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Metabolomics approach to identify therapeutically potential biomarkers of Zhi-Zi-Da-Huang Decoction effect on hepatoprotective mechanism

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Abstract: Zhi-Zi-Da-Huang Decoction (ZZDHD), a Traditional Chinese Medicine (TCM) formula, has been widely used for the treatment of alcoholic liver injury for many years. To comprehensively explore the possible mechanism of hepatoprotective effects of ZZDHD, a nuclear magnetic resonance (NMR)-based metabolomic study, ^{1}H NMR spectra combined with pattern recognition methods including PCA, OPLS-DA, was applied to identify potential plasma and liver biomarkers responsible for the positive effects of ZZDHD for alcohol-induced liver injury rats. PCA showed a global overview of control, alcohol and ZZDHD group rats. Potential biomarkers of plasma and liver that were obtained from OPLS-DA loading plots combined with the corresponding VIP values reflected the hepatoprotective effects of ZZDHD associated with alcohol-induced liver injury. Results suggested that 9 potential plasma biomarkers including lipoprotein, leucine, valine, dihydrothymine,

3-D-hydroxybutyrate, lactate, alanine, acetate and glucose, and 8 potential liver biomarkers such as triglyceride, lactate, alanine, acetate, glutamine, glucose, xanthine and hypoxanthine were identified. Related metabolic pathways analysis found that ZZDHD significantly alleviated the disturbance in energy metabolism, glucose metabolism, amino acid metabolism, lipid metabolism, and nucleic acid metabolism, ameliorating lipid peroxidation and permeability change of membrane, and oxidative stress induced by alcohol. The results indicated that ZZDHD could achieve remarkable effects on alcoholic hepatic injury through partially preventing or regulating the perturbed metabolic pathways. The study also demonstrated that NMR-based metabolomic was a useful tool for screening potential biomarkers, further helping to explain the therapeutic mechanism of TCM formula on a comprehensive scale.

Keywords: Zhi-Zi-Da-Huang Decoction, alcoholic liver injury, NMR, metabolomics, pattern recognition, potential biomarkers

Introduction

Metabolomics, a novel research method used to detect metabolic subtle changes in biological samples such as plasma or tissue, has been widely applied to the measurement of multiparametric metabolic response of living systems to disease status or biochemical effects of drugs in biological systems.[1] It can capture global metabolic changes, investigate the pathogenesis of diseases or the intervention effects of drugs through potential biomarkers identified.[2-6] Metabolomic is also an ideal tool to insight into the interaction mechanism between traditional Chinese medicine (TCM)

and biological organism.[7, 8] It can be used to assess the efficacy of TCM and the mechanism through a series of different sample spectra obtained from analytical techniques combined with pattern recognition methods.[9] Many recent researches have reported the application of metabolomic studies of TCM concentrated on exploring the molecular mechanisms of hepatoprotective effects in liver injury or liver diseases through potential biomarkers identified.[10-13]

Zhi-Zi-Da-Huang Decoction (ZZDHD), a famous Chinese traditional medicine prescription firstly recorded in Jin-Kui-Yao-Lue (Synopsis of Golden Chamber) by Zhongjing Zhang, consists of four crude herbs: *Rheum officinale* Baill. (Da-Huang), *Gardenia jasminoides* Ellis (Zhi-Zi), *Citrus aurantium* L. (Zhi-Shi) and Semen Sojae Preparatum (Dan-Dou-Chi).[14] In our prophase research, the optimal hepatoprotective effects of ZZDHD for alcohol-induced liver damage have been determined at a dose of 12 g/kg/day body weight.[15, 16] However, the remedial mechanism of the overall effect of ZZDHD remains entirely unclear.

In this study, ^{1}H NMR spectra were used to detect a global profile of endogenous metabolites; following which pattern recognition was performed to discover potential plasma and liver biomarkers of rat exposed to alcohol and hepatoprotective effects of ZZDHD. The aim of the present study was to (1) find potential biomarkers in plasma and liver and (2) improve the understanding of the therapeutic efficacy and the underlying hepatoprotective mechanism of ZZDHD.

Materials and methods

Chemicals

Analytical grade alcohol, methanol, sodium chloride, $Na_2HPO_4 \cdot 12H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ were bought from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Deuterium oxide (D_2O, 99.9 % D) and sodium 3-trimethylsilyl [2, 2, 3, 3-$^2H_4$] propionate (TSP) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of ZZDHD sample

All the herbs were purchased from Xiansheng Medicine Company (Nanjing, China) and identified by Professor Min-Jian Qin, Department of Medicinal Plants, China Pharmaceutical University. Mixture of crude herbs (48 g), *Rheum officinale* Baill., *Gardenia jasminoides* Ellis, *Citrus aurantium* L. and Semen Sojae Preparatum at the weight ratio of 1: 3: 4: 8, were extracted thrice under distilled water (1: 10; w/v), each for 30 min. Finally, the extracted ZZDHD solutions were pooled together, freeze-dried, and stored at - 20 °C before use. For the quality control of ZZDHD, six main ingredients, geniposide, naringin, neohesperidin, daidzein, rhein and emodin were determined by a HPLC-UV system on a LiChrospher-C18 column (250mm × 4.6mm, 5μm, Hanbon Science & Technology Co., Jiangsu, China). The HPLC chromatogram of ZZDHD was showed in supplemental Fig. 1 (Fig. S1).

Animal Handling and Sample Collection

Male Sprague-Dawley rats (n=18) weighted 180-220 g were purchased from Animal Experimental Centre of Qinglong Mountain (Nanjing, China). Animals were maintained at animal room with the following parameters: temperature of 20 - 25 °C, humidity of 55 - 65 % and artificial light/dark cycles from 08: 00 - 20: 00. All animal

experiments were approved strictly by the guidance for Experimental Animal Welfare of the National Guidelines (MOST of The People's Republic of China, 2006) at the Centre for SPF-grade Animal Experiments of Jiangsu Institute for Food and Drug Control.

After 7 days of acclimatization, rats were randomly divided into three groups of six rats each as follows: control group (CG), alcohol group (AG) and ZZDHD group (ZG). ZG rats were orally administered with ZZDHD solution (1.0 g/ml, 12 ml/kg/day) at 15: 00 from day 1 to day 9. Concurrently, ZG rats were given water orally at 17: 00 from day 1 to day 2, and then dosed with 50 % alcohol at a dose of 14 ml/kg/day at 17: 00 from day 3 to day 9. AG rats were orally administered 50 % alcohol at a dose of 14 ml/kg/day between 17: 00 and 18: 00 for seven consecutive days from day 3 to day 9 to induce liver injury animal model. The CG rats were always administrated with equivalent water. At 20: 00 on day 9, all animals were fasted and then euthanised following isoflurane anaesthesia after 12 hours. The surgical procedures were performed under isoflurane anaesthesia, and all efforts were made to minimize animal suffering.

The liver and blood samples from all groups were obtained immediately on day 10 (08: 00 a.m.). Blood samples were collected into Eppendorf tubes containing sodium heparin to obtain plasma samples, which was immediately snap-frozen in liquid nitrogen and stored at -80 °C for later analysis. Liver tissues were collected and divided into two parts. One part was immediately snapped frozen in liquid nitrogen and stored at - 80 °C until NMR spectroscopy, and the other part was used to

histology examination.

NMR measurements

Plasma samples were thawed at room temperature and centrifuged at 16000 rpm for 10 min. 300 μl supernatant was mixed with 150 μl phosphate buffer (0.2 mol/l, pH 7.4) and 150 μl 10 % w/v TSP (100 % D_2O), and then transferred into 5 mm NMR tubes for analysis. The liver tissues (60 mg) were homogenized three times with 600 μl of 50 % methanol and separately centrifuged at 16000 rpm for 15 min. Three combined supernatants were subjected to centrifugation (12000 rpm, 10 min). Then the resultant supernatants were dried under a stream of nitrogen. Each liver tissue extract was reconstituted into 600 μl phosphate buffer (0.15 mol/l, pH 7.4) containing 0.006 % TSP, 20 % D_2O. The solution was centrifuged at 16000 r min^{-1} for 10 min and 550 μl supernatant was transferred into 5 mm NMR tube for analysis. For plasma samples, the Carr-Purcell-Meiboom-Gill (CPMG) sequence (RD-90°-（τ-180° -τ）n- ACQ; RD = 2 s, τ = 350 μs, n = 100) with a total spin- echo delay (2 nτ) of 60 ms were used. For liver extracts, the standard noesygppr1d pulse sequence (RD-G_1-τ-90°-t_1-90°-t_m-G_2-90°-ACQ; RD = 2 s, t_1 = 4 μs, t_m = 100 ms) was used to obtain metabolic profiles. All the NMR spectra were Fourier transformed after an exponential line-broadening function of 0.5 Hz.

NMR data processing

Each spectrum was manually phased, baseline corrected and referenced to TSP (CH_3, δ 0.0) utilizing TOPSPIN software (version 2.1, Bruker Biospin, Germany). All ^1H NMR spectra were processed using the AMIX software package (Bruker Biospin).

The regions (δ 4.30-5.12, δ 5.51-6.80) in the plasma and (δ 4.50-5.20) in liver extract spectra were discarded to prevent variation in water signal. And ethyl glucuronide peaks[17] were also removed in plasma. Then all remained spectra were integrated into regions with typical bucket-width of 0.04 ppm using AMIX software package (Bruker Biospin). Each bucketed region of liver extract sample was normalized by integral normalization to compensate for the effect of variation in concentration, and the plasma spectra by probabilistic quotient normalization.

Pattern recognition processing and potential biomarker identification

Pattern recognition was performed using the SIMCA-P +13 software package (Umetrics, Umea, Sweden). A non-supervised principal component analysis (PCA) was firstly performed with mean-centered NMR data to examine group clustering. Then a supervised orthogonal projection to latent structure discriminant analysis (OPLS-DA) was further carried out with the pareto-scaled NMR data as the X-matrix and the group information as the Y-matrix to discovery potential biomarkers. All OPLS-DA models were calculated using seven-fold cross-validation method. The quality of the models was assessed by the parameters R^2X, and the predictability of models as Q^2. The models were considered as effective and reliable when these values are more than 0.5. The reliability and predictability are better when these parameters are closer to 1.0. Meanwhile, the models were also tested by the well-known cross-validated analysis of variance (CV-ANOVA) approach and $p < 0.05$ was considered to be statistically significant. In the OPLS-DA, the samples from different groups were separated into different classes to the classification, and differential

metabolites responsible for good separation of different groups were obtained from the corresponding loading S-line. The differential metabolites were further selected by variable importance in the projection (VIP) values using a VIP ≥ 1. Thus, these endogenous metabolites based on the S-line and VIP values were finally considered as potential biomarkers. Relevant heat maps of identified potential plasma or liver biomarkers were further performed to describe an unknown sample in the test set classified into the class according to the majority belongs to neighbors in the training set which are closest to this test sample. Heat map has long been used in pattern recognition, data mining and analysis.

Results and discussion

Histopathology study

Histopathology examination showed that control rats had normal liver tissue and no pathological changes (Fig. S2a). Typical pathological features of liver damage, such as fat particles of varying sizes substantial vacuolization, fatty deposition and lipid accumulation were observed in alcohol-treated rats (Fig. S2b). For ZZDHD-treated rats, pathological abnormalities were noticeably improved, which showed that the hepatoprotective function of ZZDHD (Fig. S2c). The above results indicated that the animal model of alcohol-induced liver injury was successfully, and the hepatic steatosis was ameliorated with administration of ZZDHD.

Pattern recognition analysis of plasma metabolomics data

Typical ^{1}H NMR spectra of plasma from CG, AG and ZG were shown in Fig. 1 with

major metabolites marked. Endogenous metabolites were identified by referencing previously reported the corresponding chemical shifts of the metabolites,[18-21] some publicly metabolomics databases, such as Madison http://mmcd.nmrfam.wisc.edu/, KEGG http://www.genome.jp/kegg/, HMDB http://www.hmdb.ca/, and Chenomx NMR Suite software (Version 7.5, Chenomx, Inc.). Specifically, endogenous metabolites of plasma samples mainly contained lipoprotein, dihydrothymine, 3-D-hydroxybutyrate, lactate, acetate, creatine, glucose, taurine, formate and a series of amino acids.

To obtain more details about metabolic profiles and discover potential plasma biomarkers, pattern recognition analysis was performed for the NMR metabolomics data. Firstly, the PCA of plasma profiles was performed to get a global overview of the metabolic response of rats exposed to alcohol and ZZDHD administration (Fig. 2a). In the score plot of PCA, each point represented an individual sample, the AG rats were completely separated from the CG rats, and the ZG rats were also completely separated from the AG. Due to the intervention effects of ZZDHD, the ZG rats were near to the CG rats, which indicated ZZDHD played a significant role in inhibiting alcohol induced liver injury.

Then, OPLS-DA was further conducted to explore significantly differential metabolites responsible for the group differentiation. A clear separation between the AG and CG (Fig. 2b) demonstrated remarkable metabolomic differences with good model quality ($R^2X = 0.925$, $Q^2 = 0.727$, $p = 1.64\times10^{-3}$). The corresponding loading plot of OPLS-DA indicated that metabolic changes induced by alcohol were obvious

visible including level elevations for leucine, valine, dihydrothymine, lactate, alanine, acetate and taurine together with level decrease for lipoprotein, 3-D-hydroxybutyrate and glucose compared with controls (Fig. 2c). To further valid the identified differential metabolites, variable importance to projection values (VIP) using a VIP ≥ 1 was necessary. Table 1 showed the VIPs of metabolites responsible for the loading plot. Comprehensive results identified 10 endogenous metabolites as potential biomarkers from the control and alcohol groups. Similarly, for alcohol and ZZDHD groups, a reasonably good separation was showed in the score plot of OPLS-DA with $R^2X = 0.815$, $Q^2 = 0.943$, $p = 2.74\times10^{-5}$ (Fig. 2d). Based on the corresponding loading plot (Fig. 2e) and VIP values (Table 1), 9 endogenous metabolites were selected including leucine, valine, dihydrothymine, lactate, alanine, lipoprotein, 3-D-hydroxybutyrate acetate and glucose, which could be considered as plasma potential biomarkers for the positoive therapeutic effect of ZZDHD. Then the selected 9 potential biomarkers from plasma built the prediction model using MeV software. The heat map, an unsupervised clustering, was constructed based on the identified potential significative biomarkers. The parallel heat map visualization (Fig. 3) showed distinct segregation for the alcohol and control rats, but a similar tendency to the control and ZZDHD rats.

Pattern recognition analysis of liver metabolomics data

Representative ^{1}H NMR spectra of rat liver tissue extract from CG, AG and ZG were presented in Fig. 4 with major metabolites marked. Endogenous metabolites of liver tissue extract samples mainly contained triglyceride, glucose, amino acids, purines,

and pyrimidines. In alcohol group, the increase in triglyceride was clearly observed. To investigate the significance of changes of metabolites, pattern recognition analysis was necessary. As for liver extract, the score plot of PCA was firstly conduced to get an overview of tends for normal, alcohol and ZZDHD groups rats (Fig. 5a). The result showed that CG and ZG rats had clearly separate from AG rats, and the CG was also close to ZG, indicating the hepatoprotective effects of ZZDHD rats exposed to alcohol.

The OPLS-DA model was then performed to detect differential metabolites from normal and alcohol group rats. CG and AG were clearly clustered into two groups from the corresponding score plot of OPLS-DA (Fig. 5b), which were verified with $R^2X = 0.941$, $Q^2 = 0.928$, $p = 6.06\times10^{-7}$ considered as valid. Differential metabolites of liver tissue extract from the two groups were selected through coefficient-coded loading plot (Fig. 5c). Next, the screened differential metabolites were further identified with a VIP value larger than 1.0 (Table 2). Nine metabolites containing triglyceride, lactate, alanine, acetate, glutamine, glucose, xanthine, adenosine and hypoxanthine are finally screened as potential biomarkers, which indicated alcohol disturbed their normal levels. Similarly, the plot of OPLS-DA score (Fig. 5d) obtained from ^{1}H NMR spectra of liver extract showed a clear separation between normal and alcohol rats ($R^2X = 0.899$, $Q^2 = 0.916$, $p = 4.99\times10^{-7}$). The result of the combination of the loading plot (Fig. 5e) and VIP values indicated that 8 endogenous metabolites were considered as liver potential biomarkers responsible for the positive therapeutic effect of ZZDHD. Meanwhile, the heat map of 8 liver biomarkers (Fig. 6) also

showed similar results as that of plasma biomarkers, which indicated alcohol could affect the normal metabolism of rats, and ZZDHD had clearly regulated the abnormal metabolism.

The effects of ZZDHD treatment for ALD in metabolic respects

The protective effects of ZZDHD on liver damage induced by alcohol were investigated through ^{1}H NMR spectra coupled with pattern recognition methods. The results showed alcohol affected 10 potential plasma biomarkers, 9 of which were reversed with the effects of ZZDHD intervention; alcohol altered the normal metabolic profile of 9 potential liver biomarkers, 8 of which were regulated responsible for the positive effects of ZZDHD. Related metabolic pathways were analyzed based on the identified potential plasma and liver biomarkers combined with Human Metabolome Database (http://www.hmdb.ca/), Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and MetaboAnalyst 3.0.

Energy metabolism and glucose metabolism

Lactate could reflect the supply-to-demand state of tissue.[22] It also was the end-product of glucose metabolism with anaerobic conditions.[23] In our study, lactate of AG showed increasing tendencies in plasma and liver, while the level of glucose appeared decreased. The phenomenon indicated alcohol could aggravate anaerobic metabolism, causing gluconeogenesis damage, further disrupted the levels of liver or plasma related metabolites. Additionally, alanine could be formed to pyruvate through transamination that amino acids in the muscle transferred an amino group.[24] Then alanine was transferred to liver by blood, which could deaminate back to pyruvate,

further generated glucose by gluconeogenesis pathway.[25] Our metabolomic results showed that alanine had obviously increased in liver together with decreased in plasma from AG. Evidence for gluconeogenesis damage was further supported by the distinct decrease in alanine of liver.

Alcohol disturbed the energy metabolism and glucose metabolism as revealed by remarkable increased level of lactate in liver and plasma, glucose decreased in liver and plasma, and decreased level of alanine in liver together with increased in plasma. However, the intervention effects of ZZDHD ameliorated the abnormality states by reversing these disturbed potential biomarkers.

Amino acid metabolism

In alcohol-induced liver injury, reactive oxygen species (ROS) and oxidative stress had been proven to play central roles.[26] Alcohol could increase the generation of ROS, excessive ROS disrupted the balance between the oxidation and anti-oxition systems, and further caused oxidative damage such as the lipid peroxidation of membrane and the cell membrane permeability change.[27-29] The elevated levels of amino acids (leucine, valine and alanine) suggested that alcohol altered the membrane permeability and made amino acids release into plasma from damaged hepatic cells. The reductions of glutamine and alanine in liver reconfirmed the changes of permeability of intracellular by the influence of alcohol.

ZZDHD could alleviate the membrane disruption as evidenced by regulating amino acid metabolism, such as reduced the levels of leucine, valine and alanine in plasma, and elevated levels of glutamine and alanine in liver.

Lipid metabolism and nucleic acid metabolism

Alcohol could inhibit the synthesis of protein components of lipoprotein in blood, further affected the secretion of triglyceride (TG) in liver to blood.[30] During alcohol-induced liver injury, abnormal situation of lipid metabolism could be reversed by ZZDHD, illustrated by the increasing level of lipoprotein in plasma and the reduced of TG in liver compared to CG rats. ROS from alcohol metabolism could lead to the oxidation of membrane lipid,[31, 32] disrupting both fatty acid oxidation and nucleic acid metabolism, as testified by the decreased levels of 3-D-hydroxybutyrate in plasma and xanthine, adenosine and hypoxanthine in liver, together with the increased level of dihydrothymine in plasma.

ZZDHD could partly attenuate the oxidative stress as evidenced by the elevated level of 3-D-hydroxybutyrate and lipoprotein in plasma, the reduced level of TG in liver. The regulation of the disorder of nucleic acid metabolism by ZZDHD was also indicated by restored levels of dihydrothymine, xanthine and hypoxanthine.

Conclusion

In this study, ^{1}H NMR-based metabolomic approch was used to investigate detailed information about the metabolic changes within an organism for the intervention effects of ZZDHD. ^{1}H NMR spectroscopy coupled with pattern recognition methods identified the specific metabolite changes for CG, AG and ZG, and 9 potential plasma biomarkers and 8 potential liver biomarkers responsible for the hepatoprotective actions of ZZDHD were detected. Related metabolic pathways analysis of the altered potential biomarkers indicated that ZZDHD significantly regulated lipid peroxidation

of membrane, altered cell membrane permeability, and oxidative damage induced by alcohol, alleviating the disturbance in energy metabolism, glucose metabolism, amino acid metabolism, lipid metabolism, and nucleic acid metabolism, thus achieving protective effects on the liver. The method used in this study did not only explore potential biomarkers but also could provide a comprehensive understanding of the underlying molecular mechanisms of ZZDHD for alcoholic liver disease.

Acknowledgments

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Figure Captions:

Fig. 1 Typical 500MHz ^{1}H NMR spectra of plasma from different group rats. Metabolite keys: 1, lipoprotein; 2, leucine; 3, isoleucine; 4, valine; 5, dihydrothymine; 6, 3-D-hydroxybutyrate; 7, ethyl glucuronide; 8, lactate; 9, alanine; 10, acetate; 11, N-acetyl-glycoprotein; 12, O-acetyl-glycoprotein; 13, acetoacetate; 14, pyruvate; 15, succinate; 16, α-oxoglutarate; 17, glutamine; 18, citrate; 19, creatine; 20, taurine; 21, methanol; 22, glucose; 23, phosphocholine; 24, unstaturated lipid; 25, tyrosine; 26, histidine; 27, phenylalanine; 28, formate.

Fig. 2 Multivariate analysis of plasma from CG, AG and ZG rats. A global overview from PCA analyse (a) and potential plasma biomarkers identifications from OPLS-DA model analyses (b, c, d, e).

Fig. 3 Heatmap of identified potential plasma biomarkers. Row, samples; columns, potential biomarkers. Color indicates metabolites expression value: lowest (blue) to highest (red).

Fig. 4 Typical 500MHz ^{1}H NMR spectra of liver extract from different groups. Metabolite keys: 1, isoleucine; 2, leucine; 3, valine; 4, 3-D-hydroxybutyrate; 5, triglyceride; 6,lactate; 7, alanine; 8, ornithine; 9,acetate; 10,glutamate; 11,methionine; 12,glutamine; 13,succinate; 14, citrate; 15,oxidized glutathione; 16,creatine; 17,lysine; 18, choline; 19,glucose; 20, uridine; 21, adenosine; 22, fumarate; 23, tyrosine; 24, phenylalanine; 25, uracil; 26, nicotinamide; 27, xanthine; 28, hypoxanthine; 29, formate.

Fig. 5 Multivariate analysis of liver extract from CG, AG and ZG rats. A global

overview from PCA analyse (a) and potential liver biomarkers identifications from OPLS-DA model analyses (b, c, d, e).

Fig. 6 Heatmap of identified potential liver biomarkers. Row, samples; columns, potential biomarkers. Color indicates metabolites expression value: lowest (blue) to highest (red).

Table Captions:

Table 1 List of the plasma differential metabolites identified from AG *vs*. CG and ZG *vs*. AG.

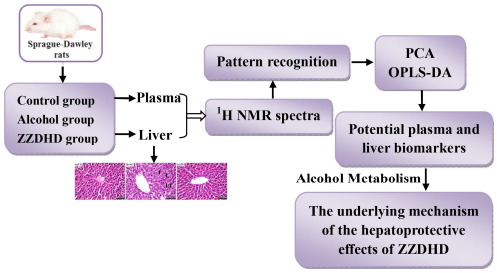
Table 2 List of the liver extract differential metabolites identified from AG *vs*. CG and ZG *vs*. AG.

GRAPHICAL ABSTRACT

Li An,[1] Qingshui Shi,[2] Fang Feng*[1, 3]

NMR-based metabolomics approach was applied to find potential plasma and liver biomarkers responsible for the hepatoprotective effects of Zhi-Zi-Da-Huang Decoction (ZZDHD).

GRAPHICAL ABSTRACT FIGURE:



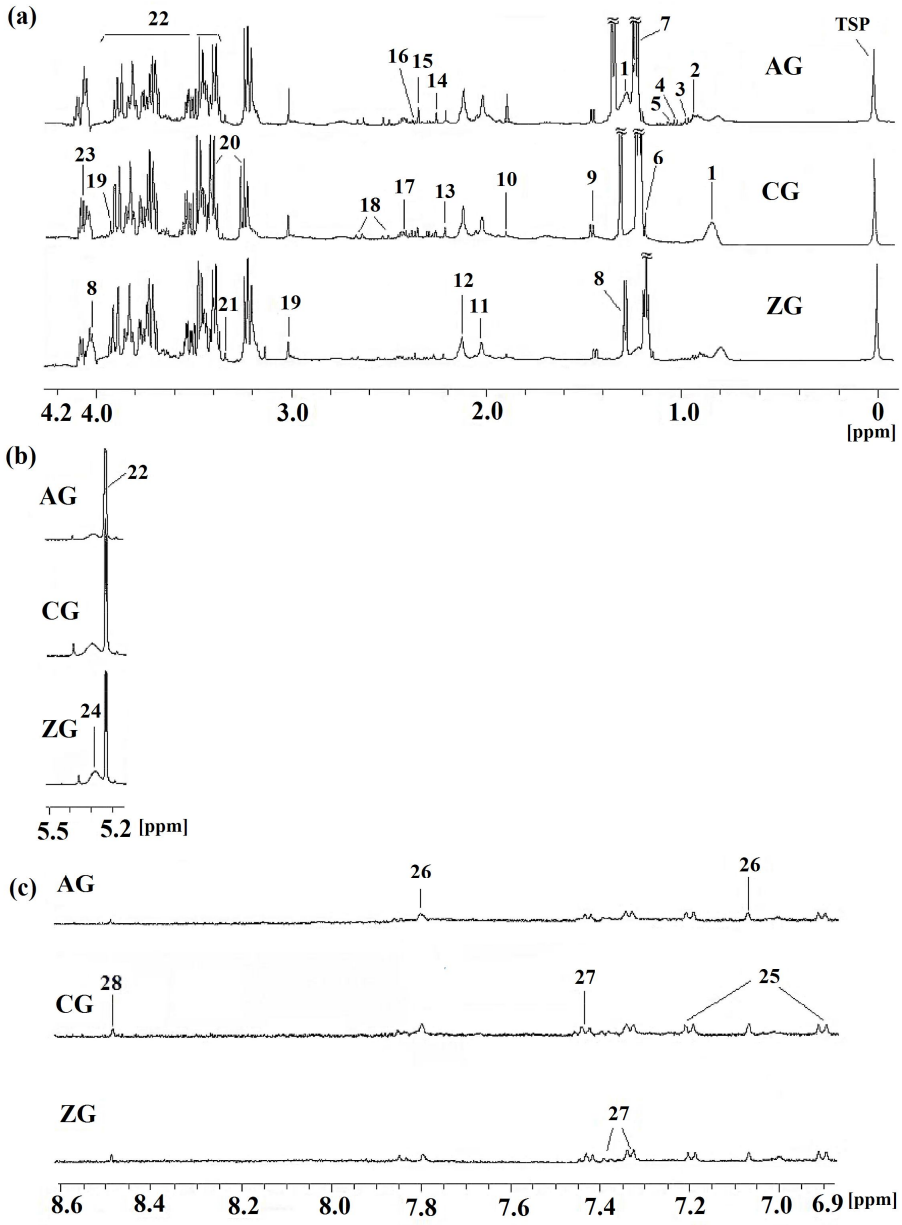


Fig. 1

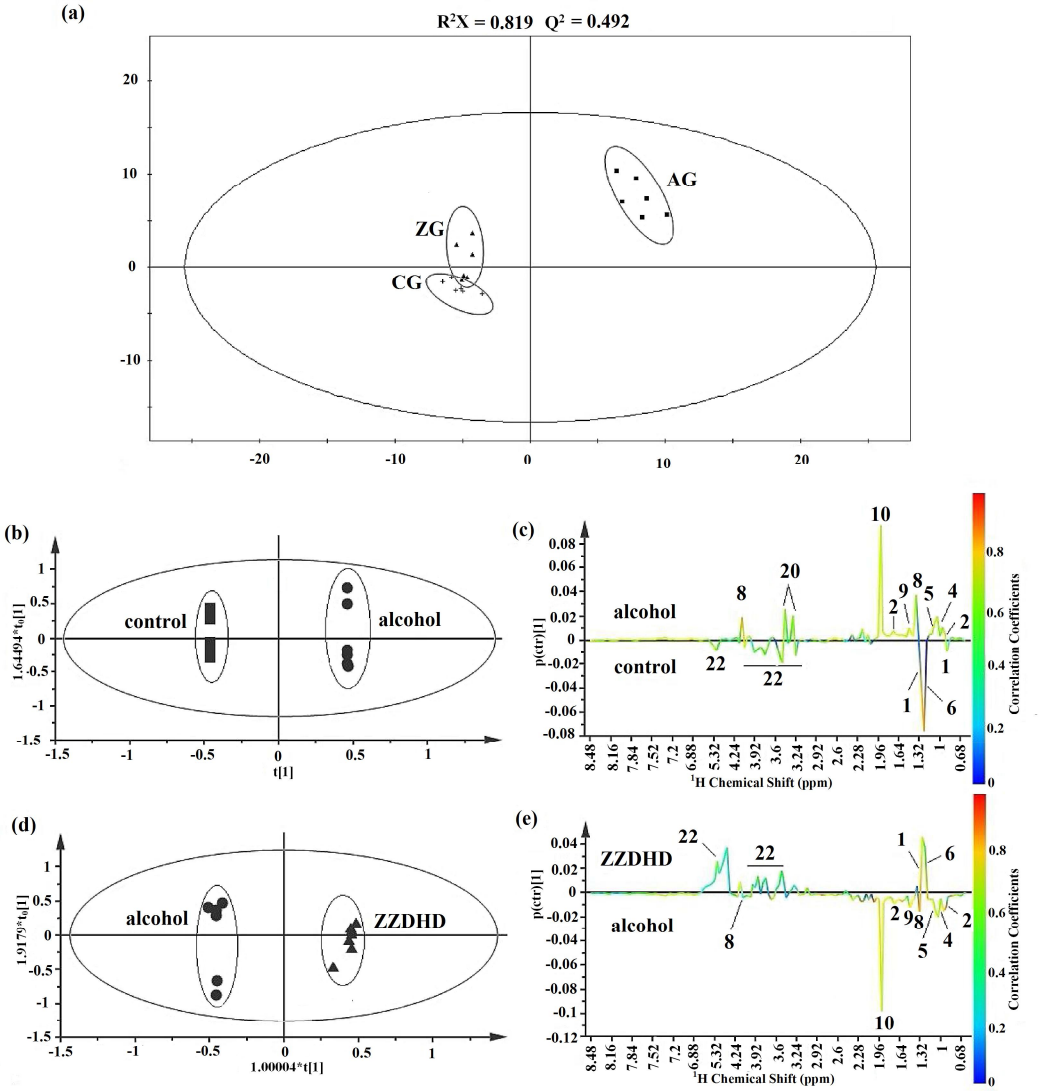


Fig. 2

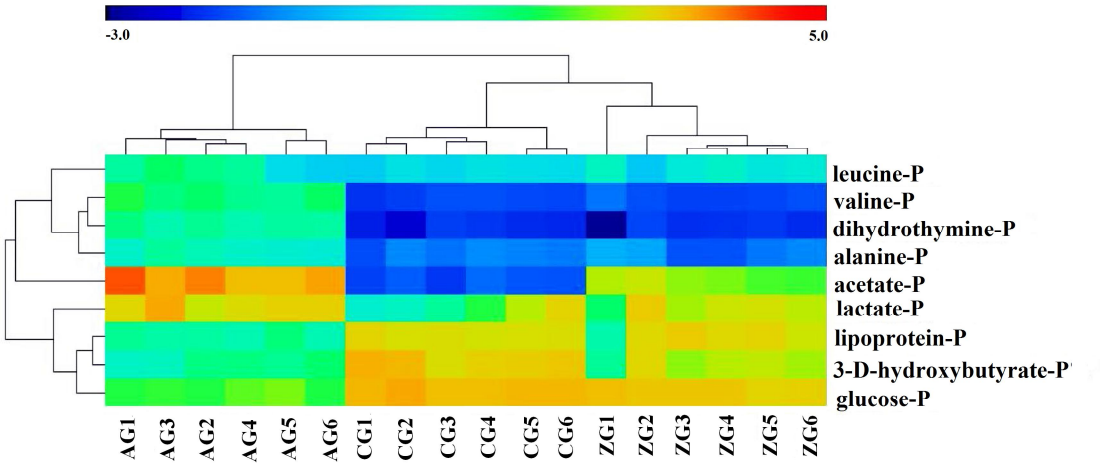


Fig. 3

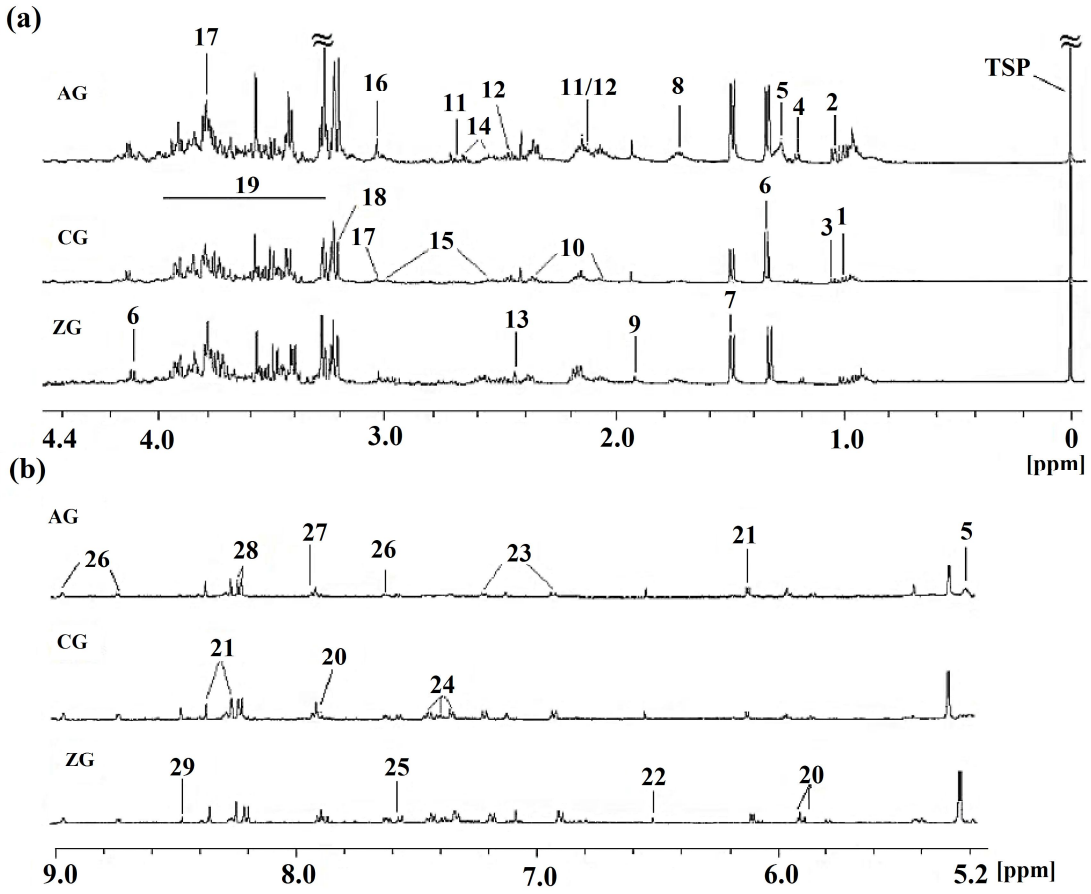


Fig. 4

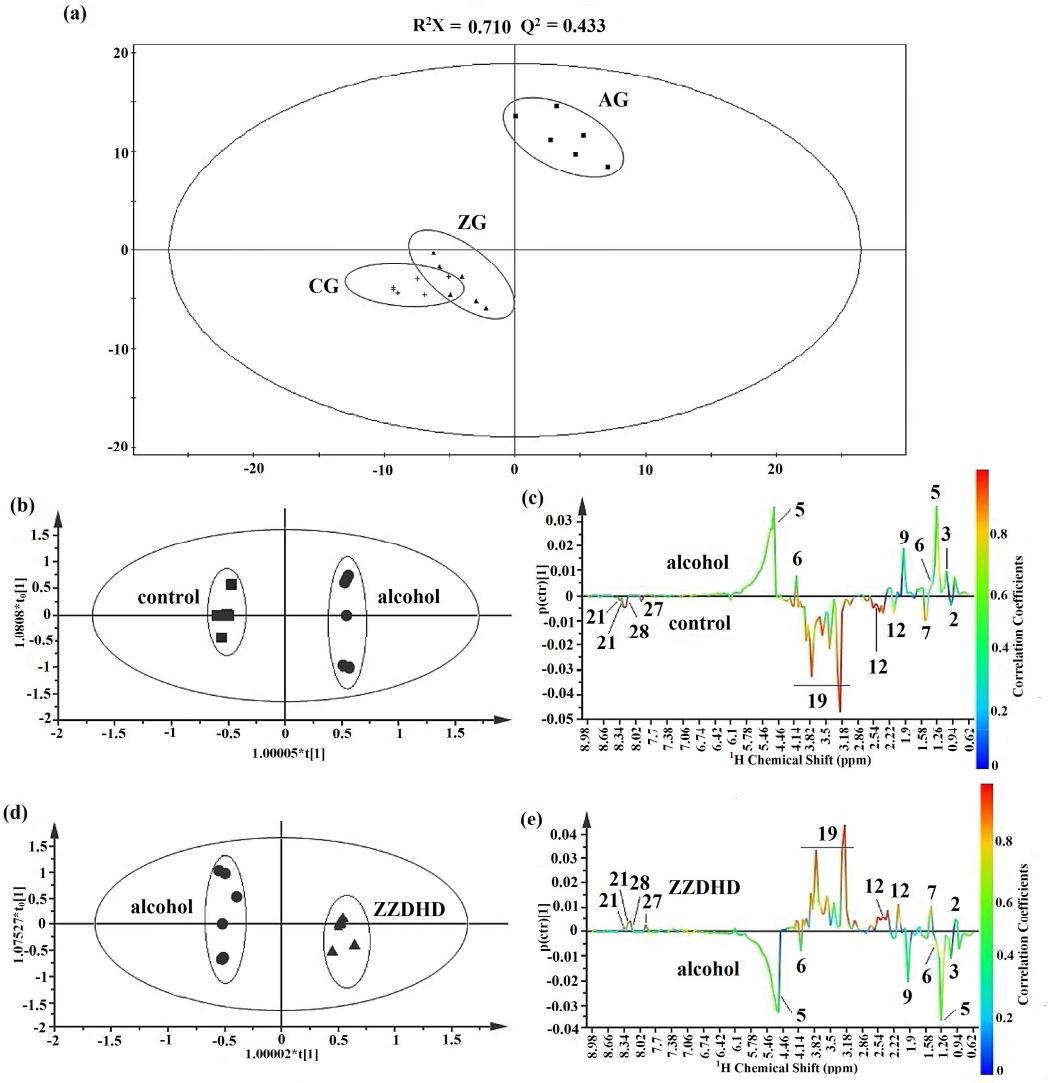


Fig. 5

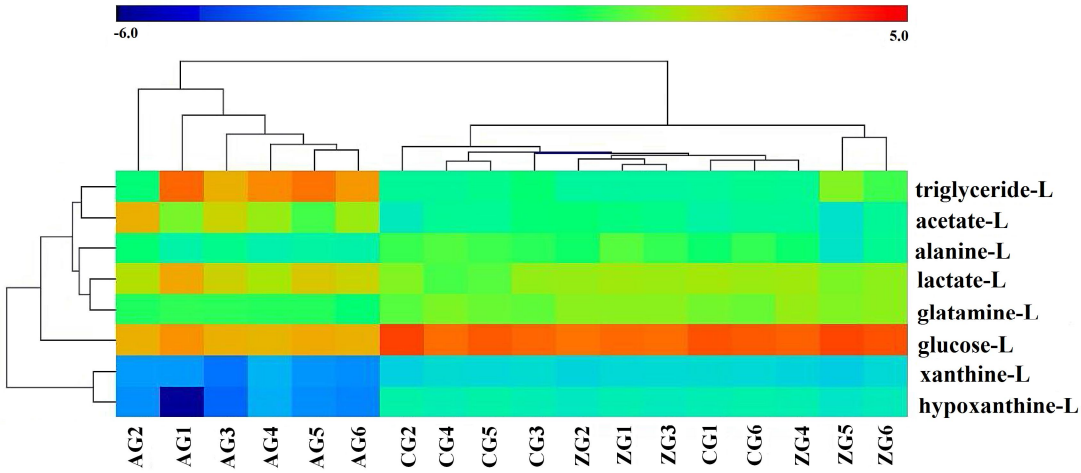


Fig. 6

Table 1 List of the plasma differential metabolites identified from AG *vs*. CG and ZG *vs*. AG.

No.	Potential biomarker	Chemical shift [a] (ppm) and multiplicity	VIP [b] AG *vs*. CG	VIP [b] ZG *vs*. AG
1	Lipoprotein	0.83-0.93(m),1.27-1.34(m)	1.82	1.94
2	Leucine	0.97(d)	1.43	1.65
3	Valine	1.04(d))	1.98	2.03
4	Dihydrothymine	1.07(d)	1.71	1.78
5	3-D-hydroxybutyrate	1.22(d)	4.29	2.44
6	Lactate	1.36(d),4.14(q)	2.42	1.75
7	Alanine	1.50(d)	1.45	1.58
8	Acetate	1.94(s)	4.39	4.55
9	Taurine	3.28(t),3.42(t)	2.06	0.60
10	Glucose	3.39(t),3.53(dd),3.73(dd),3.76(dd),3.81(m),5.26(d), 3.22(dd),3.42(t),3.47(m),3.49(t),3.72(dd),3.92(dd)	1.40	1.34

[a] s, singlet; d, doublet; t, triplet, q, quartet, m, multiplet, dd, double doublet.

[b] Variable importance in the projection (VIP) was obtained from OPLS-DA.

Table 2 List of the liver extract differential metabolites identified from AG *vs*. CG and ZG *vs*. AG.

No.	Potential biomarker	Chemical shift [a] (ppm) and multiplicity	VIP [b] AG *vs*. CG	VIP [b] ZG *vs*. AG
1	Leucine	0.98(t)	0.78	0.93
2	Valine	1.04(d)	0.86	0.93
3	Triglyceride	1.27(m),5.20(m)	2.53	2.51
4	Lactate	1.34(d),4.12(q)	1.01	1.15
5	Alanine	1.49(d)	1.71	1.14
6	Acetate	1.94(s)	1.72	1.79
7	Glutamine	2.14(m),2.45(m)	1.39	1.65
8	Glucose	3.39(t),3.53(dd),3.73(dd),3.76(dd),3.81(m),5.26(d), 3.22(dd),3.42(t),3.47(m),3.49(t),3.72(dd),3.92(dd)	2.49	2.34
9	Xanthine	7.91(s)	1.29	1.38
10	Adenosine	8.25(s),8.35(s)	1.21	0.89
11	Hypoxanthine	8.20(s),8.22(s)	1.20	1.21

[a] s, singlet; d, doublet; t, triplet, q, quartet, m, multiplet, dd, double doublet.

[b] Variable importance in the projection (VIP) was obtained from OPLS-DA.