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Urinary metabonomics study of anti-depressive effect of Chaihu-Shu-Gan-San on an experimental model of depression induced by chronic variable stress in rats

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ABSTRACT

Chaihu-Shu-Gan-San (CSGS), a traditional Chinese medicine (TCM) formula, has been effectively used for the treatment of depression in clinic. However, studies of its anti-depressive mechanism are challenging, accounted for the complex pathophysiology of depression, and complexity of CSGS with multiple constituents acting on different metabolic pathways. The variations of endogenous metabolites in rat model of depression after administration of CSGS may offer deeper insights into the anti-depressive effect and mechanism of CSGS. In this study, metabonomics based on ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–QTOF-MS) was used to profile the metabolic fingerprints of urine obtained from chronic variable stress (CVS)-induced depression model in rats with and without CSGS treatment. Through partial least squares-discriminate analysis, it was observed that metabolic perturbations induced by chronic variable stress were restored in a time-dependent pattern after treatment with CSGS. Metabolites with significant changes induced by CVS, including 3-*O*-methyldopa (**1**), pantothenic acid (**2**), kynurenic acid (**3**), xanthurenic acid (**4**), 2,8-dihydroxyquinoline glucuronide (**5**), 5-hydroxy-6-methoxyindole glucurnoide (**8**), L-phenylalanyl-L-hydroxyproline (**9**), indole-3-carboxylic acid (**10**), proline (**11**), and the unidentified metabolites (**6**, 2.11 min_*m*/*z* 217.0940; 7, 2.11 min_*m*/*z* 144.0799), were characterized as potential biomarkers involved in the pathogenesis of depression. The derivations of all those biomarkers can be regulated by CSGS treatment except indole-3-carboxylic acid (**10**), which suggested that the therapeutic effect of CSGS on depression may involve in regulating the dysfunctions of energy metabolism, tryptophan metabolism, bone loss and liver detoxification. This study indicated that the rapid and noninvasive urinary metabonomics approach may be a powerful tool to study the efficacy and mechanism of complex TCM prescriptions.

1. Introduction

Depression is a complex epidemiological psychiatric illness which is often associated with stressful events, and leads to low morale, weight loss and anhedonia [1]. Etiology of depression is not fully understood so far, but may involve changes in the nervous system, immunological system and endocrine system [2]. It has been considered as a systemic disorder caused by impairment in different biochemical pathways [3]. Though many synthetic chemical antidepressants were introduced, their therapeutic effects were not satisfying with series of side effects [4]. Alternative therapies for the treatment of depression are needed.

Traditional Chinese medicines (TCMs) are gaining more attention all over the world, due to their specific theory and long historical clinical practice. Chaihu-Shu-Gan-San (CSGS), which contains seven Chinese herbs, i.e. *Bupleurum chinense* DC, *Citrus reticulata* Blanco, *Paeonia lactiflora* Pall, *Citrus aurantium* L., *Cyperus rotundus* L., *Ligusticum chuanxiong* Hort. and *Glycyrrhiza uralensis* Fisch, is one of the most widely used TCM formulas for treating depression clinically in China [5]. It is universally acknowledged that the complexity of TCMs makes identification of chemical constituents related to their efficacy challenging, and equally challenging understanding their action mechanisms. Novel approaches suitable for complex system are in great demand to provide deeper insight into explanation of their therapeutic effects. Our previous

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study established a strategy for on-line identification of the constituents of CSGS related to intervention of oxidative stress [6]. However, its mechanism of anti-depressive effect has not yet been elucidated.

Metabonomics, focused on a holistic investigation of multiparametric metabonomics response of living systems to external stimuli (including pathological stimuli and drug treatments) based on the global metabolite profiles in biological samples such as urine, plasma or tissue [7], provides variation of whole metabolic networks for characterizing pathological states in animals and human, as well giving diagnostic information and presenting mechanistic insight into the biochemical effects of the toxins and drugs [8,9]. In agreement with the holistic thinking of TCM, metabonomics technology has shown potential in evaluation of therapeutic effects of TCMs [10], including antidepressive effect of a TCM formulas [9]. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are two analytical tools commonly used in metabonomics studies [7–9,11]. In the MS-based metabonomics, ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–QTOF-MS) has gained more application due to the high resolution of chromatographic peaks, increased analytic speed and sensitivity for complex mixtures [12]. Here, urinary metabonomics based on UPLC–QTOF-MS was applied to investigate the metabolic profiles and potential biomarkers in a rat model of chronic variable stress (CVS) [13] induced depression after treatment of CSGS, which may facilitate understanding the pathological changes of depression and antidepressive mechanism of CSGS. To our knowledge, this study is the first report of UPLC–QTOF-MS-based urinary metabonomics method used to investigate the antidepressive effect of antidepressant.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Purified water was produced by Milli-Q ultra-pure water system (Millipore, Billerica, USA). Formic acid (HPLC grade) was from Tedia (Fairfield, USA). Fluoxetine hydrochloride was purchased from Eli Lilly and Company (Suzhou, China).

2.2. Raw herbal medicines and CSGS extract

All raw herbal medicines were purchased from Beijing Tongren Tang Pharmaceutical Co. Ltd. (Beijing, China) and identified as the roots of *B. chinense* DC, the roots of *P. lactiflora* Pall, the pericars of *C. aurantium* L., the fruits of *C. reticulata* Blanco, the roots of *C. rotundus* L., the roots of *L. chuanxiong* Hort. and the roots of *G. uralensis* Fisch. Voucher specimens for these herbs are kept in our laboratory at Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, China.

The CSGS extract was prepared based on the traditional method used in TCM practice. Briefly, the above-mentioned herbs with total weight of 10 kg in the proportions of 4:4:3:3:3:3:1 by weight were crushed into small pieces and then soaked together in water (1:10, w/v) for 30 min at room temperature and then refluxed for 2 h. The filtrate was collected and the residue was then refluxed twice in the same volume of water for an additional 1.5 h. The three filtrates were combined and concentrated under vacuum to give the CSGS extract with the yield of 8%.

2.3. Rats and treatments

32 male Wistar rats, weighing 200 ± 20 g, purchased from the institute of Laboratory Animal Science, CAMS & PUMC (Beijing, China), were housed individually in cages and maintained (20–25 °C and 40–60% humidity) under a standard 12-h light/dark cycle with free access to purified water and commercial diet. Rats were habituated for 7 days before the experiment. All experimental procedures were approved by the Ethics Committee of the Institute of Medicinal Plant Development, CAMS & PUMC.

Animals were randomly divided into 4 groups: (1) naïve group, (2) CVS model group, (3) CSGS treated group with CVS (CVS + CSGS group), and (4) fluoxetine treated group (3 mg kg^{-1}) by oral gavage with CVS (CVS + fluoxetine group). All rats except those in naïve group were subjected to series of variable stimuli as previously described [13] including: (1) immobilization for 5 h, (2) swimming in 15 °C water for 5 min, (3) food withholding for 48 h, (4) thermal stimulus in 45 °C water for 5 min, (5) water intake forbidden for 48 h, (6) electric shock to pelma (electric current for 1 mA, 2 s per shock, 2 shocks per minute), (7) noise stimulus at 110 dB, and (8) stroboflash—2 flashs per second for 4 h. These stimuli were presented in an unpredictable manner with one or two of these on each rat each day. Each stimulus was performed at least 3 times in 28 days. Then the rats were administered intragastrically with CSGS at the dose of 2.5 g kg^{-1} once daily for 28 days. Untreated (CVS model) and naïve rats served as controls, and fluoxetine-treated rats as positive control.

2.4. Sample collection and preparation

All rats of each group were housed in metabolic cages (1 per cage) for collecting the 24 h urine samples at days 0, 7, 14, 21 and 28. Urine samples were stored at −80 °C until analysis. All these samples were thawed at room temperature before analysis and centrifuged at 12,000 rpm for 5 min. The supernatant was diluted at a ratio of 1:1 with water and an aliquot of 5 μL was injected for UPLC/MS analysis after filtered through a 0.22 μm membrane filter.

2.5. UPLC conditions

Separation of the metabolites was carried out on a Waters Acquity™ Ultra Performance LC system (Waters Corporation, Milford, MA, USA) equipped with HSS T3 column (100 mm × 2.1 mm, i.d. 1.8 μm). The analytical column was maintained at a temperature of 45 °C and the mobile phase was composed of acetonitrile (A) and water (B) each containing 0.1% formic acid. A solvent gradient system was used: 2% A from 0 to 0.5 min, 2–70% A for 0.5–8.0 min, 70–98% A for 8.0–10.0 min, and 98–2% A for 10.0–10.1 min. The flow rate was 0.5 mL min^{-1}.

2.6. MS conditions

MS analysis was performed on Q-TOF analyzer in SYNAPT HDMS system (Waters Corporation, Milford, MA, USA) in positive ion mode. Cone voltage was 30 V, and capillary voltage was 3000 V; cone gas rate was 50 L h^{-1}; desolvation gas rate was 800 L h^{-1} at a temperature of 420 °C and source temperature was 120 °C. The data acquisition rate was 0.15 s. Leucine-enkephalin used as the lock-mass in all analyses (in positive ion mode $[M+H]^+ = 556.2771$) was at a concentration of 0.5 μg mL^{-1} with a flow rate of 80 μL min^{-1}. The lock spray frequency was set at 20 s. Data were acquired in centroid mode. Full-scan mass range of m/z 100–1000 was scanned.

2.7. Data analysis

The raw data were analyzed using the Micromass MarkerLynx Applications Manager version 4.0, this allowed deconvolution,

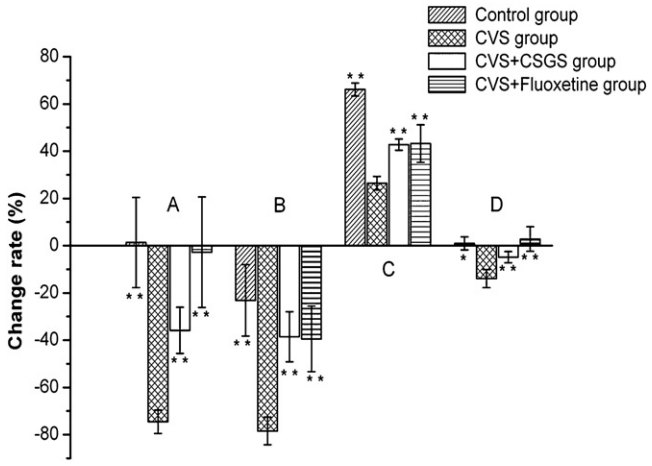

Fig. 1. The change rates (mean ± standard error) of four main indicators in each group (A: change rates of crossing activity in rats. B: Change rates of rearing activity in rats. C: Increasing rate of of body weight in rats. D: Change rates of sucrose preference in rats.). $n = 8$ in each group. Student's t-test was used for statistical analysis. *Compared with model group $p < 0.05$ and **compared with model group $p < 0.01$.

alignment and data reduction to give a list of mass and retention time pairs with corresponding intensities for all the detected peaks from each data file in the data set. The main parameters were set as follows: retention time range 1–10 min, mass range 100–1000 amu, mass tolerance 0.01, minimum intensity 1%, mass window 0.05, retention time window 0.20, and noise elimination level 6. The resulting data were analyzed by principal components analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) using SIMCA-P software (version 11.0, Umetrics AB, Sweden) after pareto-scaled procedure. Student's t-test was used for statistical analysis to evaluate the significant difference of potential biomarkers (SPSS 13.0 (SPSS Inc., Chicago, IL)).

3. Results and discussion

3.1. Ethological study of the anti-depressive effect of CSGS on CVS induced depression

Behavioral disturbances and neurochemical changes in animals were induced by stress, which mimic the symptoms and neurochemical changes of depression in human. The anti-depressive effect of CSGS was explored with the CVS model, which is becoming increasingly popular as an animal model of depression [13]. The behavioral changes in CVS-induced depression were evaluated by the open-field test [14], body weight changes [15] and sucrose preference test [16]. The corresponding formulae for quantifying the changes in crossing activity, rearing activity, body weight changes and sucrose preference in these tests are shown in supplementary material (S-A–D).

The number of rearing and crossing ($p < 0.01$), the body weight ($p < 0.01$) and the sucrose preference ($p < 0.05$) in the CVS group presented a significant decrease compared with control group. Rats in the CSGS group showed a significant increase in the sucrose preference ($p < 0.01$), the number of rearing and crossing ($p < 0.01$), and the body weight ($p < 0.01$) compared with model group. Similar changes were observed in the fluoxetine group. The results indicated that CVS led to a series of changes in rats similar to the symptoms observed in depressed patients such as lack of acute activation, weakening the function of the digestion system, and anhedonia, whereas CSGS-treatment, similar to fluoxetine-treatment, significantly prevented these abnormal ethological changes induced by CVS (Fig. 1).

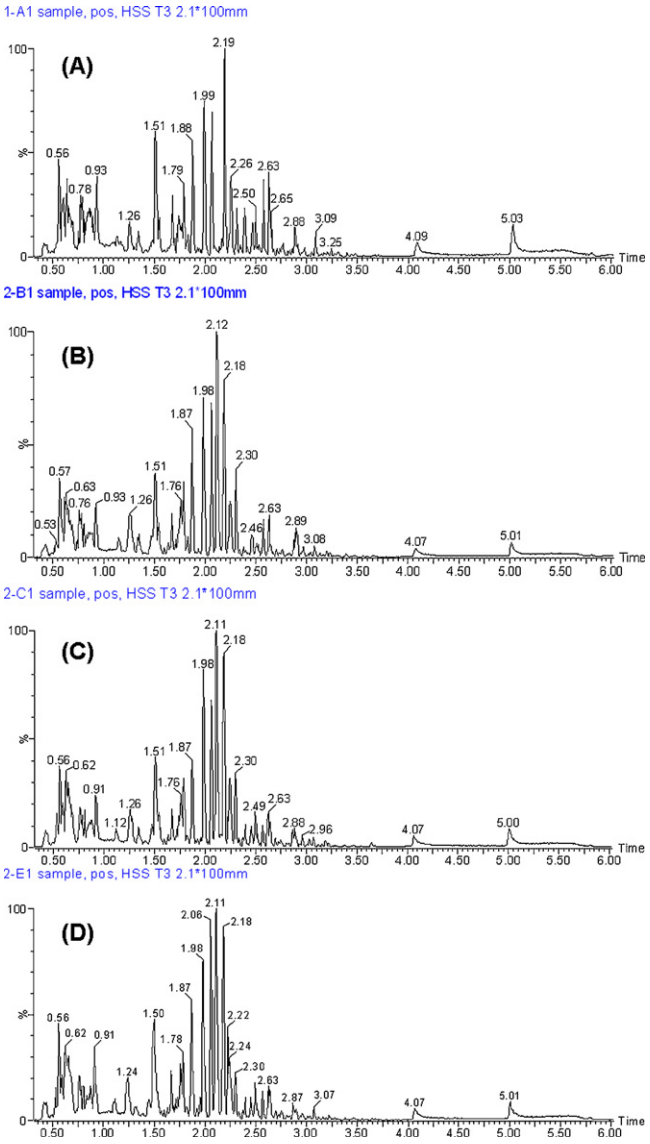

Fig. 2. The UPLC/MS total ion chromatogram of (A) control group, (B) CVS group, (C) (CVS + CSGS) group and (D) (CVS + fluoxetine) group that sampling on the 28 th day.

3.2. Method development and validation

Metabolic profiling of urinary samples was acquired using UPLC/TOF-MS/MS system in the positive ion mode. The total ion current (TIC) chromatograms of urinary samples from control group, CVS group, CSGS-treated group and fluoxetine-treated group are shown in Fig. 2(A, B, C and D). The average peak base width of 4 s was set for this separation, which generated a series of peaks with retention time and m/z pairs (t_R_m/z pair) as variables. One urinary sample was chosen as the QC sample, and the extracted ion chromatographic peaks of eleven ions (144.0799, 162.0539, 190.0479, 206.0428, 212.1004, 217.0940, 220.1163, 231.1392, 279.1338, 338.0860 and 340.1008) were selected for method validation. The repeatability of method was evaluated using five replicates of QC sample. The relative standard deviations (R.S.D.%) of peak areas, retention times and m/z were 1.37–6.05%, 0–0.92% and 0.00022–0.0032%, respectively. The post-preparation stability of samples was tested by analyzing QC sample kept in autosampler at 4 °C for 4, 8, 12 and 24 h. The relative errors (R.E.) of peak areas were 3.29% to 8.15%, demonstrating the method had excellent repeatability and stability.

Table 1
The parameters for assessing modeling quality of PCA and PLS-DA ($n = 8$ in each group).

	Components	PCA		PLS-DA		
		R^2X	Q^2 (cum)	R^2X	R^2Y	Q^2 (cum)
Conrol vs CVS group	**3**	0.594	0.337	0.559	1	0.994
CVS vs (CVS + CSGS) group	**3**	0.471	0.142	0.388	0.998	0.97
CVS vs (CVS + fluoxetine) group	**3**	0.492	0.2	0.376	0.995	0.938

3.3. Multivariate analysis of UPLC/MS data

Principal components analysis (PCA) was firstly carried out to investigate whether two groups can be separated and to find out their metabolic distinction. Then partial least squares-discriminate analysis (PLS-DA), a supervised multivariable statistical method to sharpen an already established (weak) separation between groups of observations plotted in PCA was performed. The variables responsible for differentiating CVS group and naïve control were selected as potential biomarkers of progress of diseases by variable importance of project (VIP) statistics.

7-Fold cross-validation was applied to evaluate the predictive abilities of the constructed PCA and PLS-DA models, in which the parameter R^2X and Q^2 are usually used for evaluation of PCA model, and R^2X, R^2Y, and Q^2 (cum) for evaluation of PLS-DA model. R^2X shows the capability of differentiating each group in established model, while R^2Y and Q^2 (cum) reflect the goodness of fitness and the ability of prediction of the models. The values of these parameters close to 1.0 indicating a reasonably good prediction for the constructed mathematical model. Values of the parameters for evaluating modeling quality were listed in Table 1. The results suggested that models established in this study had good fitness and prediction.

3.4. Metabolic profiles of CVS depression with CSGS treatment

Urine samples collected on the 28th day in each group were analyzed by UPLC/MS. Metabolic profiles of the CVS rats with and without treatment of CSGS were obtained based on UPLC/MS data analysis. The PCA analysis showed good separations between the CVS group and naïve control, CVS group and CSGS-treated group, CVS group and fluoxetine group, respectively (Figs. S-F-1, 2 and 3 in the supplementary material). Therefore, PLS-DA was performed and the results shown in Fig. 3(A and B) and 4, which indicated that the metabolic profile of CVS model group deviated from the naïve control, suggesting that significant biochemical changes induced by CVS. The metabolic profile of CSGS treated group fairly differed from the CVS group and close to naïve control in Fig. 4, indicating the deviations induced by CVS were significantly improved after treatment of CSGS. Similar results were observed in fluoxetine treated group except the mean center of spots in CSGS treated group was much closer to naïve control group than that of fluoxetine treated group. The score plots and S-plots between the CVS group and CSGS-treated group, CVS group and fluoxetine group processed by PLS-DA method were shown in supplementary materials (Figs. S-G-1, 2, 3 and 4).

3.5. Time-dependent route of metabolic profiles in CVS induced depression

The metabolic patterns of urine in rats of each group on the 7th, 14th, 21th and 28th day after experiment were plotted by the PLS-DA map, in which arrows depicted the variable trend of metabolic

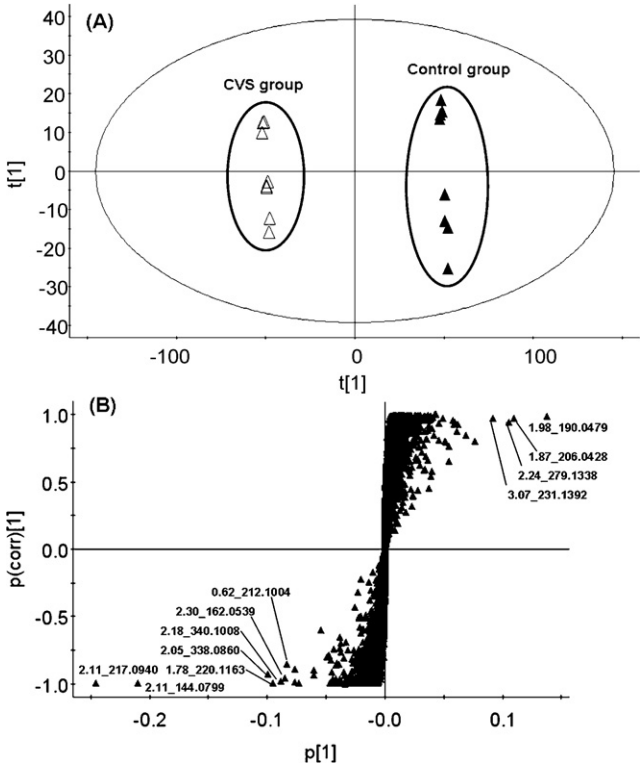

Fig. 3. (A) PLS-DA score plot based on the urine metabolic profiling of the control and CVS model rats on 28 th day by SIMCA-P 11.0 ($n = 8$ in each group). A clearly separation between the model group and control group was obtained, indicating that the urinary metabolic pattern was significantly changed with the process of CVS. (B) PLS-DA S-plot based on urine profiling of the control and CVS model rats on 28 th day by SIMCA-P 11.0 ($n = 8$ in each group). 11 variables far from the origin contributed significantly to differentiate the clustering of CVS model group from that of naïve control and were considered as potential biomarkers.

patterns at different times (Fig. 5). The mean metabolic patterns of naïve rats were used as the jumping-off point of metabolic pattern for all groups. CVS stimuli made the metabolite profile of rats deviate from the normal on day 7, and go farther on day 28, suggesting metabolism turbulence and the significant pathobiological changes induced by CVS stimuli. The intervention of CSGS or fluoxetine showed the potential correction of those deviations induced by CVS. The metabolic pattern of rats in CSGS treated group was approaching to the normal control group from day 14 and was fairly closer to the normal control group on day 28 compared to fluoxetine treated group.

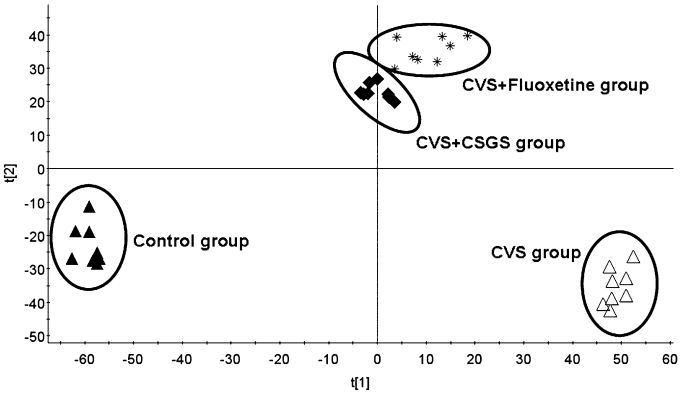

Fig. 4. PLS-DA scores plot derived from the integrated MS data of urine samples obtained from control group (▲), CVS group (△), (CVS + CSGS) group (♦) and (CVS + fluoxetine) group (✳) on 28th day by SIMCA-P 11.0 ($n = 8$ in each group).

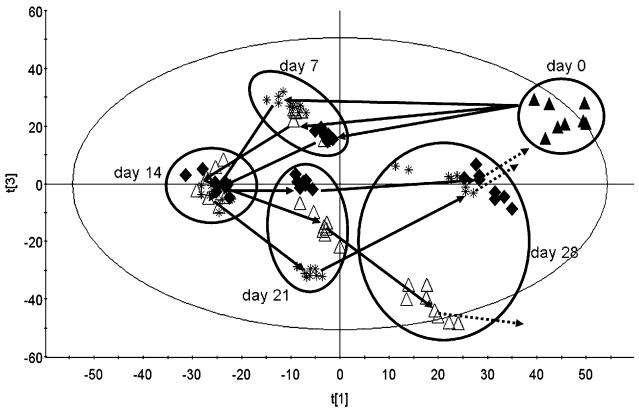

Fig. 5. Score plot of PLS-DA derived from the UPLC/MS profiles of rat urine samples in control group (▲), CVS group (△), (CVS + CSGS) group (♦) and (CVS + fluoxetine) group (✳) at days 0, 7, 14, 21 and 28 by SIMCA-P 11.0 (n = 8 in each group). The plot showed a time-related trajectory of metabolite patterns at different time points.

3.6. Potential biomarkers responsible for the depression induced by CVS and the anti-depressive effect of CSGS

Corresponding VIP statistics of PLS-DA and S-plots were used to extract the important variables responsible for the differentiation. The VIP value calculated by Simca-p software signifies the influence of metabolite ion on the classification. A VIP value >1 means that variables have above average influence on the classification. S-plot is a tool for visualizing covariance and correlation between the metabolites and the modeled class, those ions far from the origin contributing to the clustering significantly. As shown in Fig. 3B, S-plots based on urine metabolic profiles indicated 11 variables representing individual metabolites as biomarker candidate ions with retention time and m/z pairs of 0.62_212.1004 (**1**), 1.78_220.1163 (**2**), 1.87_206.0428 (**3**), 1.98_190.0479 (**4**), 2.05_338.0860 (**5**), 2.11_217.0940 (**6**), 2.11_144.0799 (**7**), 2.18_340.1008 (**8**), 2.24_279.1338 (**9**), 2.30_162.0539 (**10**), and 3.07_231.1392 (**11**). Their VIP values are list in Table S-1 in the supplementary material. Those contributed significantly to differentiate the clustering of CVS group from that of naïve control, could be considered as potential biomarkers responsible for derivations of metabolic profile induced by CVS. Their structures were tentatively identified based on accurate mass measurements via UPLC–TOF-MS, by analysis of accurate molecular weight and the MS/MS spectra. Database such as HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu) and KEGG (http://www.genome.jp/kegg/) were used for confirmation. Consequently, nine biomarker candidate ions were tentatively identified (Table S-1 in the supplementary material) as 3-*O*-methyldopa (**1**), pantothenic acid (**2**), kynurenic acid (**3**), xanthurenic acid (**4**), 2,8-dihydroxyquinoline glucuronide (**5**), 5-hydroxy-6-methoxyindole glucurnoide (**8**), L-phenylalanyl-L-hydroxyproline (**9**), indole-3-carboxylic acid (**10**) and proline (**11**). Here, a potential biomarker with m/z 212.1004 at 0.62 min is taken as an example to illustrate the identification process. The extracted ion chromatogram of ion at m/z 212.1004 was observed in Fig. S-G-5 (A) in the supplementary material. First, full-scan mass spectrum of the peak at 0.62 min (Fig. S-G-5 (B) in the supplementary material) in the positive ion mode gave the accurate molecular weight of the quasi-molecular ion at m/z 212.1004, suggested a molecular formula with $[C_{10}H_{14}NO_4]^+$ (calculated to be 212.0917). Candidates are obtained in searching molecular weight at 212.1004 Da (Positive mode, MW tolerance ± 0.05 Da) from database such as HMDB (http://www.hmdb.ca/) and METLIN (http://metlin.scripps.edu). As a result, there are two candidates with molecular formula of $[C_{10}H_{14}NO_4]^+$, which are described as 3-*O*-methyldopa and methyldopa respectively. The MS^2 spectrum of the ion at m/z 212.1004 (Fig. S-G-5 (C) in the supplementary material) generated the ions at m/z 124.1158 and m/z 109.1035 by loss of a group of $C_3H_6NO_2$ and CH_3 continuously. Compared to 3-*O*-methyldopa, methyldopa will produce fragment ion at m/z 110.11 to form a more stable structure (Fig. S-G-6 in the supplementary material). Therefore, the fragmentation mode of 3-*O*-methyldopa was matched more analogously to the observation of fragment ion in MS^2 spectrum of m/z 212.1004, which could exclude the possibility of methyldopa. By comparing the fragmentation pattern with the reference [17], this metabolite was tentatively identified as 3-*O*-methyldopa.

By comparison of the ion intensity of potential biomarkers between CVS group and naïve control, eight metabolites, **1**, **2**, **5**, **6**, **7**, **8**, **9** and **10** were up-regulated and **3**, **4** and **11** were down-regulated by CVS stimulus (Table 2). After treated by CSGS and fluoxetine, both of groups showed the tendency to correct the derivations of **2**, **3**, **4**, **5**, **8** and **9**. However, only CSGS regulated the deviations of the other four metabolites, **1**, **6**, **7** and **11**.

3.7. Biochemical interpretation

3.7.1. Tryptophan metabolism

Norepinephrine and dopamine are two important neurotransmitters as 5-hydroxy tryptamine (5-HT), which derived from tryptophan metabolism. 3-*O*-Methyldopa (**1**) is one of the main biochemical markers for aromatic L-amino acid decarboxylase (AADC) deficiency, which affects norepinephrine and dopamine biosynthesis (Fig. 6). The concentration of 3-*O*-methyldopa in CVS group increased significantly compared to the naïve rats, which was consistent with the increased level of 3-*O*-methyldopa in the AADC deficiency in urine [23]. Down-regulation of 3-*O*-methyldopa by CSGS indicated the CSGS treatment could recover the dysfunction of AADC in CVS-induced depression rats. However, the fluoxetine seemed not to regulate AADC and even had the up-regulation of 3-*O*-methyldopa compared to CVS group (Table 2).

Indole-3-carboxylic acid (ICA) (**10**), kynurenic acid (KA) (**3**) and xanthurenic acid (XA) (**4**) are the metabolites of tryptophan, the precursor of neurotransmitter 5-hydroxy tryptamine (5-HT). 5-HT showed significant lower level in depressed patients [24]. Up-regulation of ICA and down-regulation of KA and XA may be induced by excessive activation of tryptophanase (TPH) in metabolic pathway producing indole and inhibition of tryptophan 2,3-dioxygenase (TPHD) in metabolic pathway generating kynurenine in CVS process, which will cause the abnormality of tryptophan metabolism directly (Fig. 6). CSGS and fluoxetine were observed to prevent the increased tendency of ICA and decreased tendency of KA and XA in CVS process, indicating therapeutic effects of CSGS and fluoxetine may base on the regulation of the dysfunction of TPH and TPHD in tryptophan metabolism.

3.7.2. Energy metabolism

Pantothenic acid (**2**) is a precursor of coenzyme A (CoA), which derived from pantothenic acid by type I pantothenate kinase (PTK-I) and plays an important role in heme synthesis, lipid metabolism or as a prosthetic group in the tricarboxylic acid cycle (TCA cycle) [25] (Fig. 6). The increased level of pantothenic acid in CVS group by inhibition of PTK-I may be regulated by CSGS treatment. Proline (**11**), the catabolite of peptide degradation by proline iminopeptidase (PIP), is a precursor of pyruvate. Pyruvate can be converted into acetyl-coenzyme A (Acetyl-CoA), which is the main input for a series of reactions known as TCA cycle (Fig. 6). The increased level of proline in CSGS group compared to CVS group indicated CSGS may relieve the inhibition of PIP and activate the biosynthesis of pyruvate to increase carbohydrate catabolism. However, fluoxetine

Table 2
Metabolites selected as biomarkers characterized in urine profile and their change trends ($n = 8$ in each group).

Retention time (min)	m/z (amu)	VIP	Metabolites	Trend in CVS model group[a]	Trend in CVS + CSGS group[b]	Trend in CVS + fluoxetine group[b]
0.62	212.1004	14.3	3-*O*-Methyldopa [17]	↑**	↓*	↑
1.78	220.1163	15.9	Pantothenic acid [18]	↑**	↓**	↓*
1.87	206.0428	19.8	Kynurenic acid [11]	↓**	↑**	↑**
1.98	190.0479	25.8	Xanthurenic acid [11]	↓**	↑**	↑**
2.05	338.0860	18.6	2,8-Dihydroxyquinoline glucuronide [19]	↑**	↓*	↓*
2.11	217.0940	46.0	Unknown	↑**	↓*	↑
2.11	144.0799	39.2	Unknown	↑**	↓*	↑
2.18	340.1008	15.5	5-Hydroxy-6-methoxyindole glucurnoide [20]	↑**	↓*	↓*
2.24	279.1338	11.1	L-Phenylalanyl-L-hydroxyproline	↑**	↓**	↓*
2.30	162.0539	10.3	Indole-3-carboxylic acid [21]	↑**	↓	↓
3.07	231.1392	10.9	Proline [22]	↓*	↑**	↓*

[a] Change trend compared with control group.
[b] Change trend compared with CVS model group.
The levels of potential biomarkers were labeled with (↓) down-regulated and (↑) up-regulated (*$p < 0.05$; **$p < 0.01$).

showed a reversed effect on this metabolic pathway and may aggravate the disturbance in the corresponding energy metabolism.

3.7.3. Liver detoxification

2,8-Dihydroxyquinoline glucuronide (**5**) and 5-hydroxy-6-methoxyindole glucuronide (**8**), derived from the glucuronidation in the liver, can facilitate the excretion of those toxic substances or drugs not being used as energy source in liver detoxification. Uridine diphosphate-glucuronosyltransferases (UGTs) in liver catalyze the glucuronidation of xenobiotics and thus act as antioxidant while organism subjected to oxidative stress [26]. The concentration of glucuronide metabolites with significant increase in CVS group compared to the naïve control suggested the normal functioning of liver detoxification was disturbed in the depression model of rats induced by 28-days CVS, which may arise from the dysfunction of UGTs in CVS process. As shown in this study, CSGS and fluoxetine treatments may regulate this abnormality. CSGS could cause a significantly increased activity of superoxide dismutase (SOD) and catalase (CAT) in liver which indicated that CSGS had the ability of relieving oxidative injury and regulating antioxidant capacity of organisms [27].

3.7.4. Bone loss

Depression can induce bone loss through stimulation of the sympathetic nervous system. Concentration change of urinary N-telopeptide crosslinked type 1 collagen (NTx) is directly related to the bone loss process [28]. Up-regulation of L-phenylalanyl-L-hydroxyproline (**9**), a proteolytic product of collagen, suggested that CSGS and fluoxetine treatments had the potential to improve the bone loss induced by CVS in rats.

Major depressive disorder is a psychiatric disorder that encompasses different metabolic pathways. The biomarkers obtained from this study may partially elucidate the therapeutic mechanisms of CSGS, related to the regulations of the abnormality in energy metabolism, tryptophan metabolism, bone loss and liver detoxification (Fig. 6). In previous studies [9,11], researchers had found out some biomarkers related to amino acid metabolism and energy metabolism with significant changes in the process of depression,

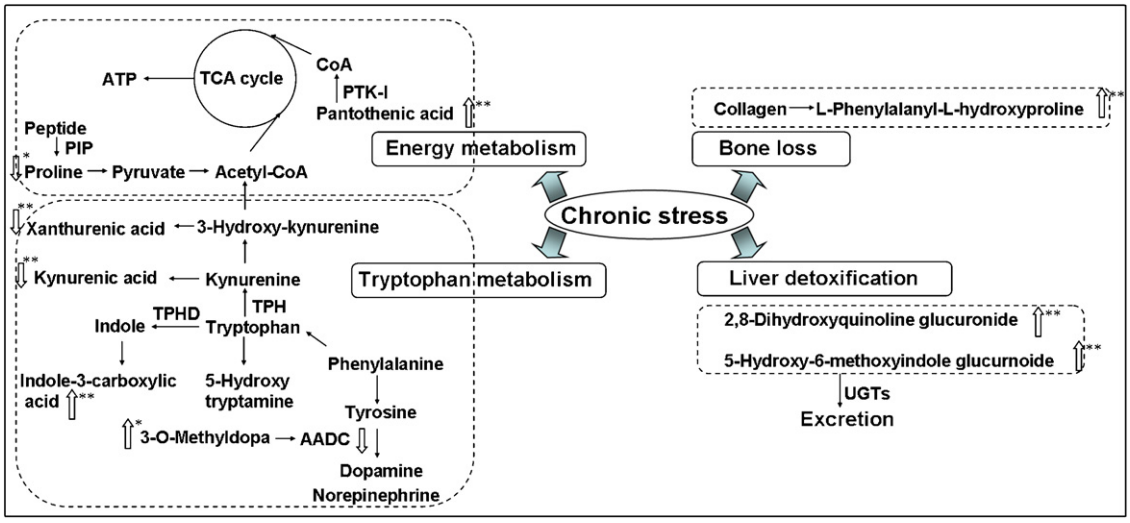

Fig. 6. The perturbed metabolic pathways in response to CVS modeling and CSGS treatment. The levels of potential biomarkers in CVS group compared to normal control group were labeled with (↓) down-regulated and (↑) up-regulated. (**Metabolites in abnormal could be regulated by both CSGS and fluoxetine; *metabolites in abnormal could be regulated by CSGS only; AADC: aromatic L-amino acid decarboxylase; TPH: tryptophanase; TPHD: tryptophan 2,3-dioxygenase; PTK-I: type I pantothenate kinase; PIP: proline iminopeptidase; UGTs: uridine diphosphate-glucuronosyltransferases.).

e.g. kynurenic acid, phenylalanine and citrate. Compared with previous studies, some metabolites concerning bone loss and liver detoxification were elucidated in our study for the first time, which indicated that more metabolic networks besides of amino acid metabolism and energy metabolism may be perturbed in the pathological process of depression. As a result, these disturbances could be rectified by CSGS which could harmonize multiple pathways to resist the CVS procedure. The metabolic fingerprints and potential markers involved in the effects of CSGS on depression were also investigated for the first time in this study. Additionally, fluoxetine did not improve metabolic disturbance of 3-*O*-methyldopa (**1**), proline (**11**), and unidentified (**6** and **7**) induced by CVS depression, which may explain why the metabolic pattern of rats in fluoxetine treated group was not closer to the normal control group than that of CSGS treated group.

4. Conclusions

A UPLC/MS-based urinary metabonomics method has been established and used for the first time to evaluate the antidepressive efficacy and mechanism of CSGS on a depression model of rats induced by CVS. Pattern recognition with multivariate statistical analysis allowed the metabolic profile of CVS-induced depression group clearly separated from naïve control, and that of CSGS group was more closer to the control group compared to fluoxetine group after 28 days treatment. Moreover, the time-dependent route study suggested that with treatments of CSGS and fluoxetine, the recovery of metabolic turbulence was observed from day 14. Eleven metabolites with significant changes in CVS group were considered as potential biomarkers to CVS depression and characterized to be 3-*O*-methyldopa (**1**), pantothenic acid (**2**), kynurenic acid (**3**), xanthurenic acid (**4**), 2,8-dihydroxyquinoline glucuronide (**5**), 5-hydroxy-6-methoxyindole glucurnoide (**8**), L-phenylalanyl-L-hydroxyproline (**9**), indole-3-carboxylic acid (**10**), proline (**11**), and the unidentified metabolites (**6**, 2.11_217.0940; 7, 2.11_144.0799), respectively. The derivations of all those biomarkers can be regulated by CSGS treatment except indole-3-carboxylic acid (**10**), which suggested that the therapeutic effect of CSGS on depression may involve in regulating the dysfunction of energy metabolism, tryptophan metabolism, bone loss and liver detoxification. This study will provide better understanding of the anti-depressive mechanism of CSGS in clinical use.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.02.013.

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