A Metabolic Phenotyping Approach to Characterize the Effects of Cantonese Herbal Tea on Restraint Stressed Rats

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Received March 4, 2014; accepted June 16, 2014; advance publication released online July 8, 2014

Restraint stressed rats were used as a model of Shanghuo and a global metabolic investigation of the effects of cantonese herbal tea (CHT) on the model rats was then performed by gas chromatography-mass spectrometry (GC/MS) metabonomics in an attempt to characterize the metabolic changes underlying such stress and any protective effects of CHT. Serum and urinary GC/MS profiling of rats exposed to 14 d of successive restraint stress for 6 h every day revealed dramatic changes as evidenced by downregulation of tryptophan metabolism, amino acid metabolism, energy metabolism and perturbation of gut microbiota composition. The administration of CHT to restraint-stressed rats ameliorated the symptoms by upregulation of energy metabolism, amino acid metabolism and modulation of the gut microbiota composition. In addition, CHT-treated rats exhibited a higher level of antioxidant production and a reduction in cholesterol level. The lower level of tryptophan metabolism caused by restraint stress, however, was not completely restored after CHT intake, indicating that the tea did not substantially improve this element. This research indicated that CHT intervention modeled Shanghuo symptoms of restraint stress through partial regulation of the perturbed metabolic pathways.

Key words  cantonese herbal tea; GC/MS; metabonomics; restraint stress

Shanghuo, a popular medical conception originating in traditional Chinese medicine (TCM), is commonly applied to describe uncomfortable sensations of heat and humidity from the body. Not defined with specific physiological indicators, the syndromes of Shanghuo can be varied from person such as gingival swelling and a sore throat that patients construes as Shanghuo. In recent years, researchers pointed out that Shanghuo is not a disease but the stress-induced physiological response and can be featured with the fatigue syndromes in both the physical and mental contexts as the combined effects of a range of external and internal factors. As such, accumulation of daily life stress, fast pace of life and lifestyle changes often cause Shanghuo. Cantonese herbal tea (CHT) is a well-known traditional herbal formula to drive away the unpleasant feelings of the heat and humidity arising from Shanghuo and has a long history of consumption in China. Indeed, the composition of herbal tea is highly complex (Table 1), which contained many kinds of phytochemicals like polyphenol and so on. As the critical metabolic interactions between the complex biologically active compounds of CHT and multiple targets in organisms is hard to classify, its related molecular mechanisms still remain unknown.

Metabonomics aims at the comprehensive characterization of biological samples under certain conditions to provide information on holistic metabolic changes with relation to different stimulus, and the interpretation of changes on metabolic pathways to decipher the physiological variation in the whole organism, which has been found widespread applications in many areas including diseases, drugs, nutrition and plant etc. Because the metabolic phenotype is closely associated with real biological end points and provides a global systematic interpretation of biological effect under a particular set of conditions, which has substantial compatibility with TCM theory, and has the potential to be able to overcome the confounding reasons and permit the study of the system-wide effects of CHT and provide an in-depth understanding of its mechanisms of action and health benefits. In the previous study, metabonomics strategies were performed to investigate the global biological characterization associated with CHT intervention in normal rats. The results showed that the overall metabolic responses to CHT intervention were reflected in the variations of energy metabolism, lipid metabolism and amino acid metabolism etc., leading to a direct elucidation of the mechanisms of action of CHT. Although the research still can’t explain why CHT alleviates the unpleasant feelings arising from Shanghuo, it clearly demonstrates that metabonomics is ideally positioned to allow further mechanistic studies of action of CHT intervention as a sensitive strategy.

Metabonomics encompasses the comprehensive and simultaneous profiling of a large number of small-molecule metabolites in all different biofluids. Mass spectrometry (MS)-based techniques and nuclear magnetic resonance (NMR) spectroscopy are usually applied for metabolites analysis. Compared to NMR, gas chromatography with mass spectrometry (GC/MS) is a productive, sensitive and high-throughput analytical technique and has been widely applied in metabonomics investigation. Furthermore the application of GC/MS-based metabonomics was facilitated by low costs compared to liquid chromatography with mass spectrometry (LC/MS).

Therefore, in the present research the proposed restraint stress was used to simulate the symptoms arising from Shanghuo and GC/MS-based metabonomics was applied to delineate the metabolic changes in related metabolic pathways underlying restraint stress and the protective effects of CHT on the restraint stress rats. Understanding the overall metabolic effects of restraint stress and dietary intervention with CHT will un-
doubtedly enrich our current knowledge of Shanghuo and provide insights into the beneficial effects of CHT intervention.

MATERIALS AND METHODS

Chemicals and Reagents N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA)+1% trimethylchlorosilane (TMCS): Fluka; pyridine: Sigma; Methoxyaminehydrochloride: Supelco; Urease: Type C-3, Sigma; Cantonese herbal tea: the product of Guangzhou Wanglaoji Pharmaceutical Company Limited; Toluene and heptane: Shanghai Chemical Reagent Company.

Animal Treatment Thirty healthy male SPF Sprague-Dawley (SD) rats approximately 3 months of age and weighing 180±20g were purchased from the animal experimental center of Sun Yat-sen University. All animals were divided into five groups, namely normal control, model control, low-dosed, middle-dosed and high-dosed group. All animals were housed in cages and had free access to water and food throughout the experiment. The light cycle consisted of 12 h of light and 12 h of dark, and the temperature was maintained at 22–24°C. All of the blood samples were collected and then centrifuged at 10000×g for 10 min at 4°C. All the supernatant was kept in the refrigerator for 10 min and then centrifuged at 12000×g for 10 min at 4°C. Five hundred microli-}

Table 1. The Herbs Involved in Cantonese Herbal Tea

<table>
<thead>
<tr>
<th>Main components</th>
<th>Family</th>
<th>Main components</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ilex asprella</td>
<td>Aquifoliaceae</td>
<td>Rosa laevigata michx</td>
<td>Rosaceae</td>
</tr>
<tr>
<td>Lophatherum gracile</td>
<td>Gramineae</td>
<td>Lygodium japonicum</td>
<td>Lygodiaceae</td>
</tr>
<tr>
<td>Vitex negundo</td>
<td>Rutaceae</td>
<td>Desmidium styracifolium merr</td>
<td>leguminosae</td>
</tr>
<tr>
<td>Microcosm paniculata</td>
<td>Tiliaceae</td>
<td>Polygonum chinense</td>
<td>Polygonaceae</td>
</tr>
<tr>
<td>Oroxyllum indicum</td>
<td>Bignoniaceae</td>
<td>Helicters angustifolia</td>
<td>Fimiana</td>
</tr>
</tbody>
</table>

with 1% TMCS at 70°C for 2 h. A volume of 50µL of heptane was added after silylation.

For urine, sample treatment and instrumental conditions for GC-MS were as previously described. Briefly, urinary samples were thawed and 100µL of urine was incubated with 150U urease at 37°C for 1 h. Then, 300µL of ice-cold methanol was added and mixed vigorously. The mixture was placed on ice for 20 min, followed by centrifugation to precipitate urease and protein. A total of 200µL of the supernatant was separated and evaporated until dry under a gentle stream of nitrogen gas. A total of 100µL of toluene (dried over anhydrous sodium sulfate) was added to the dry residue and then mixed and dried again under nitrogen gas. The dried extract was derivatized first with 30µL of dry pyridine (20mg/mL) for 16 h at 37°C, followed by trimethylsilyl-derivatization with 120µL of BSTFA for 1% TMCS at 70°C for 2 h.

GC-MS Analysis All samples were analyzed by GC/MS (Agilent 6890 GC instrument equipped with a 5975 mass detector), using a HP-5ms GC column (30 m length, 0.25 mm i.d., 0.20µm film thickness). The sample injection (1µL volume) was splitless at an injection temperature of 250°C. Helium carrier gas flow was 1.0mL/min. The ion source temperature was 230°C. The electron energy was 70eV and mass data was collected in a full scan mode (m/z 50–600). The temperature gradient of GC separation for serum samples started at 70°C for 2 min, increased from 70°C to 280°C at 7°C/min where it remained for 7 min. As for urinary samples, the temperature started at 70°C for 2 min, increased from 70°C to 140°C at 7°C/min where it remained for 2 min, then increased again from 140°C to 210 at 4°C/min. The 210°C temperature was held for 4 min, then increased from 210°C to 280°C at 7°C/min where it remained for 7 min. Metabolites were identified by matching mass spectra to the NIST05 library. The Human Metabolome Database (HMDB) was also used for compound identification and Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for pathway analysis.

Data Preprocessing Serum and urinary GC-MS files were converted into NetCDF format using Chemestation software (Agilent Inc., U.S.A.). XCMS, a freely available platform-independent R package, contains three important algorithms for peak detection, peak matching and retention time alignment to process and analyze GC/MS data. XCMS parameters were set for serum data extraction as follows: fwhm=4, method=linear, family=Gaussian, plottype=medven, and group=5. The XCMS primary parameters for urinary data extraction were as follows: fwhm=4; smthresh=6; a bandwidth of 8 for the first grouping command and 4 for the second grouping command.

Two three-dimensional data including peak index (m/z-retention time, m/z-RT), sample names and peak areas were exported from XCMS in “.tsv” format, which can be viewed...
using Microsoft Excel software. Artifacts arising from the BSTFA derivatizing reagent were removed. The m/z ions of 73, 75, and 147 were also excluded because many tetramethylsilane (TMS) derivatives produce these typical fragments. And then the representative ion for each metabolite was picked and a data matrix was formed for data analysis, containing sample names, peak indexes (m/z-RT pair) and peak areas. As variations in overall concentrations were very distinctive, normalization to the total sum of the ion fragments was performed prior to pattern recognition analysis.

Multivariate Statistical Data Analysis Normalized data were introduced into SIMCA-P 11.5 software for multivariate statistical analysis, which is developed by Umetrics for design of experiment and multivariate data analysis. Principal component analysis (PCA), a widely used statistical technique for unsupervised dimension reduction, was initially performed on mean-centered data to visualize general clustering and trends among the observations. Orthogonal projection on latent structures discriminant analysis (OPLS-DA), a supervised multivariate analysis method, was subsequently performed on autoscaled and Par-scaled data to sharpen the separation between groups of observations, by rotating PCA components such that a maximum separation among classes and to understand which variables carry the class-separating information and therefore define biomarkers.

Model Validation and Biomarkers Identification A 5-fold cross validation strategy was applied to check the model validity and produced the cross validation parameters $R^2$ and $Q^2$, which represents the variation in the data matrix and the predictability of the model, respectively. Hitherto $R^2$ and $Q^2$ were employed as first indicators of model quality. The values of these parameters close to 1.0 indicate a robust and reliable model. In order to facilitate the interpretation of the metabolic changes associated with restraint stress and the protective effects of CHT intervention, the metabolites could be identified and considered statistically significant according to the variable importance in the Projection (VIP) obtained from the O-PLS-DA models integrated with Student’s $t$-tests ($p<0.05$).

RESULTS

GC/MS Analysis of Serum and Urinary Metabolome In this study, GC-MS profiling of serum and urinary metabolite profiles was used to incorporate detailed information on the metabolic status and provide complementary information on metabolite composition. The representative GC-MS total ion chromatograms of serum and urinary samples were shown in Figs. 1A and B, which showed that there was a large dynamic range in metabolite concentration and the composition of urinary metabolome was more complicated than that of serum metabolome. Because there was variation in the concentration between samples as well as inter- and intra-individual physiological variation, visual examination of the GC-MS chromatograms from the serum and urinary samples did not reveal obvious distinguishing features among different treatment rats.

Metabolites in serum and urine were identified by mass spectra matches to the GC-MS mass spectral library NIST05 and the HMDB metabolomics database-GC/MS library (www.hmdb.ca). In all, the identified compounds included carboxylic acids, hydroxyl acids, carbohydrates, phenolics and amino acid. Worthy of special note was that some phenyl-containing compounds such as hippurate, N-phenylacetylglycine, 4-hydroxyphenylacetate, and 3-(3-hydroxyphenyl)propionic acid were identified, which are manipulated mainly by the gut microbiota in the living systems through fermentation of dietary polyphenols and aromatic amino acids. In all, the identified compounds including carboxylic acids, hydroxyl acids, carbohydrates, phenolics, and amino acids were illustrated in SI Tables 1 and 2, which contained the information about retention time (RT), metabolites name, TMS groups, main fragments and m/z-RT.

Overview of Metabolic Variation Induced by Restraint Stress The extracted data from serum and urinary samples of model control group and normal group were analyzed by SIMCA-P software. PCA models with a mean-centered scaling were used to depict the general variation and assess the inherent similarity of serum and urinary metabolic profiles (as shown in SI Figure 1). It was not at all bewildering to find out that the separation trend could not be observed between the model group and the normal group due to interindividual variation or systematic adaptation to such as environmental stimuli. Hence, OPLS-DA was further performed to maximize the separation between the groups and identify class-specific metabolites. Comparative OPLS-DA analyses of serum and urinary samples showed that the model group was markedly separated from the control group, suggesting that exposure to restraint stress may lead to metabolic variation in SD rats (as illustrated in Figs. 2A, B).

The cross validation values $R^2$ and $Q^2$ indicated the goodness of fit and predictability of the models. The corresponding VIP statistics were consequently applied to extract the metabolites contributing to the deviation of the model control group from the normal group. Variation in these metabolites was further confirmed by Students’ $t$-test (Table 2).

The corresponding OPLS-DA VIP statistics with univariate analysis indicated that restraint stress resulted in significantly decreased levels of N-phenylacetylglycine, quinolinic acid, nicotinic acid and 1-palmitoylglycerol in urine and threonine in serum, which can be used as biological markers in the restraint stress group. These significant metabolic variations were also associated with lower levels of glutamate, glucose, glycemic acid, stearic acid, 4-hydroxyphenylacetate and 3-hydroxyphenylpropionate in urine and aspartic acid and serine in serum compared to control animals.

Metabolic Effects of Restraint Stress with CHT Intervention To determine whether CHT intervention was possible to influence the metabolic pattern of the rats loaded with restraint stress and identify the metabolites with a significant contribution to the recovery of the restraint stress rats, OPLS-DA analyses of serum and urinary metabolic profiles between differently dosed rats and model rats respectively were performed. The outcome manifested that satisfactory clustering between each dosed group and model group were achieved with good model parameters for urinary samples (as illustrated in SI Figure 2), suggesting that CHT intervention with any dose could influence the urinary metabolic pattern. The serum metabolic patterns of low-dosed and middle-dosed samples differentiated from control group, however high-dosed rats didn’t deviate from the control group because of bad model quality.
Table 2. The Metabolites Responsible for the Separation between Restraint Stress Model Group and Normal Group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Corresponding metabolites</th>
<th>Change direction</th>
<th>VIP</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>M91T1497</td>
<td>N-Phenylacetylglycine</td>
<td>↓</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>M194T1275</td>
<td>Quinic acid</td>
<td>↓</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>M180T635</td>
<td>Nicotinic acid</td>
<td>↓</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>M371T2400</td>
<td>1-Palmitoyl-glycerol</td>
<td>↓</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>M179T1131</td>
<td>4-Hydroxyphenylacetate</td>
<td>↓</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>M246T1118</td>
<td>Glutamate</td>
<td>↓</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>M319T1581</td>
<td>Glucose</td>
<td>↓</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>M341T2009</td>
<td>Stearic acid</td>
<td>↓</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>M189T693</td>
<td>Glyceric acid</td>
<td>↓</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>M205T1270</td>
<td>3-Hydroxyphenylpropionate</td>
<td>↓</td>
<td>1.65</td>
</tr>
<tr>
<td>Serum</td>
<td>M218T756</td>
<td>Threonine</td>
<td>↓</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>M232T846</td>
<td>Aspicic acid</td>
<td>↓</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>M204T726</td>
<td>Serine</td>
<td>↓</td>
<td>1.67</td>
</tr>
</tbody>
</table>

VIP indicates the relative contribution of the metabolite to the separation between groups. ↑ or ↓ indicates whether there was an increase or a decrease in metabolite concentrations compared to the control, respectively. * indicates a p<0.05. NS represents no significance.
The urinary models of model rats, dosed rats and normal rats were subsequently constructed using Par scaling to identify which dosage was best suitable to act on the restraint stress rats. As illustrated in Fig. 3, it can be seen that the dosed groups were mainly located between the model group and the normal group, suggesting that the restraint stress rats treated with CHT intervention were showing a tendency of moving toward the control group. But the reversible magnitude differed among three dosed groups. We can see that there was a superimposition between low-dosed rats and model rats (Fig. 3A) and the high dosed group was mainly located between the model group and the control group (Fig. 3C). Only the subjects of middle-dosed group obtained better separation from those of the model group because they were much closer to the control group (Fig. 3B). So the middle dose of CHT intake for restraint stress rats was best effective and had the capacity of restoring the restraint stress rats to health controls. Even so, the middle dosed rats were still not completely merged with normal rats, which indicated that the restraint stress rats after CHT intervention didn’t completely restore to health.

In supporting of this, the recovery of the metabolites markedly changed in restraint stress rats after CHT consumption was illustrated in Table 3. It can be seen that the decreases of four metabolites in restraint stress rats were counteracted and these four metabolites were upregulated by CHT intervention. Even though their levels recovered after CHT intervention, intensities remained not statistically significant in low-dosed group and high-dose group. Only the urinary nicotinic acid, N-phendylacetylglycine and 1-palmitoyl-glycerol levels showed statistically significant increases in the middle-dosage group, suggesting that only middle dosage was best effective for the restraint stress rats and CHT consumption could bring partial recovery to normal, which was attesting to the above-mentioned results.

Hitherto, VIP statistics combined with Student’s t-test were used to identify the metabolites with a significant contribution to the separation of middle dosed rats from model rats in urinary and serum profiling. Several significantly perturbed metabolites were detected, including elevated levels of nicotinic acid, adipic acid, pimelic acid, 4-hydroxyphenylacetate, N-phenylacetylglycine, glucose, pantothenic acid, ferulic acid in urinary metabolome. For serum profiling, the discriminatory metabolites were elevated levels of stearic acid and proline and lower levels of leucine and cholesterol in CHT-treated rats.

![Fig. 2. OPLS-DA Score Maps between Normal Group and Restraint Stress Model Group](image1)

A: OPLS-DA analysis for rats’ serum; B: OPLS-DA analysis for rats’ urine (* for model group; ▲ for normal group).

![Fig. 3. OPLS-DA Score Maps among Normal, Model and Dosed Groups](image2)

A: OPLS-DA score map among normal, low dosed and model groups; B: OPLS-DA score map among normal, middle dosed and model groups; C: OPLS-DA score map among normal, high dosed and model groups (* for model group; ■ for dosed group; ▲ for normal group).
rats. In addition, these variations were accompanied with the higher levels of valine, 2,3,4-hydroxybutyric acid, glutamate, sorbital and hippurate in urine samples (Table 4).

DISCUSSION

Bridging the gap between traditional Chinese Medicine and western medicine, metabolomics has gained more and more appreciation in the modernization of TCM. As a “purging fire” herbal formula, CHT has efficacy in clearing internal heat, detoxifying and preventing thirsty according to TCM viewpoints. But the mode of action of CHT is unclear. The aim of this study was to investigate the protective effects of CHT on Shanghuo in restraint-stressed rats by serum and urinary GC/MS metabonomics and verify the postulation that CHT intervention could holistically protect individuals against restraint stress. Provided with complementary information, parallel application of serum and urinary metabolic profiling here demonstrated the metabolic effects of restraint stress exposure and the protection of CHT intervention against the adverse influences of stress exposure.

The Restraint Stress Rats Displayed Diverse Metabolic Phenotypic Changes In recent years, metabolomics investigation on the effects of different stress stimulus provided useful and comprehensive information in understanding biological events for the surveillance of health and diseases. In the present research, a number of important serum and urinary metabolites altered contributing to a significantly different metabolic profile of the restraint stress group compared to the control after a period of restraint stress exposure. It was thus conceivable that restraint stress exposure probably caused systematic metabolic changes involving many metabolic pathways.

In urine, quinolinic acid was observed to be downregulated in restraint stress rats, which is a metabolite involved the tryptophan metabolic pathway. The variation exhibited that restraint stress produced a pronounced effect on tryptophan metabolism. Since the level of quinolinic acid was lower in restraint stress rats, there was some concern that restraint stress might downregulate the tryptophan metabolism. Decreased
level of tryptophan metabolism might be associated with development of depressive symptoms like reduced activities and curiosities.\textsuperscript{28,29} Thus, the behavior performance of reduced activities in restraint rats probably correlated with the lower tryptophan metabolism.

Our observations indicated that restraint exposure caused significant downregulation for nicotinic acid, in accompanied with level decreases for glucose, stearic acid and glyceric acid. Nicotinic acid, also known as niacin or vitamin B\textsubscript{3}, is a water-soluble vitamin whose derivatives such as NADH, NAD, NAD\textsuperscript{+}, and NADP play essential roles in energy metabolism.\textsuperscript{30} A lower urinary excretion of nicotinic acid observed in urinary samples of the restraint-stressed rats was indicative of lower energy metabolism probably because of hypoadrenocorticism.\textsuperscript{31} Attesting to this were the concomitant decreases in the urinary levels of glucose, stearic acid and glyceric acid in model group. So, the metabolic network in restraint stress rats entered into a much lower energy consumption period. In our experiment, the restraint stress rats behaved less activity or responsive.

The significant decrease of the threonine level in serum profile accompanied with decreases for aspartic acid in serum and glutamate in urine were probably associated with the changes in immunology system because these amino acids were involved in assisting the immune system by helping the production of antibodies and promoting thymus growth and activity.\textsuperscript{32} The downregulation of these metabolites fit the biochemical response to chronic stress treatment.

Perturbations in gut microbiota-derived metabolites were observed in urine samples after the exposure to restraint stress. Significant variation of N-phenylacetylglycine in urine together with level decline for 4-hydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propanic acid was associated with disturbances in gut microbial presence. N-Phenylacetylglycine is derived from metabolism of aromatic amino acids phenylalanine and tyrosine mediated by the colonic microflora,\textsuperscript{32} which manifested that restraint stress induced the alteration of the gut microbiota composition. It has been reported that life stress impacts directly on gastrointestinal function by modulation of gut permeability and biological mediators.\textsuperscript{25,33} Thus, changes of the gut microbiota composition in restraint stress inevitably affected the global metabolism of the rat body and brought an alteration of metabolic homeostasis.

In conclusion, we identified the systematic changes of metabolic phenotyping in restraint-stressed rats, which characterized the diverse responses to restraint stress involving variation of tryptophan metabolism, energy metabolism, amino acids metabolism and perturbation of gut microbiota composition. For this reason, metabonomics provided new understandings of how stress-induced metabolic perturbations of homeostatic at the whole level.

**CHT Intervention Exhibited Partial Recovery of Restraint Stress Rats to Normal Condition** CHT intervention was hypothesized to ameliorate the press of rats loaded with restraint stress. In the present research, restraint stress rats were treated by three different doses of CHT: low dose, middle dose and high dose. All doses of CHT intervention were shown to have the effect of attenuating the response to restraint stress to some extent as evidenced by the reversible trend to normal control.

As an innovative approach, metabonomics exhibited the capacity to measure the magnitude of the biological effects of CHT intervention on restraint-stressed rats, which depicted the dose-effect relationship. The effects observed on urinary metabolome following consumption of different dosages of CHT differed slightly, suggesting that subtle variation could be detected by metabonomic strategy and the biological consequences of following CHT intervention were correctly assessed. However, only CHT intervention showed more or less limitations for restraint stress rats as dosed rats didn’t completely return stress-perturbed systems to normal function, which suggested that only CHT treatment did not completely fight restraint stress.

**CHT Restored the Perturbation of Homeostasis by Regulating Corresponding Metabolic Pathways** The recovery following CHT intervention was reflected in the return of CHT-treated groups to the normal. According to OPLS-DA analysis, middle dose was more effective than low dose and high dose and some significantly changed metabolites have been identified to explain the mechanism of action of CHT.

In middle-dosed animals, the level of nicotinic acid, glucose, and adipic acid, pimelic acid involving energy metabolism were significantly elevated, implying that CHT intervention in restraint stress rats can regulate energy metabolism. Pantothenic acid, also called vitamin B\textsubscript{5}, is a water-soluble vitamin required to sustain life. Pantothenic acid is needed to form coenzyme-A (CoA), and is thus critical in the energy metabolism and synthesis of carbohydrates, proteins, and fats. Thus, the finding of these upregulated metabolites could be explained by the recovery of energy metabolism in restraint stress rats after CHT consumption.

The recovery of gut microbiota composition was reflected in the significantly elevated levels of N-phenylacetylglycine and 4-hydroxyphenylacetic acid, which were decreased in the urinary excretion of the restraint stress rats. The significant variation of these gut microbiota-related metabolites indicated that CHT could modulate the gut microbiota composition. Since CHT was orally administrated, most active ingredients were absorbed via degradation of gut microbiota. Thus it could be implied that significant effects of CHT consumption on gut microbiota were achieved by modulation of the intestinal bacterial population as metabolic prebiotics, which was consistent with previous observations.\textsuperscript{34} It also suggested that gut microbiota somehow functioned as part of the host’s metabolic regulatory organ and further changed related endogenous metabolism. Thus, it could be implied that the protective action of CHT extended to gut microbiota from the host itself. On the basis of these results, it appeared that an important mode of action of CHT lied in the direct alteration of gut microbiota to restore the metabolic homeostasis.

Higher urinary excretion of ferulic acid was found in CHT-treated rats, a metabolite of gut-mediated bioconversion of chlorogenic acid.\textsuperscript{35} Ferulic acid was absorbed by the small intestine and exhibited significant antioxidant activities in restraint stress rats.\textsuperscript{36} Indeed gut microbiota was able to perform a range of biotransformations on xenobiotics and affect the absorption and bioavailability of active ingredients.\textsuperscript{37} Therefore, this observation suggested that the recovery of restraint stress rats after CHT treatment was involved by the gut microbiota as a superorgan.

Meanwhile, some amino acids were observed an elevated trend including glutamate and valine. Glutamate has been...
described as a key immunomodulator in the initiation and development of T-cell-mediated immunity. Valine is one of branched-chain amino acids and plays in the functioning of the immune system. Therefore the variation of these amino acids metabolism probably suggested the upregulation of organism immunity and anti-stress activity.

In addition to these variations, a significant decrease in the level of cholesterol was observed in the serum profiling. Cholesterol-lowering effect of herbal tea consumption has been reported through suppression of hydroxymethylglutaryl (HMG)-CoA reductase, which is the rate-determining enzyme for cholesterol synthesis. Thus, the finding of higher level of cholesterol in dosed rats might be associated with inhibition of HMG-CoA reductase after CHT consumption.

Based on these results, our work demonstrated that restraint stress induced multilevel homeostatic perturbations including energy metabolism, amino acid metabolism, tryptophan metabolism, gut microbiota composition. CHT intervention was effective at dampening the majority of episodes by the recovery of energy metabolism, amino acids metabolism and gut microbiota composition. But restraint stress-induced perturbation of tyrosophan metabolism could not completely restore to normal.

CONCLUSION

The potray of global metabolic status of restraint stress and the recovery of CHT intervention was delineated by metabonomics strategy in the present research. It provided deeper insight into the mechanisms of action of CHT intervention relevant to its appreciation as a “purging fire” drink. The current observations strongly supported the idea CHT intervention could ameliorate the pressure of stress systematically and relevant observations strongly supported the idea CHT intervention can provide deeper insight into the mechanisms of action of CHT intervention relevant to its appreciation as a “purging fire” drink.

Acknowledgments  We are grateful to thank for the financial supports by National Key Basic Research and Development Plan (2012CB208080) and Key Program of National Natural Science Fund of China (31130042).

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