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GC-MS based metabolomics uncovers the mechanism of *Curcumae rhizoma* and *Sparganii rhizome* on blood stasis syndrome in liver dialysis

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Abstract: Blood stasis syndrome (BSS) is characterized by blood retardation and is the major cause of some deadly diseases. Some factors that affect BSS have been identified. However, the small molecule that related to BSS is still largely unknown. Traditional Chinese Medicine (TCM), such as Sanleng and Ezhu, has been used for a long time in treating BSS and promising outcomes have been achieved. However, the mechanism of how they work is unclear. Thus, we constructed the Rat BSS model and treated them with Sanleng and Ezhu. Then, the liver dialysis of those rats was collected and the small molecule metabolites were analyzed by GC-MS based metabolomics approach. Our results showed after Sanleng and Ezhu treatment, several small molecule metabolites were significantly changed metabolites (VIP>1 and P<0.05). Pathway enrichment analysis also showed that Sanleng and Ezhu share the similar mechanism in treating BSS, such as regulating Glyoxylate and dicarboxylate metabolism pathway and energy metabolism. Besides, we also identified some key metabolites that were significantly correlated with BSS. In conclusion, those findings uncover the mechanism of Sanleng and Ezhu in treating BSS.

Keywords: Blood stasis syndrome, metabolomics, Traditional Chinese Medicine, Sanleng and Ezhu, metabolic pathway

INTRODUCTION

Blood stasis syndrome (BSS), which is called Xueyu Zheng in China, is characterized as the retardation or cessation of the blood circulation. The disturbance of the blood flow will finally lead to other deadly diseases, such as ischemic cardiac disease and stroke (Jiangquan Liao 2016). Understanding the mechanism of BSS will be helpful for the prevention and treatment of those diseases. Thus, many scientists applied system biological methods to investigate BSS, including metabolomics, proteomics, transcriptomics or other approaches (Jiangquan Liao 2016; Wen, Wang *et al*. 2017). Some specifically changed small molecule metabolites and proteins were found. However, it is still very unclear about the mechanism of BSS, especially in metabolism aspects.

Traditional Chinese Medicine (TCM) is widely used in China for the treatment of BSS for many years, and relatively good outcomes were achieved. In our previous report, the treatment with Sanleng (S, *Sparganii rhizome*) and Ezhu (E, *Curcumae rhizome*), two TCM that were used frequently in relieving the syndrome of blood cessation, significantly improved the hemorheology parameters in the model rats of BSS (Li Lin 2018). The blood dialysis samples of Sanleng and Ezhu treated rats have also been analyzed by metabolomics tools, and the potential mechanism has been illustrated. Besides of blood, the relationship between BSS and liver diseases has also been reported in many papers (Xie, Yang *et al*. 2014; Song, Chen *et al*. 2018), demonstrating a potential role of liver in the development of BSS. However, there's still no report on the metabolomics changes in liver in the background of BSS.

Metabolomics tools have been widely used in many areas of research, including cancer, liver disease, as well as BSS (Caussy, Ajmera *et al*. 2018). In the study of BSS, many researchers focused on the metabolomics profiles of blood and urine samples from patients or model animals (Yuan, Zhong *et al*. 2019). However, it is still very unclear whether Sanleng and Ezhu will affect the function of liver and what's the mechanism in BSS. In this study, we collected the micro-dialysis, which has been used in many papers to obtain more accurate data(Nakahara D 1988; Zhou Y 2017), of liver and applied GC-MS to analyze the changes of small molecule metabolites in BSS model rats and after the treatment with Sanleng and Ezhu. Specific signaling pathways, including TCA cycle, were found to be changed. Further analysis also identified the metabolites significantly associated with BSS. Our results provide a new insight on BSS and the potential treatment mechanism of Sanleng and Ezhu.

MATERIALS AND METHODS

Traditional Chinese Medicine and chemicals

The traditional Chinese medicine Sanleng and Ezhu were purchased respectively from Panan and Ruian, Zhejiang

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Province, China, and authenticated by Dr. Jianwei Chen of Nanjiang University of Traditional Chinese Medicine. To prepare the extraction, they were grinded first and extracted by heating reflux method with distilled water (plant: water, 1:15) for 2 times. Then the extractions were combined and concentrated to a final concentration of 1g medicine/ml.

Other chemicals, such as Citric acid and Glucose, were purchased from Nanjing Chemical Reagent Corporation (Nanjing, China) and Sinopharm Chemical Reagent Co., Ltd. (Beijing, China) respectively.

Animals

Male Sprague Dawley (SD) Rats with the body weight around 300 g were provided by Experimental Animal Center of Nanjing University of Traditional Chinese Medicine (License: SCXK-20080033). They were feed in a standard environment according to the instructions from "Guide for the care and use of Laboratory Animals" by the National Academy of Sciences and the National Institutes of Health. At the end of the experiment, all animals were euthanatized by submitting to CO_2.

Rat Model of BSS

Rats were randomly divided into two groups, the control group (CK) and model group (M). In model group, rats were stimulated according to previous reports(Li Lin 2018). Briefly, rats were subjected to two times sound stimulation at 70 db for 5 min, then two times of flash light stimulation at the frequency of 5 Hz and the intensity of 350 lumen for 5 min. After that, electrical stimulation (30 to 35 v, 0.3 seconds with 2 seconds' interval) was carried out for 5 min. Subsequently, 30 minutes of tail clip (about 15 N); 5 minutes of ice bath stimulation were conducted. All of those stimulations were carried out 2 times per day. Finally, Rats were constrained for 2 hours every day to limit their activities.

After 30 days of stimulation, the model rats were further randomly divided into 3 groups by feeding them with different TCM extractions at a concentration of 1 g/kg, the model group (M, fed with distilled water), Sanleng group (S, fed with Sanleng) and Ezhu group (E, fed with Ezhu). The control group was also fed with distilled water. Each group has 6 rats. The treatment lasts for one week. After that, the hemorheology parameters were measured (Li Lin 2018).

Sample collection

In this experiment, rat liver dialysis fluid was collected for further analysis. Briefly, SD rats were anesthetized with 10% chloral hydrate. After exposing liver tissue, a micro-dialysis probe with semi-permeable membrane was planted in the middle lobe of liver and tied tightly with abdominal muscle. A catheter, connected to the probe, was immobilized on the skin outside. The probe was also connected to a micro infusion pump at an infusion rate of 2 μl/min. After one hour equilibration, the dialyzate was collected for one hour and stored at -80°C for analysis.

Sample pretreatment

To prepare metabolites for further analysis, 100μl dialysis fluid was combined with 350μl methanol, and then 50μl L-2-chlorophenylalanine (4μg/ml, Shanghai Hengbai Biological Technology Co. Ltd., Shanghai, China) was added in. After brief vortex, the mixture was subjected to centrifugation with a speed of 12000 rpm at 4°C for 10 min. 350 μl supernatant was collected and placed in a sample injection tube which was placed in a vacuum concentrator to drying the supernatant. After adding Methoxyamine hydrochloride, the samples were placed in a 37°C for 2h. Then 100μl BSTFA with 1% TMCS (REGIS Technologies Inc., Morton Grove, Illinois, USA) was added quickly and incubated in 70°C for 1h. Cool down to room temperature.

Mass spectrometry

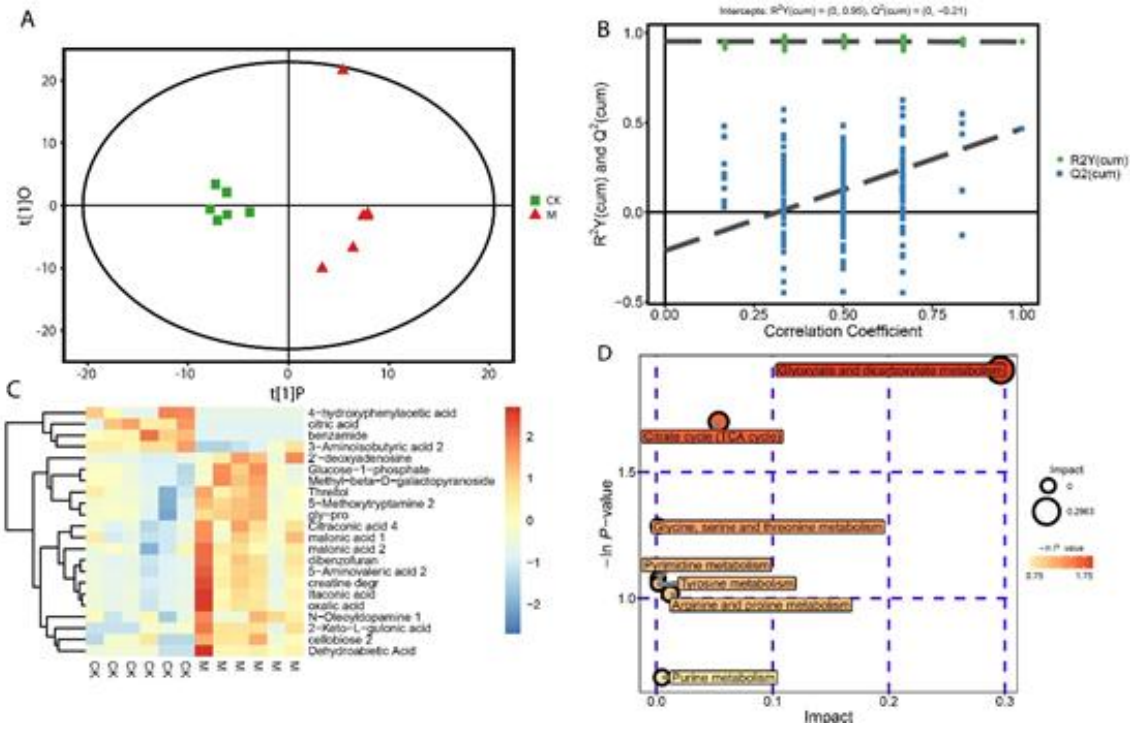
All the samples were analyzed by gas chromatography-mass spectrometry (GC-MS). Sample was injected into an Agilent 7890 GC system (Agilent; Santa Clara, CA, USA) coupled with a Pegasus 4D time-of-flight mass spectrometer (LECO; St. Joseph, MI, USA). Chromatographic separation was performed on a DB-5MS capillary column (30 m x 250 mm ID, 0.25 um film thickness; J&W Scientific; Folsom, CA, USA). Helium was used as the carrier gas with the purge flow at 3 ml/min and the flow rate of elution carrier gas at 1ml/min. To get good separation, the initial temperature was set at 80°C for 0.2 min, and gradually increased to 180°C at a rate of10°C per minute. After that, it was raised to 240°C at a rate of 5°C/min. Finally, the temperature was raised to 290°C at the speed of 20°C per minute and kept for 11min. The temperatures for injection port, transfer line and ion source were set at 280, 245 and 220°C respectively. The Bombardment voltage is 70 eV. The MS data were acquired in full-scan mode with the mass to charge ratio (m/z) range of 20-600.

Pearson correlation analysis

To identify significant correlations among significantly changed metabolites and the blood stasis parameters, we applied Pearson correlation analysis to estimate the correlation coefficiency and significance. The correlations with a P value smaller than 0.05 were considered as significant. Calculations were done by using our in-house R scripts.

STATISTICAL ANALYSIS

Chroma TOF4.3X software of LECO Corporation and LECO-Fiehn Rtx5 database were used for raw peaks extraction, calibration of the baseline and the baseline



(A) OPLS-DA score plot showed very well separation of the control group (CK) and BSS model group (M). (B) The permutation test of the OPLS-DA model. (C) Heatmap showed the significantly changed metabolites in BSS model rats. The relative expression level of metabolites is showed was plotted in the heatmap. Red, increase of metabolites; Blue, decrease of metabolites. (D) Bubble plot of the enriched pathways in BSS model rats.

Fig. 1: Untargeted GC-MS analysis identifies the differentially changed metabolites and signaling pathways in BSS model rats.

data filtering, peak alignment, deconvolution analysis, peak identification and integration of the peak area. The area of metabolite spectra was normalized to that of the spectrum of internal standard. Then the standardized data were analysis by multivariate analysis by using SIMCA 14.1 (Umetrics, Sweden), including principal component analysis (PCA) and orthogonal partial least squares discriminate analysis (OPLS-DA). The level of metabolites was compared by calculating the fold change and statistical significance by student t test. The metabolites with VIP >1 (Variable importance in projection) and P<0.05 were considered as significantly changed (Tan, He *et al*. 2018). For multiple groups comparation, posthoc Tukey's test was used, and P<0.05 was considered as significantly changed.

RESULTS

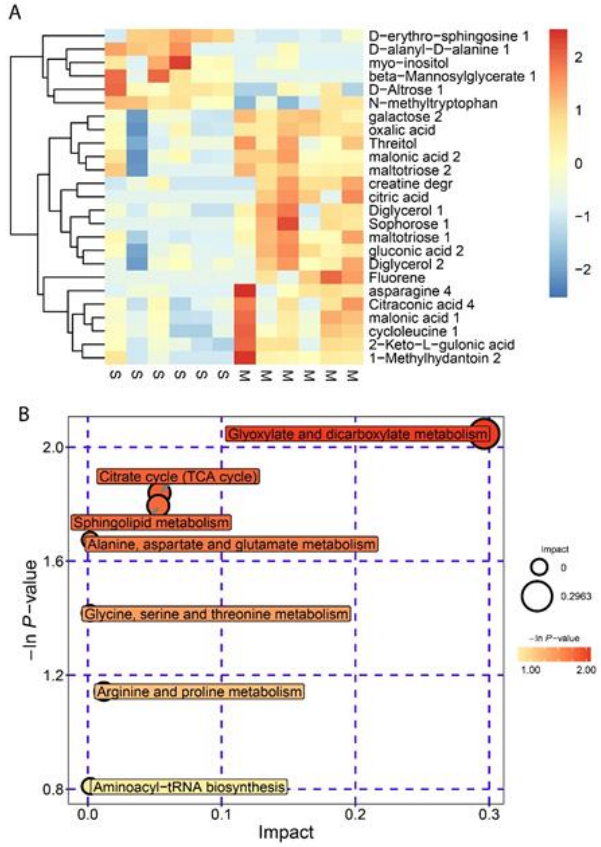
Metabolic profile alteration in Rat model of blood stasis syndrome

After 30 days of stimulation, the efficiency of BSS model construction was evaluated. As reported in our previous study, all the hemorheology parameters and blood viscosity index were significantly changed, demonstrating the success of BSS mode. At the same time, Sanleng and Ezhu treatment rescued the blood stasis phenotype. To understand the underlying mechanism, we had done the metabolomics study of blood dialysis fluid and found some key metabolic pathways (Li Lin 2018). In this study, we collected the liver dialysis fluid of the same rats and conducted metabolomics studies to further answer the question. As it is shown in fig. 1A, the model group and control group were separated very well in OPLS-DA score plot, demonstrating the different metabolic pattern between the two groups. Furthermore, the OPLS-DA model was validated by permutation test with the Q2(cum) intercept of -0.21 (fig. 1B), demonstrating the present OPLS-DA model is good to tell the difference in the metabolic profiles of the two groups. At the same time, some small molecule metabolites were identified to be significantly changed in BSS model rats (fig. 1C). By doing the KEGG pathway enrichment analysis, we found several pathways, including TCA cycle and glyoxylate and dicarboxylate metabolism, were significant changed in BSS model rats (fig. 1D).

Sanleng treatment rescued blood viscosity of BSS Rats

Sanleng is a traditional Chinese medicine that always been used in treating blood stasis syndrome. Our previous report showed the rescue effect of Sanleng on BSS model rats, and we also analyzed the metabolomics changes by using blood dialysis samples. Here, we compared the metabolomics change of liver dialysis between Sanleng treated BSS rats and model rats by GC-MS. We found that

Sanleng treatment can partially rescue the metabolism of model rats. The levels of some metabolites that are significantly changed in model rats, such as 2-keto-L-gulonic acid, 4-hydroxyphenylacetic acid and cellobiose, are rescued back by Sanleng treatment (fig. 1C & fig. 2A). Besides, Sanleng also induced the changes of some metabolites that are not changed in model rats, such as the amino acid asparagine and amino acids derivatives D-alanyl-D-alanine, N-methyltryptophan and cycloleucine (fig. 2A). These data suggest the involvement of some complementary signaling, especially amino acid metabolism. Then, we analyzed the signaling pathways enriched after Sanleng treatment. Except the TCA cycle, glyoxylate and dicarboxylate metabolism pathway, we also found the enrichment of sphingolipid metabolism pathway (fig. 2B), suggesting a potential role those signaling pathway in the effect of Sanleng on blood stasis syndrome.

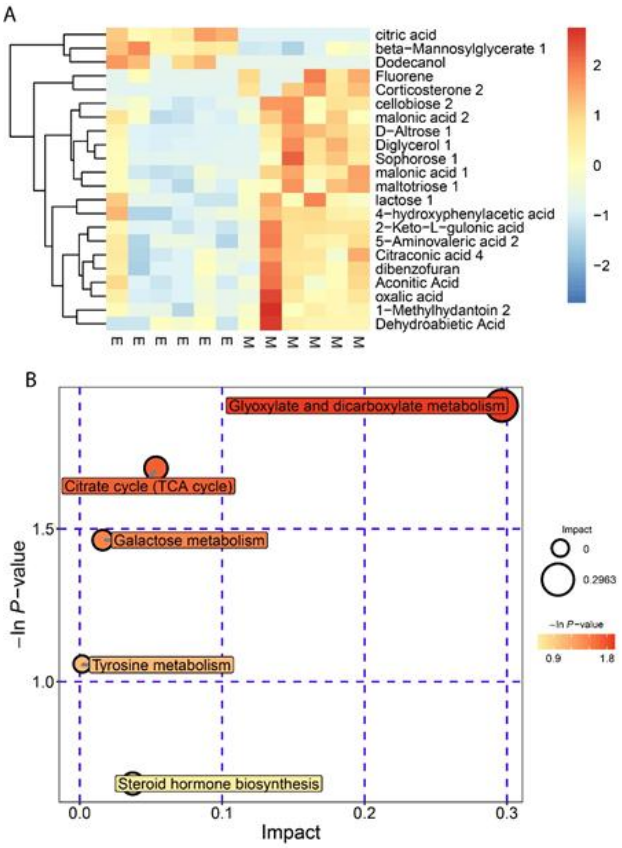


(A) Heatmap of differentially expressed metabolites in model rats after treatment with Sanleng. Red, increase of metabolites; Blue, decrease of metabolites. (B) Signaling pathway enriched in Sanleng treatment group compared with BSS model.

Fig. 2: Sanleng treatment rescued blood stasis by reverse the metabolite change caused by rat modeling.

Ezhu shares a similar mechanism with Sanleng in treatment of blood stasis syndrome

Similar with Sanleng, Ezhu is another widely used traditional Chinese medicine for blood stasis syndrome treatment. To understand the mechanism of how it works, we analyzed the metabolic changes in liver dialysis fluids. Many differentially expressed metabolites were found in Ezhu treatment group. The metabolites, such as 2-keto-L-gulonic acid, 4-hydroxyphenylacetic acid and cellobiose, which are changed in Sanleng treatment, were also significantly changed after Ezhu treatment (fig. 3A). The same with Sanleng treatment, glyoxylate and dicarboxylate metabolism pathway and TCA cycle are still the two most highly enriched metabolic pathways (fig. 3B). Those results demonstrate that Ezhu and Sanleng may share a similar mechanism in treating blood stasis syndrome by regulating the same signaling pathways.

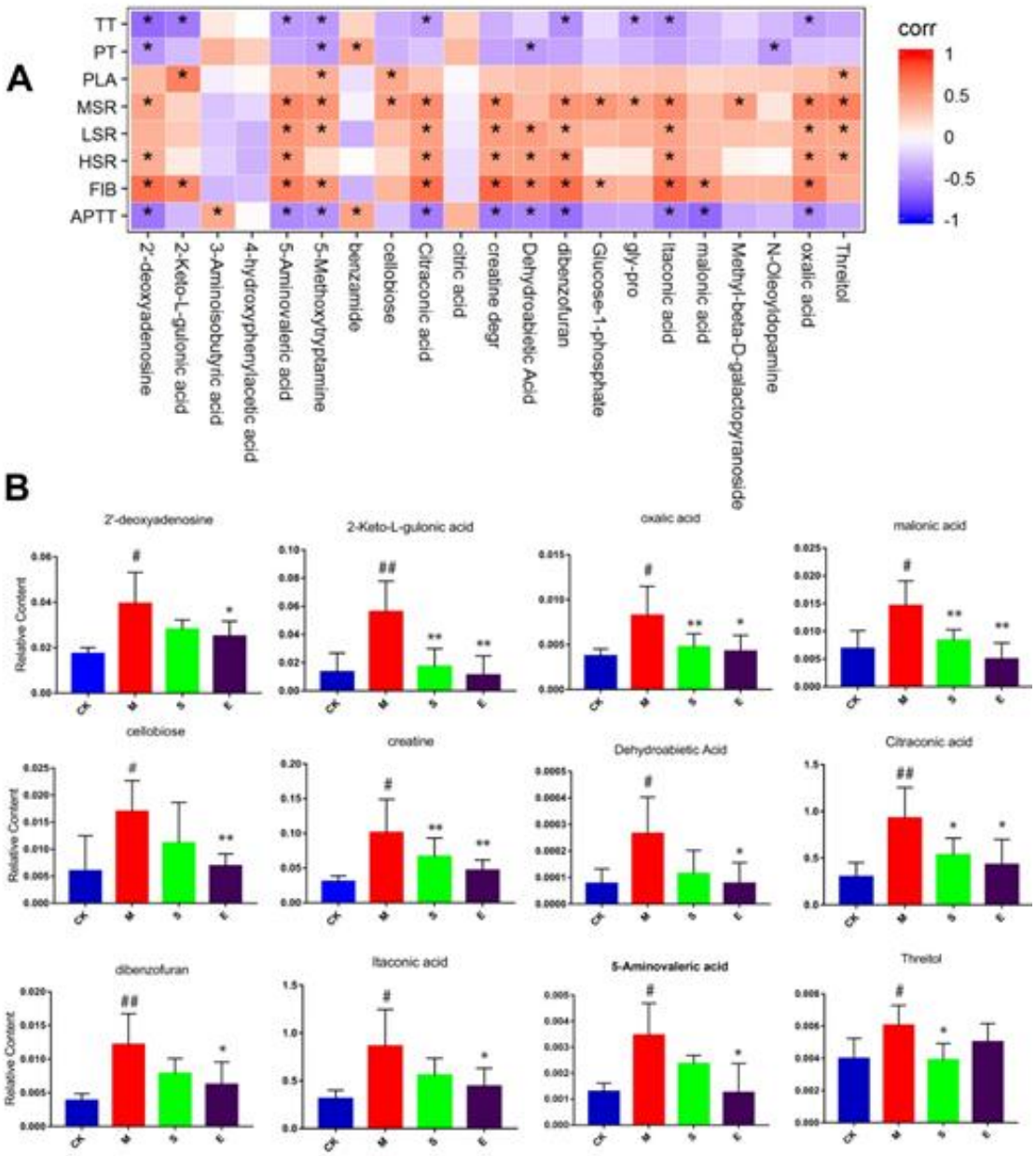


(A) Heatmap showed the differentially expressed metabolites in Ezhu treatment group compared with BSS model rats. Red, increase of metabolites; Blue, decrease of metabolites. (B) Bubble plot of the enriched signaling pathway in Sanleng treatment group compared with BSS model.

Fig. 3: Ezhu shares similar signaling mechanism with Sanleng in treating BSS.

Several metabolites are significantly associated with BSS

To answer which metabolite is responsible for the change of blood viscosity and hemorheology in BSS model rats, we applied correlation analysis of the differentially expressed metabolites and the blood parameters measured in our previous report (Li Lin 2018). Most of the



(A) Pearson correlation analysis showed the significantly correlated metabolites with blood parameters. Red, positive correlation; Blue, negative correlation, *, P<0.05. (B)Bar plot showed the expression level of metabolites that showed significant correlation with blood parameters in different groups by one-way anova analysis followed by Tukey's test. CK, control group, M, model group, S, Sanleng group, E, Ezhu group. #, P<0.05, ##, P<0.01 compared with control group. *, P<0.05, **, P<0.01 compared with model group.

Fig. 4: Some significantly changed metabolites are correlated with blood stasis parameters.

differentially expressed metabolites are positively associated with the blood viscosity in Plasma (PLA), Low shear rate (LSR), Middle shear rate (MSR), and high shear rate (HSR) level, but negatively associated with hemorheology parameters including activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT) (fig. 4A). The level of those metabolites is shown in fig. 4B. Ezhu and Sanleng treatment can rescue the level changes caused by BSS modeling. Those data suggest that modulating the level of those metabolites may be a good way to improve BSS.

DISCUSSION

In this study, we applied GC-MS based metabolomics approach to investigate the effects of Ezhu and Sanleng on BSS Rat model. Our Data showed that Ezhu and Sanleng shared similar signaling pathway in treating BSS.

Furthermore, several metabolites that significantly associated with blood stasis syndrome were identified. These data provide a new insight on the mechanism of BSS and the treating effect of Ezhu and Sanleng.

Blood stasis syndrome in traditional Chinese medicine is not just the issue of blood, but also related to many other diseases, like dermatological disease and liver diseases (Goto 2014). In our previous study, we had analyzed the metabolomics profile of blood dialysis samples from the same rats used in this study, and found several key metabolites changed. To understand the mechanism related to liver, we conducted the metabolomics study with the liver dialysis samples. Our results showed that that the differentially changed metabolites in the liver dialysis are almost totally different with blood dialysis. However, the signaling pathways that play important role in Sanleng and Ezhu treatment are similar. Analyzing the possible metabolic mechanism in liver dialysis, we found the top 2 ranked signaling pathways after the treatment of Sanleng and Ezhu are glyoxylate and dicarboxylate metabolism pathway and tricarboxylic acid (TCA) cycle (fig. 2 & fig. 3). Especially the glyoxylate and dicarboxylate metabolism pathway scored the most significantly changed and with high impact in both blood and liver dialysis. In another aspect, we also found the similar change of TCA cycle in the BSS model rats, which is very similar with the previous report by Wang Y and the colleagues that the disorder of TCA cycle also happened in blood stasis syndrome associated with coronary heart disease(WANG Yong 2016). TCA cycle, also known as Krebs cycle, is the key metabolic pathway that connects many metabolisms such as carbohydrate metabolism, lipid metabolism and protein metabolism. The dysfunction of TCA cycle in our BSS model suggests the aberrant energy metabolism. Besides, the effect of Sanleng and Ezhu on energy metabolism of BSS rats possibly responsible to their treating outcome. These results suggest the similar signaling change of Sanleng and Ezhu treatment and provide the new candidate pathways for targeting in BSS treatment.

Previous reports had shown that there're relationships between BSS and liver diseases (Liu, Liu *et al*. 2012) (Teng L 2015) (Xiao-Xi, Zhao-Xiang *et al*. 2011), suggesting a potential link of liver metabolism and BSS. In this study, we found some metabolites in the liver dialysis of BSS model rats are significantly associated with blood stasis parameters (fig. 4A). As it is shown in fig. 4B, most metabolites associated with blood viscosity are obviously increased in BSS model rats, suggesting a possible role of these metabolites in causing the blood cessation. In another aspect, Sanleng and Ezhu treatment can rescue the metabolites changes in model rats, providing a new insight of treating mechanism. However, how those metabolites regulate blood stasis and what's the action mechanism still need to be addressed.

CONCLUSION

In the present study, we discussed the metabolic mechanism of Sanleng and Ezhu in treating BSS. We found some key metabolites and metabolic signaling pathways contribute to the effect of Sanleng and Ezhu. However, what're the key components of the two TCMs that enter into the blood circulation and play the critical role in its action is still not known and need to be addressed. In conclusion, our data provide new knowledge about how Sanleng and Ezhu rescue the blood stasis syndrome, and also can improve our understanding on the role of metabolites in blood related diseases.

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