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A urinary metabolomics study of the metabolic dysfunction and the regulation effect of citalopram in the rats exposed to chronic unpredictable mild stress

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Abstract

Depression is a widespread mental disorder, however the molecular mechanism underlying depression and antidepressive effects of diverse drugs remain largely unknown. An untargeted metabolic profiling based on Ultra High Performance Liquid Chromatography coupled with Orbitrap mass spectrometry (UPLC-Orbitrap-MS) had been performed to investigate the metabolic changes between chronic unpredictable mild stress (CUMS) rats and healthy control rats, and screen for drug efficacy on these changed metabolites after administration of citalopram, then provide a new perspective into the understanding for antidepressant effect of citalopram. Behavioral tests were applied as a measurement of status of depression and the antidepressive effect. Multivariate statistics including principal component analysis (PCA), partial least squares–discriminate analysis (PLS-DA) had been applied to reveal the metabolic changes between control, model and antidepressant-treated group. Clear separation among three groups was achieved and a total of 26 metabolites had been considered as the potential biomarkers involved in the development of depression. Among them, 24 metabolites display the trend to return to normal level which may correlate to the regulation of drug treatment on depression, and specifically, 10 of them could be the key metabolites in the pathogenesis of depression and contribute to evaluate the effect of citalopram. These results provided new insight into the pathophysiological mechanism of depression and indicated that citalopram mediated synergistically abnormalities in metabolic network including tryptophan metabolism, energy metabolism and synthesis of neurotransmitters, which may be helpful to evaluate its effectiveness by a metabolomics approach.

Key words: Depression; Metabolomics; Chronic unpredictable mild stress; UPLC-Orbitrap-MS

1. Introduction

Nowadays, depression has been known as one of the most common psychiatric illnesses with prevalence of 15%-20% around the world[1]. A new WHO report predicts that depression will be the second most crucial cause of suicide and disability by 2020[2]. Different kinds of chronic stressful events have been proved to be the most significant environmental factor in the etiology of the disease[3]. Currently, the common treatments for clinical depression can be divided into two categories: psychotherapy and antidepressant medications. There are several kinds of antidepressant medications such as the monoamine oxidase inhibitors (MOIs), the selective serotonin reuptake inhibitors (SSRIs), and tricyclic antidepressants (TCAs)[4]. Among them the most widely and popular used is the SSRIs which consists of citalopram, sertraline, fluoxetine *etc*.

Chronic unpredictable mild stress (CUMS) model has been proved a valid and reliable model of depression for the reason that its symptoms are similar to human who suffer from depression[5]. Previous studies showed that

when after a period of CUMS exposure, animals would undergo a series of behavioral changes such as anxious and fretful actions along with some metabolic changes, while these changes could be reversed by antidepressive drug treatment[5, 6]. Therefore, the CUMS model has been widely used for the study of mechanism and the biochemical changes in depression.

Previously, much of attention has been paid closely to the genomics (the gene expression) and the proteomics (the protein function) due to the severity of depression[7, 8]. The emerging field of metabolomics, which devotes to capturing the metabolic information at the global level, comes into our sight and offers us a promising chance to come up with new hypotheseswhen study the molecular mechanisms of diverse diseases. Metabolomics places emphasis on the induced biochemical perturbations according to changing metabolome in a specific period, which can indicate our pathophysiological status [9]. It has been widely used to investigate the potential biomarker of several diseases and evaluate the drug effects as a versatile tool[10, 11].

It is common to combine multiple analytical techniques in order to get the biochemical information as much as possible, and Nuclear Magnetic Resonance (NMR), gas chromatography/mass spectrometry (GC/MS) and liquid

chromatography/mass spectrometry (LC/MS) are three common analytical instruments in metabolomics research[12-14]. LC/MS has advantages in that LC provides better separation capabilities than GC with complex biological samples and MS has greater sensitivity for qualitative and quantitative analysis than NMR For complex and mass data set acquired in metabolomics research, multivariate statistics have been applied to extract the important information and visualize the differences between groups by the score map[15, 16].

The primary goal of this work is to reveal the tiny changes of metabolites in the CUMS model, then screen for the related pathways and investigate the therapeutic effects of citalopram according to the changes of metabolites involved in the perturbed pathways by a metabolomics approach. Moreover, we also establish a method to rank the level of depression by combining the results of behavioral tests and the changed metabolome, and this method is validated by the antidepressant-treated group. It is very necessary and useful to discover the biomarkers which can evaluate the efficiency of the therapeutic effect of antidepressive drugs at the early stage and assess the depressive status according to the metabolome changing, so a high-resolution mass spectrometry (Orbitrap-MS) has been applied to profile the urinary samples of control, model and antidepressant-treated group. Metabolites counted in the distinction of three groups are identified as the potential biomarkers, and the regulated metabolites after drug administration are taken for the biomarkers of the treatment effect of citalopram. This study is the first attempt to combine the exploration of biomarkers of depression and evaluation of therapeutic effect of citalopram in CUMS

model rats by a metabolomics method, it can not only provide convincible biomarkers to evaluate drug effect of citalopram, but also deepen the understanding of CUMS-induced metabolic perturbation and it help to access the depressive state by combing the behavioral tests and the biomarkers.

2. Experimental

2.1. Chemical and materials

Methanol, acetonitrile, formic acid (HPLC grade) were purchased from Merck (Merck, Darmstadt, Germany). Deionized water was acquired from Milli-Q50SP Reagent system (Millipore Corporation, MA, USA). Citalopram hydrobromide was obtained from Sigma-Aldrich (MO, USA).

2.2. Animals

18 eight-week-old male Sprague-Dawley rats were commercially purchased from Shanghai Laboratory Animal Co. Ltd. (SLAC, Shanghai, China). Rats were kept at a laboratory animal barrier system with required environment (temperature of 24±1°C, relative humidity of 45±15%, and a 12h light/dark cycle) with access to food and water ad libitum. The study was approved by national legislations of China and local guidelines and the experiments were performed according to a protocol approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

2.3. Chronic unpredictable mild stress (CUMS) procedure

After 2 weeks of acclimatization, the rats were divided into three groups, control group (unstressed), model group (stressed) and model group treated with citalopram according to the results of behavioral test and each group contained 6 rats. The rats in three groups were housed individually. The control group had free access to the food and tap water, excepted for a 20 hour food and water deprivation before each sucrose preference test. The model group and the antidepressant–treated group were randomly exposed to a series of chronic unpredictable mild stressors in the first three weeks, then the antidepressant–treated group received antidepressive drug administration while both of two groups were still receiving the CUMS procedure. Citalopram was dissolved in physiological saline and intraperitoneally injected at 9:00 daily in the morning at the dosage of 10 mg/kg. Meanwhile the rats in another two groups were administered with the equivalent volume of physiological saline. The experimental design is shown in Fig.1. The CUMS procedure was conducted according to protocol[17], and stressors contained food or/and water deprivation, 45°cage tilting along the vertical axis, paired housing, soiled cage (300 ml water spilled into the padding), stroboscopic illumination (200 flashes/min), continuous overnight illumination, and white noise (85db). The detailed schedule is displayed in Table.1.

2.4. Behavioral test

2.4.1. Sucrose preference test

The sucrose preference test (SPT) was conducted as a measurement of anhedonic effect inrats which exposed to CUMS. In this test, after a 20-hour period of water and food deprivation, each rat was put into an individual cage where placed two bottles of different solution, tap water and 1% sucrose solution. During the 2-hour test, every rat stayed in a non-stressed environment and had open access to two kinds of solution. Sucrose preference (SP) means the ratio of the amount of sucrose solution to that of total solution ingested in two hour [SP = sucrose consumption/(sucrose consumption + water consumption) *100%] and it is defined as a measurement of anhedonia[17].

2.4.2. Open-field test

The open-field test (OPT) was done in a quiet environment (<65db) between 13:00 and 15:00 p.m. The open field ground consisted of a black background floor which had been divided into 25 equal-size squares and a 40-cm-high side wall. Each rat was gently put into the central square under dim light and adapted for 30 seconds, then took the 5-minutes-long test. The open-field was cleaned by 70% ethanol after each test. According to the locomotor activity such as the time spent in the center square and the frequency of rearing, they were recorded as a measurement to evaluate the status of rats[18].

2.4.3. Forced swimming test

The forced swimming test (FST) is a valid test when evaluating the status of depression in the animals which undergo the CUMS procedure and the antidepressant effects of drugs. The FST was conducted as described in the previous study[19]. Briefly, the animals were individually placed in a plexiglas cylindrical (50cm in height and 18cm in diameter) filled with water (24±1°C) up to a height of 25 cm. Water was changed after each test. The total immobility period during the 5-minutes-test was recorded with the help of stopwatch. The immobility status means that when a rat just floats passively and just moves to keep its nose above water for survival.

2.5. Behavioral data statistical analysis

All the data acquired from behavioral test were organized into the form of mean±standard deviation. Data were analyzed by using SPSS 17.0 software for Windows (Chicago, IL, USA). The behavioral data of SPT and FST were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc LSD test. The data of OPT was a non-normal distribution, so the non-parametric tests (the Kruskal-Wallis test) was applied to analyze it. The significance level was set at $p<0.05$.

2.6. Sample collection and preparation

After the final behavioral test at week 6, when the FST, OPT and SPT has been done, the collection of urine sample then begin at 9:00 next morning. All rats were transferred into the metabolic cage in a non-stressed environment to collect the urine samples. During the collection period, collecting tubes were put on ice to avoid the enzymatic degradation. All samples were stored at -80°C immediately.

Prior to analysis, urine samples were thawed at room temperature and diluted at a ratio of 1:3 with methanol (v/v) to remove the large-molecular-weight proteins, then were centrifuged at 11000g for 10 minutes. The supernatants were diluted at a ratio of 1:3 with water (v/v) and then transferred to a vial for UPLC-Orbitrap-MS analysis.

2.7. UPLC-Orbitrap-MS analysis

Metabolic profiling was performed on a UPLC Ultimate 3000 system (Dionex, Germering, Germany), coupled to an Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) in both positive and negative mode simultaneously. Both the UPLC and the Orbitrap mass spectrometer system were controlled by the Xcalibur 2.2 software (Thermo Fisher Scientific).

The chromatographic separation was performed on a 1.9μm Hypersile Gold C18 column (100mm×2.1mm) (Thermo Fisher Scientific), and the column was maintained at 40°C. A multistep gradient consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) had been applied and the gradient operated at a flow rate of 0.4 mL/min by linearly increasing solvent B from 5% to 95% over 15 min, then the column was washed with 95% solvent B for 2 min and re-equilibrated in 5% solvent B. The UPLC autosampler temperature [Ultimate WPS-3000 UPLC system (Dionex, Germering, Germany)] was set at 4 °C and the injection volume for each sample was 5 μL.

MS data were collected by the Orbitrap mass spectrometer equipped with a heated electrospray source (HESI) at the resolution of 700,000. For both positive and negative mode, the operating parameters were as follows: a spray voltage of 3 kV, the capillary temperature of 300°C, sheath gas flow of 40 arbitrary units, auxiliary gas flow of 10 arbitrary units, sweep gas of 2 arbitrary units and S-Lens RF level of 50. In the full scan analysis (70 to 1050 amu), the resolution was set at 700,000 with an automatic gain control (AGC) target of 1×10^6 charges and a maximum injection time (IT) of 120 ms. The energy of higher energy collision dissociation (HCD) is set at 70.0eV in MS/MS experiment.

The quality control (QC) samples had been prepared by pooling same volume of urine from all samples and analyzed every 10 samples to ensure the stability and repeatability. The mass spectrometry was calibrated every 24 hour while the profiling to ensure the mass accuracy.

2.8. Data analysis

All the profiling raw data files were produced by the SIEVE software (Thermo Fisher Scientific) where data pretreatment procedures such as baseline correction, peak deconvolution and peak realignment had been done. This progress produced a table organized in a three-dimensional matrix, including annotated peak indices (RT-m/z pairs), sample names (observations), and intensity of each sample (i.e. peak area). The multivariate statistics was performed by the SIMCA-P 13.0 software (Umetrics, Umea, Sweden)[20]. All data were mean-scaled and imported into the software for unsupervised PCA to identify the tiny differences among the samples. The PCA helped to reduce the high dimensional spectral variation into a two or three principal components (PCs) without losing the vast majority of information. The PCA score map was used to visualize the possible distribution the clustering or the grouping in the observations. In order to improve the classification, offer pairwise comparison between three groups, and search for the changed metabolites induced by CUMS procedure, the partial least squares discriminant analysis (PLS–DA) had been performed in this study. The dataset acquired from model and control group was chosen to conduct the PLS-DA model, then the dataset of antidepressant-treated group was used to validate and test the predictive ability of the model. Then the variable importance in the projection (VIP) analysis was applied to obtain the metabolites which counted most in the distinction of three groups and the variables with VIP values greater than 1.0 were considered statistically significant in this model.

Furthermore, unpaired Student's t-test carried out by R language (http:www.r-project.org/) was used to identify differential metabolites between groups. The q-value was employed to address the multiple testing[21]. The q-value was calculated by the package q-value of R language and indicated that an estimated possibility of the metabolites to that point were different among the three groups simply by chance. The compounds with q-value less than 0.05 and VIP values greater than 1.0 were defined to be statistically significant.

3. Results

3.1. Behavioral testing

The SPT was doing according to the experiment design mentioned above, and the SP of the three groups in different periods was displayed in Fig.2. After first three weeks of CUMS exposure, significantly decreased SP, depressive-like action such as anxious and aggressive behaviors were observed in model group and antidepressant-treated group before drug administration. In the next three weeks, after drug administration and continuously exposed to CUMS procedure, the SP of model group went down significantly ($p<0.01$), while antidepressant-treated group started to recover compared with model group ($p<0.05$), which indicated the model had been successfully conducted and citalopram did work.

For the OPT, the locomotor activity was measured twice during the CUMS period, time spent in center square and the frequency of rearing were recorded as an evaluation of stress status[17]. The results of OPT at week 3 and week 6 were shown in Fig.3. At week 3, rats in both model group and antidepressant-treated group before drug administration spent much less time in the center square ($p<0.05$ and $p<0.05$, respectively). After received the antidepressive drug treatment, the antidepressant-treated group showed a longer time staying in the center in contrast with model group ($p<0.05$). In the previous study, the time spent in the center square could be a measurement of the degree of anxiety, which indicated animals displayed high activity level in the center could be regarded as less anxious. For the frequency of rearing, it was greatly decreased in the model group compared with the control group and the antidepressant-treated group ($p<0.01$ and $p<0.05$, respectively) at week 6. The reduction of the rearing frequency and the shorter time in central area indicated a decrease in exploration ability in model group[18]. These results demonstrated that depressive-like behaviors were observed in model group and these actions improved due to the regulation of citalopram.

In the FST, the result was displayed in Fig.4. when after exposed to CUMS for three weeks, the immobility time (floating state) was significantly increased in two groups compared with control group ($p<0.05$, $p<0.05$, respectively), and then after antidepressive treatment while exposed to CUMS at the same time, the immobility time of antidepressant-treated group began to decrease. At week 6, the immobility time in the model group showed a significant increase compared with the control and antidepressant-treated group ($p<0.01$, $p<0.05$, respectively). Additionally, the immobility time between control and antidepressant-treated group did not achieve statistical significance ($p>0.05$), which indicated the increase in the immobility time was closely related to the depressive state.

3.2. Metabolomics profiling

The urinary metabolic profiling of control, model and antidepressant-treated group were obtained by UPLC-Orbitrap-MS with the pre-described condition. Full scan mode was engaged to investigate the urinary metabolites in both positive and negative modes simultaneously. A total of 3525 features, 2507 in positive mode and 1018 in negative mode were obtained. A three-component PCA model was initially obtained with dataset which was extracted and pretreated by SIEVE software. 66.7% of the total variations were accumulated within the first three components. The score plot（Fig.5a.）showed a relative good separation between three groups which preliminarily indicated the different metabolic pattern between groups.

3.3. Prediction of depression ranking The PLS-DA score plots showed that the metabolic profiles of model rats deviated from those of control and antidepressant-treated group ($R^2Y = 0.969$, $Q^2 = 0.861$; Fig.5b.) and

suggested that significant biochemical changes were induced by CUMS. More specifically, we tried to combine results of all the different behavioral test so as to get a comprehensive indicator to reflect the depression status, but unfortunately, it is difficult to find a gold standard to give the weight of each result of the behavioral test. In order to state the depression level as accurate as possible according to practical situation, we chose the SP as the measurement of depression for its wide use in the CUMS model. So in the PLS-DA model, we defined the SP as a Y variable, and the metabolic dataset of three groups was set as the X variable, then autofit the model in order to study the relationship between the changed–metabolome and the depression status.

3.4. The score plot showed that the control and model group were clearly distinguished from each other and the antidepressant–treated group also had distinctive pattern compared with the model group, while after drug administration, the clusters of antidepressant-treated group was moving towards the control group especially in the first component. The key parameters for assessing modeling quality (R^2Y=0.910 and Q^2=0.733) both were larger than 0.5, which suggested that this model was predictive and robust. Furthermore, we applied a permutation test to validate the PLS-DA model (Fig.5.c), and the validation plot showed that Q^2 and R^2 were higher than those corresponding permutated Q^2 and R^2 values at left side. These results not only mean that the PLS-DA model was robust and had a relative good predictive power when facing new observations, but also demonstrate that it is practicable to rank the level of depression and evaluate the drug effect according to the metabolome changes along with the behavioral test results by a metabolomics method.**Searching for identity of biochemical changes**

The variable importance for projection (VIP) statistics was obtained from the PLS-DA and had been applied for the selection of discriminational variables among the three groups. By considering the q-value, along with the VIP-value, we revealed that there were 26 metabolites were significantly changed (q-value<0.05, VIP-value>1.0) when exposed to the CUMS and then after drug administration, 24 metabolites changed before turned out a trend to return to normal. Specifically, 10 of them were significantly changed (q-value<0.05) in the urine of antidepressant-treated group compared with model group and had no statistically significant difference with control group. These results indicated that the 26 metabolites could be the potential biomarkers of depression and the 10 significant changed metabolites were the key metabolites and could be the biomarkers to evaluate the drug effect of citalopram from the perspective of metabolomics.

3.5. Metabolite identification

The significantly changed metabolites indicated by q-value and VIP-value were presented in the form of retention time and m/z pairs. A library consisting 493 authentic chemicals with high accurate m/z and retention

time in pre-described condition were established in our laboratory for identification of metabolomics analysis. The library was conducted with the same column for metabolomics analysis and a model transfer protocol was created to ensure the retention time shift when changing column. Briefly, high retention time reproducing chromatography columns were carefully selected with no supplier change; the standards were carefully grouped and retention time were re-acquired when columns changed. By comparing the retention time and high accurate m/z with specific authentic standards, the identification was then delivered. For the high resolution of Orbitrap mass spectrometry, it is exact enough for the identification by using the retention time and high accurate m/z and MS/MS spectra are further compared with those in our library.

21 of the compounds were confirmed by comparing retention time and high accurate m/z with the local library and 16 of them were further validated by MS/MS spectra. The rest 5 metabolites without authentic chemicals was identified by searching the HMDB (http://www.hmdb.ca/) database for candidates with similar molecular weight in the tolerance of 5ppm. According to the fold change and q-value, there were 10 metabolites significantly changed after antidepressant treatment including glycerol, creatine, quinolinic acid, L-phenylalanine, kynurenic acid, Gamma-aminobutyric acid, L-kynurenine, L-isoleucine, N-acetyl-L-aspartic acid, indoleacetic acid. The list of 26 significantly changed metabolites after exposed to CUMS and the 10 key biomarkers that could be useful to offer a better understanding of antidepressant effect of citalopram was shown in Table.2.

4. Discussion

4.1. CUMS-induced metabolomic changes

After the CUMS exposure, metabolic changes were observed and this was consisted with the behavioral tests. In our research, compared with the control group, 26 metabolites were significantly altered in the model group and the related metabolic pathways were shown in Fig.6.

4.2. The therapeutic effect of citalopram in the rats exposed to CUMS

When rats in antidepressant-treated group received the administration of citalopram, 24 of the 26 significantly-changed metabolites mentioned above displayed the trend to return to normal level, and specifically, the level of glycerol, creatine, quinolinic acid, phenylalanine, kynurenic acid, gamma-aminobutyric acid, kynurenine, isoleucine, N-acetyl-L-aspartic acid, indoleacetic acid was significantly changed compared with model group due to the regulation of citalopram, which meant they were useful in evaluation of citalopram's therapeutic effect.

Furthermore, the PLS-DA showed that the cluster of antidepressant-treated group was moving towards to the control group especially in the first component, which was consist with the results of behavioral test, and this

297 indicated that the PLS-DA model has a good predictive ability and meant is was promising to combine the changed
298 metabolome and the behavioral test to rank the level of depression in the following research.

299 **4.3 Biochemical interpretation**

300 **4.3.1. Amino acids metabolism**

301 Tryptophan (Trp) and the related metabolites kynurenine (Kyn), kynurenic acid (KA), 5-hydroxyindoleacetic
302 acid (5-HIAA), 4,6-dihydroxyquinoline, quinolinic acid (QUIN), indoxyl sulfate, indole-3-carboxylic acid and
303 xanthurenic acid (XA) were significantly changed in the urine of rats in model group. Trp is an essential amino
304 acid and the metabolism of it is quite complicated in our body. Except for synthesizing proteins, there are three
305 main metabolic pathways: indoleacetic acid (IAA) pathway, Kyn pathway and serotonin (5-HT) pathway[22]. Trp
306 can be converted into formylkynurenine by L-tryptophan-2, 3-dioxygenase (TDO) or indoleamine-2,
307 3-dioxygenase (IDO), and then kynurenine formamidase converts it into Kyn. Kyn is the first key branch point in
308 Trp's catabolic pathway which undergoes a series of catabolic reactions producing KA, XA or the QUIN. Previous
309 study has reported that when suffering depression, the increasing proinflammatory cytokines can active IDO,
310 which results in the decrease of Trp[23]. It is consistent with the results in our study, which shows a large decline of
311 Trp in urine of model group compared with the control group. 5-HIAA is a main breakdown product of serotonin
312 that is excreted in the urine, and the significant decline of 5-HIAA indicates the low level of its precursor 5-HT. As
313 a monoamine neurotransmitter, the lower level of 5-HT has been reported in the previous study which has the
314 relationship with the development with depression according to the monoamine neurotransmitter hypothesis[24, 25].
315 The decrease of 5-HIAA and Trp, combined with the increasing concentration of Kyn, we can infer that the Kyn
316 pathway has been promoted which leads to reduce the availability of Trp for conversion into 5-HT. In the
317 xanthurenic pathway, the increased concentration of XA may indicate that the level of its precursor
318 L-3-hydroxykynurenine (L-3-HK) had also risen. An elevation of L-3-HK levels had been shown to constitute a
319 significant hazard in situations of excitotoxic injury and cause the death of neuronal cells which may be the
320 inducement of depression and some other CNS diseases, and the increasing level of XA founded in our research
321 was also consists with the results in the depression patients.

322 When Trp is catabolized into Kyn, the metabolism of Trp mainly comes into the kynurenine pathway in which
323 the product of it can affect the neuroprotective–neurodegenerative balance in the brain. Kyn can be further
324 metabolized into two main pathways, the toxic quinolinic pathway and the kynurenic pathway. Firstly, Kyn can be
325 converted into 3-hydroxy kynurenine which is the bioprecursor of QUIN by kynurenine-3-monoxygenase.
326 3-hydroxy kynurenine is a free radical generator, and it can cause neurons apoptosis and neurodegenerative

changes in brain. 3-hydroxy kynurenine can be further catabolized into QUIN, which is a kind of endogenous excitotoxic material and can lead to many neurodegenerative disorders. However, Kyn can also be metabolized to KA which proved to be a NMDA receptor antagonist and have protective effects against QUIN by kynurenine aminotransferase[26]. The metabolic abnormalities of Kyn can disequilibrate the balance of the neuroprotective–neurodegenerative metabolites. In our research, the concentration of KA in model group was significantly lower than control group, which suggested that the metabolism of Kyn was mainly going into the quinolinic pathway. The lower level of Kyn and the up-regulation level of QUIN was consist with it. The increasing level of QUIN and the decreasing level of KA led to the imbalance in the neuroprotective and neurodegenerative metabolites which could be a hypothesis to further study the mechanism of depression and on this basis to evaluate the effect of citalopram.

KA is an endogenous antagonist of the excitatory amino acid receptors which plays an important role in the protection of nervous system and anticonvulsive activities. In some animal models of neurodegenerative diseases, KA has been proved that it has the anticonvulsive and neuroprotective functions. As is mentioned above, the higher level of QUIN could cause the apoptosis of astrocytes, and this would lead to the lower neuroprotective activity against QUIN, and worse still, this may further caused the apoptosis of astrocytes, then result in consecutive down-regulation of KA, which makes the balance of neuroprotective–neurodegenerative metabolites worse and worse in the brain[27]. As a neuroprotective metabolite, KA has been reported that it is closely connected to the pathogenesis of some neurological diseases with age, such as the Parkinson's disease and Alzheimer's disease and patients suffered severe metabolic disorder of KA in the ageing progress[28, 29]. This remarkable profile of KA metabolism alterations in the mammalian brain has been suggested to result from the development of the organization of neuronal connections and synaptic plasticity, development of receptor recognition sites, maturation and ageing. When after the drug administration, it was observed that the therapy could prevent the down-regulation of KA which indicated that citalopram could have effect on the regulation of the dysfunction in the tryptophan metabolism. When the Kyn metabolizes into the toxic quinolinic pathway, in which the excitotoxic QUIN is produced and this may leads to an imbalance of neuroprotective and the excitotoxic metabolites. When released by the activated macrophages, QUIN can act as a kind of endogenous excitotoxic material which is related to many psychiatric disorders. Previous study proved that in the urine of depressed patients, there was a significant higher level of 3-hydroxykynurenine (3-HK) that could result in the producing of QUIN than normal people[30]. And in our study, although we did not observe the up-regulated level of 3-HK, we found the level of QUIN in the CUMS group was significant higher than the control group, and after treatment it had been down-regulated in

antidepressant-treated group compared with the model group, which indicated QUIN could be a biomarker of the disorder of tryptophan metabolism and then served as a tool for investigation of the pathogenesis of depression and pharmaceutical effect of citalopram. A lot of researches to study the mechanism of depression mechanism have concentrated on the changed level of 5-HT, and have proved that depression has a close relation to the decline of 5-HT[31, 32]. Our research showed that the metabolic disturbance of tryptophan not only led to the decline of 5-HT, but also a significant increase of QUIN. When the increased level QUIN reaches the pathological concentration, it can over-activated the NMDA receptor and the metabotropic glutamate receptors which can cause the nerve damage. Moreover, the up-regulation of QUIN can also inhibit the reabsorption of glutamic acid in the gliocyte, which will intensify the excitotoxic effect in the CNS.

Isoleucine (Ile), as a kind of branched chain essential amino acid, plays a critical role in the human life and has been proved particularly involved in stress, energy and muscle metabolism. In our body, Ile can provide the amino when in the synthesis of glutamate, which has been regarded as an important neurotransmitter in the central nervous system, by passing the blood-brain-barrier. It has been reported that the damaged homeostasis of glutamate and glutamatergic neurotransmission may be closely linked to the development of depression[33]. Moreover, Ile can combine with glutamine to form the glutaminyl-isoleucine, which is a kind of dipeptide and play an important role in the locomotor behavior. Previous researches have shown that when the rats received the injection of glutaminyl-isoleucine into ventral tegmental area, which can transmit afferent glutamatergic projections to the prefrontal cortex, and this can greatly improve the locomotor behavior. In our research, after drug administration, the significantly down-regulated level of Ile had return to normal, and before treatment the low Ile level was accompanied by the low degree of locomotor behavior in CUMS model group. On account of the finding, it is necessary to further look into the relationship between Ile and the glutamatergic activity during the development of depression. Besides, Ile can be converted into both carbohydrates and fats as it is an essential and ketogenic amino acid, which means it can also affect the energy metabolism. In the citric acid circle, it can be converted into succinyl CoA in the presence of alpha-ketoglutarate, which suggests that CUMS may also has impact on the energy metabolism.

L-Serine can be biosynthesized from glycine which is a well-acknowledged inhibitory neurotransmitter. Furthermore, a D/L-Serine racemase can link the formation of D-Serine and glycine to the L-Serine metabolism[34, 35]. In our body, the function of L-Serine is not only offering the nucleotide precursors during the cell proliferation, but also has the trophic effects[36]. Glycine is one of the well-known inhibitory neurotransmitter and the role of glycine is to regulate the locomotor behavior. It has been reported that in the plasma and urine of the patients who

suffered the schizophrenia, the concentration of serine and glycine had evaluated[37]. However, the changes of the serine and glycine in patients suffering psychiatric disorders have not been confirmed because there are some different results in other study[38-40], which means it is necessary to focus on the regulation of the pathways involved and the specific activity of particular enzymes to get a better understanding and biochemical interpretation of L-Serine, D-Serine and glycine.

4.3.2. Synthesis of neurotransmitter

In the metabolic pathway of phenylalanine (Phe) and tyrosine (Tyr), the concentration of Phe and its metabolite phenylpyruvic acid was significantly increased, and the level of dopamine and its precursor L-dopa was decreased in the model group compared with the control group. Phe is an essential amino acid which can be incorporated into cellular proteins, or converted to phenylpyruvic acid, and it is the precursor for the amino acid tyrosine (Tyr). Phe can be converted into Tyr in the liver and then, as a precursor for L-dopa, Tyr can be further metabolized into the neurotransmitters dopamine, norepinephrine and epinephrine. Half of the Phe would go into the biosynthesis of Tyr through phenylalanine hydroxylase. Research showed that depressed patients had a higher ratio of Phe-Tyr, which meant there could be a dysfunction of phenylalanine hydroxylase[41, 42]. According to the results, it could be found that more Phe had been metabolized into phenylpyruvic acid, along with the low level of L-dopa and its metabolite dopamine, we could deduce that CUMS caused the metabolic disturbance of Phe and led to the low level of Tyr. The reason why we didn't detect Tyr may be that Tyr had been rapidly metabolized, so it was not found in large concentrations throughout the body. When the Tyr has been down-regulated, the biosynthesis of its metabolites such as L-dopa, dopamine, norepinephrine and epinephrine could be disturbed, and lower level of L-dopa is consist with this. When under stress, it is suggested that people need more Tyr so that the supplement of it could prevent the stress-induced depletion of norepinephrine. The metabolic disturbance of Phe and Tyr has closely relationship with depression, and could be the biomarkers to study the mechanism of depression along with to evaluate the effect of the treatment for depression by citalopram.

Gamma-aminobutyric acid (GABA) is a chief inhibitory neurotransmitter in the nervous system, which can act at inhibitory synapses in the brain. GABA works by binding to specific receptors in the membrane of both pre- and postsynaptic neurons. GABA can be synthesized from glutamate with the action of L-glutamic acid decarboxylase and pyridoxal phosphate. The drugs which can increase the available level of GABA or work as the agonists of GABA receptors have been reported have anti-anxiety and anti-convulsive effects. It has been reported that the dysfunction of GABA neurotransmitter system has great relationship with depression and has been observed that there is an increased level of GABA in occipital cortex of depressed patients when receive the

treatment of SSRI[43, 44]. Moreover, previous study also showed that GABA was involved in the pathogenesis of some anxiety and mood disorders, while the mechanisms of the decreasing level of GABA was not quite sure[44]. The significantly reduced concentration of GABA observed in our research could be due to the dysfunction of GABA synthesis which resulted from the reduction of glutamatergic stimulation along with the decreasing level of necessary substrate involved in the synthesis of GABA. Moreover, the reduced level of GABA also can partly reflect the abnormal activity of glutamic acid decarboxylase which has been observed that in depressed patients and some environmental factors also can influence the regulation of the glutamic acid decarboxylase activity[45]. In this research, the result is consists with the previous study, which shows a significant down-regulation level of GABA, and moreover, the GABA level then up-regulated after treatment, which meant the urinary level of GABA could be very useful in the diagnosis of depression and be the trait biomarker for evaluating the effect of citalopram by a metabolomics method.

N-Acetylaspartic acid (NAA) is a derivative of aspartic acid which is a major excitatory neurotransmitter and can provide resistance to fatigue. NAA can be biosynthesized by aspartic acid and acetyl-CoA in neuronal mitochondria. In the CNS, NAA is the second most concentrated molecule in the brain just after glutamate which is the most abundant fast excitatory neurotransmitter. As a neuronal osmolyte, NAA can maintain the fluid balance in the brain, and it is a source of acetate for lipid and myelin synthesis in oligodendrocytes, the glial cells that myelinate neuronal axons. Moreover, it is also the precursor in the synthesis of N-acetylaspartylglutamate which is an important neuronal dipeptide, and NAA play role in the energy and lipid metabolism. It has been observed that the level of NAA is significantly down-regulated in depressed patients and in patients with brain atrophy[46]. However, the dysregulation of NAA in CUMS rats is not consistent with each other for the differences in measurement equipment and samples[47-49]. In our study, we observed a significant down-regulation level of NAA in CUMS group and then it had been corrected after drug administration, the difference among the CUMS group, control group and drug administration group had statistical significance.

4.3.3. Energy metabolism

The pyruvic acid, citric acid, creatine, hypoxanthine and succinic acid are another five key metabolites which play an important role in the discrimination of the three groups. These metabolites are crucial in the citric acid cycle which is associated in the energy metabolism. The pyruvic acid is one of the most important metabolic intermediates in the basic metabolisms in our body and it can link the transformation of carbohydrates, lipids and amino acids by converting into acetyl-CoA and participating in the citric acid cycle. In the aerobic condition, pyruvic acid can be converted into acetyl-CoA by pyruvate dehydrogenase system in mitochondria. The high level

of pyruvic acid along with the low level of citric acid and succinic acid indicated that the citric acid cycle had been affected by the CUMS procedure and caused the energy metabolism perturbation. More specifically, the deficiency of citric acid and succinic acid resulted in the energy deficiency which manifest as exhaustion and fatigue. One of the most frequent symptoms of depression is fatigue and this is consistent with our results which indicate there is an energy metabolism perturbation in the pathogenesis of depression.

Creatine is an amino acid that occurs in vertebrate tissues and in urine. In our body, creatine is synthesized mainly in the liver and most of it is stored in skeletal muscle and the rest of it is stored in brain and heart. The main function of creatine is participating in the transport of the cell's energy and the high energy phosphate group of ATP is transferred to creatine to form phosphocreatine which is reversibly catalyzed by creatine kinase. In this study, significantly down-regulated level of creatine was observed in the model group compared with the control group, and when received the drug administration, the concentration of creatine in antidepressant-treated group was associated with a significant rise at the side of the model group and had no statistical significance with the control group. Although the mechanism of creatine is not clearly enough in depression, it has been proved that creatine is closely associated with the energy deficiencies. While in depression, there are many common symptoms such as extreme fatigue, psychomotor retardation and lethargy which is consistent with energy deficiencies. This may be the joint result of the changed level of some energy-metabolism-related materials. The foregoing result implies that creatine can be a potential biomarker for depression and also can evaluate the effect of citalopram by the metabolomics method.

Glycerol is an important component of the phospholipids and triglycerides, which is a three-carbon substance that forms the backbone of fatty acids in fats. When the body uses stored fat as a source of energy, glycerol and fatty acids are released into the bloodstream. The glycerol component can be converted to glucose by the liver and provides energy for cellular metabolism. Previous studies showed that there did occur the lipid metabolic disorder in patients suffering from depression, along with the perturbations of some protein related to the lipid metabolism in CUMS rats[50, 51]. So in this study the significantly down-regulated level of glycerol discovered in the model group may due to the perturbation of lipid metabolism, and after treatment, the glycerol level returned to normal which indicated it could be a biomarker to evaluate the drug effect.

4.3.4. Intestinal flora variation

In the model group, the concentration of indoleacetic acid (IAA) and indoxyl sulfate was significantly decreased and up-regulated respectively. These changed metabolites was closely linked to the metabolism of amino acids through the intestinal flora[52, 53], and the perturbation of them may indicated the imbalance of intestinal

flora. The imbalance of intestinal flora could have an impact on the appetite, and previous research has reported the symptom of the loss of appetite in depressed patients[54]. IAA is a breakdown product of tryptophan metabolism and is often produced by the action of bacteria in the mammalian. It may be produced by the decarboxylation of tryptamine or the oxidative deamination of tryptophan. The reduced concentration of IAA had been observed in the model group, and this may be due to the loss of appetite which is a clinical symptom in depressed patients. The induced changes of IAA along with indoxyl sulfate could be partly explained by the disturbance of enteric flora when facing CUMS procedure, which has been reported that there is a connection between irritable bowel syndrome and depression. Previous research has reported that some depressed patients had gastrointestinal symptoms due to the intestinal flora disturbance[55]. Moreover, some research also reported a two-way link between gut and brain which was the synergistic effect of immune system, endocrine system and gut [56, 57]. The two-way link can associate depression with the alteration of intestinal flora, and the metabolism imbalance of IAA and indoxyl sulfate in model group could provide a new insight to study the aetiopathogenesis of depression and learn the effect of citalopram from a metabolomics method.Gut flora also plays an important in many essential physiological functions even in the biosynthesis of 5-HT and the metabolism of tryptophan[58]. Gut flora can regulate the tryptophan metabolism by affecting the production of proinflammatory cytokine or cortisol. Recently, an article in cell shows that most of 5-HT is produced in intestinal and stored in enterochromaffin cell[59]. By comparing the mice which don't have gut flora in intestinal with the healthy control, the level of 5-HT is about 60% lower than normal mice, and when injected with the probably-related gut flora, 5-HT is significantly up-regulated. All these results demonstrates that tryptophan metabolism can also be affected by gut flora, and this link the intestinal flora variation with tryptophan metabolism which helps to give an overall understanding of depression.

5. Conclusion

In conclusion, our study focused on the metabolomic changes induced by CUMS procedure and firstly studied whether the metabolome changes could correctly reflect the status of depression and obtained promising results. Furthermore we searched for the potential biomarkers of depression and evaluate the therapeutic effect of citalopram according to the regulated metabolites by a metabolomics method. A total of 26 metabolites accounted for the difference between the control, model and antidepressant-treated group had been found and identified, and then 24 of them appeared a trend to return to normal level. We also gave the appropriate interpretations for these potential biomarkers. According to our study, the metabolic changes in the model group showed that the depression induced by CUMS were mainly associated with the perturbation of amino acids metabolism, energy metabolism, synthesis of neurotransmitter and intestinal flora disturbance. After drug administration, the

therapeutic effects of citalopram on CUMS rats was observed and resulted in the regulation of 10 key metabolites involved in the perturbative pathways. Meanwhile, the PLS-DA model showed a good predictive ability which meant it is feasible to evaluate the status of depression and the therapeutic effect of citalopram according to the changed-metabolites at a specific period by a metabolomics method. This is the first attempt to combine the exploration of biomarkers of depression and evaluating the effect of citalopram by a metabolomics method, and then use the method to access the depression status according to the changed metabolome. It is promising to use the metabolomics tools to evaluate the relationship between depression and relevant somatic symptoms by testing the metabolic changes of the urine, and it has bright future in making a definite evaluation the therapeutic effect of antidepressant drugs. Moreover, this untargeted metabolomics study can lay the foundation for our next targeted metabolomics study of the patients suffering depression or other neurological disorders.

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Figure Legends

Fig.1 Experimental design for the present study.

Fig.2 The sucrose preference at week3 (a), week6 (b) of the control, model, and treated group. Data are represented as mean±SD.

* means a statistically significant difference at $p<0.05$, ** means a statistically significant difference $p<0.01$

Fig.3 Measurement of locomotor activity in the open-field test. The time spent in the in the central area at week3 (a), week6 (b), the frequency of rearing at week3 (c), week6 (d).

* means a statistically significant difference at $p<0.05$, ** means a statistically significant difference $p<0.01$

Fig.4 The immobility time in the forced swimming test at week3 (a), week6 (b).

* means a statistically significant difference at $p<0.05$, ** means a statistically significant difference $p<0.01$

Fig.5 (a) The score plot from a PCA model distinguishing the control, model and antidepressant-treated group (green circle, blue square, brown triangle) at week6. (b) PLS-DA score plots for pair-wise comparisons among the control, model and antidepressant-treated group (green circle, blue square, brown triangle) at week6. (c)Correlation coefficient between original and permuted data.

Fig.6 The perturbed metabolic pathways in response to CUMS and treatment of citalopram. The levels of potential biomarkers in the model group compared to the control group were labeled with (↑) down-regulated and (↓) up-regulated.

Table 1. Stressor weekly schedule

Time	Stressor	Lasting period
Monday	Cage cleaning, changing the soiled padding and weighing the rats	8:00
	Food and water deprivation	13:00
	Sucrose preference test	9:00-11:00
Tuesday	Paired housing	15:00-8:00
	Stroboscopic illumination	20:00-23:00
	Remain single housing	8:00
Wednesday	Soiled cage	15:00-8:00
	White noise	20:00-23:00

Thursday	Cage cleaning and changing the solid padding	8:00
	Water deprivation	8:00-20:00
	Cage tilting	20:00-8:00
	Food deprivation	8:00-20:00
Friday	Stroboscopic illumination	20:00-23:00
	Cage tilting	23:00-11:00
Saturday	Paired housing	8:00-20:00
	Overnight illumination	20:00-8:00
Sunday	Soiled cage	15:00-8:00
	White noise	20:00-23:00

693 **Table.2** List of candidate biomarkers for depression and evaluating the effect of citalopram

No	Metabolite	m/z (amu)	t_R (min)	VIP score	Model vs. Control		Model vs. Treat		Corresponding metabolic pathway
					fold change	q-value	fold change	q-value	
1	Glycerol[a,b]	92.0470594	1.34	2.84	0.51	0.008	0.66	0.021	Glycerolipid metabolism
2	4,6-Dihydroxyquinoline[c]	161.0472132	6.10	2.58	0.49	0.002	0.85	N.S.	Tryptophan metabolism
3	Succinic acid[a,b]	118.0262727	0.66	2.47	0.54	0.001	0.80	N.S.	Energy metabolism
4	Creatine[a,b]	131.0696566	5.49	2.30	0.24	0.002	0.44	0.016	Energy metabolism
5	Quinolinic acid[a,b]	167.0217195	0.99	2.20	2.34	<0.001	1.64	0.019	Tryptophan metabolism
6	L-Phenylalanine[a,b]	165.0797272	6.49	2.17	3.12	<0.001	1.34	0.031	Synthesis of neurotransmitter
7	Indole-3-carboxylic acid[c]	161.0470437	5.47	2.08	0.24	0.003	1.14	N.S.	Energy metabolism
8	Kynurenic acid[a]	189.0404926	4.87	2.03	0.26	0.001	0.43	0.006	Tryptophan metabolism
9	Indoxyl sulfate[c]	213.0090460	2.72	1.93	0.48	0.003	0.87	N.S.	Tryptophan metabolism
10	Gamma-Aminobutyric acid[a,b]	103.0635047	7.30	1.91	0.19	0.009	0.49	0.024	Synthesis of neurotransmitter
11	L-Kynurenine[a]	208.0842484	5.72	1.89	3.09	<0.001	0.43	0.015	Tryptophan metabolism
12	Dopamine[a,b]	153.0785807	5.25	1.85	0.26	0.004	0.72	N.S.	Synthesis of neurotransmitter
13	L-Tryptophan[a,b]	204.0902216	4.89	1.84	0.58	0.023	0.83	N.S.	Tryptophan metabolism
14	L-Isoleucine[a,b]	131.0952197	1.24	1.81	0.59	0.009	0.52	0.022	Isoleucine degradation
15	L-Serine[a,b]	105.0428964	1.11	1.80	0.49	0.002	0.86	N.S.	Glycine and serine metabolism
16	Hypoxanthine[a,b]	136.0380725	1.34	1.79	0.50	0.004	0.88	N.S.	Energy metabolism
17	5-hydroxyindoleacetic acid[a]	191.0586587	3.72	1.79	0.42	0.009	0.69	N.S.	Tryptophan metabolism
18	N-Acetyl-L-aspartic acid[a]	175.0476473	4.36	1.77	0.59	0.011	0.75	0.018	Synthesis of neurotransmitter
19	Pyruvic acid[a,b]	88.0158195	0.70	1.75	1.94	0.028	1.08	N.S.	Energy metabolism
20	Phenylpyruvic acid[c]	164.0469798	0.74	1.72	1.24	0.006	1.16	N.S.	Synthesis of neurotransmitter
21	Xanthurenic acid[c]	205.0369086	5.62	1.67	1.33	0.012	0.96	N.S.	Tryptophan metabolism
22	Phenylacetylglycine[a]	193.0735057	5.53	1.66	1.39	0.008	1.23	N.S.	Glycine metabolism

23	Indoleacetic acid[a,b]	175.0629743	6.74	1.64	0.46	0.006	0.65	0.013	Tryptophan metabolism
24	Citric acid[a,b]	192.0264797	6.55	1.59	0.72	0.027	0.89	N.S.	Energy metabolism
25	L-Dopa[a,b]	197.0683015	5.35	1.54	0.60	0.007	0.90	N.S.	Synthesis of neurotransmitter
26	Glycine[a,b]	75.0323353	3.22	1.53	0.43	0.017	073	N.S.	Glycine and serine metabolism

694 a Metabolites identified by comparing with authentic standards.

695 b Metabolites further validated by MS/MS spectra with authentic standards.

696 c Metabolites identified by comparing with the HMDB database.

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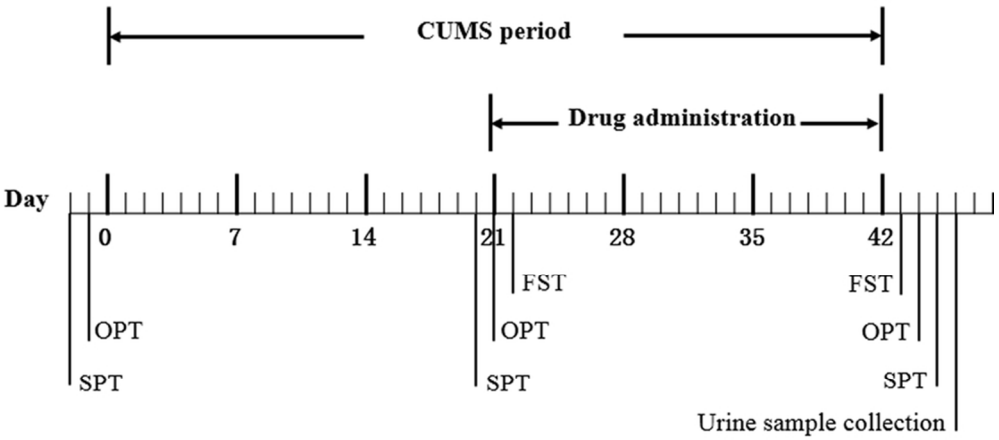

Fig.1 Experimental design for the present study.
82x42mm (300 x 300 DPI)

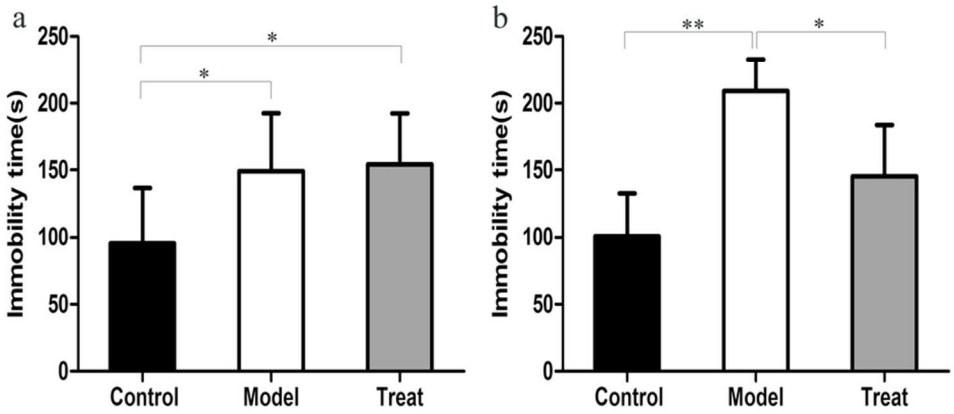

Fig.4 The immobility time in the forced swimming test at week3 (a), week6 (b).
* means a statistically significant difference at $p<0.05$, ** means a statistically significant difference $p<0.01$

82x36mm (300 x 300 DPI)

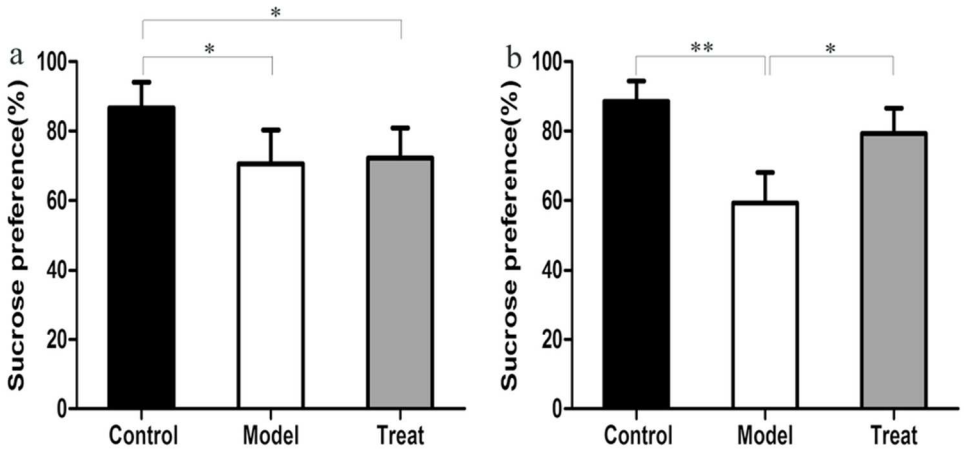

Fig.2 The sucrose preference at week3 (a), week6 (b) of the control, model, and treated group. Data are represented as mean±SD.
* means a statistically significant difference at $p<0.05$, ** means a statistically significant difference $p<0.01$

82x38mm (300 x 300 DPI)

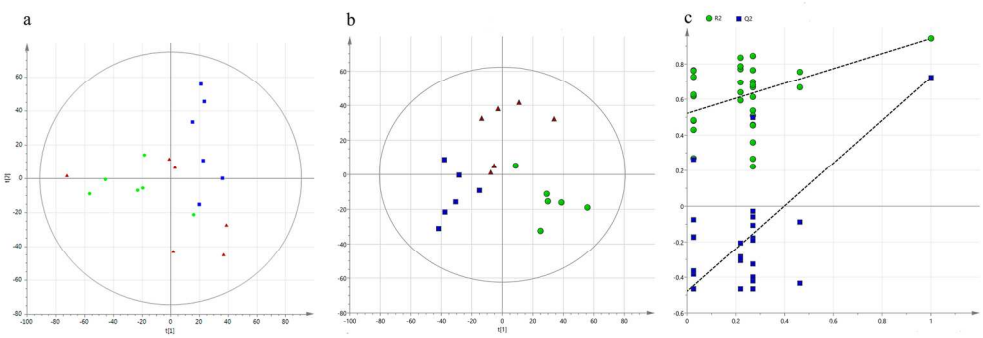

Fig.5 (a) The score plot from a PCA model distinguishing the control, model and antidepressant-treated group (green circle, blue square, brown triangle) at week6. (b) PLS-DA score plots for pair-wise comparisons among the control, model and antidepressant-treated group (green circle, blue square, brown triangle) at week6. (c)Correlation coefficient between original and permuted data.
82x28mm (600 x 600 DPI)

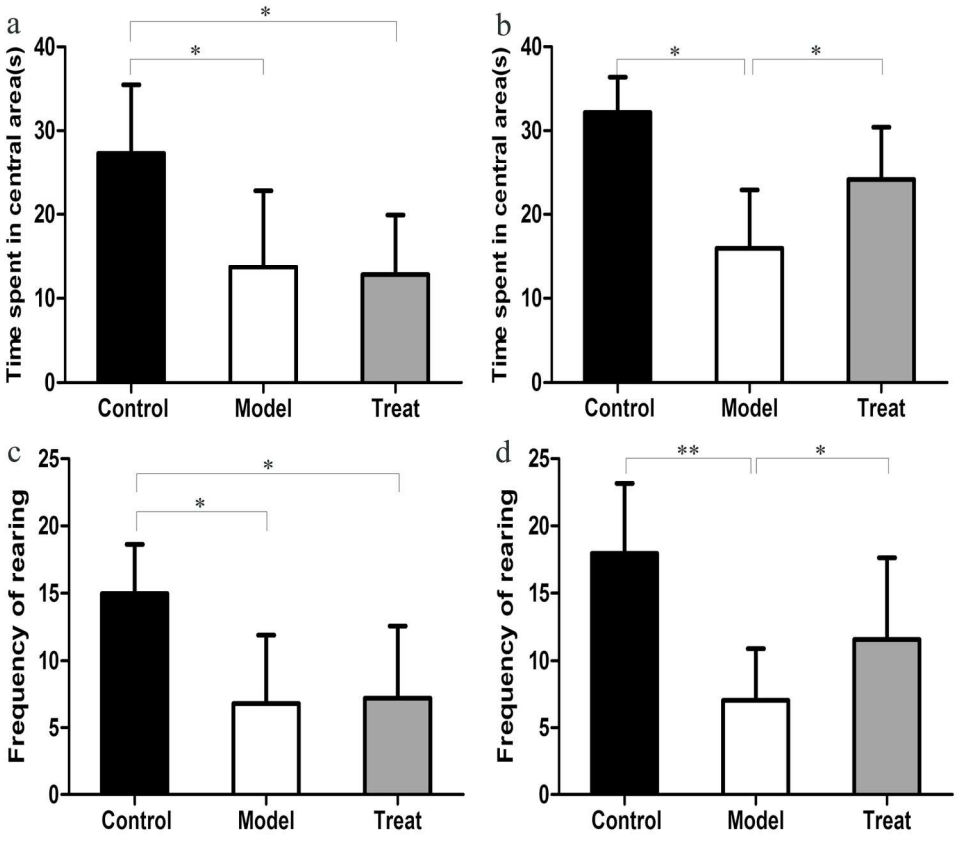

Fig.3 Measurement of locomotor activity in the open-field test. The time spent in the in the central area at week3 (a), week6 (b), the frequency of rearing at week3 (c), week6 (d).
* means a statistically significant difference at $p<0.05$, ** means a statistically significant difference $p<0.01$

170x157mm (300 x 300 DPI)

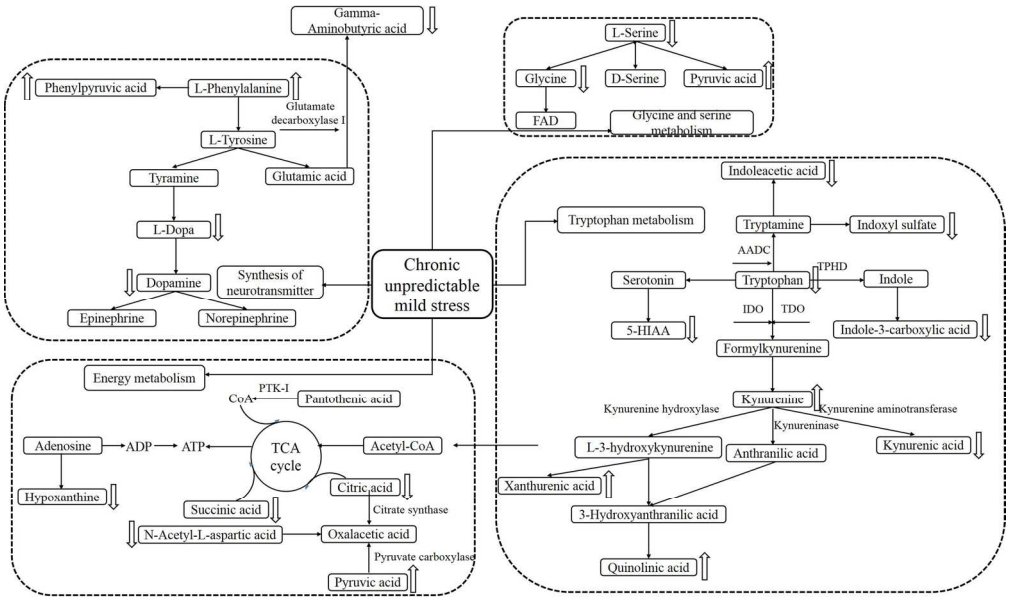

Fig.6 The perturbed metabolic pathways in response to CUMS and treatment of citalopram. The levels of potential biomarkers in the model group compared to the control group were labeled with (↑) down-regulated and (↓) up-regulated.
172x103mm (300 x 300 DPI)