知症の原因遺伝子の一つプレセニリン1の変異体（deltaE9）発現神経細胞において，抑肝散および川芎が小胞体ストレス応答経路を介して細胞死を抑制するという分子機序も見出している[1]．

また，認知症BPSDの治療にはリスペリドンなどの非定型抗精神病薬だけでなく，抗うつ薬も効果があるとの報告があることから，抗うつ薬と類似した成分が抑肝散の中にも含有されている可能性がある，すなわち抑肝散の構成成分の中にうつ病態の一つである不安や気分の落ち込みなどの症状にも有効な成分が含まれている可能性がるのではと著者らは考えた．そこで著者らは，環境ストレス曝露によるうつ病モデルマウスを作成し，抑肝散の効果の有無について検討を行った．各種環境ストレス暴露動物は不安や攻撃性など様々なストレス症状を呈する．これらのストレス暴露に対する生体反応系として視床下部－下垂体－副腎軸（Hypothalamic-Pituitary-Adrenal axis; HPA axis）が知られており，通常はこのHPA axisは負のフィードバック機能により過剰な刺激が持続しない．しかし，うつ病をはじめとする一部のストレス性の精神疾患では，慢性的な繰り返しのストレス暴露により，HPA axisが過剰に反応し続け，負のフィードバックシステムが機能不全に至るこ

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とが大きく関連すると考えられている．著者らは環境ストレス曝露によるうつ病モデルマウスのHPA axis応答経路を中心に検討を重ねた．その結果，抑肝散投与群ではストレスに上昇した血中コルチコステロン（グルココルチコイド）値が非ストレス群と同程度にまで低下すること．HPA axis制御に深く関わるGlucocorticoid Receptor（GR）タンパク量は視床下部のparaventricular nucleus（室傍核；PVN）および脳梁オリゴデンドロサイトにおいて有意に増加することを見出した．次にこの分子機序について検討したところ，タンパク発現量を転写後に調節するnon-coding RNAのひとつであるmicroRNA（miR）としてmiR-18又はmiR-124aによるGRタンパク質の翻訳レベルの制御機構を明らかにした．すなわち，抑肝散投与により，ストレス負荷マウスの視床下部神経細胞ではmiR-18，脳梁オリゴデンドロサイトではmiR-124aの発現が低下し，GR翻訳抑制が解除され，その結果GRタンパク量を正常量に増加させることでHPA axisを正常化するという抑肝散の新たな抗ストレス効果の分子機序を明らかにすることが出来た[4)5)]．更に，抑肝散の構成生薬の中で柴胡を含む数個に抗ストレス効果がある可能性まで見出している．

加味逍遥散の抗ストレス効果について

女性の月経困難症や月経前症候群は特定の性周期に表出することで日常生活に支障をきたす事があり，近年のジェンダーインクルージブな社会環境の安定的構築にとって，

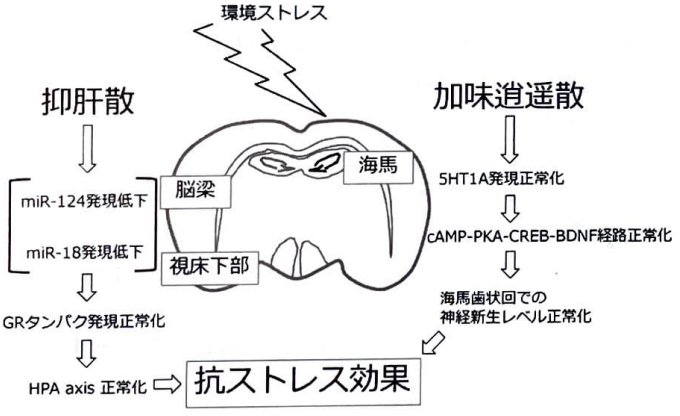

図1 著者らが見出した抑肝散と加味逍遥散の抗ストレス効果の分子機序．（左）抑肝散によるmiR-18制御で視床下部PVN neuronの，miR-124a制御で脳梁オリゴデンドロサイトのGR protein levelをそれぞれ正常化することで抗ストレス効果を発揮する．（右）加味逍遙散による海馬神経細胞でのセロトニン5HT1A受容体発現量正常化による細胞内シグナル伝達を活性化しストレスにより低下した神経新生レベルを正常化する．PVN: paraventricular nucleus, GR: glucocorticoid receptor, HPA axis: hypothalamic-pituitary-adrenal axis, PKA: protein kinase A, CREB: cAMP response element binding protein, BDNF: brain-derived neurotrophic factor.

それら症状の治療・予防は大きな課題となっている．更に，更年期障害では卵巣機能低下によるエストロゲン分泌減少という生物学的要因に加えて，社会心理的ストレスなどの環境要因が複雑に関与する事で，のぼせ・冷えなどの身体的症状や不安，イライラなどの精神的症状を呈することから，この症状の治療・予防も女性活躍社会構築における課題の一つである．これらの症状に対しては，女性三大漢方薬が処方されることが多く，特に精神的症状が強い場合には加味逍遥散が臨床的に使用される事が多い．

加味逍遥散は柴胡，当帰，芍薬，茯苓，白朮，甘草，生姜，薄荷，牡丹皮，山梔子の10種類の生薬からなる多成分合剤であり，抑肝散と一部構成生薬が重なっている．そこで著者らは抑肝散研究と同様に精神症状に効果を示す加味逍遥散には不安や気分の落ち込みなどの症状にも有効な成分が含まれている可能性があるものと考え，更年期障害モデルマウスへの環境ストレス曝露により不安，イライラなどの精神的症状を呈する更年期障害モデルマウスを作成し，加味逍遥散の効果の有無について検討を行った．その結果，加味逍遥散投与群ではストレスに上昇した血中コルチコステロン（グルココルチコイド）値が非ストレス群と同程度にまで低下すること，海馬歯状回おける神経幹細胞からの神経新生レベルが正常化することを見出した．次にこの分子機序について検討したところ，海馬歯状回の神経細胞におけるセロトニン受容体の一つ5-HT1A受容体の発現量が正常化することが明らかになった．うつ病患者における死後脳解析から海馬や扁桃体などのうつ病と関連する領域で5-HT1A受容体の密度低下が報告されており，更年期障害時でも海馬歯状回の神経細胞で5-HT1A受容体が発現低下することによりうつ病の症状に関連する様な精神症状が表出する可能性が考えられた．今回の著者らの検討により，加味逍遥散はこの海馬歯状回の5-HT1A受容体発現レベルを正常化することによりHPA axisを正常化し，神経症状を改善するという新規分子機序を見出した[3)]．更に著者らは詳細な解析を実施し，加味逍遥散が5-HT1A受容体の下流シグナルであるcAMP-PKA-CREB-BDNF経路を正常化するという事実も見出している[3)]．この加味逍遥散は，抑肝散と同様に構成生薬の中に柴胡を含んでおり，加味逍遥散の抗ストレス効果への柴胡の関与の可能性に今後の興味がもたれた．

おわりに

主に精神症状に有効であり臨床適用されている抑肝散と加味逍遥散の抗ストレス効果についての著者らの基礎研究は現在も進行中で，更なる分子機序の解明を実施しており，両者の漢方薬の根本的な作用機序に少しでも近づきたい所である（図1）．しかし，どちらの漢方薬も多成分合

剤でありトランスレーショナルリサーチとして探索する必要のある有効成分の同定という点からは，単独の成分なのかくつかの成分が複合的に関与して効果を示しているのか等についてはっきりしないことが多く，古くから議論の余地が大きい課題でもある．著者らだけでなく，全世界的観点から今後の漢方薬の基礎研究およびトランスレーショナルリサーチの進展に期待したい．

利益相反について：すべての著者に開示すべき利益相反はない．

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Abstract

Translational research based on functional mechanisms of Kampo medicine

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Major depressive disorder (MDD) is one of the leading causes of disturbances in emotional, cognitive, autonomic, and endocrine functions, according to the large amount of information on MDD that has been accumulated during recent years. Although dysregulation of the HPA axis by chronic stress is indicative of MDD, the molecular mechanisms and functional changes in the brain underlying depression are largely unknown. Yokukansan (YKS) can affect behavioral and psychological symptoms such as aggression, anxiety, and depression in patients with Alzheimer's disease and other forms of dementia, and Kamishoyosan (KSS) is widely used for the treatment of various neuropsychiatric symptoms in perimenopausal and postmenopausal women. In the previous study, we developed specific animal models and indicated molecular mechanisms of the YKS and KSS functions for these various neuropsychiatric symptoms. These results suggest that one or more active ingredients in YKS and KSS could be used as a possible alternative to current antidepressant drugs.

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