Original Article
Effects of 919 syrup and D101 on immobilization stress response in postpartum mice

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Abstract: Aim: 919 syrup (919 TJ) affects immobilization stress (IS)-induced anorexia related gene expression in postpartum mice. The present study aimed to explore possible mechanisms. Methods: Mice were divided into 5 groups: control, IS, IS + D101, IS + 919 TJ, and IS + D101 + 919 TJ (n = 8 per group). Repeated IS stimulation was applied to maternal mice. Moreover, 919 TJ was intragastrically administrated daily and D101 (5-Hydroxytryptamine [5-HT] agonist) was administered every two days via intraperitoneal injection. Food intake and body weights were recorded daily. Protein expression was detected by Western blotting and immunohistochemical staining. Additionally, mRNA expression was assessed by real-time PCR. Results: IS-induced inhibition of weight gain and food intake was not affected by 919 TJ and D101 in postpartum mice. Results suggest that 919 TJ and D101 reversed the inducing effects of IS on ghrelin protein expression in the stomach and hypothalamus, as well as the inhibitory effects of IS on 5-HT2b receptors (5-HT2bR) in the stomach and 5-HT2c receptors (5-HT2cR) in the hypothalamus. Moreover, IS affected mRNA expression of several appetite regulation and nutrient response-related genes in the stomach and hypothalamus, which was partially reversed by 919 TJ and D101. Conclusion: 919 TJ, similar to D101, can reverse the effects of IS on appetite regulation and nutrient response-related genes. 919 TJ may function through regulating 5-HT receptors.

Keywords: Postpartum stress, 919 syrup, D101, ghrelin, 5-HT2bR, 5-HT2cR

Introduction
Postpartum depression (PPD) is highly prevalent in postpartum women, worldwide [1]. Approximately 50-70% of women experience “baby blues” with the birth of a child, showing unstable emotions, including depression and crying. Approximately 10-15% of mothers have a strong clinical manifestation, including slow reactions and a tendency to commit suicide or infringe on the baby [2]. Clinical studies have suggested that PPD not only impairs maternal health, but also retards the growth and development of the offspring [3-5]. Mothers with PPD are more likely to stop breastfeeding earlier [6]. Their infants are more likely to have decreased prolactin and growth hormone [7]. Evidence has shown that PPD may lead to maternal and child connection disorders, resulting in slow weight gain in the offspring, making them more susceptible to diabetes and heart disease [8-10]. The effects of PPD on physical and mental development of the offspring often continues to adulthood [11].

Ghrelin is a hunger signal, produced mainly by X/A-like cells of the gastric mucosa and pancreatic cells. Ghrelin is also produced in the hypothalamus arcuate nucleus, which stimulates the production of growth hormone in the anterior pituitary [12, 13]. Evidence has demonstrated that ghrelin may enhance appetite and increase food intake [14]. Previous studies have shown that stress increases expression of ghrelin in postpartum mice, but has little effect on body weight and food intake [15, 16]. These results indicate that ghrelin is not the main driving force of postnatal maternal feeding.

5-Hydroxytryptamine 5-HT, also known as serotonin, is a neurotransmitter mainly distributed in the pineal gland and hypothalamus [17]. 5-HT uptake in the body has been associated with anxiety and widely used as an antidepres-
sant and anti-anxiety drug [18]. Previous experiments have proven that PPD leads to low expression of 5-HT\textsubscript{2c} receptors (5-HT\textsubscript{2c}R) in the hypothalamus and 5-HT\textsubscript{2b} receptors (5-HT\textsubscript{2b}R) in the stomach [19]. Studies have shown that medication should be used with caution during lactation [20, 21]. A Traditional Chinese herbal medication, known as jiubaiyishijiu tang jiang (919 TJ), has been found to be effective in treating PPD [22]. A previous study with an immobilization stress (IS)-induced mice model has shown that PPD causes a decline in body weight and food intake of maternal mice, as well as low weight gain for the offspring. 919 TJ reverses IS-induced anorexia related changes, including increased expression of ghrelin and decreased expression of 5-HT\textsubscript{2c}R in the hypothalamus and 5-HT\textsubscript{2b}R in the stomach [15]. The present study investigated the effects of D101 (DOI, a 5-HT receptor agonist [23]) and 919 TJ on appetite-related responses to IS, hypothesizing that 919 TJ might exert its function through 5-HT\textsubscript{2c}R and 5-HT\textsubscript{2b}R.

**Materials and methods**

**Animals**

Initially, ICR mice (SCXK, Shanghai, China) were housed in the Animal Laboratory Building in Shanghai Public Health Clinic (Shanghai, China) at 21°C ± 1°C, with a 12/12-hour light-dark cycle. Standard food and drinking water were given ad libitum at all times, except during the IS procedure. The animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University.

**Maternal separation and immobilization stress experiments and 919 TJ medication**

After acclimatization for 7 days, each female was placed in a cage with 2 males for a continuous 4-day period to allow for mating. Subsequently, female mice were separated from the males. The pregnant females (n = 40) of similar body weights were divided into 5 groups: control, IS, IS + D101, IS + 919 TJ, and IS + D101 + 919 TJ, with 8 mice in each group. Following parturition, body weights and food intake were recorded daily. Within 24 hours of parturition, each mouse in the IS, IS + D101, IS + 919 TJ, and IS + D101 + 919 TJ group was separated from her pups and placed in a separate cage from 09:00 to 12:00 every day for 21 days. Each mouse was immobilized during this period by placing it inside a 50-mL centrifuge tube (Dow Corning, Midland, Michigan, USA), which had a 5 × 110 mm longitudinal slit cut at the base of the tube for ventilation and extricating the tail. The tube was placed vertically inside the cage during the treatment period.

Postpartum mice in IS + 919 TJ and IS + D101 + 919 TJ groups were intragastrically administered with 919 TJ (27 g/kg body weight; Shanxi Province Shangmao Medicine; Xian, Shanxi, China) once a day. Postpartum mice in IS + D101 and IS + D101 + 919 TJ groups were intraperitoneal administered with D101 (3 mg/kg body weight; Sigma, St. Louis, MO, USA) every two days.

On postnatal day 22, the mice were humanely killed by intraperitoneal injections of 10% chloral hydrate in normal saline. The stomach and hypothalamus were surgically removed from each mouse.

**Quantification of mRNA expression**

The mRNA levels of ghrelin, 5-HT\textsubscript{2c}R, interleukin 1 beta (IL1B), and peroxisome proliferator activated receptor gamma (PPARG) in the stomachs and those of ghrelin, 5-HT\textsubscript{2c}R, agouti-relat-
ed protein (AgRP), retinoid X receptor alpha (RXRA), neuropeptide Y (NPY), and adrenoceptor beta 3 (ADRB3) in the hypothalamus were determined by reverse transcription and real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from the tissue samples using a RNeasy Mini Kit (Qiagen, Hilden, Germany), from which complementary DNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA), according to manufacturer instructions. qRT-PCR analysis was performed in triplicate using the primers listed in Table 1 and the QuantiFast SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Thermal cycling was performed in an iCycler iQ™ Real-Time PCR System (Bio-Rad Laboratories) at 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. After normalization based on GAPDH transcription, relative mRNA expression was determined using the 2^ΔΔCt method [29].

**Western blotting**

Tissue samples were lysed in RIPA buffer. Lysates were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and electro-transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in 1% bovine serum albumin and 1% nonfat dry milk for 1 hour and probed overnight at 4°C with anti-ghrelin (MAB10404, EMD Millipore, Bredford, MA, USA), anti-5-HT\textsubscript{2b}R (Orb11593, Biorbyt, Cambridge, UK), anti-5-HT\textsubscript{2c}R (Ab197776, Abcam, Cambridge, MA, USA), or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Following incubation with peroxidase-conjugated secondary antibodies, signaling was detected by enhanced chemiluminescence substrate (ECL, Bio-Rad). Western blotting was repeated three times and the bands were quantified using Image J (National Institutes of Health).

**Immunohistochemical staining**

Tissues samples were fixed in 10% formaldehyde, paraffin-embedded, and cut into 5-μm-thick sections using routine protocol. Following dewaxing and dehydrating, the sections were immersed in 3% hydrogen peroxide for 10 minutes to block endogenous peroxide activity. Subsequently, antigen retrieval was conducted in citrate buffer, pH 6.0. The sections were incubated with antibodies against ghrelin, 5-HT\textsubscript{2b}R (Ab197776, Abcam, Cambridge, MA, USA), or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Following incubation with peroxidase-conjugated secondary antibodies, signaling was detected by enhanced chemiluminescence substrate (ECL, Bio-Rad). Western blotting was repeated three times and the bands were quantified using Image J (National Institutes of Health).

**Statistical analysis**

Statistical analysis was performed using SPSS for Windows software (Version 10.0. Chicago, SPSS Inc.). Data are presented as mean ± stan-
Figure 2. Effects of 919 TJ and D101 on IS-induced changes in ghrelin expression in the stomach and hypothalamus of mice. (A, B) Expression levels of ghrelin in the stomach (A) and hypothalamus (B) of postpartum mice were examined using Western blotting on day 22 postpartum. (C, D) Ghrelin in the stomach (C) and hypothalamus (D) of postpartum mice was detected using immunohistochemical staining on day 22 postpartum. Magnification: 200× (**P < 0.01, ***P < 0.001 vs IS group).
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standard deviation (SD). Intergroup differences were evaluated using one way ANOVA analysis, with the level of statistical significance set at \( P < 0.05 \).

Results

IS-induced inhibition of weight gain and food intake was not affected by 919 TJ and D101 in postpartum mice

Mean body weight of mice in the IS group was significantly lower from day 4 to day 15 postpartum than mice in the control group (**\( P < 0.01 \), ***\( P < 0.001 \); Figure 1A). Mean food intake of the mice in the IS group was significantly lower from day 1 to day 15 postpartum than mice in the control group (**\( P < 0.001 \); Figure 1B). Body weight and food intake in the IS + TJ, IS + D101, and IS + D101 + TJ groups did not differ significantly from those in the IS group (\( P > 0.05 \)). Thus, IS inhibited weight gain and food intake in postpartum mice, which was not affected by 919 TJ and D101.

IS-induced increases in ghrelin protein expression in the stomach and hypothalamus of postpartum mice was inhibited by 919 TJ and D101

Protein levels of ghrelin in the stomach and hypothalamus were measured on day 22 postpartum. Western blotting analyses showed that levels of ghrelin protein in the stomach (Figure 2A) and hypothalamus (Figure 2B) of the IS group were significantly higher than the control group (**\( P < 0.001 \)). Moreover, 919 TJ, D101, and 919 TJ + D101 could reduce ghrelin protein in both the stomach and hypothalamus of postpartum mice subjected to IS (**\( P < 0.001 \)). IHC staining demonstrated that ghrelin mainly presented in the cytoplasm (Figure 2C, 2D) and the change trend of the ratio of ghrelin positive cells per square millimeter was similar to the results of Western blotting.

Decreased expression of 5-HT\(_{2b}\)R in the stomach and 5-HT\(_{2c}\)R in the hypothalamus of IS mice was improved by 919 TJ and D101

Levels of 5-HT\(_{2b}\)R in the stomach (Figure 3A, 3B) and 5-HT\(_{2c}\)R in the hypothalamus (Figure 3C, 3D) were measured on day 22 postpartum. Western blotting analyses showed that levels of 5-HT\(_{2b}\)R in the stomach (**\( P < 0.001 \), Figure 3A) and 5-HT\(_{2c}\)R in the hypothalamus (*\( P < 0.05 \), Figure 3C) of the IS group were significantly reduced, compared to the control group. 919 TJ, D101, and 919 TJ + D101 attenuated the effects of IS, with the most significant effects obtained in the IS mice with 919 TJ and D101. IHC staining further validated the changes of 5-HT\(_{2b}\)R in the stomach (Figure 3B) and 5-HT\(_{2c}\)R (Figure 3D) in the hypothalamus.

Effects of 919 TJ and D101 on IS-induced changes in appetite related gene expression in the stomach and hypothalamus of postpartum mice

The mRNA expression levels of appetite-related genes in the stomach (Figure 4) and hypothalamus (Figure 5) were measured on day 22 postpartum using qRT-PCR. In the stomach of the IS group, mRNA levels of IL-1β and ghrelin were remarkably elevated, compared to the control group (Figure 4A), while mRNA levels of PPARγ and 5-HT\(_{2b}\)R were significantly decreased (Figure 4B). In the hypothalamus of the IS group, mRNA levels of AgRP, RXRA, NPY, and ghrelin were remarkably elevated, compared to the control groups (Figure 5A), while mRNA levels of ADRB3 and 5-HT\(_{2c}\)R were significantly decreased (Figure 5B). 919 TJ, D101, and 919 TJ + D101 weakened the effects of IS, with 919 TJ + D101 showing the most significant effects.

Discussion

D101 (D01), a 5-HT receptor agonist, can act not only on 5-HT\(_{2b}\) receptors but also 5-HT\(_{2c}\) receptors. D101 is clinically used to treat schizophrenia [23]. The present study explored the effects of D101 and 919 TJ on appetite-related responses to IS. Similar to 919 TJ, D101 had no effects on weight gain or food intake in IS mice. D101 had similar effects to 919 TJ, which is known to increase expression of ghrelin and decrease expression of 5-HT\(_{2c}\)R [15]. These findings suggest that 919 TJ may exert its functions through regulating 5-HT receptors.

In the present study, mice were divided into Control, IS, IS + 919 TJ, IS + D101, and IS + 919 TJ + D101 groups. Abnormal food intake, body weight, and expression of related genes, such as ghrelin, 5-HT\(_{2b}\)R, 5-HT\(_{2c}\)R, NPY, and AgRP, in IS mice were consistent with previous experimental results [7, 15, 22], indicating that the postpartum stress model was repeatable.

Compelling evidence has emerged suggesting that ghrelin is a relevant regulator of appetite,
Figure 3. Effects of 919 TJ and D101 on IS-induced changes of 5-HT\(_{2a}\)R in the stomach and 5-HT\(_{2c}\)R in the hypothalamus of mice on day 22 postpartum. (A, B) Expression levels of 5-HT\(_{2a}\)R of postpartum mice were examined using Western blotting (A) and immunohistochemical staining (B). (C, D) Expression levels of 5-HT\(_{2c}\)R of postpartum mice were examined using Western blotting (C) and immunohistochemical staining (D). Magnification: 200× (**P < 0.01, ***P < 0.001 vs IS group).
food intake, and energy homeostasis [14]. IS could increase mRNA and protein expression of ghrelin in both the stomach and hypothalamus of postpartum mice [15, 16]. Here, real-time PCR, Western blotting, and immunohistochemical staining analyses also validated the increased expression of ghrelin in the stomach and hypothalamus of IS mice. 919 TJ, D101, and 919 TJ + D101 could partially reverse the effects of IS on ghrelin expression.

5-HT is a widely used antidepressant and anti-anxiety drug [18]. Consistent with a previous study [19], IS led to low expression of 5-HT$_{2c}$R in the hypothalamus and 5-HT$_{2b}$R in the stomach, as indicated by real-time PCR, Western blotting, and immunohistochemical staining analyses. 919 TJ, D101, and 919 TJ + D101 could partially reverse the effects of IS on 5-HT receptors.

Several appetite regulation and nutrient response-related genes were detected in the stomach and in the hypothalamus by real-time PCR (Figures 4, 5). IL1B is an anorectic gene [24]. PPARG contributes to control of food intake and appetite [25]. The present study found that IS upregulated IL1B and downregulated PPARG in the stomach (Figure 4). Increased expression of AgRP and NPY, two anabolic signaling molecules, in the hypothalamus can stimulate food intake [26]. RxRA, an important adipogenesis regulator [27], was found to be significantly increased in the peripheral blood mononuclear cells (PBMCs) of the IS group [28]. Adrb3 is an energy expenditure gene, expressed in the hypothalamus [24]. The present study found that IS upregulated mRNA expression of AgRP, NPY, and RxRA, but downregulated that of Adrb3 in the hypothalamus (Figure 5). More importantly, 919 TJ, similar to D101, can improve the abnormal expression of appetite regulation and nutrient response-related genes induced by IS, suggesting that 919 TJ may exert its functions through regulating 5-HT receptors.
Conclusion

In summary, 919 TJ and D101 had similar effects on maternal mice subjected to IS. The present study revealed potential mechanisms involved in the effects of 919 TJ on appetite and ghrelin pathways in PPD.

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Disclosure of conflict of interest

None.

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