Urinary metabonomic study using a CUMS rat model of depression

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ABSTRACT: Chronic unpredictable mild stress (CUMS) is a well-validated model of depression. In this study, a urinary metabonomic method based on the NMR spectrometry was used to study the metabolic perturbation in CUMS-induced rat depression model. With pattern recognition analysis, a clear separation of CUMS rats and healthy controls was achieved, and nine endogenous metabolites contributing to the separation were identified. CUMS-treated rats were characterized by the increase of glycine, pyruvate, glutamine, and asparagines, as well as the decrease of 2-oxoglutarate, dimethylglycine, citrate, succinate, and acetate. The urinary biochemical changes related to the metabolic disturbance in CUMS induced depression, and the possible correlations with live qi stagnation in traditional Chinese medicine are discussed. The work shows that CUMS is a reliable model for studying depression, and the noninvasive urinary metabolomic method is a valuable tool to investigate the biochemical perturbations in depression as an early diagnostic means. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: NMR; 1H; metabonomics; depression; urine; CUMS

Introduction

Depression, with the complex mechanism and etiology, is a psychiatric disorder characterized by anhedonia and feelings of sadness.[1,2] Etiology of depression is not fully understood but may involve changes in the nervous system, immunological system, and endocrine system.[3] It has been considered as a systemic disorder caused by impairment in different biochemical pathways.[4] Currently, diagnosis of depression remains subjective based on descriptive symptoms by depression patients.

Chronic unpredictable mild stress (CUMS), a well-validated model of depression, has been widely used for studying depression and evaluating antidepressant effects of diverse drugs.[5] The CUMS procedure, in which rats are exposed sequentially to a variety of mild stressors, mimics the role of chronic stress in precipitating depression and induces various long-term physical, behavioral, neurochemical, and neuroendocrine alterations that resemble those observed in depressed patients.

Metabonomics is a systematic approach to study the in vivo metabonomic profile, defined as the quantitative measurement of the dynamic multi-parametric metabolic responses of living systems to patho-physiological stimuli or genetic modifications.[6] Urine and blood are the most frequently profiled biological matrices for exploring the systematic modification of the metabolome. Compared with the blood samples, the advantage of urinalysis is that sample collection is noninvasive to the body.[7] A number of analytical tools, including NMR spectrometry, liquid chromatography–mass spectrometry (LC-MS), and gas chromatography–mass spectrometry (GC-MS), have currently been employed, which are the three techniques mostly used.[8] For metabonomic studies, NMR spectrometry provides rapid, non-destructive and high-throughput methods, requiring minimal sample preparation.[9] In addition, detailed structural information, including chemical shifts and coupling constants, regarding the components of the biological sample, can be interpreted from the spectra.

The complexity of spectra and the presence of biological variation between individuals across a set of samples often make it difficult to obtain valuable information from simple inspection of spectra. Consequently, it is necessary to use data reduction and pattern recognition methods to uncover the latent biochemical information from spectral data. Principal components analysis (PCA), allowing the identification of groups of variables that interrelated via phenomena that cannot be directed observed, is one of such methods widely used.

Recently, GC/MS-based metabonomic studies on the plasma of elderly depression,[10] as well as brain tissue,[11] plasma[12] and...
urine\textsuperscript{13,14} from CUMS-treated rats, have been reported. In addition, UPLC/MS also was applied to investigate the urinary metabolomic changes in CUMS model.\textsuperscript{13} However, there is currently no single technique that fulfills all the requirements of an ideal global metabolite profiling tool, and the judicious use of a suite of metabolite profiling techniques is most likely to result in comprehensive metabolite profiles.\textsuperscript{13} So complementary techniques should be used to investigate the perturbed metabolic pattern in the depression state comprehensively. To our knowledge, no NMR-based metabolomic study of CUMS-induced depression has been reported. The primary goal of this work is to characterize the urinary metabolic changes in CUMS-treated rats by NMR.

Compared with our previous study by GC-MS, NMR spectrometry, which is more reproducible than the chromatographic methods, was used, and biochemical interpretation of possible biomarker induced by CUMS was discussed. In addition, the proper time of carrying out the CUMS model also was investigated.

Materials and Methods

Animals handling

The protocol of the study was approved by national legislations of China and local guidelines. The investigation was conducted in accordance with the ethical principles of animal use and care. A total of 12 male Sprague–Dawley rats, weighing 200 ± 20 g, were purchased from the Experimental Animal Center of Military Medical Sciences Academy (no. SCXK2005-0004). Throughout the study, all animals were kept under appropriate conditions (22 ± 2 °C and 45 ± 10% relative humidity). Air was replaced with adequate frequency, and the rats were maintained under a 12-h light–darkness cycle. Animals were housed in metabolism cages, and all had free access to tap water and food.

Chronic unpredictable mild stress procedures

The animals in the chronic unpredictable mild stress (CUMS) model group were individually housed and repeatedly exposed to a set of chronic unpredictable mild stressors as follows:\textsuperscript{13} cage tilting and damp sawdust for 24 h (200 ml of water per individual cage, which is enough to make the sawdust bedding wet), noises for 1 h (alternative periods of 60 dBA noise for 10 min, and 10 min of silence), swimming in 4 °C cold water for 5 min, exposure to an experimental room at 50 °C for 5 min, 24 h of food deprivation and 24 h of water deprivation, tall clamp for 1 min, 15 unpredictable shocks (15 mA, one shock/30 s, 10 s duration), and restricted movement for 4 h. One stressor was applied per day, and the whole stress procedures lasted for 3 weeks with a completely random order. The healthy control rats were housed in groups of three per cage without disturbing except for necessary procedures such as weighting or cage cleaning. They had free access to water and food except for the period of water and food deprivation prior to the sucrose preference test.

Animal and sample collection

After 2 weeks habituation, the rats were randomly divided into two groups (n = 6) according to the body weights and behavior scores in open-field experiment: healthy control group (NS, no stress plus 0.9% NaCl solution) and CUMS-model group (MS, stress plus 0.9% NaCl solution). Animals were administered separately via gastric intubation with the administration of volume of 10 ml/kg, which were started at the beginning of stress procedures and lasted for 3 weeks. Samples of 12-h urine were collected pre-dose and at days 7, 14, and 21, with plastic bottles kept on ice. After centrifugation at 4000 g for 10 min to remove residues, urine samples were immediately stored frozen at −80 °C until NMR analysis.

Behavioral analysis

An open-field test, performed as previously described,\textsuperscript{13} was used to study the exploratory and anxiety behavior of rats. Open-field test was conducted between 7:00 and 11:00 h in a quiet room pre-dose and at days 7, 14, and 21. The open field apparatus consisted of a square arena 100 × 100 cm, with an opaque wall 40 cm high. The floor was marked with a grid dividing it into 25 equal-size squares. Each animal was placed in the central square of the apparatus and observed for 5 min, and the following behaviors were recorded: crossing – the number of grid lines it crossed with all the four paws, rearing – by counting the number of times the animal stood on its hind limbs, and immobility time. At the end of each test, the whole area was cleaned to eliminate odors for the next test.

The depressive-like behavioral state was assessed by a sucrose preference test described as follows. Rats were trained to consume 1% (w/v) sucrose solution before the experiment. They were exposed to two bottles of 1% sucrose solutions in a 24-h period and exposed to one bottle of 1% sucrose solution and one bottle of drinking water for the next 24-h period. Formal tests were carried out pre-dose and at days 7, 14, and 21. After a 24-h period of water and food deprivation, each rat was subjected to an individual metabolic cage, in which two bottles containing water and 1% sucrose solution were placed. The ratio of the amount of sucrose solution to that of total solution ingested within 4 h represented the parameter of hedonic behavior, using the following formula:

\[
\text{sucrose preference} = \frac{\text{sucrose consumption}}{\text{water consumption} + \text{sucrose consumption}} \times 100\%
\]

All data in the figures or tables were presented as means ± SD, analyzed using SPSS (version 11.5) statistical package. Independent-samples t-test were used for statistical evaluation, and p value lower than 0.05 were considered statistically significant.

\( ^{1}H \) NMR spectroscopic analysis

Urine samples were thawed, and aliquots of 500-μl urine were mixed with 50-μl phosphate buffer (81:19, v/v mixture of 0.2 M Na\textsubscript{2}HPO\textsubscript{4} and 0.2 M NaH\textsubscript{2}PO\textsubscript{4} in D\textsubscript{2}O; pH 7.4), containing 0.1% 3-trimethylsilyl-(2,2,3,3-\textsuperscript{13}C\textsubscript{4})-1-propionate (TMSP). Phosphate buffer minimized chemical shift variation because of different pH in urine samples, with D\textsubscript{2}O as a field lock and TMSP as a chemical shift reference. Any resulting precipitate was removed by centrifugation at 11400 g for 20 min. An aliquot of 500 μl was transferred into a 5-mm NMR tube for NMR analysis. \( ^{1}H \) NMR spectra were acquired on a Varian VNMR-600-MHz NMR spectrometer (Varian Inc., USA) operating at 600.095 MHz. \( ^{1}H \) frequency and a temperature of 298 K, using a 600-MHz 5-mm \( ^{1}H \) (13C/15N) PFG high-field triple resonance probe. Urine samples were analyzed using one-dimensional (1D) NOESY
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(RD-90º-t1-90º-tm-90º-acquire). On resonance, saturation was used to suppress the water signal during the recycling delay (RD) of 2.0 s and mixing period ($t_m = 0.10 s$). A total of 64 transients were collected into 32k time domain data point, with a spectral width of 8000 Hz. Prior to Fourier transformation, the free induction decay was multiplied by an exponential weighting function corresponding to 0.5 Hz line broadening and zero filled by a factor of 2. Spectra were manually phased and baseline corrected and referenced to TMSP at 0.0 ppm.

NMR spectral data reduction and data analysis

All NMR data acquired above were then subjected to multivariate statistical analysis. The $^{1}H$ NMR spectra data were imported into the SIMCA-P+ (version 11.0; Umetrics, Umea, Sweden) software for the multivariate analysis. Prior to PCA, all NMR data variables were mean centered and Pareto scaled. Scores plots of the first two principal components (PCs) were used to visualize the separation of groups. The peak areas of the differential metabolites identified from loadings plot were further compared by independent-samples t-test, using SPSS 11.5 (SPSS, Chicago, IL, USA) with the threshold of $p$ value set at 0.05.

Results and Discussion

Behavior tests on CUMS rats and healthy controls

Body weight, sucrose preference test, and open-field test were measured during the experimental period to assess the depression model. Before the CUMS procedures, the mean body weight, sucrose preference percentage, crossing, rearing and immobility time between the stressed and control groups showed no significant difference (Table 1). After 3 weeks of experiment, the mean body weight increased in both groups, but the weight gain was significantly less in the CUMS group compared with the normal controls ($p < 0.01$). The reduced body weight gain induced by chronic stress was similar to the loss of appetite experienced by patients with depression.[14] Anhedonia, as previously described,[16] is a core symptom of depression, and sucrose preference is the hedonic measure that has been widely adopted. When faced with the choice between bottles of pure water and sucrose solution, rats normally prefer to drink the sucrose solution. In this study, sucrose consumption in stressed rats was significantly lower than that in the healthy controls ($p < 0.01$), indicating a state of CUMS-induced anhedonia. Meanwhile, the marked decrease of crossing ($p < 0.01$) and rearing numbers ($p < 0.01$), as well as increasing of immobility time ($p < 0.01$) in CUMS-treated rats, also were observed by day 21, as compared with the non-stressed rats. The reduced activity and curiosity of model animals also fit the clinical psychomotor symptoms of depression. In summary, the marked decrease of body weight gain, sucrose preference percentage, crossing and rearing numbers, as well as the significantly increased immobility time, all similar to the clinical symptoms of depression in humans, suggested the rats depression models were achieved after 3 weeks of CUMS treatment.

The perturbation of urinary metabolic profiling of CUMS rats

Figure 1 displayed the representative $^{1}H$ NMR spectra of urine samples from rats of the CUMS group and healthy controls after 3 weeks of stress procedures. Major identified endogenous metabolites were assigned and labeled in the spectra. The identification was based on chemical shifts, coupling constants, and multiplicity as described in previous reports.[9,17–21] In all, 20 endogenous urinary metabolites were identified, and each spectrum could be considered as a fingerprint of endogenous metabolites in urine. Visual inspection of these spectra did not give any clear differences between them. To extract any possible subtle change because of CUMS treatment, PCA analysis was applied. Before CUMS treatment, little separation between the two groups was observed (Fig. 2). During 3 weeks of exposure to CUMS procedures, clear separations between the stress and non-stressed groups were gradually achieved, and the maximum metabolic changes were observed at day 21 (Fig. 2D). The marked separation between the two groups, which was along the loadings of CUMS rats significantly increased immobility time, all similar to the clinical symptoms of depression in humans, suggested the rats depression models were achieved after 3 weeks of CUMS treatment, and the

Table 1. The dynamical changes of behavior scores of healthy control and model group

<table>
<thead>
<tr>
<th>Test items</th>
<th>–1 d</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight(g)</td>
<td>NS</td>
<td>239.81 ± 9.92</td>
<td>284.56 ± 13.50</td>
<td>294.81 ± 32.80</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>235.56 ± 18.16</td>
<td>264.75 ± 25.76</td>
<td>268.18 ± 26.75</td>
</tr>
<tr>
<td>Sucrose preference (%)</td>
<td>NS</td>
<td>79.69 ± 32.21</td>
<td>76.84 ± 2.83</td>
<td>76.71 ± 10.42</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>73.73 ± 11.83</td>
<td>78.29 ± 5.35</td>
<td>66.50 ± 7.07</td>
</tr>
<tr>
<td>Open-field test</td>
<td>Crossing numbers</td>
<td>NS</td>
<td>71.50 ± 37.96</td>
<td>63.00 ± 40.41*</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>90.13 ± 17.43</td>
<td>20.1 ± 14.43</td>
<td>19.37 ± 11.41</td>
</tr>
<tr>
<td>Rearing numbers</td>
<td>MS</td>
<td>11.66 ± 6.83</td>
<td>10.00 ± 7.48*</td>
<td>9.00 ± 5.04*</td>
</tr>
<tr>
<td>Immobility time (s)</td>
<td>NS</td>
<td>18.60 ± 44.42</td>
<td>49.32 ± 10.23</td>
<td>18.88 ± 23.59**</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>0.14 ± 0.39</td>
<td>70.23 ± 54.59</td>
<td>129.25 ± 59.95</td>
</tr>
</tbody>
</table>

The behavior scores in the control group (NS) and CUMS model group (MS) were expressed as mean ± SD ($n = 6$). Compared with CUMS group: * $p < 0.05$, ** $p < 0.01$. 


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differences between CUMS group and normal control group were more remarkable than individual differences of the rats. The significant urinary metabolic variation between the two groups also suggested that the CUMS depression model was achieved by day 21, which was in agreement with the results of behavior tests. The corresponding loadings plot, as shown in Fig. 3, was used to find the metabolites responsible for separation between the two groups. Independent-samples t-tests also were conducted on the normalized integral data of selected regions, which represented these metabolites, to detect the significance of these changes. The results showed that after exposure to CUMS procedures for 3 weeks, a number of important metabolites had altered significantly (Table 2). The CUMS group had higher levels of glycine, pyruvate, glutamine, and
asparagines and lower levels of 2-oxoglutarate, dimethylglycine, citrate, succinate, and acetate in urine. The marked changes of these endogenous metabolites were correlated with CUMS-induced depression.

In this study, the concentration of nine endogenous urinary metabolites, including organic acids, amino acids, and amines, were significantly affected by CUMS treatment. Citrate, 2-oxoglutarate and succinate, key intermediate products of tricarboxylic acid cycle (TCA), are associated with energy metabolism. Pyruvate can be converted by the pyruvate dehydrogenase complex to acetyl CoA, which further enters the TCA cycle. The TCA cycle is an important biological metabolic pathway in the body; it involves not only the glucose aerobic oxidation but also the major pathways for fat and amino acid metabolisms.[22] The lower level of citrate, 2-oxoglutarate, and succinate and high level of pyruvate in the urine of CUMS-treated rats were indicative of TCA cycle dysfunction. The energy deficiency, one of the most represented depressive symptoms, is connected with reduced activity and curiosity in some way.[31] Acetate is produced from acetyl CoA through acetyl phosphate, and the decreased urinary level also revealed a disorder of energy metabolism.[23]

Glycine, a nonessential amino acid, is served as an inhibitory neurotransmitter in the central system.[11] The increased urinary level of glycine in CUMS rats may suggest the injury of hepatic mitochondria, which causes suppressed dynamic glycine cleavage system.[24] Dimethylglycine (DMG), produced when the body metabolizes choline into glycine,[25] the decreased level in the urine of CUMS rats suggested that the biosynthetic pathway of glycine and choline also may be disturbed by CUMS procedures. In addition, the glutamine and asparagine were increased in the CUMS group, as compared with the healthy controls, suggesting that the glutamine and asparagine biosynthesis also may be affected by CUMS procedures.

The concentrations of some unidentified sugars in the CUMS group were increased significantly. It was reported that depression was associated with glucose metabolism in the biological mechanisms, and glucose metabolism may be affected by the abnormal secretion of depression-related hormone.[26]

We found that many altered metabolites were involved in liver metabolism. Increase of glycine and decrease of TCA cycle intermediates suggested the injury of hepatic mitochondrial.[24] Previous studies have shown that injury of liver function was related to the symptom of liver qi stagnation,[27] which was thought to be the onset of depression according to the TCM theory.

### Conclusion

A metabonomic method based on NMR has been used to study the urinary metabolic variation in CUMS-induced rat depression model. Combined with the results of behavioral tests, we could conclude that the depressive disorders were induced by CUMS. With multivariate statistical analysis, a clear separation between model group and healthy controls was observed after 3 weeks of CUMS treatment, and 9 endogenous urinary metabolites contributing to the separation were identified.

Compared with our previous urinary metabonomic study of CUMS by GC-MS, there are some differences in this study. First, although NMR and MS have been successfully used for metabolic fingerprinting analysis, these two techniques have their respective advantages and limitations and are often discussed as being complementary.[28,29] In this study, the levels of nine urinary

### Table 2. Endogenous urinary metabolites responsible for the classification of CUMS group

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Peak regions</th>
<th>Change trend compared with controlsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>3.52 d</td>
<td>↑</td>
</tr>
<tr>
<td>2-OG</td>
<td>2.44 t, 3.00 t</td>
<td>↓</td>
</tr>
<tr>
<td>DMG</td>
<td>2.92 s</td>
<td>↓</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.52 d, 2.68 d</td>
<td>↓</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.40 s</td>
<td>↓</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.92 s</td>
<td>↓</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>2.36 s</td>
<td>↑</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.48 m, 3.80 m</td>
<td>↑</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.88 m, 2.96 m, 4.00 m</td>
<td>↑</td>
</tr>
</tbody>
</table>

Peak area of endogenous urinary metabolites in the control group (NS) and CUMS model group (MS) were expressed as mean ± S.D (n = 6). “↑” and “↓” denote higher or lower amounts relative to the healthy controls.

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![Figure 3. The loadings plot of PCA from the control group (■) and the CUMS group (△) at day 21.](image-url)
metabolites were found significantly changed in CUMS rats. Although the GC-MS-based study revealed 13 altered urinary metabolites including four unknown metabolites in CUMS-induced depression rats. Obviously, the biomarkers identified in the two studies were different. In addition, sugars could not be detected by the derivatization method in GC-MS. However, 1H NMR spectra of urine clearly revealed the presence of sugars, such as α-glucose and β-glucose. Second, the dynamical changes of behavior scores and scores plots of healthy controls and the model groups at different time points were analyzed in this study, and the results of both behavior and metabonomic analysis proved that the depression model was achieved after 3 weeks study, and the results of both behavior and metabonomic analysis proved that the depression model was achieved after 3 weeks of CUMS treatment. Third, biochemical interpretation of possible biomarkers of CUMS-induced depression was discussed, and the possible relationship between liver qi stagnation and the state of depression was analyzed.

This work shows that CUMS may be employed as a reliable model for depression research, and the noninvasive urinary metabonomic method is a valuable tool to investigate the biochemical perturbations in depression as an early diagnostic means.

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