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Original Article

Effects of the Chinese herbal medicine mixture 919 syrup on the isolation stress response in postpartum mice

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Abstract: Objective: Because 919 syrup (919 TJ) improved the appetite of chronic liver disease patients, we examined whether it had an effect on appetite and isolation stress (IS)-induced anorexia related gene expression in postpartum mice. Methods: Mice puerperas and offspring were separated for 3 h/day. Weight gain, serum ghrelin concentrations as well as hypothalamic and gastric ghrelin, growth hormone secretagogue receptor (GSHR), leptin, neuropeptide Y (NPY), agouti-related protein (AgRP), corticotrophin-releasing factor (CRF), proopiomelanocortin (POMC) and serotonin receptors 5-HT_{2c}R (hypothalamus) and $5HT_{2b}$R (stomach) transcriptions were monitored in IS exposed postpartum mice treated with high and low dose 919 TJ. Results: Over the course of 21 days, IS inhibited feeding and weight gain, increased the serum levels of ghrelin, upregulated ghrelin and GHSR expression in the stomach and hypothalamus, downregulated leptin and 5-HT_{2b}R expression in the stomach, upregulated NPY and AgRP expression in the hypothalamus and downregulated CRF, POMC, and 5-HT_{2c}R expression in the hypothalamus. Although 919 TJ did not improve food intake or weight gain, it reduced the serum concentration of active ghrelin, down regulated ghrelin and GHSR, and upregulated leptin and 5-HT_{2b}R transcription in the stomach, down regulated ghrelin and GHSR, and upregulated CRF, POMC and 5-HT_{2c}R transcription in the hypothalamus of postpartum mice. Conclusion: 919 TJ reversed all IS-induced anorexia related changes other than weight gain and food intake.

Keywords: Anorexia, appetite regulation, ghrelin, leptin, maternal stress, postpartum mice

Introduction

Depressive disorders are highly prevalent worldwide [1]. Although the incidence of depressive disorders is similar between adolescent males and females, the incidence among women after menarche is approximately two-fold higher than that among their male counterparts [2], and varies thereafter with hormonal fluctuations, such as in puerperium, during which the relative risk of depression increases significantly [3]. Approximately 50-75% of puerperas present with symptoms of depression such as inexplicable crying and depressed mood, with 10-15% receiving a diagnosis of postpartum depression (PPD) [4].

Previous studies have shown that PPD is associated with loss of appetite, reduced postpartum weight gain, and an increased risk of substance and alcohol abuse and suicide [5-9]. The effects of PPD on the neonate can be significant and long-lasting. Children born to mothers with PPD often suffer poor growth rates, higher levels of childhood malnutrition and increased incidences of cardiovascular [10], respiratory, and bowel diseases later in life [11], and are themselves at a 5-fold higher risk of developing depression compared to children born to mothers without depression [12]. Although the onset of PPD correlates with changes in hormones related to reproduction [13], the physiological manifestations of PPD, especially changes in appetite, suggest that other factors may be involved.

High calorie food intake has been shown to attenuate responses to restraint stress that are

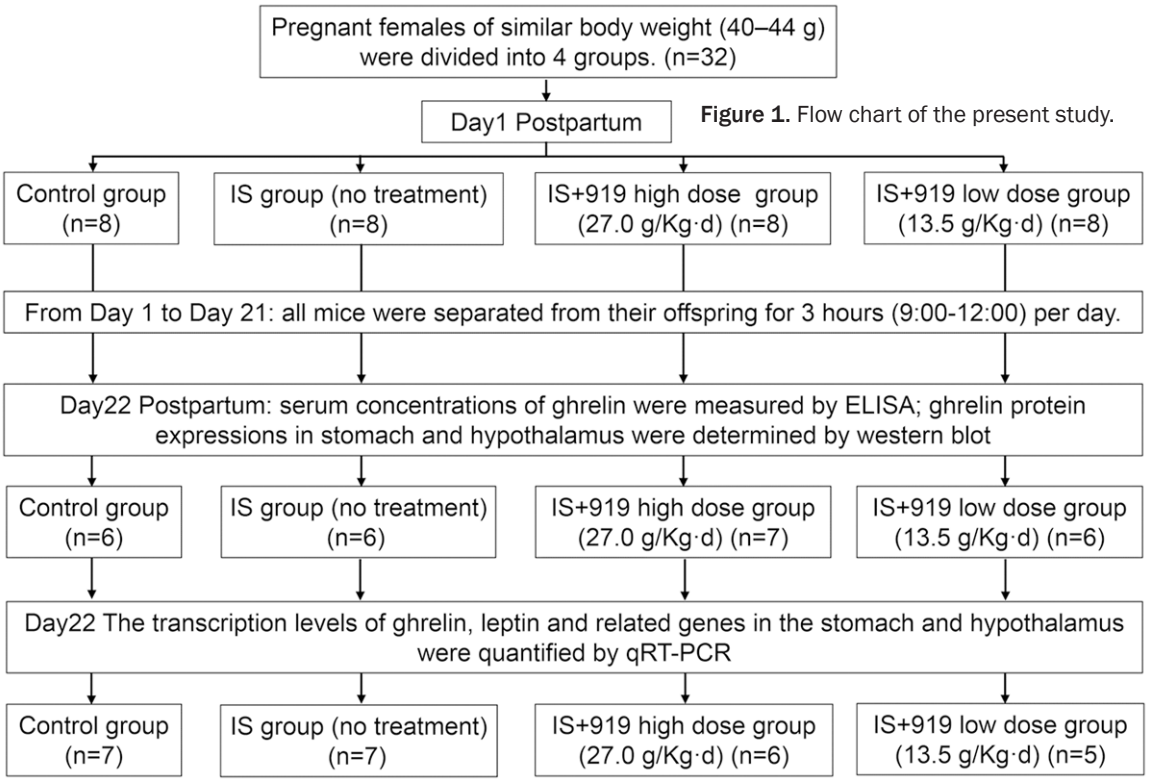

Figure 1. Flow chart of the present study.

mediated by the HPA axis [14]. Ghrelin is a 28-amino-acid peptide hormone that is secreted primarily by endocrine cells in the gastric and intestinal mucosa [15-17] and is an endogenous ligand at the growth hormone secretagogue receptor (Ghsr), through which it stimulates feeding [18, 19]. However, ghrelin has also been shown to modulate anxiety levels in response to stress via the HPA axis in mice [20]. In a mouse model of depression, ghrelin levels have been shown to rise in response to stress, and mice with null mutations in the *Ghsr* gene exhibited more negative effects in response to stress compared with mice expressing the wild-type Ghsr protein [21]. Given that ghrelin functions in both appetite regulation and the stress response, it is possible that ghrelin may be involved in the physiological manifestations of postpartum depression.

To test this hypothesis, we investigated changes in body weight, food intake and the expression levels of important neuropeptide regulators of appetite and energy metabolism in response to maternal separation and immobilization stress (IS), which have been shown to reduce feeding in postpartum mice [22]. In previous studies, a traditional Chinese herbal medication, known as jiubaiyishijiu tang jiang (translated as 919 syrup (tang jiang) in English) (919 TJ) has been shown to improve appetite in patients with chronic liver disease [23, 24], and rikkunshito, a traditional Japanese herbal medication that shares a number of components with 919 TJ [25, 26], has been reported to enhance ghrelin activity in healthy human subjects [27]. In the present study, we investigated the effects of 919 TJ on feeding and appetite-related responses to IS in postpartum mice and hypothesized that 919 TJ might have an influence on ghrelin activity.

Materials and methods

Animals

Initially, 42 female ICR mice (SCXK, Shanghai, China) used in our experiments were housed in the animal care facility of Jinshan Hospital of Fudan University (Shanghai, China) at 21°C ± 1°C with a 12/12 h light-dark cycle. Standard food and drinking water were provided *ad libitum* at all times, except during the IS procedure.

Male and female mice were at first housed separately, with 5 mice per cage (28 × 20 × 13 cm) and after acclimatization for 7 days, each female was placed in a cage with 2 males for a

continuous 4-day period to allow mating. Subsequently, female mice were separated from the males and finally pregnant females (*n* = 32) of similar body weight (40-44 g) were used for further examinations and divided into control, IS, IS + 919 TJ high dose, and IS + 919 TJ low dose groups, with 8 mice in each group (**Figure 1**). Following parturition, body weight and food intake were recorded daily. The animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University and performed in compliance with the guidelines presented in Animal Research: Reporting of *in vivo* Experiments (http://www.nc3rs.org.uk/arrive-guidelines) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023).

Maternal separation and immobilization stress experiments and 919 TJ medication

Within 24 h of parturition, each mouse in the IS, IS + 919 TJ high, and the IS + 919 TJ low groups 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.

The 919 TJ was obtained from Shanxi Province Shangmao Medicine (Xian, Shanxi, China) and consisted mainly of a mixture of extracts from *Radix salviae miltiorrhizae*, *Aurantii nobilis pericarpium* and *Fructus schisandrae*.

Using a 1.35 g/mL decotion stock solution of 919 TJ, the herbal mixture was administered intragastrically once a day to each postpartum mouse at doses of 27 g 919 TJ (IS + 919 TJ high dose) and 13.5 g of 919 TJ (IS + 919 TJ low dose) per kg mouse body weight for 21 days post-delivery. On postnatal day 22, blood was collected from each mouse into heparinized tubes and combined with EDTA and aprotinin before storage at -20°C. Immediately after blood collection, each mouse was humanely killed by an intraperitoneal injection of 10% chloral hydrate in normal saline and the stomach and hypothalamus surgically removed.

Identification of ten compounds using HPLC/ESI-MS

919 TJ samples were analyzed using an Agilent 1100 HPLC system containing a quaternary pump, an auto sampler, a degasser, an automatic thermostatic column compartment and a diode array detector (DAD) (Agilent Technologies, MA, USA) as well as a LC/MSD Trap XCT ESI mass spectrometer (Agilent Technologies, MA, USA). For proceedings see Supplementary Data 1.

Quantification of ghrelin protein

In serum, ghrelin occurs primarily as a highly active n-octanoylated form (active ghrelin) and a des-octanoylated (desacyl) form with limited biological activity [28]. The serum levels of active and desacyl-ghrelin in postpartum mice were determined separately by enzyme-linked immunosorbent assay (ELISA). Blood samples were centrifuged to obtain the plasma fraction. The levels of active and total ghrelin protein in serum were measured using the Mouse Active Ghrelin ELISA Kit and Mouse Desacyl Ghrelin ELISA Kit, respectively (both obtained from Merck Millipore, Billerica, MA, USA).

Western blot

The levels of ghrelin protein expression in stomach and hypothalamus tissues collected from postpartum mice were measured by western blotting. Tissue samples were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na_3PO_4, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF and 10 μg/mL aprotinin. Lysates were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) on a 12% acrylamide gel and the resolved protein bands were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were soaked in blocking buffer consisting of 1 × Tris-buffered saline, 1% bovine serum albumin and 1% nonfat dry milk for 1 h, and probed overnight at 4°C with a rabbit anti-ghrelin monoclonal antibody (MAB10404, EMD Millipore) and a rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (control). Primary antibody reactivity was detected using peroxidase-conjugated anti-rabbit IgG antibodies and a chemiluminescent detection

Table 1. Primers used for qRT-PCR analysis of mRNA expression

Gene	Primer sequences	
Ghrelin	Forward	5'-AAGAAGCCACCAGCTAAAC-3'
	Reverse	5'-ATCGAAGGGAGCATTGAAC-3'
Leptin	Forward	5'-TCTGTCTGGTGCTGTGAG-3'
	Reverse	5'-GCCCTGAAATGCGGTATG-3'
5-HT_{2b}R	Forward	5'-GATGCCGATTGCCCTCTTGAC-3'
	Reverse	5'-CTGGGATGGCGATGCCTATTG-3'
5-HT_{2c}R	Forward	5'-CATTCTTCATCCCGTTGAC-3'
	Reverse	5'-TTCCTCATCACCCTTCTTG-3'
CRF	Forward	5'-TTCTGCGGGAAGTCTTGG-3'
	Reverse	5'-ATCGGAGCTGCGATATGG-3'
POMC	Forward	5'-TTGGAAAGATAGCGGGAGAG-3'
	Reverse	5'-GCAGAGGCAAACAAGATTGG-3'
NPY	Forward	5'-GGTGATGGGAAATGAAAC-3'
	Reverse	5'-CAACAACAAGGGAAATGG-3'
AgRP	Forward	5'-CCACCTTTGCAGCATTCC-3'
	Reverse	5'-GTGCCAACAGCAGAACAC-3'
GHSR	Forward	5'-ATTTCCAATGCCCTGGTC-3'
	Reverse	5'-CCTTGAACTCCTGGTAATCC-3'
GAPDH	Forward	5'-ATCACTGCCACCCAGAAG-3'
	Reverse	5'-TCCACGACGGACACATTG-3'

system (Santa Cruz Biotechnology, Dallas, TX, USA). Band intensity was quantified by densitometry using the ChemicDoc XRS System and Quantity One imaging software (both from Bio-Rad Laboratories).

Quantification of mRNA transcription

The mRNA transcription levels of ghrelin, leptin, growth hormone secretagogue receptor (GHSR) and the 5-HT_{2b}R receptor in the stomach and those of ghrelin, neuropeptide Y (NPY), agouti-related protein (AgRP), proopiomelanocortin (POMC), corticotrophin-releasing factor (CRF) and the 5-HT_{2c}R receptor in the hypothalamus of postpartum mice were determined using a 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 forward primers listed in **Table 1** and the Improm-IITM Reverse Transcription System (Promega, Madison, WI, USA), according to the manufacturer's instructions. The qRT-PCR analysis was performed in triplicate using the forward and reverse primers listed in **Table 1** and the QuantiFast SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). Thermal cycling was performed in an iCycler iQTM Real-Time PCR System (Bio-Rad Laboratories) at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. After normalization based on GAPDH transcription, relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method [29]. The identities of the various complementary DNAs were confirmed by DNA sequencing using the same primers used for amplification (**Table 1**).

Statistical analysis

Statistical analysis was performed using SPSS for Windows software (Version 10.0. Chicago, SPSS Inc.). Data are presented as the mean ± standard deviation (SD). Intergroup differences were evaluated using the Fisher exact test or unpaired *t*-tests, with the level of statistical significance set at $P < 0.05$.

Results

Composition of 919 TJ derived from HPLC/ESI-MS analysis

We found 10 main chemicals in 919 TJ including angeloylisogomisin O, rosmarinic acid, narirutin, salvianolic acid B, dihydrotanshinone I, hesperitin, schizandrol A, neohesperidin dihydrochalcone, cryptotanshinone and tanshinone IIA. (Supplementary Figures 1 and 2).

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

Weight gain is considered a normal short-term response to childbirth [30]. The mean body weight of mice in the IS group was significantly lower from day 3 to day 16 postpartum than mice in the postpartum control group ($P < 0.01$, **Figure 2**). Although no clear pattern emerged, the mean food intake of the mice in the IS group was significantly lower on postpartum days 1, 3, 4, 6, 7, 13 and 14 than in the control group ($P < 0.05$). Body weight and food intake in the IS + 919 TJ high and IS + 919 TJ low dose groups did not differ significantly from those in the IS group ($P > 0.05$). These results demonstrated that IS inhibited feeding and weight gain in postpartum mice and that 919 TJ had no effect on the IS-induced suppression of appetite or weight gain.

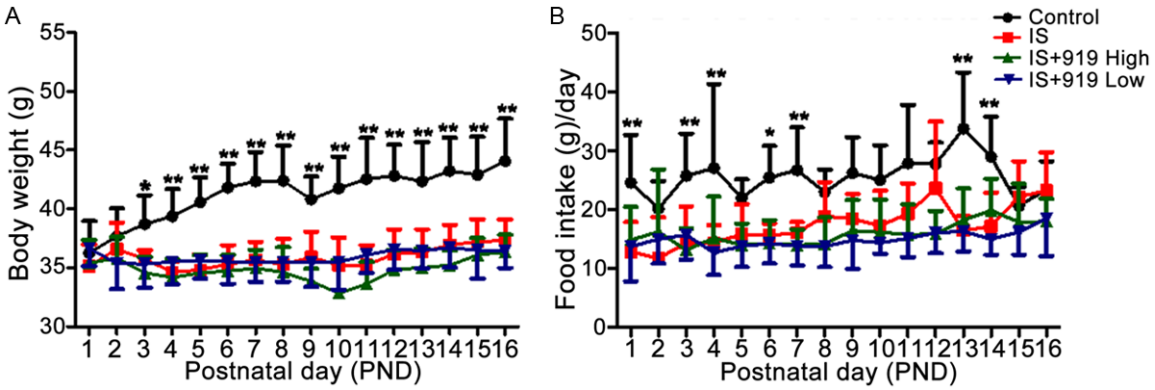

Figure 2. Effects of 919 TJ on IS-induced suppression of appetite and weight gain in mice from day 1 to day 16 postpartum. Pregnant females of similar body weight were divided into the control, IS, IS + 919 TJ high, and IS + 919 TJ low groups. Following parturition, the puerperas in the IS, IS + 919 TJ high, and IS + 919 TJ low groups were subjected to maternal isolation and immobilization stress for 3 h each day, and mice in the IS + 919 TJ high and IS + 919 TJ low groups were treated with 919 TJ at a dose of 0.270 and 0.135 g of 919 TJ per gram of body weight, respectively. (A) Body weight and (B) food intake were recorded daily (*$P < 0.05$ and **$P < 0.01$ *vs* IS group; control: n = 7; IS: n = 7; IS + 919 TJ high dose: $n = 8$; IS + 919 TJ low dose: $n = 8$).

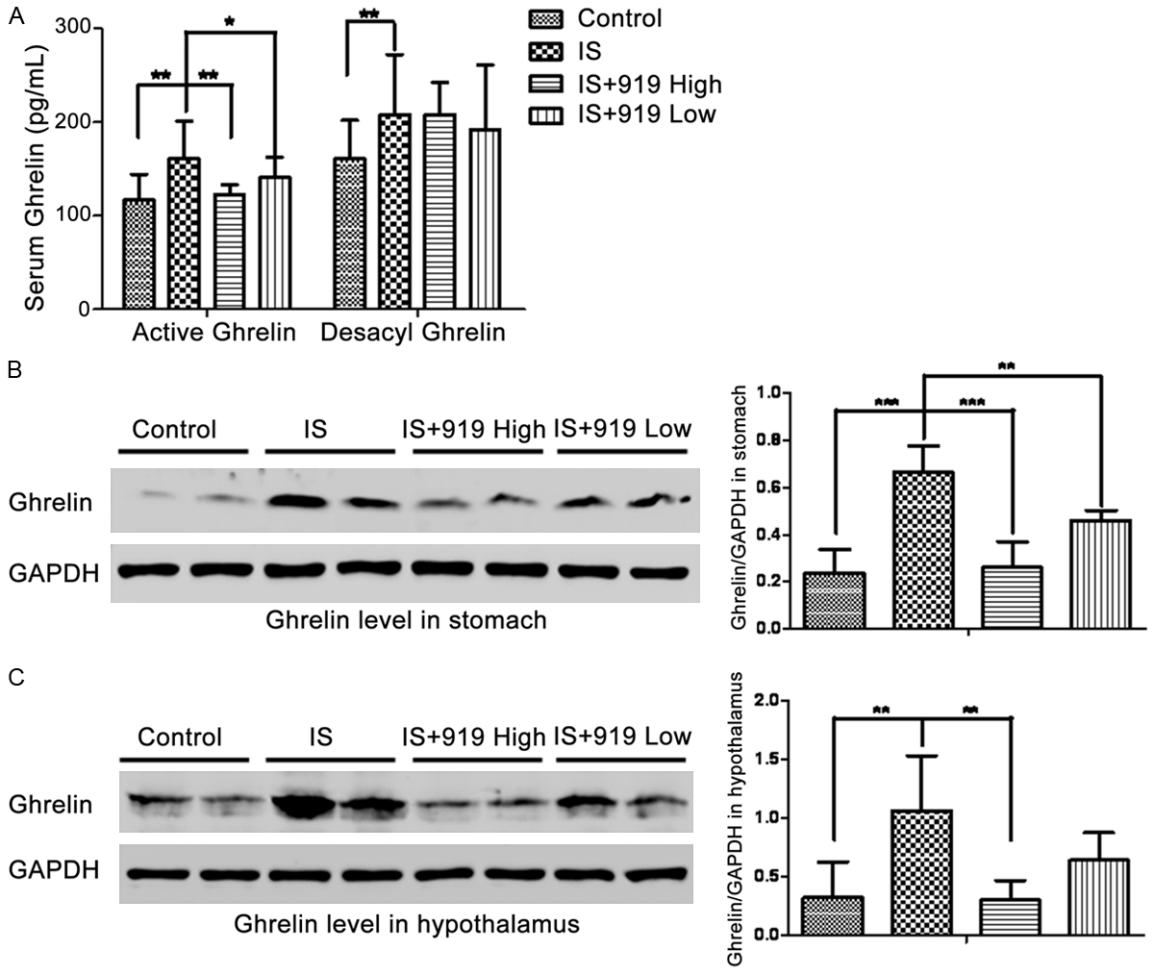

Figure 3. Effects of 919 TJ on IS-induced changes in serum ghrelin concentrations and ghrelin expressions in the stomach and hypothalamus of mice on day 22 postpartum. A. Serum levels of active and desacyl ghrelin were measured by ELISA (*$P < 0.05$, **$P < 0.01$). B, C. The level of ghrelin protein in the stomach and hypothalamus of postpartum mice subjected to IS was quantified by western blotting relative to that in the IS group (**$P < 0.01$ and ***$P < 0.001$ *vs* IS group).

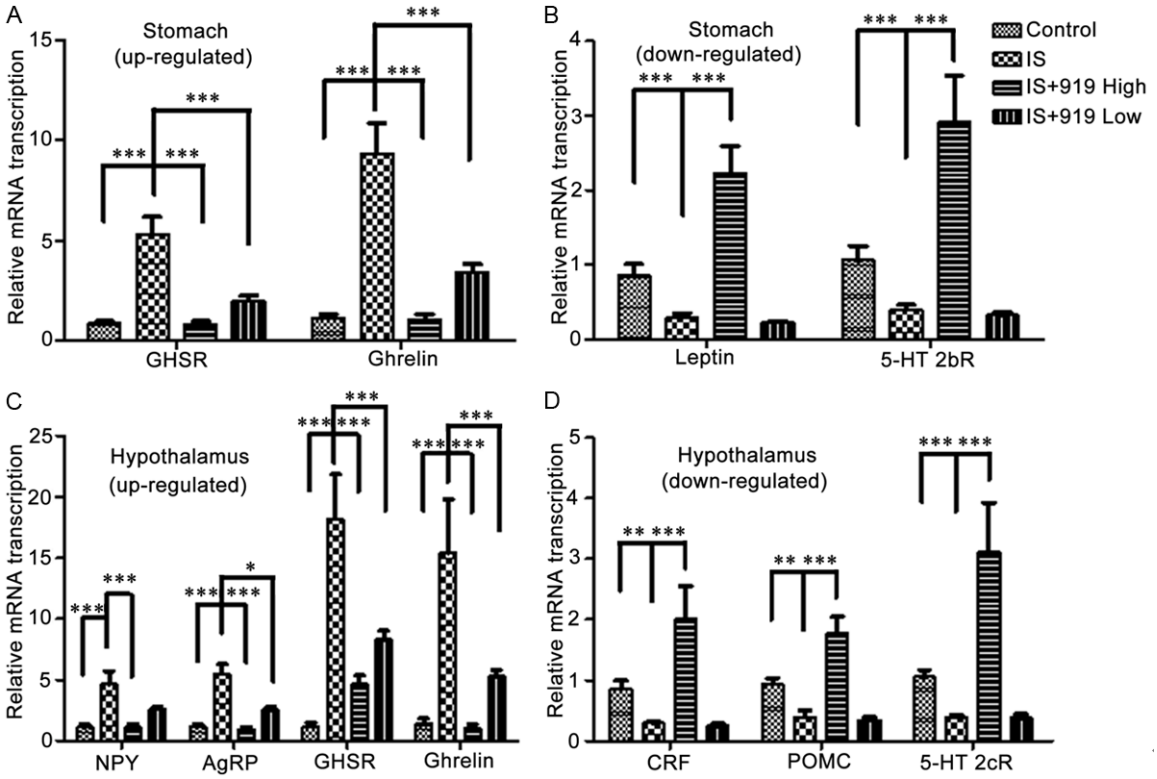

Figure 4. Effects of 919 TJ on changes in the expression levels of appetite and metabolism related genes in postpartum mice were examined using qRT-PCR on day 22 postpartum. A, B. Gene expression profiles of ghrelin, leptin, GHSR, and 5-HT_{2b}R in the stomach. C, D. Gene expression profiles of NPY, AgRP, GHSR, CRF, POMC, and 5-HT_{2c}R in the hypothalamus (*P < 0.05, **P < 0.01, and ***P < 0.001 *vs* IS group). GHSR: Growth hormone secretagogue receptor; 5-HT_{2b}R: Serotonin receptor in stomach; NPY: Neuropeptide Y; AgRP: Agouti-related protein; CRF: Corticotrophin-releasing factor; POMC: Proopiomelanocortin; 5-HT_{2c}R: Serotonin receptors in hypothalamus.

IS-induced increase in serum concentrations of active ghrelin in postpartum mice is inhibited by 919 TJ

The blood serum levels of active and desacyl ghrelin protein were measured on day 22 postpartum. The serum concentrations of both proteins were significantly higher in the IS group than in the control group (P < 0.01, **Figure 3A**). In the IS + 919 TJ high and IS + 919 TJ low dose groups, the serum level of active ghrelin was significantly lower than that in the IS group (P < 0.05), with the lowest level of active ghrelin observed in the IS + 919 TJ high dose group, whereas the levels of desacyl ghrelin in the IS + 919 TJ high and IS + 919 TJ low dose groups were not significantly different from the IS group (P > 0.05). These results demonstrated that IS increased serum concentrations of both active and desacyl ghrelin in postpartum mice, indicating that ghrelin may not be the principal driver of feeding in postpartum mice. The data also showed that 919 TJ reduced the serum level of active ghrelin in postpartum mice, without affecting the level of desacyl ghrelin.

IS-induced increase in ghrelin protein expression in the stomach and hypothalamus of postpartum mice is inhibited by 919 TJ

The level of ghrelin protein expression in the stomach and hypothalamus were measured on day 22 postpartum. Western blotting analyses showed that the level of total ghrelin protein in the stomach and hypothalamus of mice in the IS group were significantly higher compared to the control group (P < 0.01, **Figure 3B**, **3C**), and that the level of total ghrelin in the stomach and hypothalamus of mice in the IS + 919 TJ high dose group was significantly lower than in the IS group (P < 0.01). In the IS + 919 TJ low dose group, the level of total ghrelin in the stomach was significantly lower than that in the IS group (P < 0.01), whereas the level of ghrelin in the

Table 2. Effect of IS on the indicated factors. All effects beside the concentration of serum concentrations of desacyl ghrelin protein were significantly reversed by high dose 919 TJ

	Effects of IS	Effects of high dose 919 TJ
Serum		
Concentration of active ghrelin protein	↑	X
Concentration of desacyl ghrelin protein	↑	+/-
Stomach		
Ghrelin protein	↑	X
Gene transcription of Growth Hormone Secretagogue Receptor (GHSR)	↑	X
Gene transcription of ghrelin	↑	X
Gene transcription of leptin	↓	X+
Gene transcription of serotonin receptor 5-HT_{2b}R	↓	X+
Hypothalamus		
Ghrelin protein	↑	X
Gene transcription of Neuropeptide Y (NPY)	↑	X
Gene transcription of Agouti-Related Protein (AgRP)	↑	X
Gene transcription of Growth Hormone Secretagogue Receptor (GHSR)	↑	X-
Gene transcription of ghrelin	↑	X
Gene transcription of Corticotrophin-Releasing Factor (CRF)	↓	X+
Gene transcription of Proopiomelanocortin (POMC)	↓	X+
Gene transcription of serotonin receptor 5-HT_{2c}R	↓	X+

Note: ↓ downregulation; ↑ upregulation; +/- no effect; X reversal of IS effects to same levels as control; X+ reversal of IS effects exceeding the control; X- partly reversal of IS effect (less than control).

hypothalamus was not ($P > 0.05$). These data suggest that 919 TJ downregulates ghrelin protein expression in the stomach and hypothalamus in postpartum mice subjected to IS.

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

The mRNA expression levels of appetite and metabolism related genes in the stomach and hypothalamus were measured on day 22 postpartum using qRT-PCR. In the stomach tissues of mice in the IS group, the levels of ghrelin and GHSR mRNAs were significantly higher than in the control group, which was completely reversed by high doses of 919 TJ ($P < 0.001$, **Figure 4A**). In contrast, transcriptions of leptin and the serotonin receptor 5-HT_{2b}R were significantly lower in the stomach tissues of IS mice compared to the control group ($P < 0.001$), but they were extremely upregulated particularly in high dose 919 TJ treated IS mice, to levels far exceeding the control ($P < 0.001$, **Figure 4B**).

In the hypothalamus of mice in the IS group, transcription of ghrelin, GHSR, NPY and AgRP mRNAs were significantly higher ($P < 0.001$) compared to control mice, which could be partly reversed by application of 919 TJ (**Figure 4C**). The transcription rates of CRF, POMC and 5-HT_{2c}R mRNAs were significantly lower in IS mice compared to the control group ($P < 0.01$), but could be enhanced to 1-3 fold of control rates particularly with high doses of 919 TJ ($P < 0.001$, **Figure 4D**).

These data showed that in postpartum mice, IS induced increased activity of ghrelin in the stomach, serum and hypothalamus, GSHR in the stomach and hypothalamus as well as NPY and AgRP in the hypothalamus. In contrast, activity of leptin in the stomach, and CRF and POMC in the hypothalamus were reduced by IS, and the serotonin receptor 5-HT_{2b}R was less expressed in the stomach and the 5-HT_{2c}R in the hypothalamus of IS mice. Application of 919 TJ could reverse most of the changes to control or above control levels (**Table 2**).

Discussion

We investigated the effects of restraint stress during maternal separation on appetite and

weight gain in postpartum mice. Our results showed that over the course of 21 days daily 3-h IS exposure inhibited feeding and weight gain, increased the serum concentrations of both active and desacyl ghrelin, upregulated ghrelin and GHSR transcription in the stomach and hypothalamus, upregulated NPY and AgRP transcription in the hypothalamus, as well as downregulated leptin and 5-HT_{2b}R transcription in the stomach and CRF, POMC, and 5-HT_{2c}R transcription in the hypothalamus (**Table 2**).

Stress has been shown to reduce ghrelin secretion in the stomach and is thought to suppress the orexigenic effects of ghrelin via the HPA axis, which is characterized by hypersecretion of CRF [31-33]. Activation of the hypothalamic CRF type 1 receptor has been shown to contribute to anorexia in response to novelty stress, which is manifested by a reduction in serum ghrelin [31]. A reduced serum level of active ghrelin further suppresses appetite by reducing afferent vagal stimulation via reduced ghrelin-GHSR binding [34]. Increased serum serotonin also occurs in response to stress. Activation of 5-HT_{2b} receptors inhibits the secretion of active ghrelin in the stomach [35]. The activation of 5-HT_{2c} receptors by serotonin on hypothalamic neurons expressing CRF or POMC also contributes to appetite suppression [35, 36], but the mechanisms by which 5-HT_{2c} receptor agonists/antagonists influence appetite remain unclear.

Despite the wealth of evidence supporting the above mentioned paradigm, multiple studies have also shown that stress upregulates the secretion of ghrelin in the stomach of rodents [21, 37, 38]. We also observed that IS during maternal separation caused an increase in the serum levels of both active and desacyl ghrelin. The anticipatory response could be mistaken for an increase in serum ghrelin in response to stress [39, 40], but we provided food *ad libitum* to the postpartum mice in our study, which has been shown to eliminate the anticipatory response in mice [40]. Combined with those of previous studies, our findings suggest that mechanisms other than those involved in the HPA axis-mediated responses to stress may be involved in the suppression of appetite under certain biological conditions, such as those occurring during the postpartum period. However, the suppression of CRF, POMC and 5-HT_{2c}R expression in the hypothalamus of postpartum mice subjected to IS nonetheless indicates the involvement of a hypothalamic mechanism. Future studies are warranted to investigate further how the hormonal changes induced by IS suppressed feeding and weight gain in postpartum mice.

We also investigated the effects of the traditional Chinese herbal medication, 919 TJ on stress-induced suppression of appetite and weight gain in postpartum mice. The 919 TJ formulation shares herbal components with the traditional Japanese herbal medication rikkunshito, which has been shown to ameliorate appetite suppression caused by reduced serum ghrelin in mice subjected to novel-environment stress [41, 42]. Although 919 TJ had no significant effect on food intake or weight gain in postpartum mice subjected to IS, it reduced the serum level of active ghrelin and suppressed ghrelin and GHSR expression in the stomach and hypothalamus. These results suggested that ghrelin is not the primary orexigenic driver of feeding behavior in mice. Treatment with 919 TJ reversed the suppressive effects of IS on CRF, POMC, and 5-HT_{2c}R activity in the hypothalamus of postpartum mice. We also found that 919 TJ increased leptin and 5-HT_{2b}R in the stomach of postpartum mice subjected to IS, both of which have been shown to suppress directly ghrelin secretion in the stomach in *ex vivo* studies [43, 44] (**Table 2**).

Our results indicated that the effects of 919 TJ in postpartum mice subjected to stress differ from those of rikkunshito in other animal models of stress, highlighting the need for further research specifically addressing the role of stress in hormonal changes that contribute to postpartum anorexia. Future studies are also warranted to identify the molecular targets of 919 TJ, as these may be relevant to the treatment of ghrelin-mediated obesity. However, our findings are subject to certain limitations. The sample sizes used in our experiments were relatively small, ranging from 5 to 8 mice in each group, because a number of the mice died during the course of the experiments. Future studies are required to confirm our results and to investigate the mechanism(s) underlying increased ghrelin expression in postpartum mice subjected to stress.

We found that subjecting postpartum mice to IS suppressed feeding and weight gain incre-

ased the serum concentrations of both active and desacyl ghrelin, upregulated ghrelin and GHSR expression in the stomach and hypothalamus, upregulated NPY and AgRP expression in the hypothalamus as well as down-regulating leptin and 5-HT_{2b}R expression in the stomach and CRF, POMC, and 5-HT_{2c}R expression in the hypothalamus. Application of high 919 TJ doses could significantly reverse the IS induced changes although 919 TJ treatment did not improve food intake or weight gain, indicating that ghrelin is not the principal driver of feeding behavior in postpartum mice. Since the biochemical alterations caused by IS are not in agreement with the classical hypothalamic-pituitary-adrenal (HPA) axis stress response, other mechanisms might be involved in IS induced metabolic changes.

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Disclosure of conflict of interest

None.

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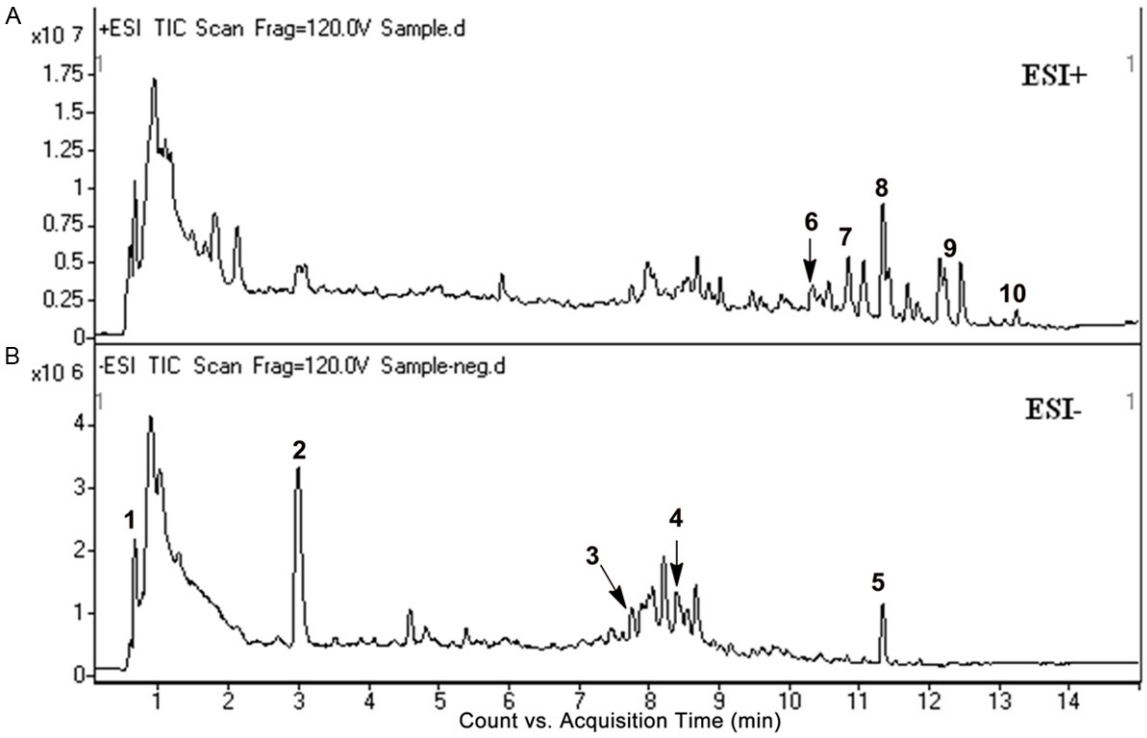
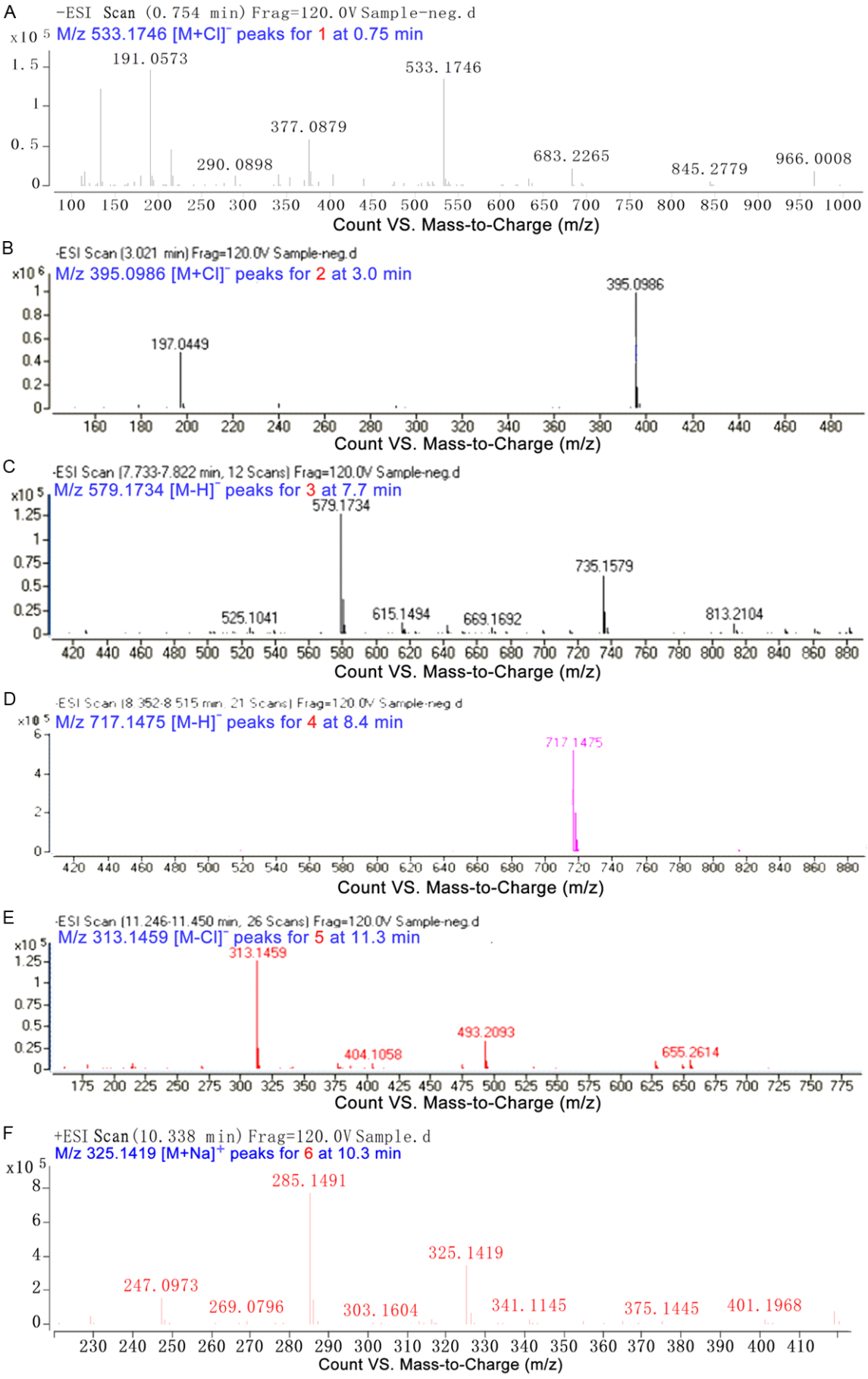
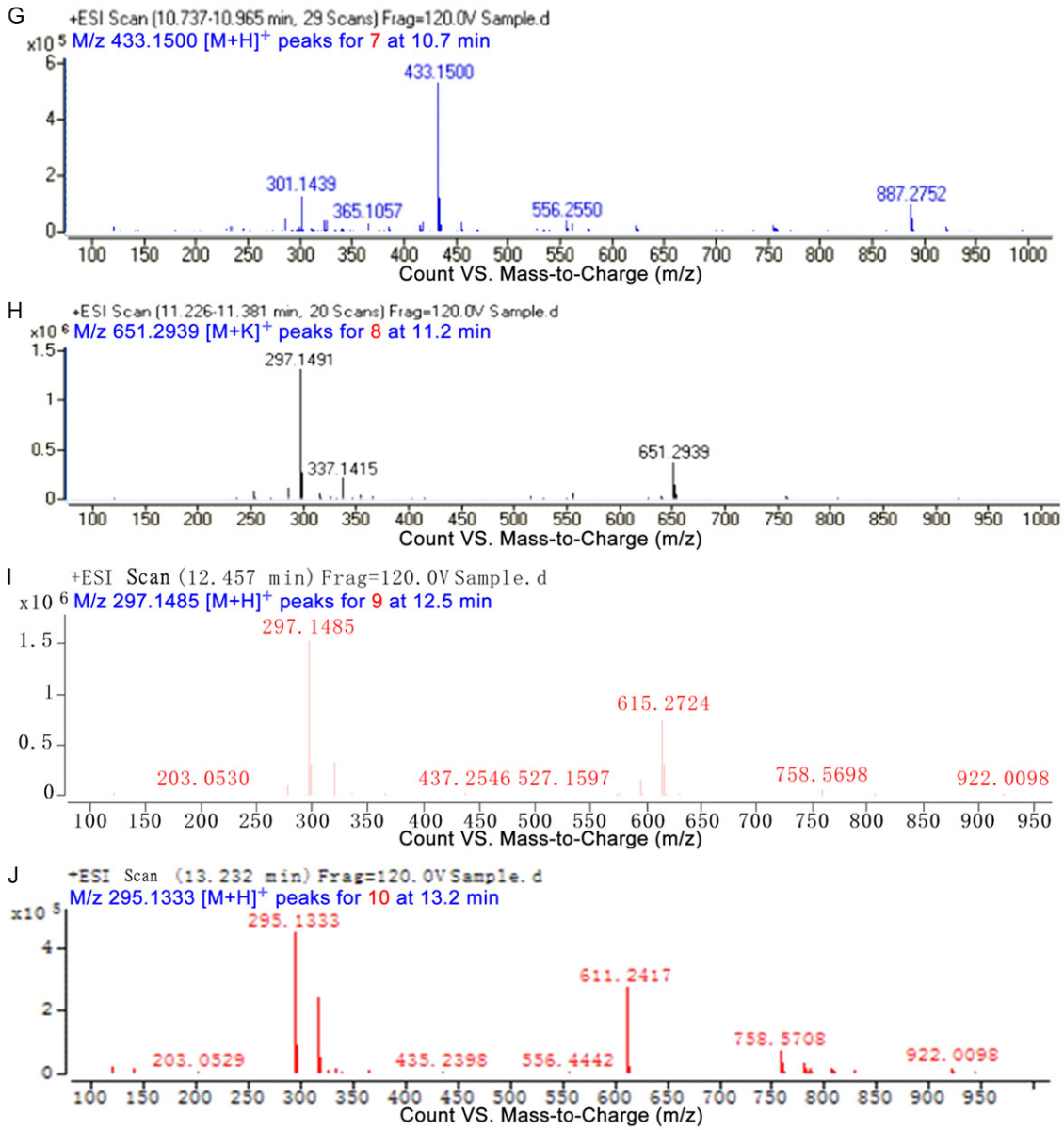
Supplementary Data 1

HPLC-ESI-MS/MS analysis. The separation was performed on a GS-120-5-C18-BIO chromatographic column (5 μm, 250 × 4.6 mm i.d.) with the column temperature set at 35°C. A linear gradient elution of A (0.1% formic acid water) and B (acetonitrile) was used with the gradient procedure as follows: 0 min, B 5%, to 60 min B 40% (v/v). The flow rate was 1.0 mL/min and the injection volume was 10 μL. DAD was on and the target wavelength simultaneously set at 210 nm. The split ratio to the mass spectrometer was 1:3. The acquisition parameters for negative ion mode were: collision gas, ultra high-purity helium (He), nebulizer gas (N2), 35 psi, drying gas (N2), 10 L/min, drying temperature, 350°C, HV, 3500 V, mass scan range, m/z 100-2200, target mass, 500 m/z, compound stability, 100%, trap drive level, 100%. All the data were analyzed by Chemstation software (Agilent Technologies, MA, USA).



Supplementary Figure 1. HPLC/ESI-MS chromatogram of the aqueous extract in negative and positive mode (A, B).


A
-ESI Scan (0.754 min) Frag=120.0V Sample-neg.d
x10 5
M/z 533.1746 [M+Cl]⁻ peaks for 1 at 0.75 min
191.0573
533.1746
377.0879
290.0898
683.2265
845.2779
966.0008
Count VS. Mass-to-Charge (m/z)
B
-ESI Scan (3.021 min) Frag=120.0V Sample-neg.d
x10 6
M/z 395.0986 [M+Cl]⁻ peaks for 2 at 3.0 min
395.0986
197.0449
Count VS. Mass-to-Charge (m/z)
C
-ESI Scan (7.733-7.822 min, 12 Scans) Frag=120.0V Sample-neg.d
x10 5
M/z 579.1734 [M-H]⁻ peaks for 3 at 7.7 min
579.1734
735.1579
525.1041
615.1494
669.1692
813.2104
Count VS. Mass-to-Charge (m/z)
D
-ESI Scan (8.352-8.515 min, 21 Scans) Frag=120.0V Sample-neg.d
x10 5
M/z 717.1475 [M-H]⁻ peaks for 4 at 8.4 min
717.1475
Count VS. Mass-to-Charge (m/z)
E
-ESI Scan (11.246-11.450 min, 26 Scans) Frag=120.0V Sample-neg.d
x10 5
M/z 313.1459 [M-Cl]⁻ peaks for 5 at 11.3 min
313.1459
493.2093
404.1058
655.2614
Count VS. Mass-to-Charge (m/z)
F
+ESI Scan (10.338 min) Frag=120.0V Sample.d
x10 5
M/z 325.1419 [M+Na]+ peaks for 6 at 10.3 min
285.1491
325.1419
247.0973
269.0796
303.1604
341.1145
375.1445
401.1968
Count VS. Mass-to-Charge (m/z)



Supplementary Figure 2. HPLC/ESI-MS chromatogram of the aqueous extract. A: ESI-MS spectra of $[M + Cl]^-$ ion of compound 1 (retention time: 0.75 min). B: ESI-MS spectra of $[M + Cl]^-$ ion of compound 2 (retention time: 3.0 min). C: ESI-MS spectra of $[M - H]^-$ ion of compound 3 (retention time: 7.7 min). D: ESI-MS spectra of $[M - H]^-$ ion of compound 4 (retention time: 8.4 min). E: ESI-MS spectra of $[M + Cl]^-$ ion of compound 5 (retention time: 11.3 min). F: ESI-MS spectra of $[M + Na]^+$ ion of compound 6 (retention time: 10.3 min). G: ESI-MS spectra of $[M + H]^+$ ion of compound 7 (retention time: 10.7 min). H: ESI-MS spectra of $[M + K]^+$ ion of compound 8 (retention time: 11.2 min). I: ESI-MS spectra of $[M + H]^+$ ion of compound 9 (retention time: 12.5 min). J: ESI-MS spectra of $[M + H]^+$ ion of compound 10 (retention time: 13.2 min). The ingredients including angeloylisogomisin O (1), rosmarinic acid (2), nariruti (3), salvianolic acid B (4), dihydrotanshinone I (5), hesperitin (6), schizandrol A (7), neohesperidin dihydrochalcone (8), cryptotanshinone (9), and tanshinone IIA (10) in **Figure 1**, on the basis of the observation of the pseudomolecular ion peak at *m/z* 533.1746 $[M + Cl]^-$ (1), *m/z* 395.0986 $[M + Cl]^-$ (2), *m/z* 579.1734 $[M - H]^-$ (3), *m/z* 717.1475 $[M - H]^-$ (4), *m/z* 313.1459 $[M + Cl]^-$ (5), *m/z* 325.1419 $[M + Na]^+$ (6), *m/z* 433.1500 $[M + H]^+$ (7), *m/z* 651.2939 $[M + K]^+$ (8), *m/z* 297.1485 $[M + H]^+$ and 615.2724 $[2M + Na]^+$ (9), *m/z* 295.1333 $[M + H]^+$ and 611.2417 $[2M + Na]^+$ (10).