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Tao-Hong-Si-Wu decoction improves depressive symptoms in model rats via amelioration of BDNF-CREB-arginase I axis disorders

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ABSTRACT

Context: The traditional Chinese medicine formula Tao-Hong-Si-Wu decoction (TSD), used for treating ischaemic stroke, has the potential to treat depressive disorder (DD).

Objective: To explore the effective targets of TSD on DD animal models.

Materials and methods: Sprague-Dawley (SD) rats were modelled by inducing chronic unpredictable mild stress (CUMS) during 35 days and treated with three dosages of TSD (2.5, 5 and 10 g/kg) or fluoxetine (10 mg/kg) by oral gavage for 14 days. Bodyweight measurements and behavioural tests were performed to observe the effect of TSD on the CUMS animals. A gas chromatography coupled with mass spectrometry (GC–MS)-based metabolomic analysis was conducted to reveal the metabolic characteristics related to the curative effect of TSD. Levels of the proteins associated with the feature metabolites were analysed.

Results: Reduced immobile duration and crossed squares in the behavioural tests were raised by 48.6% and 32.9%, on average, respectively, by TSD treatment (ED_{50}=3.2 g/kg). Antidepressant effects of TSD were associated with 13 decreased metabolites and the restorations of ornithine and urea in the serum. TSD (5 g/kg) raised serum serotonin by 54.1 mg/dL but suppressed arginase I (Arg I) by 47.8 mg/dL in the CUMS rats. Proteins on the brain-derived neurotrophic factor (BDNF)-cAMP response element-binding protein (CREB) axis that modulate the inhibition of Arg I were suppressed in the CUMS rats but reversed by the TSD intervention.

Discussion and conclusions: TSD improves depression-like symptoms in CUMS rats. Further study will focus on the antidepressant-like effects of effective compounds contained in TSD.

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Metabolomics; GC–MS; traditional Chinese medicine; depressive disorder

Introduction

Depressive disorder (DD) is a common illness that severely limits psychosocial function and increases the risk of suicide (Moussavi et al. 2007). According to a report by the World Health Organization (WHO) in 2017, about 322 million patients have depression worldwide. The high prevalence and the risk of disability make the disease a serious global health problem (Friedrich 2017).

The pharmacotherapies for DD are primarily based on enhancing monoamine neurotransmission until the present day. However, few drugs combine effectiveness and tolerability (Malhi and Mann 2018). This is partly due to the unclear pathology of DD. Typically, more than the reductions in the levels of monoamine neurotransmitters, physiological disorders are found in different DD pathways, such as dysfunctions of the hypothalamic-pituitary-adrenal axis, inflammation, inhibition of neurogenesis, loss of brain function, and genetic modifications. Thus, multi-target medications may be a promising therapeutic strategy for DD treatment.

Emerging evidence shows that ethnomedicines, which are generally multi-targeted, especially traditional Chinese medicine (TCM), serve as critical complementary therapies in DD treatment (Li et al. 2020). Several inherited prescriptions from TCM have been reported to be useful in improving depressive symptoms (Li et al. 2003; Zhu et al. 2016; Chi et al. 2019; Sun et al. 2021). Most herbal drugs show the effect of serum serotonin enrichment, which is likely to be a common effect of most TCM prescriptions for DD (Wang et al. 2019). According to the theory of TCM, DD is associated with the deficient circulation of blood and *qi*, invasion of detrimental *qi* (Yeung et al. 2015), and organ weakness, which can be implicated with the depressive symptoms of anaemia and malnutrition, inflammation and impaired organ functions, respectively (Kostev et al. 2017; Ahmed and Vasiliadis 2021; Shen et al. 2021). Interestingly, these conditions were reported to be improved using Tao-Hong-Si-Wu decoction (TSD), a classical formula recorded in TCM, which consists of

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Angelica sinensis (Oliv.) Diels, (Umbelliferae), *Rehmannia glutinosa* (Gaert.) Libosch. ex Fisch. et Mey. (Scrophulariaceae), *Ligusticum sinense* cv. Chuanxiong S. H. Qiu et al. (Apiaceae), *Paeonia lactiflora* Pall. (Paeoniaceae), *Prunus persica* L. (Rosaceae) and *Carthamus tinctorius* L. (Asteraceae). The traditional use of TSD was to treat ischaemic stroke (Li et al. 2015). In the last decades, bioactive compounds contained in these herbs, such as ferulic acid and albiflorin, were found to improve inflammation and depressive symptoms (Chen et al. 2015; Zhao et al. 2018). Interestingly, these herbs were known as inhibitors of serotonin reuptake. However, no previous studies focussed on the antidepressant function of TSD.

Clarifying the mechanism behind the curative effect of a TCM formula is challenging because of the comprehensive ingredients in the mixture of various herbs. To this end, metabolomics is a promising tool because the improvement in disease-induced dysregulations can be witnessed sensitively and simultaneously by restoring metabolic disorders (Nicholson et al. 2012). Also, the variations in inherent metabolites can appear as individual responses to treatments, thereby providing evidence for personalized therapy. In addition, an overview of the metabolome regulations follows the TCM theory, which emphasizes restoring the integral balance and harmony in the internal environment of patients (Wang et al. 2019). However, metabolic profiling is prone to variations by environmental variations and individual differences. Therefore, further verifications regarding the upstream proteins related to the drug effect-induced metabolic variations are necessary to ascertain the medicinal actions and corresponding drug targets (Tuyiringire et al. 2018).

In the above context, the present study hypothesized that TSD was useful to improve depression-like symptoms in model rats and metabolomics analysis might help to uncover the underlying targets of the pharmaceutical effect. To prove the hypothesis, this study confirmed the antidepressant effect of TSD on the DD animal model using bodyweight measurements and behavioural tests. Subsequently, a series of serum metabolomic analyses were conducted to reveal discriminant metabolites and varied metabolic pathways associated with CUMS modelling and the effect of TSD on depression-like symptoms. Feature metabolites associated upstream proteins in the serum and hippocampus were tested to explore the mechanism behind the effects of TSD on DD-like animal models.

Materials and methods

Experimental animals

Three cohorts of Sprague-Dawley (SD) rats (weighting 210 ± 12 g) were purchased from the Experimental Animal Center of Anhui Medical University (Hefei, China). The first rat cohort (nine groups, $n = 5$ for each group) was for the determination of ED_{50} and appropriate dosages of TSD in CUMS rats. The second cohort (eight groups, $n = 5$ for each group) was for the analyses of 14-d acute toxicity according to OECD guidelines 420 and LD_{50} in healthy rats. The last cohort (six groups, each group = eight rats) served for the study of the effect of TSD on CUMS rats. The animals were housed with free access to food and water for 1 week. All the procedures in the animal experiments followed the guidelines from the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and comply with the rules of the Ethics Committee and the Animal Experimental Committee of Anhui Medical University (LLSC20220590).

Medication

The herbs used for gavage were purchased in 2019 from Anhui Baixingyuan Pharmacy, Hefei, China. The herbs were mixed before preparing the decoction with the mass proportion of each herbal medicine: *Semen Prunus* 12 g, *Flos Carthami* 18 g, *Rhizoma Chuanxiong* 18 g, *Radix Angelicae sinensis* 18 g, *Radix Rehmanniae praeparata* 18 g and *Radix Paeoniae Alba* 18 g according to the original record of 'Yuji Weiyi,' a Chinese classical medical book. The herbs were identified by referring to the corresponding voucher specimens (nos. 03152019, 03122011, 03172020, 02212006, 04532018 and 05012009) deposited in the Exhibition Center of School of Pharmacy, Anhui Medical University. Further, the herbs were standardized by comparing the high-performance liquid chromatographic (HPLC) analysis of each herb and that of the corresponding medicinal compound. The relevant spectra are displayed in the supplementary information.

Chemicals

HPLC-grade methanol, isopropanol, acetonitrile, methoxamine salt, pyridine, pentobarbital and N-paraffin mix used for calculating retention index were purchased from J&K Scientific Ltd. (Shanghai, China). HPLC-grade *N*-methyl-*N*-trimethylsilyltrifluoroacetamide and the standard substances including L-norvaline, L-lysine, L-tyrosine, L-phenylalanine, L-serine, L-valine, L-glutamic acid, L-alanine, L-proline, L-ornithine, D-glucose, sucrose, urea, lactic acid and fluoxetine were purchased from Sigma-Aldrich Ltd. (Shanghai, China). The standard products of the six herbs included in TSD were bought from Desite Company (Chengdu, China). Ultrapure water was prepared by the Milli-Q system (Merck, Darmstadt, Germany).

Preparation and evaluation of the homogeneity of TSD

The herbs contained in TSD were soaked in double-distilled water (the mass ratio of medication and water was equal to 1:8) for more than 2 h. The mixture of medications was decocted for 50 min. The resulting decoction was filtered with gauze, and the filtrates were centrifuged at 3000 rpm and 4 °C for 10 min. The supernatants of the two parts of aqueous solutions were combined, lyophilized into powder, and stored at −80 °C before administering medication. The internal standard L-norvaline (1 mg/L, relative standard divergence (RSD)=0.09) added to the prepared TSD samples was evaluated every three days before the next gavage to ensure the homogeneity of TSD used during the 2-week intervention. The sample preparation and the determination of the internal standard based on gas chromatography coupled with mass spectrometry (GC–MS) are described in the supplementary data. The standard products of the six herbs included in TSD were compared with the nontargeted metabolomic analyses to authenticate all the herbs bought from the pharmacy. The standard products of the six herbs were extracted in the same way as our medications. The extracts of both the tested samples and the standard products were prepared using the same method: an equal mass of lyophilized TSD extract powder was re-dissolved in isopropanol solution:acetonitrile:water = 3:3:2. An equal volume of internal standard (1 mg/L) was added

to each sample. The samples were then filtered using a microfiltration membrane and subsequently freeze-dried. The extracted samples were stored at −80 °C until GC–MS analysis. The derivatization reactions for the extracts were the same as the sample preparation of the serum samples. The gradient temperature program was as follows: the column temperature was set at 70 °C at the start of the analysis with the maintenance of 5 min; it was then augmented to 150 °C by the gradient of 4 °C/min; after holding for 5 min, the column temperature was raised to 230 °C and then held for 5 min; and the column temperature was then increased to 300 °C at the rate of 5 °C/min and held for 3 min. Other instrumental conditions were similar to the determination of rat serum metabolome described in the manuscript.

Determination of ED_{50} of TSD in depression-like model rats

Forty-five SD rats were equally divided into nine groups. The rats were all subject to chronic unpredictable mild stress (CUMS) stimulations (Kim et al. 2007) for establishing depression-like models. The operations included water deprivation, reversal of day and night, shaking, dampness, crowding, fasting (24 h) and ice water stimulation (4 °C, 5 min) in turn (detailed in the supplementary data). The model groups were repeatedly stimulated for 35 days for which different stimulations in each week have been listed in the supplementary document. Nine doses including 0.25, 0.5, 1, 2, 5, 10, 20, 30 and 40 g/kg TSD were given to the nine groups of rats after the modelling for 14 days. The serum of each rat was taken after dosing. Serum serotonin was measured by enzyme-linked immunosorbent assay (ELISA) kits.

Determination of the acute toxicity and LD_{50} of TSD in healthy rats

The acute oral toxicity and LD_{50} of TSD were determined according to the Organization for Economic Co-operation and Development (OECD) test guidelines 420 (shown in the supplementary information) in another cohort of rats (eight groups, $n = 5$ for each group, shown in the supplementary information). The experimental procedures complied with the rules of the Ethics Committee and the Animal Experimental Committee of Anhui Medical University, China (LLSC20180399).

Animal modelling and treatment

Another cohort of rats received an adaptive feeding 1 week before further treatments. They were randomly divided into six groups as follows: the blank control group (B), the model group (M), the model group treated with fluoxetine (FX, 10 mg/kg) and the model groups treated with TSD (2.5 (TL), 5 (TM) and 10 g/kg (TH)) by oral gavage ($n = 8$). Two deaths occurred after 14 d TSD administration only for the TH group). Except for the blank controls, the DD was imitated by inducing CUMS stimulation (Kim et al. 2007) in rats. The operations included water deprivation, reversal of day and night, shaking, dampness, crowding, fasting (24 h) and ice water stimulation (4 °C, 5 min) in turn. The model groups were repeatedly stimulated for 35 days for which different stimulations in each week have been listed in the supplementary document. Of the five model groups, three groups of rats with CUMS were treated with low (2.5 g/kg/d), medium (5 g/kg/d) and high (10 g/kg/d) doses of TSD, while the rats in the FX group were subject to fluoxetine (positive control, 10 mg/kg/d). Before medication administration, the prepared TSD powder and fluoxetine were dissolved in 0.5% sodium carboxymethyl cellulose (CMC-Na) suspension. The other two groups were subjected to gavage of 0.5% CMC-Na solution.

Bodyweight measurements and behavioural tests

The animal experiment process is displayed in Figure 1(a). The bodyweight measurement and the behavioural tests were first performed before medication interventions to verify the successful establishment of the model. The same tests were repeated after a 2-week medication therapy to observe the effect of TSD on depression-like symptoms. The open-field test (OFT) was carried out with light alteration as described previously (Liu L et al. 2019). Briefly, the OFT apparatus was a black wooden box (100 cm long × 100 cm wide × 50 cm high). The floor of an arena was divided into 25 equal-size squares with white lines. Groups of rats were placed individually in the corner of the apparatus back to the wall and allowed to explore freely for 5 min. A minimal amount of light (60 Lx) was used to avoid anxiety behaviour. The apparatus was cleaned with 90% ethanol and dried before the next test to remove the smell from the former animal. The numbers of crossed squares, and rearing and grooming times were manually recorded by trained observers blinded to the experiment. Forced swimming tests (FSTs) were conducted as described previously (Zhang et al. 2020). All the rats were separated and individually put into a transparent glass cylinder (60 cm tall × 25 cm inner diameter) filled with water. The water temperature was maintained at 25 ± 1 °C during the experiments. The immobility time was defined as the period during which no body motion occurred with only the tiny necessary movements such as taking a breath above the water surface. A pre-test before data recording was done and the FST test lasted 5 min 12 h after the preliminary test.

Sample collections from rats

The animals were sacrificed immediately under anaesthesia by pentobarbital sodium (45 mg/kg) 24 h after the final behavioural tests. The rat blood was sampled from the abdominal aorta. The serum was kept after centrifugation at 3000 rpm and 4 °C for 15 min. The brain tissues of the rats were sampled from which the hippocampi were dissected in an ice bath. The hippocampal samples were subsequently snap-frozen with liquid nitrogen and subsequently stored at −80 °C until analysis.

Serum sample preparations

For the metabolomic analyses, the samples were first thawed at room temperature. A series of metabolite extraction and derivatization reactions were performed with 100 μL of each serum sample from each rat following the same method applied in our previous study (Liu Z et al. 2018). An equal volume of each collected sample was taken out to mix up the quality control (QC) pool. The QC samples were prepared in the same way as other real samples during the metabolite extraction and derivatization reactions. The QC samples were used to balance the instrument and estimate the stability of the analyses. In the sequence of GC–MS analyses, the QC samples were randomly inserted after every five real samples.

GC–MS conditions

A Shimadzu GC-MS 2010 (Kyoto, Japan) was used to determine the metabolic profiling in each sample. The GC–MS condition referred to our previous study (Liu Z et al. 2018) except for the gradient temperature program. For this study, the gradient temperature program was set as follows: the inlet temperature was set at 300 °C, the interface transfer temperature was 280 °C and the ion source temperature was 230 °C. The column flow was 1.2 mL/min, and the split ratio was set as 10:1. The event time was set to 0.2 s. Ions with their mass-to-charge ratios ranging from 50 to 600 *m*/*z* were detected under full-scan mode. The injection volume was 1 μL for each data acquisition. The gradient temperature program was set as follows: the initial column temperature was set to 70 °C and maintained for 3 min. The temperature was then increased to 200 °C with a gradient of 3 °C/min and held for 5 min. A gradient of 5 °C/min was applied while the temperature was raised to 300 °C and the column temperature was finally maintained for 3 min.

Data processing and data analysis for the metabolic profiling

The acquired data were first processed with the steps of ion peak alignment, deconvolution and normalization as described previously (Liu Z et al. 2021). Briefly, the normalized peak areas were used for the relative quantification of each ion peak. The annotation was done with the NIST database (version 2019) and by comparing it with the spectra of the standard substance. Data were screened within the QC samples in light of the RSD and the ratio of signal to noise (S/N) of each ion peak. Metabolites with their S/N inferior to 3 or RSD superior to 30% across all the QC samples were removed. After pre-processing and screening, the data set was subjected to Metaboanalyst software (version 5.0, www.metaboanalyst.ca) for further analyses (Pang et al. 2021).

Enzyme-linked immunosorbent assay

The serum and hippocampus levels of serotonin and Arg I were evaluated using ELISA kits produced by Abcam Company (Cambridge, UK) following the manufacturer's protocols. The concentration of serotonin was measured at 405 nm, while the concentration of serum Arg I in rats was measured at 450 nm.

Western blot assays

The tissue samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM Na_3VO_4). Before homogenization, a protease inhibitor cocktail (Servicebio Technology Co. Ltd., Ghent, Belgium) and a phosphatase inhibitor PhosSTOP (Servicebio Technology Co. Ltd., Ghent, Belgium) were added. Protein quantitation was conducted using a BCA protein concentration assay kit (Sparkjade Science Co. Ltd., Shanghai, China). The same quantity (approximately 50 μg) of protein from each sample was loaded, separated using 12% SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was blocked with 5% skimmed milk for 1 h, incubated with antibodies targeting brain-derived neurotrophic factor (BDNF) (1:500; Servicebio Technology Co. Ltd., Ghent, Belgium), TrkB (1:500; Servicebio Technology Co. Ltd., Ghent, Belgium), CREB (1:1000; Abcam, Cambridge, UK), ERK1/2 (1:1000; Abcam, Cambridge, UK), p-ERK1/2 (1:1000; Abcam, Cambridge, UK) or β-actin (1:1000; Kangchen Biotechnology, Inc., Shanghai, China) at 4 °C overnight. Next, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) at 37 °C for 2 h. The blots were developed with an Easy Enhanced Chemiluminescence Western Blot Kit (Pierce Biotechnology, Rockford, IL). The relative expression levels of proteins were determined using a densitometer and normalized by referring to β-actin. ImageJ software (NIH, Bethesda, MD) was used to quantify relative protein expressions. One-way analysis of variance (ANOVA) with the Tukey HSD correction was used to determine the significant differences in protein expression between the groups.

Statistical analyses

All the univariate statistical analyses were conducted using SPSS software (version 20, IBM, Armonk, NY). The dose–effect curve was drawn by GraphPad Prism software (version 7.0, GraphPad Software, La Jolla, CA). Paired non-parametric analysis was used for the analyses of significant differences between the rats before and after TSD or FX treatment. The two-group *p* values for the differentiation analyses of metabolites were calculated using non-parametric tests. Bonferroni's corrections were used for false discovery rate (FDR) correction for the *p* value calculated between the groups using MultiExperiment Viewer software (Mev, version 4.9.0, Dana-Farber Cancer Institute, Boston, MA). The same method was applied to interpret the medicinal effects on serotonin and Arg I. For the analyses of WB, protein expressions were analysed with three repeats of results. One-way analyses of variance were used for analysing the intergroup differences of protein expressions. The online open-source software assumed multivariate analyses such as principal component analysis (PCA), orthogonal projection to latent structures-discriminatory analysis (OPLS-DA) and metabolic pathway analysis.

Results

Analyses of ED_{50}, LD_{50} and acute toxicity of TSD

Given that previous studies documented that some of the component herbs in TSD were effective to inhibit serotonin reuptake, we first attempted to determine the ED_{50} of TSD by measuring increased serum serotonin in CUMS rats, to reveal appropriate dosages of TSD for the following interventions. As the administration of FX at the commonly used dose of 10 mg/kg raised serum serotonin by 60%, which was regarded as the desired effect for the treatment (Sommi et al. 1987). The percentages of rats whose serum serotonin reached the threshold were recorded for the calculation of ED_{50}. As shown in Figure 1(b), the ED_{50} calculated was equal to 3.2 g/kg (detailed data are available in the supplementary document).

The second cohort of rats served to analyse the acute oral toxicity and the LD_{50} of TSD. Eight dosages from 0.005 to 30 g/kg of TSD were given to different groups ($n = 5$ in each group) of healthy rats by gavage. Only 2/5 deaths occurred in the rats treated with the dose of 30 g/kg, which was not superior to 50% (detailed in the supplementary information). Hence, the LD_{50} was not achieved herein. Such findings emerged that the oral administration of TSD was not toxic.

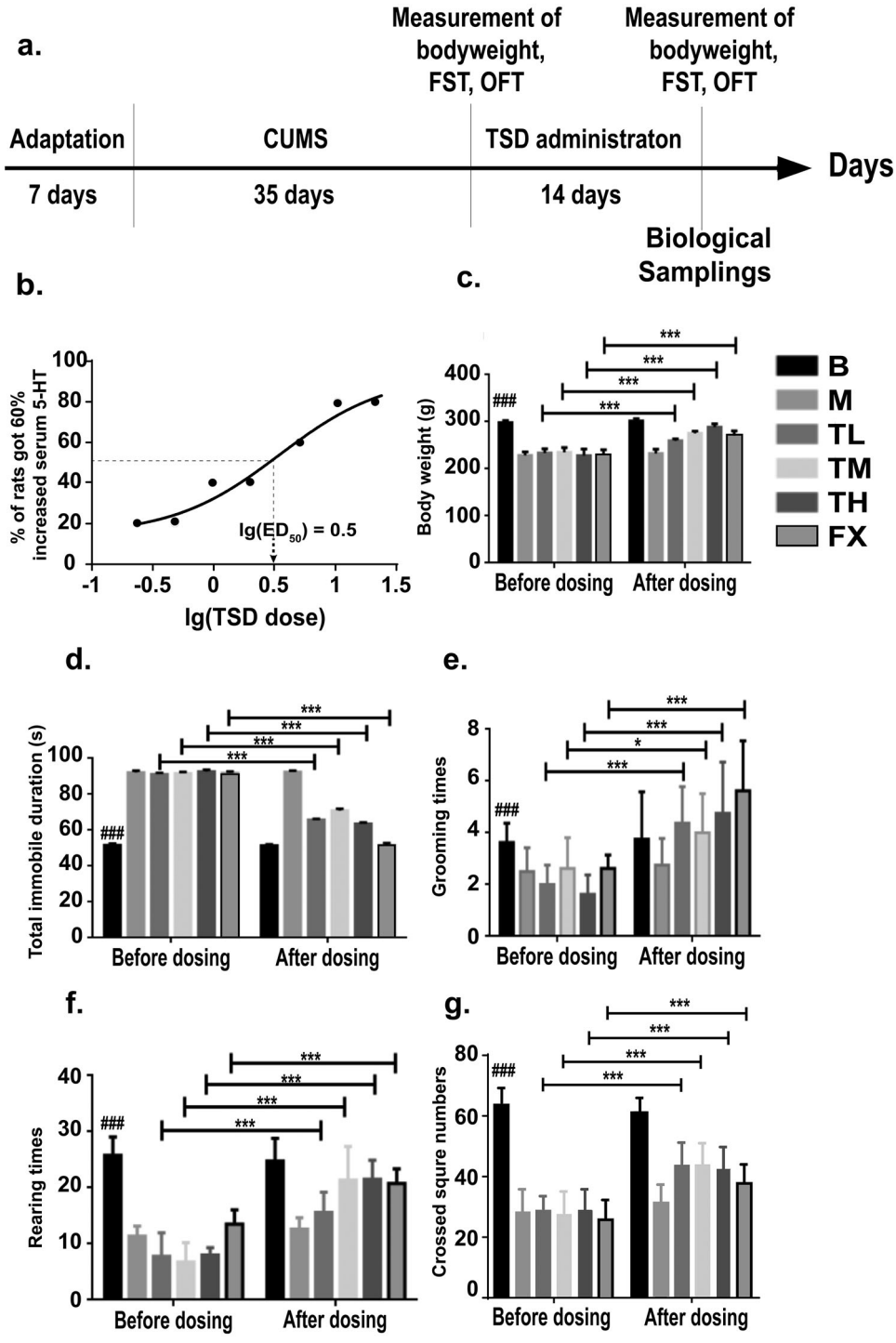

Figure 1. The comparison of body weight and the findings of behavioural tests before and after dosing of TSD. (a) A schema displaying the process of animal experiments; (b) ED_{50} of TSD determined by measuring serotonin in another cohort of CUMS rats. Sixty percent increased serotonin in the rat serum is considered as desired response after pharmaceutical treatments. (c) Bodyweight changes along with the TSD dosing for the groups of rats; (d) the total immobile time of rats recorded in the forced swimming test; (e–g) grooming times, rearing times, and numbers of crossed squares recorded in the open field test, respectively. *Significance between the treated and non-treated CUMS rats; #significance between the model and common rats; $^{*/\#}p < 0.05$; $^{***/\#\#\#}p < 0.001$.

Effect of TSD on depressive behaviour in CUMS model rats

Another cohort of rats was used for the study of the pharmacological effect of TSD on depression-like symptoms in rats. Inhibited anabolism and malnutrition are common symptoms of DD. We, therefore, measured the weights of all the rats before and after medication interventions. Decreases in general body weight were observed in CUMS model rats compared with the nontreated controls. The body weights of rats treated with TSD (2.5, 5 and 10 g/kg) and fluoxetine (FX, positive control, 10 mg/kg) significantly increased compared with those of the model rats (Figure 1(c)). OFT and FST were also performed, accompanied by weight measurements. The first behavioural tests aimed to confirm the successful establishment of the model, while the repeated actions after medication administrations served to visualize the effect of TSD on depression. Accordingly, the model rats before pharmacotherapies presented extended periods of

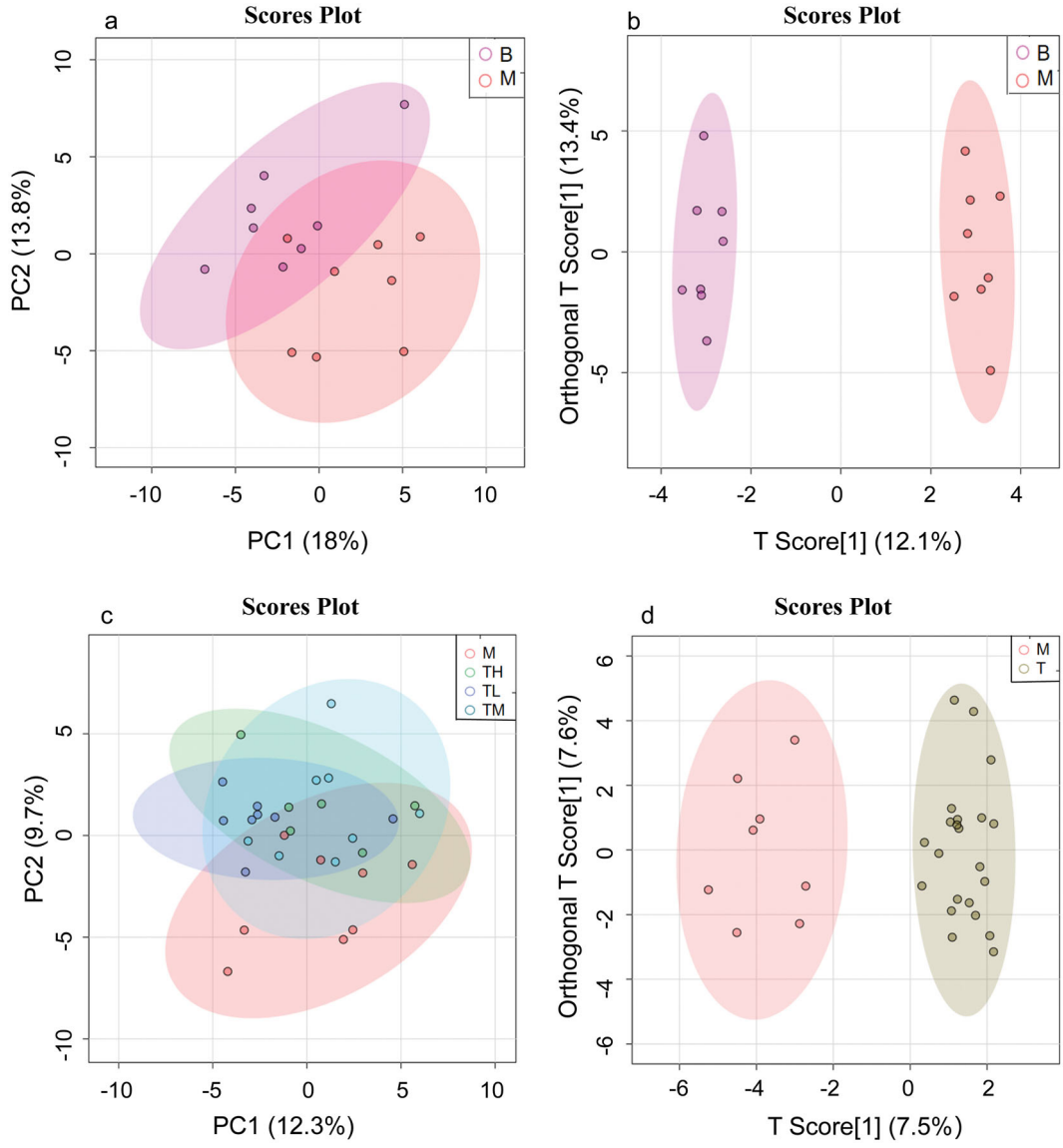

Figure 2. Score plots of PCA and OPLS-DA for the separations of serum metabolome between different groups of rats. (a, b) Score plot of PCA and OPLS-DA for the separations between the CUMS model group and the blank group; (c) a PCA showing the separation between the CUMS model group and the CUMS rats treated with different dosages of TSD. (d) OPLS-DA showing the separation between CUMS model rats and the integration of TSD-treated rats. B: The blank group; M: the model group; TL: the model animals treated with a low dose of TSD; TM: model animals treated with a medium dose of TSD; TH: rats treated with a high dose of TSD (the same indications are used below); T: TSD-intervened group.

immobility in the FST and reduced activities in the OFT compared with controls in the behavioural tests, showing that the model was successfully established. The episodes of immobility reduced while the activities increased significantly in the groups treated with TSD or FX. Concretely, bodyweight losses, decreased immobility, in the FST, and activity in the OFT were raised by 15.1%, 48.6% and 32.9% on average, respectively, by the treatment of TSD which confirmed the antidepressant effect of TSD in rats, as shown in Figure 1(d–f).

Effect of TSD on the serum metabolome of rats with CUMS

After the samplings of rat serum, we applied the GC–MS-based metabolomics method to the rats to study the metabolic characteristics of rats in each group, especially to have an insight into the metabolic alterations caused by TSD administration. Before data analysis, the stability of instrumental data acquisition was assessed with the QC samples. Consequently, we identified 82 metabolites among the retained 166 ion peaks. MetaboAnalyst 5.0 software was employed to analyse the data corresponding to the refined and normalized peak areas of metabolites. Figure 2 shows the differences in the metabolic profiles between different groups obtained using the score plots of PCA and OPLS-DA. Concretely, a PCA separating the blank control group from the CUMS model group is shown in Figure 2(a) (PC1 = 18%, PC2 = 13.8%). The same separation based on OPLS-DA (two components, $R^2X = 0.511$, $R^2Y = 0.993$, $Q^2Y = 0.685$) also showed clear discrimination between these two groups (Figure 2(b)). Another PCA was conducted between the model group (red dots illustrated in Figure 2(c), PC1 = 12.3%, PC2 = 9.7%) and TSD treatment groups with different dosages (presented by blue, cyan and green dots, respectively), aiming to understand the effect of TSD on the metabolome of rats with CUMS. Correspondingly, the model group samples in the PCA were shown to be separated from those of TSD-treated groups.

However, unexpectedly, few metabolic differences were present for the model rats fed different dosages of TSD. Clear

Table 1. Discriminant metabolites refined in both the comparisons B versus M and M versus TSD (TL + TM + TH).

	Metabolites	tR (min)	RI	*m/z*	Formula	P1	P2	FC1	FC2
Common discriminant metabolites refined in M vs. TSD and M vs. B	Phosphate	7.369	1035	73	H_3PO_4	0.008	0.005	1.50	10.12
	Lactic acid[a]	7.704	1039	73	$C_3H_6O_3$	0.002	0.002	1.53	1.67
	Glycine[a]	9.545	1098	102	$C_2H_5NO_2$	0.000	0.005	1.62	1.84
	3-Hydroxybutyric acid[a]	10.873	1129	147	$C_6H_{12}O_3$	0.008	0.014	1.45	1.84
	Urea[a]	13.931	1270	147	$C_{13}H_{20}N_8O_{10}$	0.002	0.012	0.59	0.62
	3-Aminoisobutyric acid[a]	15.767	1335	174	$C_4H_9NO_2$	0.033	0.019	2.49	1.47
	Unknown 2	23.435	1486	263	$C_{15}H_{24}O$	0.004	0.003	1.54	1.36
	L-Phenylalanine[a]	23.567	1491	120	$C15_5H_{18}N_4O_3$	0.029	0.028	1.39	1.59
	L-Orinthine[a]	29.725	1633	174	$C_5H_{12}N_2O_2$	0.034	0.026	0.55	0.52
	Citric acid[a]	31.71	1712	73	$C_6H_8O_7$	0.049	0.007	1.21	1.49
	L-Tyrosine[a]	34.432	1887	73	$C_9H_{11}NO_3$	0.000	0.003	1.83	2.17
	L-Lysine[a]	34.805	1893	218	$C_6H_{14}N_2O_3$	0.002	0.006	1.56	1.67
	myo-Inositol	39.444	2035	73	$C_6H_{16}O_{18}P_4$	0.011	0.027	1.29	1.36
	Unknown 3	39.832	2046	73	$C_{15}H_{24}N_4S_2$	0.001	0.015	1.86	2.38
	Pseudouridine	45.607	2215	217	$C_{10}H_{14}N_2O_6$	0.011	0.015	1.39	1.45
M vs. TSD	Glycolic acid	8.17	1041	73	$C_4H_6O_4S_2$	0.012		1.25	
	Benzeneacetic acid	12.603	1225	73	$C_{15}H_{19}N_3O_2$	0.024		1.91	
	L-Valine[a]	13.565	1267	144	$C_{25}H_{39}N_7O_5$	0.019		1.54	
	L-Serine[a]	14.12	1289	116	$C_{13}H_{13}NO_7$	0.025		2.98	
	L-Hydroxyproline[a]	23.107	1478	230	$C_5H_9NO_3$	0.026		1.44	
	L-Glutamic acid[a]	26.052	1585	73	$C_{13}H_{13}NO_7$	0.046		1.30	
	D-Allose	33.516	1809	73	$C_6H_{12}O_6$	0.040		7.83	
	D-Glucose[a]	36.169	1920	204	$C_6H_{12}O_5$	0.045		2.50	
	beta-D-Galactofuranoside	36.448	1927	73	$C_{19}H_{28}O_{10}$	0.006		0.50	
	Arachidic acid	47.749	2356	73	$H_4N_9O_4Si$	0.006		3.97	
	Cholesta-3,5-diene	55.33	NA	368	$C_4H_{10}ClNO_3$	0.002		2.58	
	1-Palmitoyllysophosphatidic acid	57.278	NA	299	$C_7H_{15}NO_3S$	0.014		1.80	
	Sucrose[a]	51,197	NA	361	$C_3H_5O_2$	0.019		1.37	
M vs. B	Propionic acid	7.369	1035	73	$C_3H_6O_2$		0.013		1.51
	L-Alanine[a]	8.97	1051	116	$C_3H_7O_2N$		0.002		2.09
	L-Proline[a]	11.275	1187	70	$C_5H_9NO_2$		0.002		2.04
	Unknown 1	11.545	1201	98	$C_9H_{20}N_2O$		0.003		1.55
	1,4-Dithiane-2,5-diol	22.633	1456	180	$C_8H_{12}O_4S_2$		0.043		1.36
	Dopamine[a]	48.014	2373	174	$C_8H_{11}NO_2$		0.033		1.37

tR: retention time; RI: retention index; NA: retention time non-analysed because the retention time of metabolite was superior to that of n-tetracosane; P1: *p* value calculated by comparing the model and the integration of TSD-treated groups; P2: *p* value calculated with the comparison between the model and the blank control groups. All the *p* values were corrected using FDR based on Bonferroni's correction. FC1: fold change of the comparison between the model and the TSD-treated groups; FC2: fold change of the comparison between the model and the blank control group. All the fold changes were calculated by the same dominator, which was the corresponding level of each metabolite in the model group.
[a]Identification of metabolite verified by the spectrum of corresponding standard chemical.

discrimination of metabolome was otherwise confirmed between the rats with CUMS and overall TSD-treated rats using another OPLS-DA ($R^2X = 0.075$, $R^2Y = 0.811$, $Q^2Y = 0.588$, Figure 2(d)). Differential metabolites ($p < 0.05$ calculated by non-parametric tests), associated with CUMS modelling and with the effect of TSD intervention are shown in Table 1. The features that contributed to the separation of B versus M and M versus overall TSD-treated rats are listed in the Venn diagram (Figure 3(a)). Fifteen common discriminants were observed for the separation of blank versus model and the separation of model versus TSD-treated groups. Among these key metabolites, the levels of urea and ornithine (f) significantly increased in the model group compared with the control group but significantly decreased in the TSD treatment group. Other primary discriminants comprised four amino acids (AAs), phosphate, citrate acid, lactate, pseudouridine, *myo*-inositol and 3-aminoisobutyric acid (3-AIB). The levels of these components decreased in the model group but were restored in the treatment groups, as shown in Figure 3(d–p). An enrichment of primarily varied metabolic pathways was performed along with the determination of metabolic features between the groups. As shown in Figure 3(q,r), various metabolic pathways associated with depression (comparison between the M and S groups) were similar to those associated with the effect of TSD on depression (comparison between the M and integrated groups of rats treated with TSD). Notably, arginine-related pathways were determined as primary characteristics in both pathway analyses.

Effects of TSD on the serum levels of serum arginase I and serotonin

Arginase I (Arg I) is the enzyme that contributes to the conversion of arginine into urea and ornithine. We assessed Arg I level in the serum of all the groups of rats because consistent enrichments were found in urea and ornithine in rats with CUMS, but this effect was alleviated by TSD administration. Our data showed that the Arg I level increased in the model group but decreased again in the TSD-fed groups (Figure 4(a)), which was consistent with the aforementioned two products of arginase degradation. Meanwhile, considering that increasing evidence showed the enhancement of serotonin level by herbal medications, we also tested the serum serotonin level using another ELISA. As shown in Figure 4(b), an apparent decrease in the serotonin level was observed in rats with CUMS without treatments compared with the blank controls. In contrast, the depletion of serotonin was attenuated in the rats with TSD interventions. Such effect of TSD on serotonin was found to be dose-dependent. Even a more adequate recovery of serum serotonin was found in rats with CUMS treated with FX because 5-

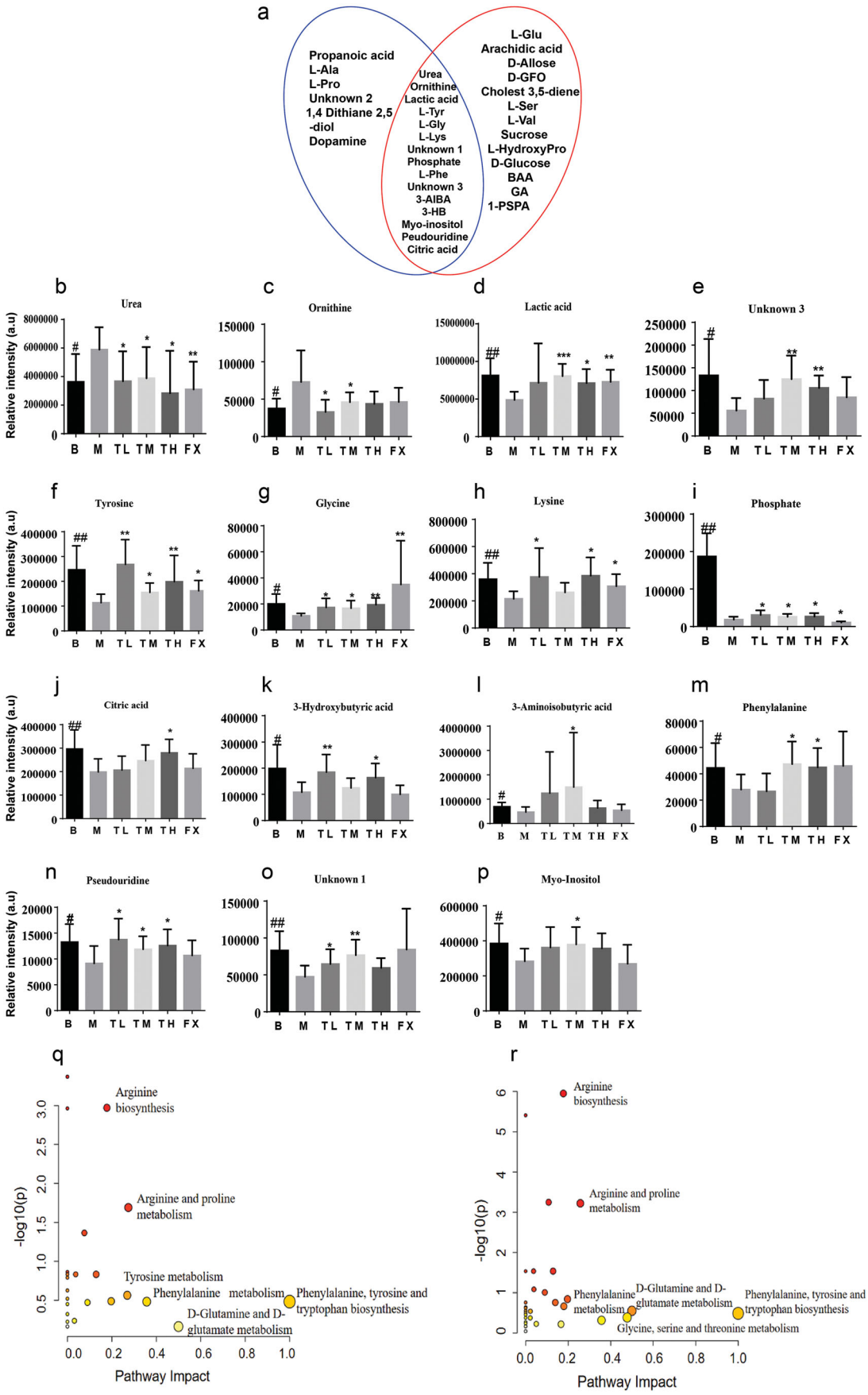

Figure 3. Metabolites with different characteristics between groups of serum metabolism of rats. FX: CUMS rats treated with fluoxetine. (a) A Venn diagram showing the differential metabolites associated with CUMS modelling and with the effect of TSD. Metabolites encompassed within the left oval: characteristic metabolites involved in the CUMS modelling; metabolites encompassed within the right oval: feature metabolites linked to the effect of TSD on CUMS rats; metabolites included the intersection of the two ovals represent the key metabolites contribute to both the differential analyses; (b–p) key discriminant metabolites that varied both in the comparison between B vs. M and M vs. TSD, a.u.: arbitrary unit; L-Ala: L-alanine; L-Pro: L-proline; L-orn: L-ornithine; L-Tyr: L-tyrosine; L-Gly: L-glycine; L-Glu: L-Phe: L-phenylalanine; 3-AIBA: 3-aminoisobutyric acid; 3-HB: 3-hydroxybutyric acid; L-Glu: L-glutamic acid; L-Ser: L-serine; L-Val: L-valine; L-HP: L-hydroxyproline; D-GFO: β-D-galactofuranoside; BAA: benzeneacetic acid; 1-PSPA: 1-palmitoyllysophosphatidic acid; (q, r) pathway analyses showing the primarily varied metabolic pathways involving the CUMS modelling and the TSD effect, respectively; (q) metabolic pathway analyses for the comparison of B vs. M; (r) metabolic pathway analysis for the comparison between the model group and the integrated groups of rats treated with TSD.

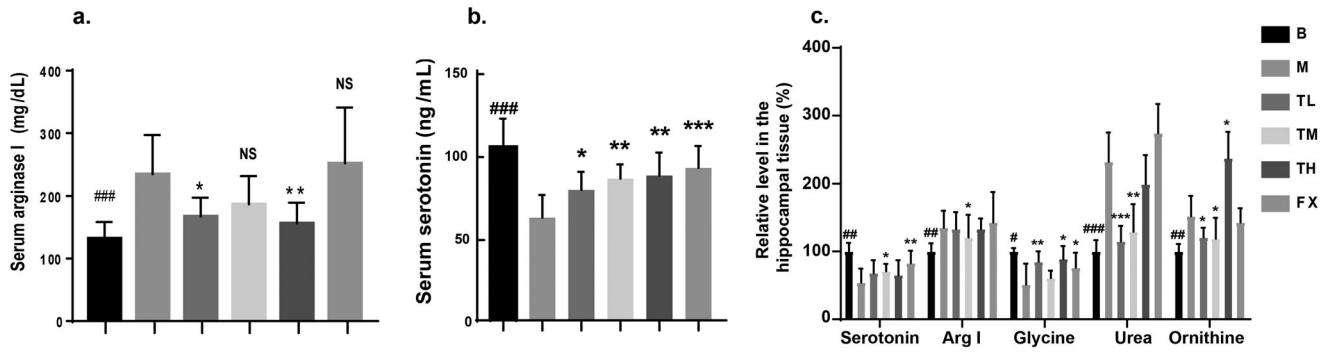

Figure 4. Measurements of serotonin, Arg I and their related metabolites in different groups. (a) Serum level of arginase I; (b) serum level of serotonin; (c) hippocampal levels of serotonin, Arg I, glycine, urea and ornithine in the groups of rats. The indicators */**/*** stand for the significance by the comparison between the medicine-treated group and the non-treated group; the indicators #/##/### represent significant variations between the model group and the control; $^{*/\#}p < 0.05$; $^{**/\#\#}p < 0.01$; $^{***/\#\#\#}p < 0.001$; NS: non significant.

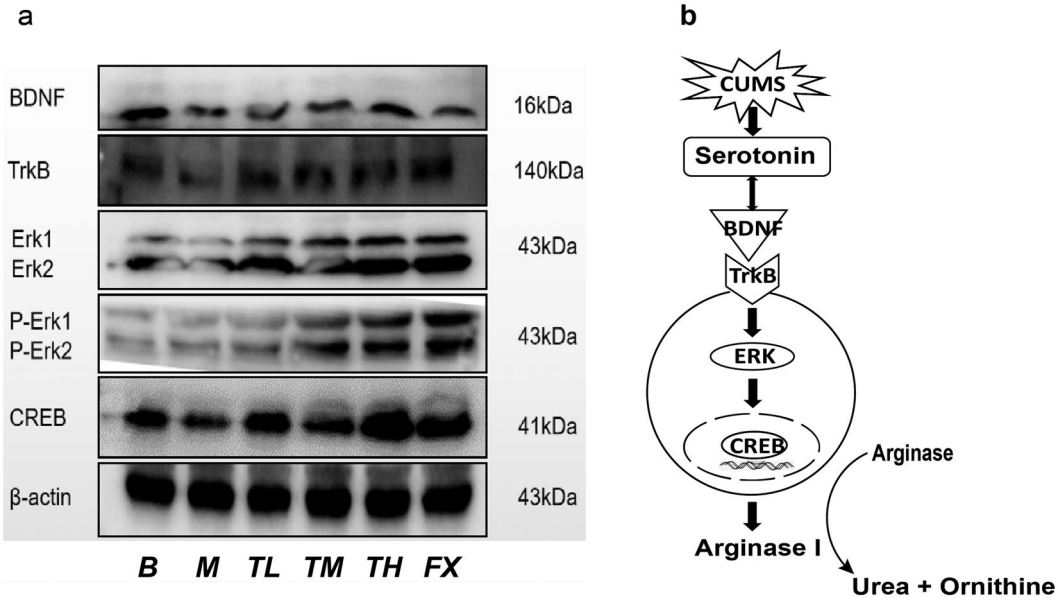

Figure 5. The effect of TSD on the expressions of upstream proteins of Arg I. (a) Targeted hippocampus protein expressions comprised in the BDNF/ERK/CREB signalling. (b) Summary schema of determined varied metabolites and proteins involved in the BDNF-CREB-Arg I axis. Arg I is the enzyme that catalyses the conversion from arginine to ornithine and urea. The transcription of Arg I is regulated by the expression of CREB, which is downstream of serotonin/BDNF/TrkB signalling. The BDNF-CREB-Arg I axis is downregulated after the induction of CUMS modelling and can be reversed by TSD administration.

hydroxytyptophan (5-HT) receptors are known targets of FX. As to the intervention of TSD, TSD (5 g/kg) raised serum serotonin by 54.1 mg/dL but suppressed Arg I by 47.8 mg/dL in the CUMS rats.

Effects of TSD on the modulations of hippocampal serotonin, glycine, Arg I and related metabolites

Hippocampal levels of above determined neural transmitters and metabolites were also measured based on similar methods hereinbefore. By comparing to their levels determined in the control group, relative levels of these targeted molecules in the hippocampus from groups of rats are shown in Figure 4(c). Interestingly, like the findings in the serum, serotonin and glycine deprivations were found to be improved in the hippocampus by TSD interventions. We also observed significantly decreased hippocampal Arg I, urea and ornithine in the rats treated with the medium dosage of TSD.

Effects of TSD on BDNF, TrkB, ERK, PERK and CREB protein expression in the hippocampus

Caldwell et al. showed that cAMP response element-binding protein (CREB) was involved in the transcription of Arg I (Caldwell et al. 2018). CREB is modulated by the BDNF-TrkB-ERK signalling cascade. Strikingly, we found that the BDNF-CREB axis was closely related to the pathology of depression (Wang et al. 2017). Therefore, we evaluated the levels of related target proteins in the hippocampus of rats by Western blot analysis to determine whether the effect of TSD on Arg I release was due to the regulation of the BDNF-CREB axis (Figure 5(a)). The results of ANOVA tests displayed that the expression levels of BDNF and TrkB proteins were significantly downregulated compared with the levels in the normal group (BDNF: (F (5, 17)=5.025, $p<$ 0.05); TrkB: (F (5, 17)=33.093, $p<$ 0.001); CREB: (F (5, 17)=35.045, $p<$ 0.001)), and the effect was effectively reversed by TSD (2.5, 5 and 10 g/kg) treatment. Also, the expression of ERK1/2 was decreased in the CUMS model (F (5, 17)=15.190, $p<$ 0.01). TSD treatment increased the phosphorylation level of

hippocampal ERK1/2. These results suggested that TSD might exert its antidepressant effect by inhibiting arginase, which was related to the upregulation of the BDNF/ERK/CREB signalling pathway. Together, a schema summarizing the possible protein targets of TSD belonging to the BDNF-CREB-Arg I axis is shown in Figure 5(b).

Discussion

The incidence of DD has increased globally in recent years, which has become a worldwide public health problem. Therefore, safe and effective anti-depression drug development is always required. Recent studies have shown that ethnomedicine is a valuable tool for developing effective drugs against depression (Shao et al. 2020). In particular, some formulas of TCM have been reported to be helpful to improve DD (Zhang and Cheng 2019). We first reported the effect of TSD on depression-like symptoms.

In the present study, depression-like symptoms were induced with CUMS, a model that imitated the occurrence and pathogenesis of human depression (Geng et al. 2019). After the modelling within rats, the results of behavioural experiments showed that such a modelling method was reliable. Also, rats treated with TSD had significant decreases in the time of immobility in the behavioural experiments.

FST and OFT were designed herein to evaluate despair and anxiety-like behaviours in model animals. Active swimming was the normal behaviour of the non-CUMS rats in the FST. The reduction of the movements in the water was considered a depression-like symptom in the CUMS rats, which were significantly improved by TSD interventions. The OFT was used to evaluate anhedonia and anxiety-like behaviours. Increased rearing and grooming times, and the numbers of squares crossed in the TSD-treated rats provide evidence that locomotor and exploratory activities of the CUMS rats were improved by TSD treatments. It is worth noting that the sucrose preference test (SPT) is another essential test for the observation of anhedonia-like behaviours. However, SPT was not performed in the rats because sucrose intake might extensively alter the metabolome of the rats. Instead of SPT, anhedonia-like behaviours could be also estimated by the grooming times recorded during the OFT. The increased grooming frequency in the TSD-treated rats compared with the rats in the non-treated group was another signal of the antidepression-like effect of TSD.

A further investigation based on metabolomic techniques was conducted to better understand the aberrant metabolic variations associated with CUMS and the pharmacological effect of TSD in CUMS rats. The dysregulation of two urea cycle intermediates, urea and ornithine, was a primary factor for the discrimination between rats with and without TSD gavage, and between the blank group and the model group. The increases in the levels of these two metabolites in the serum of model group rats indicated enhanced arginase degradation, which was ameliorated after TSD administration. The effect was further demonstrated by the results of the serum levels of Arg I, which showed that the variations in serum Arg I were in line with those of urea and ornithine. It was documented previously that the increase in the arginase level in the blood was associated with the occurrence of depression (Cao et al. 2020).

Interestingly, the level of Arg I was also regarded to be predicable for liver and heart injury (Sims et al. 2017). Correspondingly, TSD was widely used to treat heart diseases and was found to be effective in improving liver injury (Xi et al. 2016; Tao et al. 2020). Therefore, the restoration of arginase after the dosing of TSD might also indicate the reversals of organ injuries caused by CUMS. In addition, the upregulation of arginase was also related to immune defection and aberrant responses to inflammation (Kim et al. 2020). The reversal of arginase disorder within TSD-treated rats might be partly owing to the anti-inflammatory effect of TSD, which was documented in another study (Yuan et al. 2018).

The general declines in the levels of AAs (glycine, alanine, proline, phenylalanine, glutamate, lysine and tyrosine) were remarkable features of CUMS. After treatment with TSD, the levels of most AAs recovered in the serum of rats. First, the decreases in the levels of AAs suggested a malnutrition situation in rats with CUMS, which was reported previously (Tan et al. 2016). Such a symptom was evidenced by the weight losses in the model group (Figure 1(a)). The impaired anabolic metabolism could be partly reflected by the lower levels of energetic metabolites such as citrate and lactate in rats with CUMS than in other groups. Notably, common discriminants between M versus B and M versus TSD-treated rats, such as alanine and glutamate, were AAs involved in the anaplerotic reactions from AA to the TCA cycle, which is the core metabolic pathway for energy supply. Second, the amelioration of the defective Tyr/Phe pathway was also found in the TSD-treated rats, which was in accordance with the study by Liu CC et al. (2015). Finally, glycine as well as glutamate, which could be converted into GABA, were associated with neurotransmitters. Thus, besides the diminished secretion of glycine and 5-HT, depressive symptoms in rats with CUMS were firmly attributed to the inhibited transmitter synthesis.

Other common discriminant metabolites between M versus B and M versus TSD included 3-AIB, pseudouridine and 3-HB. 3-AIB is known as a critical synthetic precursor of AAs. Its variation pattern is in line with the pattern form most serum AAs in rats. Pseudouridine was reported recently as a potential biomarker of post-stroke depression (Cho et al. 2020). 3-HB is an end-product of β-oxidation in fatty acids. A decrease in the level of 3-HB in rats with CUMS was consistent with the diminished serum levels of fatty acids such as propionic acid compared with the blank controls, and lower levels of arachidic acid found in the comparison between CUMS and nontreated rats.

Our data also suggested that the dramatic dysregulation of Arg I in rats with depression was involved in the inhibited BDNF/CREB signalling and was reversible after the intervention of TSD. BDNF and its membranal receptor (TrkB) in the hippocampus were implicated in both the pathogenesis of depression and the antidepressant actions (Yu and Chen 2011). Several studies showed that the medications targeting the BDNF-TrkB-CREB axis improved depression-like symptoms in animal models (Lee et al. 2020). Interestingly, a putative compound hydroxysafflor A issued from safflower was reported to up-regulate BDNF expression (Xing et al. 2016). Similar results were available in another recent study showing the effect of safflower yellow, a drug extracted from safflower, on the activation of BDNF/TrkB/ERK signalling (Pang et al. 2020). Alternatively, the ethanolic extract of *Radix Rehmanniae praeparata* also helped enhance BDNF levels in the serum of rats with CUMS (Wang et al. 2018). Other potential compounds contained in TSD involving depression therapy encompassed albiflorin from *Radix Paeoniae Alba* (Wang et al. 2016), ferulic acid from both *Rhizoma Chuanxiong* and *Angelica sinensis* (Liu Y-M et al. 2017), and ligustrazine from *Rhizoma Chuanxiong*. Remarkably, ferulic acid could increase BNDF expression and reduce oxidative stress induced

by chronic corticosterone stimulation in mice (Zeni et al. 2017). Given that Arg I is a pivotal marker of NO production, the effect of TSD on depression may be due to the anti-inflammatory effect. Ligustrazine was also reported to attenuate inflammation and oxidative stress in rats (Li et al. 2019). Furthermore, neurotrophic effect of amygdalin, one of the main ingredients from *Semen Pruni Persicae* was previously documented (Park et al. 2018). Hence, the anti-depressive effect of TSD might attribute to all the components of TSD.

Our findings also suggested that the effect of TSD in CUMS rats was linked to the regulation of neurotransmitter metabolism including serotonin and glycine. Quantifications of these two metabolites in the serum were in accordance with the findings in the hippocampal tissue, whose variations between the model and TSD-treated groups were similar to Arg I and its products. Emerging data showed close interactions between BDNF and serotonin. Some studies suggested that BDNF was the target of serotonin (Martinowich and Lu 2008). However, the reverse regulation of serotonin receptor 5-HT1A by BDNF expression was also reported by Homberg et al. (2014). Together, our data implied that the actions of TSD on BDNF and serotonin were interdependent.

Our study had some limitations. The contributions and the interplays between each component of TSD for treating depression-like symptoms remain unclear. Second, the sample size of model rats was limited. Besides, our present study only analysed the 14-day acute toxicity of TSD. The subacute toxicity was not measured, which is important for further clinical applications of TSD.

To summarize, our findings showed that the TCM formula TSD helped to improve depression-like symptoms in CUMS rats. The mechanism underlying the efficacy of TSD on depression-like symptoms was related to the enhancement of neural transmitters such as glycine and serotonin, as well as to the improvement in disorders on the BNDF-CREB-Arg I axis. Our results also provide evidence that metabolomics is a useful tool for uncovering medicinal effective targets. Further studies should investigate the antidepressant-like effects of the six component medications of TSD using a larger number of samples.

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Author contributions

Xiaoping Zhang: conceptualization, writing-original draft, formal analysis and methodology; Zeng Li: conceptualization and writing-original draft; Chuanpu Shen: conceptualization and investigation; Jinzhi He: writing-original draft, investigation and validation; Longfei Wang: data curation, validation and methodology; Lei Di: data curation and methodology; Bin Rui: data curation and validation; Ning Li: conceptualization and project administration; Zhicheng Liu: conceptualization, data curation, writing-original draft, validation and project administration. All the authors contributed to the review and editing.

Disclosure statement

The authors declare that there is no conflict of interest.

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Data availability statement

All the data generated or analysed during this study are included in the manuscript and the supplementary data.

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