Integrated metabonomic study of the effects of Guizhi Fuling capsule intervention on primary dysmenorrhea using RP-UPLC-MS complementary with HILIC-UPLC-MS technique

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Abstract

*Guizhi Fuling* capsule (GFC), developed from the traditional Chinese prescription of *Guizhi Fuling Wan*, has been commonly used for the treatment of primary dysmenorrhea (PD). However, the intervention effective mechanism *in vivo* has not been well elucidated. In this study, an integrated plasma metabonomic strategy based on RP-UPLC-MS coupled with HILIC-UPLC-MS technique has been developed to investigate the global therapeutic effects and intervention mechanisms of *Guizhi Fuling* capsule (GFC) on dysmenorrhea rats induced by oxytocin. The total twenty potential biomarkers were identified and primarily related to sphingolipid metabolism, steroid hormone biosynthesis, glycerophospholipid metabolism, amino acid metabolism, lipid metabolism and energy metabolism. The results showed that the GFC has therapeutic effects on rat with dysmenorrhea via the regulation of multiple metabolic pathways. Some new potential biomarkers associated with primary dysmenorrhea such as phenylalanine, tryptophan, taurine, carnitine, betaine, creatine and creatinine have been discovered in this study for the first time. This study provides a metabonomic platform based on RP-UPLC-MS complementary with HILIC-UPLC-MS technique to investigate both nonpolar and polar compounds, so as to get a more comprehensive metabolite information for yielding insight into the pathophysiology of PD and assessing the efficacy of GFC on PD rats.

Key words: *Guizhi Fuling* capsule, primary dysmenorrhea, RP-UPLC-MS, HILIC-UPLC-MS, metabonomics
Introduction

Primary dysmenorrheal (PD) is a common gynaecological disease characterized by lower abdomen pain and back pain involved in the menstrual period without pelvic abnormality (Doubova et al. 2007). It was reported that the morbidity of PD is estimated to be 20-90% of childbearing women worldwide and more than 15% patients described the pain was extremely severe which results in a significant absenteeism from work or school and other profoundly negative impact on the community economy (De et al. 2017, French 2005). The possible pathophysiology concluded from the previously proposed theory was considered as the excessive secretion of prostaglandins (PGs) associated with the sex-hormone, leukotrienes, vasooressin and oxytocin related disorder which eventually stimulates hyperalgesia, myometrial contraction, vasoconstriction, inflammatory pain and ischemia (Dawood 2006, Liedman et al. 2008, Michael et al. 2016, Wallace et al. 2010). Nonsteroidal anti-inflammatory drugs (NSAIDs) and oral contraceptives are the principal pharmacologic treatment for dysmenorrheal in clinic (Davis et al. 2005, Lefebvre et al. 2005, Zahradnik et al. 2010). However, NSAIDs have serious adverse effects on human body, including kidney, liver and digestive tract, and Oral contraceptives also have potential side effects such as the water retention, nausea and disorder of whole endocrine metabolism (Davis et al. 2005, Lefebvre et al. 2005). Because of the shortcomings mentioned above, the Traditional Chinese Medicine (TCM) is considered as a well-accepted therapy of PD.

*Guizhi Fuling* capsule (GFC) is developed from the traditional Chinese prescription of *Guizhi Fuling Wan*, originally came from Jingui Yaolue written by Chinese physician Zhongjing Zhang in the Eastern Han Dynasty (third century A.D.). The formula with the traditional efficacy of dissolving stasis, invigorating blood and resolving mass, consists of five herbs (1:1:1:1:1, g/g), namely *Cinnamomum cassia* (L.) J.Presl (*Cassia bark*), *Poria cocos* (Schw.) Wolf (*Poria*), *Paeonia lactiflora* Pall (*Herbaceous peony*), *Paeonia suffruticosa* andrews (*Moutan Cortex*) and *Amygdalus persica* L. (*Persicae Semen*) (Liu et al. 2013). GFC has been widely applied in the clinical prevention and treatment of dysmenorrheal, uterine fibroids, endometriosis, ovarian cysts and pelvic inflammatory disease, which are considered to be caused by the disharmony patterns of blood stasis in TCM (Hu et al. 2014, Li et al. 2007, Yao et al. 2008). Our previous studies have shown that GFC could significantly inhibit the expression of Cyclooxygenase-2 (COX-2) and oxytocin receptor (OTR), reduce the level of prostaglandin F$_{2a}$ (PGF$_{2a}$), intracellular Ca$^{2+}$ and NO in uterine tissue of oxytocin induced rats (Lan et al. 2016). GFC contained numerous bioactive compounds, including gallic acid, amygdalin, albiflorin, paeoniflorin, paenonol and cinnamic
acid etc (Zhao et al. 2015). However, the therapeutic mechanism of GFC for PD is still necessary to have a further comprehensive investigation.

Metabonomics, based on the analysis of the dynamic changes of low molecular weight compounds in organism, provide a powerful approach to investigate the entire physiological status of metabolic responses in biological systems related to the pathophysiological stimuli or other factors, such as genes, environment and lifestyle (Sun et al. 2012). In particular, the comprehensive therapeutic efficacies and action mechanisms of TCMs would be elucidated by analyzing the drug-target interactions through the metabolic pathway of identified biomarkers (Tan et al. 2012). Many metabonomic studies now utilize ultra performance liquid chromatography-mass spectrometry (UPLC-MS) due to its high sensitivity, selectivity, resolution and reproducibility of analysis. Most of the studies are performed using reverse-phase liquid chromatography (RPLC, mainly C18 columns) to generate reproducible data for the non- and moderately polar compounds (Want et al. 2010, WB et al. 2011). However, many of the water soluble, ionic and polar metabolites are not retained on RP-UPLC columns and would be hindered from identification and accurate quantification (Boudah et al. 2014). Hydrophilic interaction liquid chromatography (HILIC) offers a complementary selectivity to RPLC to analysis the polar metabolites, thus expanding the range of obtained metabolites compared to just using RP approaches (Cubbon et al. 2009, Spagou et al. 2015, Tang et al. 2016). A UPLC-MS metabonomic approach based on RP and HILIC separations to investigate the global serum metabolic profile associated with myocardial infarction was developed by Tan et al. In their study, twenty-one potential biomarkers were identified, and further demonstrated the effects of sini decoction on myocardial infarction (Tan et al. 2012).

As a consequence, we combined the characteristics of RP-UPLC-MS and HILIC-UPLC-MS on metabonomics platform, in order to find more comprehensive metabolite profile information and investigate the global therapeutic effect and mechanisms of GFC on PD model rats in this paper.

2. Materials and methods

2.1. Chemicals and reagents

*Guizhi Fuling* capsule (No. 16021720407) was purchased from Kanion Pharmaceutical Co., LTD. (Jiangsu, China). Estradiol benzoate (No. 150509) and Oxytocin injection (No. 160108) were obtained from Ningbo the Second Biochemical Pharmaceutical Co., LTD. (Ningbo, China).

HPLC grade acetonitrile was obtained from Tedia (Fairfield, OH, USA). Formic acid of
HPLC grade was supplied by Dikma Corp (Richmond Hill, NY, USA). Ultra-pure water used in this study was purchased from Wahaha Co, LTD. (Hangzhou, China). All other chemicals and reagents used were of analytical grade.

The prostaglandin F2a (PGF2a) and prostaglandin E2 (PGE2) assay kits used in the experiment were supplied by Meilian Biotechnology Co., LTD. (Shanghai, China).

2.2. Animals and treatments

Female wistar rats weighing 180-220 g were obtained from the Central Animal House of Shenyang Pharmaceutical University. The rats were housed in the SPF grade Experimental Animal House (temperature of 20 ± 2°C, relative humidity of 50% ± 5%, and 12 h/12 h light/dark cycle) with free access to food and drinking water. Before the treatment, all the animals were allowed to acclimatize for a week. The experiments were in accordance with the regulations of animal care issued by the National Institute of Health's Guidelines and admitted by the Medical Ethics Committee of Shenyang Pharmaceutical University.

The 24 female rats were randomly divided into 3 groups (n=8 rats/group): control group, model group and treatment group. Except for the control group, the remaining 2 groups were produced dysmenorrheal model using estradiol benzoate and oxytocin injection. Estradiol benzoate (0.01 g/kg/d) was injected subcutaneously once daily for 6 days and on the seventh day oxytocin (10 ml/kg) was administrated by peritoneal injection, while the control group injected saline solution. Rats in the treatment group were consecutively administered orally with GFCC suspended with 0.30% CMC-NA (0.25 g/kg/d) from the first day producing dysmenorrheal model for 7 days, while the control and model group received the same volume of 0.30% CMC-NA solution during this period.

2.3. Sample collection and preparation

After intraperitoneally injection of oxytocin for 30 min, the blood was collected from the suborbital vein into heparinized tubes and immediately centrifuged at 4000 rpm for 10 min, 10 μL of plasma was used for biochemical analysis. The plasma samples were transferred into tubes and stored at -80°C before analysis.

Prior to analysis, plasma samples were thawed at room temperature. 600 μL acetonitrile was added to the 200 μL aliquot of plasma and then vortexed for 30 s. After centrifugation at 13,000 rpm for 10 min, the supernatant was transferred and concentrated to dryness under a gentle stream of nitrogen. The dry residue was reconstituted in 100 μL of acetonitrile-water (10:90, v/v) and vortexed for 30 s. The content was transferred to the sampling vial and aliquot of 10 μl was injected for UPLC-MS analysis.
2.4. Behavioral and biochemical analysis

The numbers of writhing times of each rat in 30 min after oxytocin injection were observed to calculate the inhibition ratio. The plasma levels of PGF$_2\alpha$ and PGE$_2$ were determined using commercial kits, according to the manufacturer’s instructions.

2.5. UPLC-MS analysis

The UPLC-MS analysis was carried out using a Waters ACQUITY™ ultra performance liquid chromatography system (Waters Corp., Milford, USA) coupled with a Micromass Quattro Micro™ API mass spectrometer (Waters Corp., Milford, MA, USA). The plasma metabonomic method was developed by using RP-UPLC-MS and HILIC-UPLC-MS. The UPLC column used was a 100 mm×2.1 mm-1.7 μm C$_{18}$ column (Waters Corp., Milford, MA, USA) and the HILIC column used was a 100 mm×2.1 mm-1.7 μm HILIC column (Waters Corp., Milford, MA, USA), which were held at 40°C. The gradient mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). In RP-UPLC-MS mode, the column was eluted with a linear gradient of 0–95% B over 0.5–20 min, the composition was held at 95% B for 1 min then returned to 0% B and re-equilibrated for an additional 3 min before injection of the next sample. In HILIC-UPLC-MS mode, A was linearly increased from 5% to 20% in 2-10 min, 20% to 50% in 10-12.5, and then A was decreased to 5% in 3 min. The flow rate was set at 0.20 mL/min and the autosampler was maintained at 4°C.

An electrospray ionization source (ESI⁺) interface was used. The following parameters were employed: the source temperature and the cone gas flow rate were 120°C and 30 L/h, the desolvation gas flow rate was set to 450 L/h at a temperature of 350°C. The capillary voltage was 3.0 kV and the cone voltage was 35 V. MS data were collected in the full scan mode from $m/z$ 100 to 1000 amu over 24 min. The collision gas in the MS/MS experiments to identify the potential biomarkers was argon and the collision energy was altered between 15 and 25 eV.

2.6. Method validation

The applied method was validated prior to analysis of experimental samples, including the precision of injection, method repeatability, stability of freeze-thaw process, post-preparative stability, and the system stability. Quality control (QC) samples were prepared by the mixture of 300μL of each plasma for method validation. 200 μL aliquot of the pooled sample from plasma was extracted using the same preparation method described above. Six replicated injections from the same QC sample were used for the precision of instrument. The method repeatability was tested by analysis of six replicated QC samples on
one day. The post-preparative stability was evaluated by analyzing six QC samples left at autosampler (maintained at 4°C) for 24 h with the freshly prepared QC samples (n=6) continuously in a single batch. The stability of the freeze-thaw process was tested by analyzing the QC samples undergone from 1 to 3 freeze-thaw cycles together with the freshly thawed QC samples (n=6) in a single batch. The system stability was carried out by injecting a QC sample every 4 samples during the whole sample analysis.

2.7. Data analysis

Each sample was represented by a total ion chromatogram (TIC) chromatogram. The integrated and centroided raw mass spectrometric data of plasma were processed using the Micromass MarkerLynx Applications Manager version 4.0 (Waters Corp., Milford, USA). The intensity of the ions, after peak recognition and peak alignment, were normalized to the total to generate a three-dimensional data matrix that consisted of the retention times, m/z values, and the normalized ion intensities. The multivariate data matrix was then introduced to SIMCA-P 11.0 software package (Umetrics, Umea, Sweden) for principal component analysis (PCA) and partial least squared discriminant analysis (PLS-DA). PCA as an unsupervised method was applied to filter out the noise and reduce the dimension of data to visualize the clustering, trends and outliers in the observations. PLS-DA, as a supervised method was used to enhance the classification performance and was applied to obtain a better discrimination of the different groups. In this study, the variable importance in the projection (VIP) was used to select potential biomarkers. Variables with the VIP value larger than 1 were showed higher influence on the classification than average (Lenz et al. 2007). The loading plots as the most discriminatory metabolite resonances were normalized and integrated to the sum of total spectral integral. A paired-sample t-test was carried out to estimate the variables between groups were statistically significant difference (p< 0.05).

3. Results

3.1. Behavioral and biochemical results

The results of the rat’s writhing times and the plasma biochemical assay are presented in Table 1. After oxytocin injection, obvious symptoms of torsion in model group appeared showed that the PD model was successfully established. The writhing times were significantly decreased in treatment group as compared to that of the model group (p<0.01) and the inhibition ratio was 63.90%. Compared with the control group, the PGE$_2$ level of the model group was markedly decreased (p<0.01). Furthermore, the level of PGF$_2\alpha$ in the model group significantly increased beyond the control group (p<0.01). Notably, the PGE$_2$ and
PGF$_{2a}$ levels were markedly restored in the treatment group compared with the model group ($p<0.01$). The above results not only validated the establishment of PD rat model but also implied GFC has the therapeutic effect on PD rats.

3.2. Validation of analytical performance

In the RP-UPLC-MS mode, extracted ion chromatographic peaks of six ions (with the retention time and $m/z$ pairs of 1.11-132.2, 5.22-120.0, 6.07-205.4, 10.86-218.2, 15.78-545.4, 22.39-954.1) were selected to validate the analytical method. In the HILIC-UPLC-MS mode, extracted ion chromatographic peaks of six ions were selected for method validation. The pairs with the retention time and $m/z$ value were 1.22-415.2, 3.86-167.0, 5.54-830.0, 7.69-118.1, 9.01-162.4, 11.61-258.2. The relative standard deviations (RSDs) or relative errors (REs) of peak intensity and retention time for the selected ions in pooled plasma samples were calculated. The results obtained are presented in Table 2, the RSD and RE values were all less than 15%, indicated that this method could be utilized to the analysis of experimental plasma samples.

3.3. Analysis of metabolic pattern and identification of potential biomarkers

The representative ion based peak intensity (BPI) chromatograms of plasma in RP-UPLC-MS and HILIC-UPLC-MS modes are shown in Fig. 1 and 2, respectively. The dataset was further analyzed using the unsupervised PCA to investigate the subtle changes among the groups and the therapeutic effects of GFC in PD rats. According to the score plots from PCA (Fig. 3), the model group and control groups were separated obviously in both RP-UPLC-MS and HILIC-UPLC-MS modes, which suggests that plasma biochemical perturbation significantly happened in model group. In accordance with the score plots from PLS-DA of the three groups, a clear separation was observed and the treatment group was restored back to the control-like level, which indicated that GFC did have therapeutic effects on the PD rats. The parameters of the PLS-DA model presented in Fig. 4 are as follows: A($R^2_X=0.503$, $R^2_Y=0.938$, $Q^2=0.817$), C($R^2_X=0.442$, $R^2_Y=0.849$, $Q^2=0.537$). A response permutation test showed no overfitting in the model: A($R^2_Y$-intercept of 0.352, $Q^2_Y$-intercept of -0.242), C($R^2_Y$-intercept of 0.232, $Q^2_Y$-intercept of -0.152).

Corresponding loading plots (Fig. 5) were used to indicate the variable responsible for the separation. The metabolites furthest away from the center contribute to the clustering significantly and these metabolites with VIP values over 1 and $p$ values lower than 0.05 were regard as the potential biomarkers.
The structure identification of the potential biomarkers were performed according to their molecular ion masses and the MS/MS fragmentation patterns comparing with the available reference standards or various databases including HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/), KEGG (http://www.genome.jp) and PubChem (http://ncbi.nlm.nih.gov/).

Take the potential biomarker at \(m/z\) 166 as an example to illustrate the identification process. In the positive ion spectrum (Fig. 6A), besides the base peak ion at \(m/z\) 120, the ions at \(m/z\) 166, \(m/z\) 188, \(m/z\) 331 and \(m/z\) 353 were also found. Thus, we inferred that the quasimolecular ion was \(m/z\) 166 ([M+H]⁺), and the adduction ions were \(m/z\) 188 ([M+Na]⁺), \(m/z\) 331 ([2M+H]⁺) and \(m/z\) 353 ([2M+Na]⁺). Furthermore, the fragmentation of the ion at \(m/z\) 166 (Fig. 6B) from tandem MS in positive ions was further investigated. Four major fragment ions were found at \(m/z\) 149, \(m/z\) 131, \(m/z\) 120 and \(m/z\) 103 and \(m/z\) 120 was the main fragment ion in them. Finally, it was identified as phenylalanine by comparing with the fragmentation pattern of compound (HMDB00612) in HMDB database. The possible metabolic pathway of phenylalanine was shown in Fig. 6C.

As the result, the detected significant variables in the RP-UPLC-MS and HILIC-UPLC-MS mode were summarized in Table 3 and 4 respectively. Thirteen biomarkers based on the data from the RP-UPLC-MS separation mode and nine biomarkers based on the data from the HILIC-UPLC-MS separation mode were selected. Some biomarkers, such as betaine and LPC(16:0), were found as potential biomarkers in both RP-UPLC-MS and HILIC-UPLC-MS modes. It was noteworthy that most of the potential biomarkers found in the HILIC-UPLC-MS model were polar compounds, supplementary to those from the RP-UPLC-MS. Compared to control group, the metabolites of choline, proline, betaine, phenylalanine, tryptophan, LPC(16:0), LPC(18:1), LPC(18:0), LPC(20:4), sphingosine, progesterone, carnitine, estrone, phytosphingosine, sphinganine were observed to be up regulated in model group compared with control group, whereas the metabolites of creatine, creatinine, asymmetric dimethylarginine, taurine, 17-hydroxyprogesterone were down regulated. As shown in Table 3 and 4, the levels of these metabolites in the model group were significantly altered comparing with the control group. The endogenous metabolites in the treatment group were restored back to the control-like level and there were no significantly differences in the GFC-treatment group compareds to the control group.

3.4. Metabolic pathway and function analysis

The related pathways of each biomarker are listed in Table 3 and 4 by searching the KEGG (http://www.genome.jp). The metabolic pathway also analysis with MetaboAnalyst.
3.0 (http://www.metaboanalyst.ca/) indicated that identified biomarkers were critical for the effects of GFC intervention on PD rats, which was responsible for sphingolipid metabolism, steroid hormone biosynthesis, glycerophospholipid metabolism, arginine and proline metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, taurine and hypotaurine metabolism (Fig. 7). The pathway impact value calculated from pathway topology analysis above 0.1 was filtered out as potential target pathway.

4. Discussion

Metabonomic technology has been considered as an efficient tool for diagnosis and evaluation of TCM therapy, because its systemic feature was consistent with the comprehensive therapeutic efficacies and mechanisms of TCM action (Su et al. 2011, Tan et al. 2012). GFC is a historically documented formula for prevention of PD with no adverse syndrome (Hu et al. 2014, Lan et al. 2016). In our study, an RP-UPLC-MS coupled with HILIC-UPLC-MS based metabonomics approach was used to analysis metabolite profiling before and after treatment by GFC in PD rats, as well as the possible intervention mechanism of GFC. It was implied that the pathway of sphingolipid metabolism (impact value 0.50), steroid hormone biosynthesis (impact value 0.43), glycerophospholipid metabolism (impact value 0.40), arginine and proline metabolism (impact value 0.17), phenylalanine, tyrosine and tryptophan biosynthesis (impact value 0.14), taurine and hypotaurine metabolism (impact value 0.12) were disturbed. After treatment with GFC these disturbed metabolic pathways were restored back to the control-like levels. Several GFC-regulated specific biomarkers were identified, such as sphingosine, phytosphingosin, estrone, 17-hydroxyprogesterone, LPCs, asymmetric dimethylarginine, phenylalanine, tryptophan, taurine, etc. These promising biomarkers candidates verified that the intervention mechanism of GFC was closely related to the multiple etiologies and pathogenesis of PD.

Sphingolipid is a type of phospholipids synthesized from sphingosine, contains ubiquitous components in eukaryotic cells cellular membranes (Bettiga et al. 2017). Previous studies demonstrated that both peripheral and central sensitization resulted in the enhanced pain perception of PD patients (Tu et al. 2009). G.J.Patti et al. used a rat model of neuropathic pain to detect metabolomics changes in the dorsal horn, found conspicuous up-regulated in sphingosine and its metabolites which induced mechanical hypersensitivity in vivo (Patti et al. 2012). Phytosphingosine are converted into ceramide and sphingosine 1-phosphate (S1P), ceramide is then converted into sphingomyelins and sphingosine (Gault et al. 2010). S1P plays an important role in vascular maturation and it is closely related to the pathophysiology of wound healing, cancer and atherosclerosis (Kim et al. 2011). Sphingosine
as a nerve receptor ligand is involved in the binding of PGF$_{2\alpha}$ and PGE$_2$ to appropriate receptor during signal transduction processes (Arulkumaran et al. 2012). In our study, GFC reduced the amount of phytosphingosine, sphingosine and sphingine in PD rats which was agreed with the discoveries we mentioned above. The level of sphingolipids in plasma also has been reported to be the risk of polycystic ovary syndrome and endometriosis (Sun et al. 2012).

Steroid hormone disorder has been suggested to be an important causation of PD (Marsh et al. 2011). Cholesterol is converted into pregnenolone and then converted into progesterone under the effect of mitochondrial enzymes and isomerases. Progesterone is further transformed into 17-hydroxyprogesterone by 17-hydroxylase. After the onset of progesterone withdrawal, the arachidonic acid is released which causes a cascade of leukotrienes (LT) and prostaglandins (PG) in uterus. These LTs and PGs mediate the inflammatory response, such as cramps, nausea, bloating, headaches and vomiting in PD patients (Xue et al. 2014). Previous study has reported that reduction of progesterone in rats led to the decrease of pain threshold, when progesterone section increase the rats pain disappeared (Sternberg et al. 1995). In addition, estrone can stimulate the release of vasopressin in uterine spiral arteries, similar to the role of PGF$_{2\alpha}$, vasopressin can cause a strong contraction of the uterine small blood vessels during dysmenorrheal (Mechsner et al. 2010).

Glycerophospholipids are precursors for lipid mediators, such as LPC(16:0), LPC(18:1), LPC(18:0) and LPC(20:4), which were considered as biomarkers between normal and PD rats. In our study, the concentrations of these markers were significantly increased in model group compared with the control group and restored back to the normal level after administration of GFC. Hydrolysis of the glycerophospholipids by phospholipase A2 (PLA2) generates arachidonic acid (AA) and lysophosphatidylcholine (LPC) (Jabbour et al. 2009). Arachidonic acid is then oxygenated to prostaglandins (PGs) through cyclooxygenase (COX) to induce the potent vasoconstriction and myometrial contractions during menstruation pain (Marsh et al. 2011). LPC has some functions in signal transduction process and specific receptors (coupled to G proteins). LPC activates the specific phospholipase C to release diacylglycerols and inositol triphosphate, which leads to the increase of intracellular Ca$^{2+}$ concentration (Kabarowski 2009). It is now established that the mechanism of myometrium smooth muscle contraction is a rise in intracellular Ca$^{2+}$ (Kupittayanant et al. 2002). Consistent with our results, the formula of GFC has an inhibitory effect on LPC, thereby reducing the level of AA, PGF$_{2\alpha}$ and intracellular Ca$^{2+}$ in PD rats.

Phenylalanine is the precursor of tyrosine and both of them are essential aromatic amino
acids (AAA). One of the most important roles of tyrosine is the conversion to catecholamines, such as dopamine, norepinephrine and epinephrine (Fernstrom et al. 2007). An increase concentration of phenylalanine or phenylalanine/tyrosine ratio can lead to an inhibition of DA and NE synthesis in brain, which results in the depression, irritability, insomnia and other clinical symptoms in PD patients (Hanley et al. 2000). Tryptophan, as another aromatic amino acid can generate 5-hydroxy tryptophan (5-HT) by tryptophan hydroxylase. 5-HT is a neurotransmitter induced the vascular smooth muscle contraction (Molins - Cubero et al. 2014). Arginine is a basic semi-essential amino acid synthesized from glutamine and proline. NO, which is produced from arginine, is a neurotransmitter involved in modulating peripheral and central pain level (Z et al. 2002). NO plays a dual role in pain and analgesia through the NO-cyclic guanosine monophosphate pathway: when NO value reduced, it induces pain and results in dysmenorrheal, but when increased it inhibits pain (Rocha et al. 2015, Yun et al. 2016). Our study showed a decreased level of arginine in plasma of model group, inferred that the generation of NO produced from aginine was suppressed which maybe the cause of pain during primary dysmenorrhea. Taurine, also known as β-aminobutyric acid, is a kind of sulfur-containing non-protein amino acid. Taurine can protect the antioxidant activity of blood cells and prevent the tissue from oxidative and free radicals (Cui et al. 2017). The down regulation of phenylalanine, tyrosine and tryptophan as well as the up regulation of arginine, praline and taurine in GFC treated group implied that GFC had significant therapeutic effects on PD by regulating amino acid metabolism.

Carnitine is a kind of coenzyme related to lipid metabolism in the human body, involved in the transport and oxidation of fatty acids, lipids synthesis and the utilization of ketone bodies (Weng et al. 2016). Betaine as a methyl donor, which is an oxidation product of choline, also playing an important role in lipid metabolism (Bai et al. 2004). Creatine is an amino acid derivative synthesised by arginine, glycine and methionine synthesis, can supplement energy quickly and accelerate fatigue recovery in human body. Creatinine is a nonenzymatic breakdown product of creatine and phosphocreatine, and the creatine-phosphocreatine system is crucial for cellular energy transport action (Yáñezsilva et al. 2017). As the results of our study, the concentration of carnitine, betaine, creatine and creatinine in plasma of PD rats were significantly restored after GFC intervention supported that GFC might regulate lipid metabolism and energy metabolism in PD rats.

5. Conclusion

A metabonomic method based on RP-UPLC-MS coupled with HILIC-UPLC-MS has been
established to profile the metabolic alternations of oxytocin induced dysmenorrhea. In our study, we studied the plasma samples for comprehensive metabolites of dysmenorrhea to demonstrate that GFC had preventive effects on primary dysmenorrhea. As a result of this approach, 20 metabolites, primarily involved in sphingolipid metabolism, steroid hormone biosynthesis, glycerophospholipid metabolism, amino acid metabolism, lipid metabolism and energy metabolism, have been identified as potential biomarkers. Seven new potential biomarkers of primary dysmenorrhea, phenylalanine, tryptophan, taurine, carnitine, betaine, creatine and creatinine were reported for the first time, they are all reflective of dysmenorrhea-related change at metabolic level. The GFC treatment group obviously decreased the metabolic deviations in rats with oxytocin induced dysmenorrhea, indicating the therapeutic effect of GFC on primary dysmenorrhea. Elucidating the mechanism and metabolic pathway of GFC in the treatment of primary dysmenorrhea from the perspective of plasma metabolites is important for accurate diagnosis of primary dysmenorrhea and development of treatment strategies.

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**Table 1**

The results of biochemistry (mean ± SD, n=7).

<table>
<thead>
<tr>
<th>Groups</th>
<th>PGF$_{2a}$ (pg/ml)</th>
<th>PGE$_2$ (pg/ml)</th>
<th>PGF$_{2a}$/PGE$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>21.94 ± 0.23</td>
<td>23.24 ± 0.27</td>
<td>0.85 ± 0.14</td>
</tr>
<tr>
<td>Model group</td>
<td>22.83 ± 0.18*</td>
<td>22.07 ± 0.21*</td>
<td>1.13 ± 0.08*</td>
</tr>
<tr>
<td>Treatment group</td>
<td>22.35 ± 0.16▲</td>
<td>22.69 ± 0.12▲</td>
<td>0.98 ± 0.10▲</td>
</tr>
</tbody>
</table>

* p < 0.01, compared with control group
▲ p < 0.01, compared with model group
Table 2
The analytical performance of plasma samples

<table>
<thead>
<tr>
<th>tr/m/z</th>
<th>Precision (RSD,%)</th>
<th>Repeatability (RSD,%)</th>
<th>System stability (RSD,%)</th>
<th>Post-preparative stability (RE,%)</th>
<th>Freeze-thaw cycles stability (RE,%)</th>
</tr>
</thead>
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<tr>
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<td>ts</td>
<td>Peak area</td>
<td>ts</td>
<td>Peak area</td>
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<tr>
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<td></td>
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<tr>
<td>1.11-132.2</td>
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<td>5.4</td>
<td>0</td>
<td>3.6</td>
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<td>5.22-120.0</td>
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<tr>
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<td>7.7</td>
<td>0.2</td>
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<td>11.1</td>
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<td>0.1</td>
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<td>9.2</td>
<td>0</td>
<td>6.5</td>
<td>0.1</td>
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<td>HILIC-UPLC-MS</td>
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<td>1.22-415.2</td>
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<td>9.01-162.4</td>
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<td>0</td>
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<td>0.2</td>
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<td>11.61-258.2</td>
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<td>0</td>
<td>7.1</td>
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</tr>
</tbody>
</table>

RSD: Relative standard deviation. RE: Relative error
<table>
<thead>
<tr>
<th>tR</th>
<th>m/z</th>
<th>metabolites</th>
<th>VIP</th>
<th>Relative intensity in control group</th>
<th>Relative intensity in model group</th>
<th>Relative intensity in treatment group</th>
<th>change trend of model group vs control group</th>
<th>change trend of treatment group vs model group</th>
<th>Related pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.08</td>
<td>104.91</td>
<td>Choline</td>
<td>1.94</td>
<td>14.02 ± 0.18</td>
<td>25.78 ± 0.18</td>
<td>14.94 ± 0.21</td>
<td>↑*</td>
<td>↓*</td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>1.09</td>
<td>203.18</td>
<td>Asymmetric dimethylarginine</td>
<td>5.17</td>
<td>187.93 ± 0.10</td>
<td>150.02 ± 0.12</td>
<td>189.18 ± 0.05</td>
<td>↓**</td>
<td>↑**</td>
<td>Arginine and proline metabolism</td>
</tr>
<tr>
<td>1.14</td>
<td>116.06</td>
<td>Proline</td>
<td>6.23</td>
<td>23.38 ± 0.18</td>
<td>42.53 ± 0.15</td>
<td>24.62 ± 0.16</td>
<td>↓***</td>
<td>↓**</td>
<td>Arginine and proline metabolism</td>
</tr>
<tr>
<td>1.15</td>
<td>132.15</td>
<td>Creatine</td>
<td>5.94</td>
<td>262.51 ± 0.11</td>
<td>185.13 ± 0.19</td>
<td>264.02 ± 0.10</td>
<td>↓*</td>
<td>↑*</td>
<td>Energy metabolism</td>
</tr>
<tr>
<td>5.20</td>
<td>166.19</td>
<td>Phenylalanine</td>
<td>6.01</td>
<td>110.32 ± 0.18</td>
<td>153.21 ± 0.12</td>
<td>117.77 ± 0.03</td>
<td>↑***</td>
<td>↓**</td>
<td>Phenylalanine, tyrosine and tryptophan biosynthesis</td>
</tr>
<tr>
<td>5.20</td>
<td>120.08</td>
<td>fragment of Phenylalanine</td>
<td>8.68</td>
<td>206.76 ± 0.03</td>
<td>276.33 ± 0.16</td>
<td>210.40 ± 0.18</td>
<td>↑***</td>
<td>↓**</td>
<td>Phenylalanine, tyrosine and tryptophan biosynthesis</td>
</tr>
<tr>
<td>6.01</td>
<td>146.15</td>
<td>fragment of Tryptophan 2</td>
<td>3.02</td>
<td>12.53 ± 0.16</td>
<td>18.67 ± 0.19</td>
<td>12.64 ± 0.10</td>
<td>↑**</td>
<td>↓*</td>
<td>Phenylalanine, tyrosine and tryptophan biosynthesis</td>
</tr>
<tr>
<td>6.01</td>
<td>188.19</td>
<td>fragment of Tryptophan</td>
<td>7.02</td>
<td>190.83 ± 0.13</td>
<td>232.40 ± 0.14</td>
<td>199 ± 0.03</td>
<td>↑*</td>
<td>↓***</td>
<td>Phenylalanine, tyrosine and tryptophan biosynthesis</td>
</tr>
<tr>
<td>7.85</td>
<td>300.28</td>
<td>Sphingosine</td>
<td>3.1</td>
<td>58.16 ± 0.15</td>
<td>94.20 ± 0.06</td>
<td>60.35 ± 0.10</td>
<td>↑***</td>
<td>↓**</td>
<td>Sphingolipid metabolism</td>
</tr>
<tr>
<td>9.53</td>
<td>316.24</td>
<td>Progesterone</td>
<td>2.49</td>
<td>48.27 ± 0.06</td>
<td>74.19 ± 0.10</td>
<td>51.15 ± 0.08</td>
<td>↑**</td>
<td>↓*</td>
<td>Steroid hormone biosynthesis</td>
</tr>
<tr>
<td>15.77</td>
<td>544.53</td>
<td>LPC(20:4)</td>
<td>2.51</td>
<td>18.17 ± 0.14</td>
<td>44.19 ± 0.08</td>
<td>18.95 ± 0.09</td>
<td>↑**</td>
<td>↓**</td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>16.55</td>
<td>496.54</td>
<td>LPC(16:0)</td>
<td>3.07</td>
<td>59.78 ± 0.07</td>
<td>79.42 ± 0.09</td>
<td>60.42 ± 0.10</td>
<td>↑**</td>
<td>↓**</td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>16.98</td>
<td>522.51</td>
<td>LPC(18:1)</td>
<td>2.91</td>
<td>18.51 ± 0.19</td>
<td>34.37 ± 0.16</td>
<td>20.33 ± 0.14</td>
<td>↑*</td>
<td>↓***</td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>18.54</td>
<td>524.53</td>
<td>LPC(18:0)</td>
<td>2.82</td>
<td>63.39 ± 0.08</td>
<td>98.58 ± 0.12</td>
<td>66.54 ± 0.08</td>
<td>↑***</td>
<td>↓*</td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>22.27</td>
<td>118.01</td>
<td>Betaine</td>
<td>3.62</td>
<td>17.06 ± 0.14</td>
<td>32.71 ± 0.14</td>
<td>19.36 ± 0.16</td>
<td>↑***</td>
<td>↓***</td>
<td>Lipid metabolism</td>
</tr>
</tbody>
</table>

* Metabolite has significant difference between two groups (p < 0.05).
** Metabolite has significant difference between two groups (p < 0.01).
*** Metabolite has significant difference between two groups (p < 0.001).
Table 4
Identification of potential biomarkers in HILIC-UPLC-MS mode

<table>
<thead>
<tr>
<th>t&lt;sub&gt;R&lt;/sub&gt;</th>
<th>m/z</th>
<th>metabolites</th>
<th>VIP</th>
<th>Relative intensity in control group</th>
<th>Relative intensity in model group</th>
<th>Relative intensity in treatment group</th>
<th>change trend of model group vs control group</th>
<th>change trend of treatment group vs model group</th>
<th>Related pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.36</td>
<td>302.31</td>
<td>Sphinganine</td>
<td>3.21</td>
<td>68.23 ± 0.05</td>
<td>94.79 ± 0.19</td>
<td>70.15 ± 0.10</td>
<td>↑***</td>
<td>↓***</td>
<td>Sphingolipid metabolism</td>
</tr>
<tr>
<td>1.46</td>
<td>318.31</td>
<td>Phytosphingosine</td>
<td>2.67</td>
<td>18.17 ± 0.16</td>
<td>44.19 ± 0.10</td>
<td>19.65 ± 0.11</td>
<td>↑*</td>
<td>↓*</td>
<td>Sphingolipid metabolism</td>
</tr>
<tr>
<td>2.83</td>
<td>114.04</td>
<td>Creatinine</td>
<td>5.3</td>
<td>94.06 ± 0.44</td>
<td>121.39 ± 0.46</td>
<td>96.75 ± 0.56</td>
<td>↑***</td>
<td>↓***</td>
<td>Energy metabolism</td>
</tr>
<tr>
<td>3.45</td>
<td>331.24</td>
<td>17-Hydroxyprogesterone</td>
<td>4.98</td>
<td>37.59 ± 0.47</td>
<td>12.49 ± 0.26</td>
<td>33.42 ± 0.33</td>
<td>↓***</td>
<td>↑**</td>
<td>Steroid hormone biosynthesis</td>
</tr>
<tr>
<td>3.96</td>
<td>126.04</td>
<td>Taurine</td>
<td>3.45</td>
<td>44.31 ± 0.48</td>
<td>27.49 ± 0.32</td>
<td>40.04 ± 0.17</td>
<td>↓**</td>
<td>↑***</td>
<td>Taurine and hypotaurine metabolism</td>
</tr>
<tr>
<td>6.20</td>
<td>118.06</td>
<td>Betaine</td>
<td>2.99</td>
<td>26.93 ± 0.58</td>
<td>46.04 ± 0.41</td>
<td>34.15 ± 0.24</td>
<td>↑**</td>
<td>↓**</td>
<td>Lipid metabolism</td>
</tr>
<tr>
<td>7.53</td>
<td>496.53</td>
<td>LPC(16:0)</td>
<td>1.83</td>
<td>37.51 ± 0.45</td>
<td>62.49 ± 0.24</td>
<td>39.32 ± 0.31</td>
<td>↑***</td>
<td>↓***</td>
<td>Glycerophospholipid metabolism</td>
</tr>
<tr>
<td>8.63</td>
<td>271.17</td>
<td>Estrone</td>
<td>3.42</td>
<td>42.11 ± 0.26</td>
<td>66.46 ± 0.15</td>
<td>49.02 ± 0.12</td>
<td>↑*</td>
<td>↓**</td>
<td>Steroid hormone biosynthesis</td>
</tr>
<tr>
<td>8.97</td>
<td>162.23</td>
<td>Carnitine</td>
<td>4.27</td>
<td>40.19 ± 0.45</td>
<td>60.78 ± 0.13</td>
<td>49.23 ± 0.12</td>
<td>↑***</td>
<td>↑*</td>
<td>Lipid metabolism</td>
</tr>
</tbody>
</table>

*Metabolite has significant difference between two groups (p < 0.05).
**Metabolite has significant difference between two groups (p < 0.01).
***Metabolite has significant difference between two groups (p < 0.001).
Fig. 1. Representative ion based peak intensity (BPI) of rat plasma in RP-UPLC-MS mode. Control group (A), Model group (B), Treatment group (C).
Fig. 2. Representative ion based peak intensity (BPI) of rat plasma in HILIC-UPLC-MS mode. Control group (A), Model group (B), Treatment group (C).
Fig. 3. Score plot for PCA model between Control group and Model group in RP-UPLC-MS mode (A) and HILIC-UPLC-MS mode (B).
Fig. 4. Score plot for PLS-DA model between three groups in RP-UPLC-MS mode (A) and HILIC-UPLC-MS mode (B).
Fig. 5. Corresponding loading plots of rat plasma in RP-UPLC-MS mode (A) and HILIC-UPLC-MS mode (B).
Fig. 6. (A) Mass spectrum of m/z 166. (B) Product ion spectrum of biomarkers at m/z 166 in positive ion mode. (C) The possible fragment mechanism of phenylanine.
Fig. 7. Summary of pathway analysis with MetaboAnalyst 3.0. (A) Sphingolipid metabolism, (B) Steroid hormone biosynthesis, (C) Glycerophospholipid metabolism, (D) Arginine and proline metabolism, phenylalanine, (E) Phenylalanine, tyrosine and tryptophan biosynthesis, (F) Taurine and hypotaurine metabolism
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