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Metabolomics Study of Amenorrhea After the Administration of Shimshin-6 Traditional Prescription in Rats Using the Ultra High Performance Liquid Chromatography Coupled With Time of Flight Mass Spectrometry

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Objectives: The objective of this study is to determine metabolomics changes in the blood of amenorrhea model rats treated with Shimshin-6, a traditional Mongolian medicine, and search the important biomarkers using the UHPLC/TOF MS platform. **Methods:** The influence of a traditional Mongolian Shimshin-6 preparation administered to rats with a dexamethasone induced amenorrhea model was assessed to evaluate the physiological changes and the drug's metabolites in biological samples. The metabolites responsible for the differentiation of metabolic profiles between the two groups were obtained based on a multivariate statistics variable importance in the projection (VIP) threshold of 1.0 from the PLS-DA model. The analyses were carried out using an UHPLC-TOF-MS platform. One-way analysis of variance (ANOVA) was applied to test the significance of differences between groups. **Results:** The metabolic pathways were identified through the online databases Metlin and HMDB. Metabolic pathways analysis found tryptophan metabolism, phenylalanine metabolism, D-Glutamine and D-glutamate metabolism, Sphingolipid metabolism, Ubiquinone and other terpenoid-quinone biosynthesis and Fatty acid biosynthesis were mainly enriched based on the differential metabolites in rats of AG. Some of these pathways were markedly altered, such as the Tryptophan metabolism and phenylalanine metabolism pathway. **Conclusions:** A variety of potential biomarkers of Shimshin-6 physiological activity have been identified in the tested samples. According to the results it could be clearly seen, that the administration of this traditional medication affected the metabolism of amino acids, fatty acids and energy, to improve the condition of the animals suffering from amenorrhea..

Keywords: Shimshin-6, Amenorrhea, *Rheum Undulatum* L, *Hippophae Rhamnoides* L,

Introduction

Amenorrhea is one of the most common symptoms of gynecology. Modern medicine believes that the reproductive system includes the hypothalamic-pituitary-ovarian axis (hereinafter referred to as the gonadal axis) and the reproductive tract, and amenorrhea can occur if any link is maladjusted or defective [1]. At present, there are two types of amenorrhea: primary and secondary. Primary amenorrhea is very rare and the common causes are Turner syndrome, androgen insensitivity syndrome as well as Mullerian defects. On the other hand, secondary amenorrhea can result from a gynecological disorder, physical stress as well as low body mass index [2].

The treatment of amenorrhea depends on its cause. Some causes can be treated by medical therapy. Dopamine agonists or metformin medroxyprogesterone may be prescribed in order to restore normal ovarian endocrine function and ovulation. While especially in secondary amenorrhea, shifting to a healthier lifestyle, diet as well as stress reduction will be a key point for bring back a regular cycle. There are approaches where the alternative medicine remedies are used. Takahashi et al. demonstrated that TJ-68, shyakuyaku-kanzo-to, increases the activity of the aromatase, which in turn promotes estradiol synthesis and decreases serum testosterone level [3]. *Vitex agnus-castus*, a popular plant used in herbal medicine, demonstrated positive effects for amenorrhea in several clinical trials. It contains several compounds which bind to dopamine receptors in rat pituitary cell cultures and lower prolactin level [4]. Further, *Tribulus terrestris*, a potential stimulator of testosterone production, has also been effective in polycystic rat ovaries [5].

Takeuchi et al. also revealed that *Glycyrrhiza* can be used for lowering androgen effects through the enhanced aromatization of testosterone to 17-beta estradiol [5]. Shimshin-6 is a traditional medicine that is used in Mongolian medicine for a number of years. It is mainly administered to improve blood circulation, and to alleviate menstrual discomfort and irregularities. According to the previous reports, Shimshin-6 has also been useful for blood clotting in females, for back, waist, bladder, and groin areas pain treatment, and regulation of a menstrual cycle. Shimshin-6 contains a mixture with equal quantity of powdered plants, that includes *Rheum undulatum* L., *Hippophae rhamnoides* L., *Ammonium chloride, Aucklandia lappa Decne., Tronae veneni, and Kaempferia galanga* L [6, 7].

The analysis of pharmacokinetics and drug metabolism performed on traditionally used medicines is particularly important now days, when more advanced analytical tools are available to exclude intoxications and provide information on drug metabolism and its influence on a living organism. There are several studies using liquid chromatography-mass spectrometry, the method which has advantages because of its high selectivity and sample preparation simplicity with reasonable low cost, to identify the level of endogenous metabolites in the body after drug administration in order to reveal its regulatory effects and may lead to a clarification of the mechanisms of action [8]. Ren et al. developed the UHPLC-Q-TOF MS method to identify the metabolites of rats treated with the medicinal herb *Lycopus lucidus* Turcz extract [9, 10].

The analysis of a total of 36 metabolites in feces and urine samples revealed *in vivo* metabolism processes included sulfate, glucuronide, hydrogenated, methylated, and dehydroxylated reactions of the *Lycopus lucidus* Turcz extract. Further, Takiyama et al. performed LC–MS/MS to investigate the pharmacokinetics of shimotsuto, a traditional japanese kampo medicine used to treat gynecological diseases. After oral administration of shimutsudo, the authors measured the plasma level of 7 candidates absorbed into the systemic circulation in rats [11].

Shimshin-6 is the Mongolian traditional medicine which has been developed over a number of years and has been used for blood circulation in menstrual cycles. However, the active ingredients and mechanism of action of Shimshin-6 is still unclear. The objective of this study, thus, is to determine metabolomics changes in the blood of diseased rats, and to search for potentially important biomarkers that appear after the administration with the help of principal component analysis. We have used the UHPLC/TOF MS platform in this study to validate Shimshin-6 on amenorrhea by comparing blood changes in healthy and diseased rats.

Materials and Methods

Chemicals and reagents

HPLC grade methanol, acetonitrile, ammonium acetate, and ammonium hydroxide were purchased from CNW Technologies (CNW, Germany). All aqueous solutions were prepared with ultrapure water produced by a Milli-Q water purification system

(Millipore Corp., Billerica, MA, USA). Dexamethasone solution for injection was purchased from Shanghai Xinyi Pharmaceutical Co (Shanghai, China).

Animal experiments and sample collection

Thirty-three-month-old Sprague dawley (SD) rats were purchased from Shanghai SLAC Laboratory Animals Co., Ltd., Shanghai, China and were maintained at 23±1 °C, at relative humidity of 55-65 %, in a standard 12 h/ 12 h day and night cycle, with constant access to food and water. After 4 days of adaptive feeding, the rats were randomly divided into 2 groups with 15 rats in each group: Control group (CG), Model group (MG) and administration group (AG). All rats from the AG have been subcutaneously (s.c.) injected with dexamethasone at a concentration of 0.675 mg/kg for 14 days to trigger the model of amenorrhea. From the fifteenth day on, the AG group was subcutaneously given Shimshin-6 at a concentration of 6.3 g/kg for 21 days. The animals from the CG group were given 0.5% of sodium carboxymethylcellulose solution at the same time. On day 22, all rats from CG and AG groups were euthanized by intraperitoneal injection of 10% chloric acid hydrate. Afterwards, blood samples were collected from the abdominal aorta and the serum was separated and stored at -80 ^{0}C.

Metabolites extraction

All samples were thawed at 4 ^{0}C on ice. Then 100 µL of each sample was taken and placed in an Eppendorf (EP) tube, then vortexed with 400 µL of extraction liquid (methanol: acetonitrile= 1:1) for 30 seconds and then ultrasound-treated for 5 min, incubated in ice water, and then incubated for 1h at -20^{0}C to precipitate proteins. The samples were then centrifuged at 12000rpm for 15 min at 4 ^{0}C and the supernatant was transferred (425 µL) into fresh EP tubes. The samples were dried using a vacuum concentrator without heating and redissolved prior to the chromatographic analysis. For this purpose, 100 µL of acetonitrile: water (1:1 v/v) was added and the samples were vortexed for 30 seconds and ultrasonicated for 10 min in a 4 ^{0}C-water bath. Again, the EPs were centrifuged for 15min at 12000 rpm, 4 ^{0}Cand the supernatant (60 µL) was finally transferred to 2 mL LC/MS glass vials for the UHPLC-TOF-MS analysis.

UHPLC/ TOF-MS profiling analysis

UHPLC/TOF MS analyses were performed using a UHPLC system (1290, Agilent Technologies) with a UPLC BEH Amide column (d = 1.7 µm, 2.1x100 mm, Waters, USA) coupled to a Triple TOF 6600 (Q-TOF, AB Sciex). The mobile phase consisted of 25 mM NH4OAc and 25 mM NH4OH in water (pH = 9.75) (A) and acetonitrile (B). The analysis was carried out with the following elution gradients: 0 min, 95% B; 7 min, 65% B; 9 min, 40% B; 9.1 min, 95% B; and 12 min, 95% B, which were delivered at 0.5 mL min-1. The injection volume was 2 µL. The Triple TOF mass spectrometer was used for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during a LC/ MS experiment. In this mode, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluated the full scan survey MS data as it collected and triggered the acquisition of MS spectra depending on the preselected criteria. In each cycle, 12 precursor ions whose intensity was greater than 100 were chosen for fragmentation at collision energy (CE) of 30 V (15 MS/MS events with product ion accumulation time of 50 msec each). ESI source conditions were set as following: ion source gas 1 as 60 psi, ion source gas 2 as 60 psi, curtain gas as 35 psi, source temperature 650 ^{0}C, Ion Spray Voltage Floating (ISVF) 5000 V or 4000 V in positive or negative modes, respectively.

Statistical analysis

Our original data did not follow the bell curve, so we carried out log transformation to make it as "normal" and used the log transformed data for our analysis. The main effects of time, treatment type and their interaction were determined using a mixed two-way ANOVA with a Greenhouse-Geiser adjustment for lack of sphericity. A critical p-value of < 0.05 was used. The repeated measurements within subjects were then compared to the previous time interval using paired t-tests. The control and study groups' differences at each time interval were tested using one-way ANOVA followed by multiple comparison test. A Bonferroni-type correction was applied to all t-test results resulting in a significance level set at $p < 0.017$ (= 0.05/3). SPSS version 24 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Ethical statement

The animal study was carried out in accordance with laboratory protocol. Ethical approval to conduct the experiments was obtained from the Mongolian National University of Medical Sciences (Protocol No.2018/3-08). All efforts were made to minimize the number of animals used and their suffering.

Results

The changes of biochemical indexes

Body weight and biochemical indicators in the tested animals were analyzed, and results were significantly different between control, model, and medicine groups. At 7d, 14d and 21d, the weight of rats from CG and AG increased rapidly, and AG was significantly higher than that of MG (Figure 1). Compared with the control group, the biochemical indexes of MG and AG rats all showed significant changes (Figure 2). The biochemical index alterations were clear between CG and MG. FSH, LH, E2, T, T3, TSH, GnRH, and 5-HT in Model group were significantly higher than those in the control group, and T4 showed a decreasing trend. After administration, all the indexes of rats in the administration group were significantly reduced compared with those in the Model group. Each index of the low dose group was close to that of the control group.

Table 1. The list of differential serum metabolic profiles.

Metabolite	m/z	Retention time (min)	VIP	Trend	p-value
Alpha-ketoisovaleric acid	115.038	33.304	1.629	↓	0.009
Taurine	124.005	194.369	1.576	↓	0.015
ketoisocaproic acid	129.054	44.299	1.396	↓	0.029
L-Norleucine	130.084	165.383	1.423	↓	0.028
L-Carnitine	142.084	102.562	1.366	↓	0.016
alpha-ketoglutarate	145.012	348.488	1.625	↑	0.010
L-Glutamine	145.059	258.176	1.788	↓	0.002
2-Hydroxyphenylacetic acid	151.037	102.495	1.461	↑	0.030
Ribitol	151.058	235.083	1.301	↑	0.038
Salicylic acid	154.050	238.460	1.422	↓	0.035
5-Hydroxymethyluracil	158.058	149.206	1.442	↓	0.043
7-Methylxanthine	165.042	20.910	1.526	↑	0.030
Acetyl-DL-Leucine	172.094	169.977	1.460	↑	0.004
Indole	176.068	103.879	1.572	↑	0.010
D-Fructose	179.052	195.561	1.495	↑	0.024
Glucosamine	179.079	166.409	1.478	↑	0.029
L-Tyrosine	180.063	281.595	1.459	↑	0.023
L-Fucose	180.084	378.299	1.444	↑	0.029
3-Phosphoserine	185.009	58.721	1.530	↑	0.020
N6-Acetyl-L-lysine	187.106	370.256	1.330	↓	0.048
3-Hydroxycapric acid	187.130	70.844	1.372	↑	0.041
Phenylpropionylglycine	188.072	296.513	1.622	↑	0.027
5-Hydroxyindoleacetate	190.054	29.110	1.380	↑	0.027
Galactonic acid	195.048	357.042	1.508	↑	0.011
Homoveratric acid	196.069	210.539	1.388	↑	0.031
L-Tryptophan	203.081	238.211	1.901	↑	0.000
N-Acetyl-L-phenylalanine	206.078	164.821	1.788	↑	0.029
trans-cinnamate	207.065	200.090	1.428	↑	0.023
L-Kynurenine	207.074	240.937	1.567	↓	0.005
Succinylacetone	217.074	227.272	1.430	↑	0.019
Deoxycytidine	226.081	196.155	1.533	↓	0.033
L-Cystine	239.019	289.968	1.450	↑	0.023

Continued

5-Methylcytidine	256.090	215.064	1.278	↓	0.048
Ribothymidine	257.074	84.257	1.319	↑	0.036
Acetylcarnitine	262.126	288.669	1.416	↑	0.038
Glycerophosphocholine	274.137	447.105	1.602	↓	0.006
Linoleic acid	281.245	102.427	1.500	↓	0.049
trans-Dehydroandrosterone	287.198	235.567	1.935	↓	0.001
Erucic acid	337.307	39.378	1.969	↓	0.000
Nervonic acid	365.338	38.812	1.254	↑	0.046
Lithocholic acid	375.285	47.159	1.546	↑	0.046
L-Thyroxine	775.674	183.364			

Analysis of blood metabolites' profile

The total ion chromatograms of blood samples from 2 groups of 30 amenorrhea rats were obtained (model including positive ion mode (ESI+) and negative ion mode (ESI-). We analyzed and selected metabolites with significant changes in blood levels. Firstly, we used supervised multivariate statistical analysis (principal component analysis (PCA)) to find out and exclude outliers. The difference between the two groups of samples were statistically significant, and the samples were all within the 95% confidence interval (Hotelling's t-squared ellipse). The observed trends in the positive and negative ion patterns were consistent.

Table 2. The changes of body weight.

Experimental days	Control[a,b,c] (n=9)	Model[d,e] (n=9)	s-6 small[f,g] (n=9)	s-6 medium[h] (n=9)	s-6 high[k,l] (n=9)	*p-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
1 day	202.73 ± 0.74	204.55 ± 2.0	200.66 ± 2.09	196.5 ± 1.57	199.06 ± 1.41	0.000
7 days	231.45 ± 2.06	198.17 ± 1.76	196.42 ± 171	200.86 ± 0.84	193.94 ± 1.97	0.000
14 days	242.24 ± 5.93	197.85 ± 1.24	200.66 ± 1.06	205.53 ± 1.64	201.46 ± 0.70	0.000
21 days	259.42 ± 1.59	221.41 ± 4.80	230.79 ± 1.97	224.5 ± 3.29	225.56 ± 1.58	0.000

Two-way mixed ANOVA results: Interaction of time and treatment $F(1.919, 338.59) = 23.166$, $p < 0.041$; Main effect of time $F(1.938, 336.49) = 334.32$, $p < 0.012$; Main effect of treatment $F(1,186) = 0.666$, $p = 0.567$; *One-way ANOVA; Paired t-test: aday1 vs. 7, $p = 0.010$, bday1 vs. 14, $p = 0.045$, cday7 vs. 21, $p = 0.051$ in control; dday1 vs.14, $p = 0.030$, eday7 vs. 14, $p = 0.041$ in model; fday1 vs. 7, $p = 0.001$, gday7 vs. 14, $p = 0.004$ in s-6 small; hday7 vs. 14, $p = 0.026$ in s-6 medium; kday1 vs. 7, $p = 0.001$, lday1 vs. 21, $p = 0.031$ in s-6 high.

In the permutation test, the obtained R2X, R2Y, and Q2 were 0.36, 0.995, and 0.796 respectively. No overlapping was found in the positive and negative ion alignment tests. According to the above methods, we identified a total of 42 metabolites (Table1) that could become biomarkers after administration.

Table 3. The changes of biochemical indexes in each group of rats.

Experimental days	Control (n=9)	Model (n=9)	s-6 small (n=9)	s-6 medium (n=9)	s-6 high (n=9)	*p-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
FSH (IU/L)	0.54 ± 0.01	0.65 ± 0.02	0.45 ± 0.02	0.54 ± 0.03	0.64 ± 0.04	0.471
LH (mIU/ml)	2.11 ± 0.16	3.13 ± 0.07	2.24 ± 0.12	2.35 ± 0.06	2.71 ± 0.13	0.393
E2 (pmol/L)	0.26 ± 0.02	0.28 ± 0.01	0.26 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.407
GnRH (mIU/ml)	0.32 ± 0.04	0.36 ± 0.09	0.33 ± 0.01	0.33 ± 0.03	0.40 ± 0.23	0.069
5-HT (ng/ml)	0.22 ± 0.01	0.24 ± 0.02	0.23 ± 0.07	0.25 ± 0.01	0.25 ± 0.01	0.098
T (nmol/l)	0.24 ± 0.00	0.29 ± 0.01	0.26 ± 0.11	0.28 ± 0.01	0.31 ± 0.51	0.386
T3 (ng/ml)	0.22 ± 0.03	0.24 ± 0.02	0.22 ± 0.03	0.27 ± 0.09	0.27 ± 0.07	0.101
T4 (ng/ml)	0.44 ± 0.05	0.35 ± 0.04	0.44 ± 0.08	0.48 ± 0.04	0.46 ± 0.02	0.121
TSH (mU/L)[a,b,c,]	0.34 ± 0.09	0.37 ± 0.00	0.34 ± 0.06	0.34 ± 0.03	0.41 ± 0.05	0.051

*ANOVA; multiple comparison test results: a: control vs. model, $p = 0.04$; b: control vs. s-6 medium, $p = 0.05$; c: model vs. s-6 small, $p = 0.016$.

Metabolite differences

The metabolic pathways were identified through the online databases Metlin and HMDB. Metabolic pathways analysis found that Tryptophan metabolism, phenylalanine metabolism, D-Glutamine and D-glutamate metabolism, Sphingolipid metabolism, Ubiquinone and other terpenoid-quinone biosynthesis and Fatty acid biosynthesis were mainly enriched based on the differential metabolites in rats of AG group. Some of these pathways were markedly altered, such as Tryptophan metabolism and phenylalanine metabolism pathways.

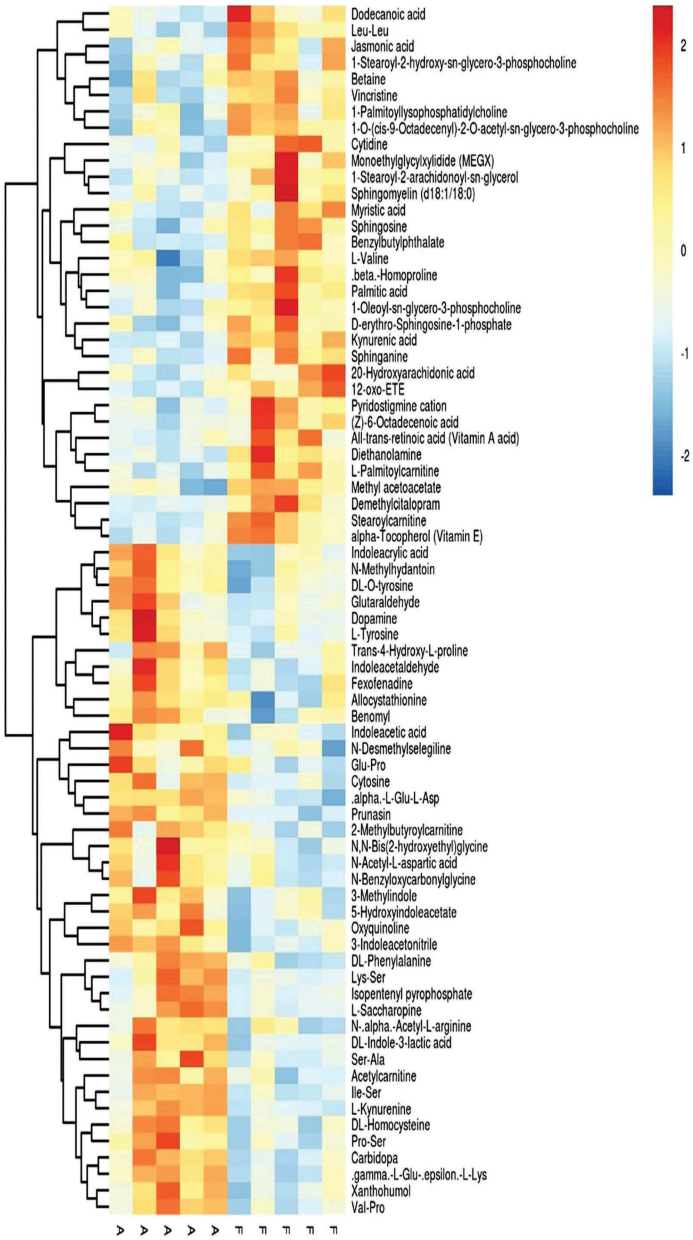

Figure 1. Heatmap of hierarchical clustering analysis for group A vs F.

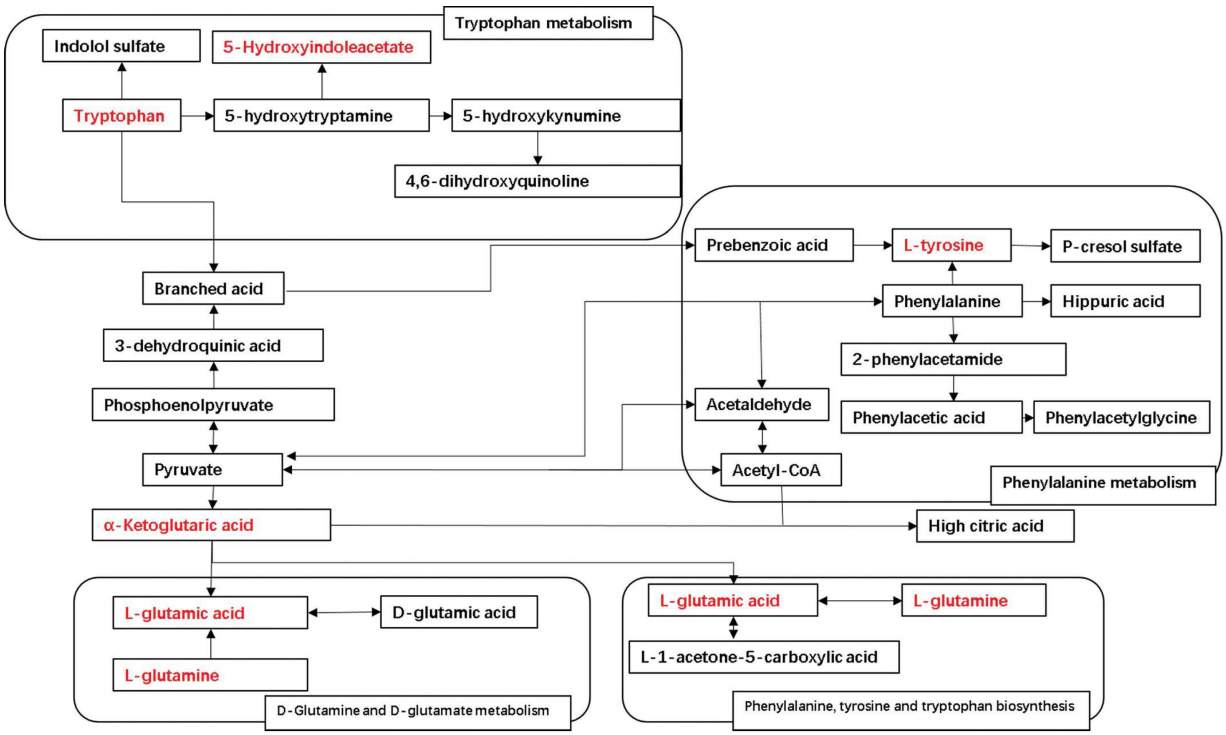

Figure 2. Pathway analysis of metabolomics alterations associated with model vs. administration rats

Discussion

WBlood is the most important body fluid in the biological system. It contains many types of nutrients, such as inorganic salts, sugars, amino acids, lipids and hormones. Blood is not only responsible for the real-time replenishment and transportation of various nutrients t different tissues and organs, but also for transporting various metabolites out of the production site or to the next site. The change of blood phase parameters reflects the change of organ function in real time. In addition, our data suggest that the metabolomics technology to study the pathogenesis of diseases and evaluate the pharmacological activity and efficacy mechanism of traditional Chinese medicine is an advanced and reasonable method [12-15].

Modern medicine thinks that endocrine maladjustment causes ovarian function to decline bring about amenorrhea. Decrease of the level of oestrogen in the ovary leads to imbalance of the hypothalamic-pituitary-ovarian (HPO) axis, which leads to the dysfunction of the autonomic nervous system [16]. Basophilic cells in the anterior pituitary secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). Under the action of FSH and LH, the follicles are developed and mature, and estrogens are synthesized. Meanwhile, the estrogens secreted by the ovary are negatively feedback regulated by the pituitary secreting FSH and luteinizing hormone (LH) [17]. In the present study, we suggested that the metabolomics technology to study the pathogenesis of diseases and evaluate the pharmacological activity and efficacy mechanism of traditional Chinese medicine is an advanced and reasonable method.

We have demonstrated in the present study that Shimshin-6, a Mongolian traditional medicine, is effective to adjust the amino acid levels and consequently, improve amenorrhea. Different body weight, behavioral and biochemical indicators in the tested animals were analyzed to evaluate the pharmacodynamics of Shimshin-6. On the other hand, using LC-MS tandem technique combined with multivariate statistics, it was found that the metabolites in the serum samples of the control group and the administration group were obviously divided into two categories. All the data from different perspectives showed that Shimshin-6 was effective in the treatment of amenorrhea. VIP values

showed that 42 metabolites could be potential biomarkers. There are very few similar reports of herbal preparations on gynecological syndromes where the metabolites were analyzed by mass spectrometry. For example, Xu et al. demonstrated that oral administration of Wen-Jing decoction, a traditional Chinese medicine formula used to treat gynecological syndromes such as amenorrhea and menstrual disorders, 27 compounds, including 9 prototype compounds and 18 metabolites were detected in rat plasma through the UPLC-Q-MS [18]. The analysis of the flavonoids revealed that there were 13 flavonoid-related metabolites in the rat plasma and the main biotransformation reactions included hydrolysis, hydrogenation, hydroxylation, sulphation, and glucuronidation. Li et al. also developed a sensitive and reliable LC-MS/MS method to determine the metabolism of *Corydalis yanhusuo* extract, a herb that is widely used as an analgesic agent for treating spastic, abdominal, and menstrual pains, and for pain due to injury, in rat plasma [19].

In the present study, based on the results of biochemical pathway analysis, it could be seen that the majority of pathways were linked with alanine, aspartic acid, glutamic acid, and tryptophan metabolism, and with phenylalanine, tyrosine and tryptophan biosynthesis pathways. Tryptophan is a necessary amino acid, that maintains human protein biosynthesis and ensures the synthesis of neurotransmitters (serotonin and melatonin) [20]. Tryptophan and its metabolites play an important role in the life processes of behavior, emotion, growth and immune response [21]. 5-hydroxyindoleacetic acid is the downstream product of 5-hydroxytryptamine pathway. 5-hydroxytryptamine (5-ht) can promote the release of GnRH (Gonadotropin releasing hormone). GnRH is a small peptide synthesized by hypothalamic neuroendocrine cells, which has a variety of biological functions. Jiang et al. represented that bu-shen-zhu-yun decoction (BSZY-D), a traditional Chinese preparation which has lowering effect in the expression of estrogen receptor and progesterone receptor, can reverse the cetrorelix induced reducing effect of the expression of the GnRHR, LH, and FSH in rats, which in turn results to the increased levels of GnRHR, transcription factors, and secretory vesicles leading to increased secretion of FSH and LH [22]. On the other hand, Comai et al. revealed that an increase of 5-HT in serum was observed in amenorrhea with the highest value in hyperprolactinemic patients [23]. In our study, 5-HT and GnRH were significantly down-regulated after administration. Therefore, Shimshin-6 may achieve the purpose of treating amenorrhea by regulating GnRH. L-glutamine and L-ketoglutaric acid exist in the metabolic pathways of d-glutamine and d-glutamate and the metabolic pathways of alanine, aspartic acid, and glutamate, respectively. Alpha-ketoglutaric Acid (AKG) is a key intermediate product of bioactive metabolism, which plays an important role in regulating muscle and bone development, stem cell differentiation and cell metabolism, as well as immune and inflammatory responses. A recent study showed that the content of AKG in the intestine was related to the increase of estrogen level, and estrogen directly or indirectly affected the levels of GnRH (gonadotropin releasing hormone), T3, TSH and other hormones [24]. Further, the decrease in isoleucine level in the administration group suggested that Shimshin-6 had a certain regulatory effect on the energy metabolism and central nervous system. As we known, the central nervous system plays an important regulatory role in food intake, energy balance, glucose homeostasis and other vital activities. Leucine and isoleucine play an important role in brain nitrogen homeostasis and neurotransmitters' recycling. Also, the modulation of indoxyl sulfate level has an influence on tryptophan metabolism, and neurotransmitter and intestinal homeostasis regulation.

Song et al. demonstrated that the follicular fluid analyzed by UPLC-MS showed significant changes in phenylalanine, tryptophan, hemolysis phosphatidyl choline chloride, linoleic acid, oleic acid, arachidonic acid, docosahexaenoic acid, vitamin D, and 28 other metabolites. The biochemical changes involved 12 different metabolic pathways, including metabolism of fatty acids and amino acids, and biosynthesis of bile acids [25-27]. In our study, compared with the model group, leucine and tryptophan levels decrease in the Shimshin-6 administration group. This data inferred that Shimshin-6 improved amenorrhea by adjusting the amino acid metabolic pathways.

A limitation of this study is that the current study aimed to determine the effects of a single dose of Shimshin-6 in amenorrhea. Thus, it has led to the future direction involving dose-dependent experiments in order to establish the effectiveness of Shimshin-6. Moreover, further analysis is required to determine the precise mechanism of active compounds against amenorrhea. Our report presents the first novel formulation of WLT with neuroprotective effect and ease of use, which has potential for clinical applications.

Conclusion

A variety of potential biomarkers of Shimshin-6 physiological activity have been identified in the tested samples. According to the results it could be clearly seen that the administration of this traditional medication affected the metabolism of amino acids, fatty acids and energy, to improve the condition of the animals suffering from amenorrhea.

Conflict of interest

The authors declare that they have no competing interests.

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