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A metabolomics study: Reveals the protective effect and mechanism of *Terminalia chebula Retz* on the cardiotoxicity induced by radix *Aconiti kusnezoffii Reichb*

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Abstract: To reveal the protective effect of *Terminalia chebula Retz* (TCR) on cardiotoxicity induced by radix of *Aconitum kusnezoffii Reichb* (AKR). Control, AKR, AKR-TCR 1:3, AKR-TCR 1:1, AKR-TCR 3:1 and TCR-prepared AKR groups were set up. After treatment, the heart tissues were observed by H&E staining and transmission electron microscope. Serum myoglobin (MB) and troponin (cTn) were detected by ELISA. UPLC-Q Exactive/MS analysis was performed to detect the metabolic difference among the groups. ELISA results showed that the MB and cTn values of AKR group were significantly higher than Control group (P<0.05), while those of the other groups were lower than AKR group. TCR-prepared AKR group had similar MB and cTn contents to the Control group. Histopathological examination also indicated better detoxifying effects in the TCR-prepared AKR and AKR-TCR 1:1 group. The serum metabolomics analysis showed obvious distinction between the AKR and Control groups, while AKR-TCR combination reversed the metabolomics changes induced by AKR. Through multivariate statistical analysis, 9 metabolic markers related to energy, nucleic acid and amino acid metabolism were identified. Conclusively, AKR-induced cardiotoxicity may be related to energy, nucleic acid and amino acid metabolism, and TCR can reduce the cardiotoxicity by regulating the relative metabolism pathways.

Keywords: Metabolomics, *Terminalia chebula* Retz, *Aconitum kusnezoffii* Reichb, cardiac toxicity.

INTRODUCTION

Radix *Aconitum kusnezoffii Reichb (*AKR), a commonly used Traditional Mongolian Medicine, is extracted from the dried root of *Aconitum kusnezoffii Reichb* (2010). AKR has cardiotoxic, anti-inflammatory and analgesic effects and can be used to treat wind cold damp impediment symptom, joint pain, abdominal pain with cold sensation, and anesthesia pain (Huang, 1988, Nesterova *et al*., 2014). There are various toxic alkaloids in AKR, such as hypaconitine and aconitine Yang *et al*., 2018). Interestingly, diester-type alkaloids, including aconitine, hypaconitine and mesaconitine, are not only the active ingredients but also the toxic components, since the therapeutic dose is close to the toxic dose (Zong *et al*., 2019, Ma *et al*., 2018). If overdosed, AKR can cause paralysis of respiratory muscle, and aconitine result in arrhythmia, ventricular ectopic beats, ventricular tachycardia, and ventricular fibrillation through up-regulating the Ca^{2+} level in intracellular fluids (Sun *et al*., 2014, Fu *et al*.,2007).

The processing of Mongolian herbal medicine is strictly required, which is an efficient way to enhance efficacy and reduce toxicity (Zhao *et al*., 2010, Wang *et al*., 2016). AKR is commonly used in combination with *Terminalia* to achieve the detoxification effect. *Terminalia chebula Retz* (TCR) has astringent nature and can be used as adjuvant in hemorrhages. It is used to treat asthma, sore throat and chronic cough, and is also useful in renal calculi, retention of urine, dysuria, and urticaria (Liu *et al*., 2014, Bag *et al*., 2013).

There are a lot of studies on the toxicity of *Aconitum* (Wangchuk *et al*., 2015, Sun *et al*., 2012), but few focused on the metabolomics after *Aconitum* treatment. In the study of metabolomics, Ultra Performance Liquid Chromatography (UPLC) combined with Mass Spectrometry (MS) improves the speed of analysis, resolution, detection sensitivity and MS ionization efficiency, and reduces the matrix effect, and thus can fully adapt to the qualitative and quantitative detection of the complex traditional Chinese medicine system (Ma *et al*., 2014, Liu *et al*., 2016;, Yang *et al*., 2012).

In this study, UPLC-MS was used to investigate the overall metabolic changes underlying the toxic effect of AKR and the detoxification effect of TCR in rats. The metabolic changes of rats after the treatment with AKR and AKR+TCR were evaluated. The results suggest that TCR may relieve the AKR-induced cardiotoxicity through regulating the energy, nucleic acid and amino acid metabolism.

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MATERIALS AND METHODS

Preparation of AKR and TCR decoction

The dried roots of AKR were made into powder by grinding and mixed with 0.5% sodium carboxymethyl cellulose (CMC-Na) (Fuchen Chemical Reagent Co., Ltd, Tianjin, China) to prepare AKR suspension.

The dried roots of AKR and TCR were mixed at the ratio of 1:1, 1:3, and 3:1 (w/w) and then ground into powders (about 100 meshes). The AKR-TCR powder was mixed with 0.5% CMC-Na solution to prepare AKR-TCR suspension.

TCR-prepared AKR was processed as follows: TCR was first broken into small pieces and soaked with 40 times of water for 12 h, and then reflux decocted for 1h. TCR decoction was obtained by filtration after cooling. An appropriate amount of AKR (1/3 weight of TCR) was taken and soaked with TCR decoction for 3 days, changing the TCR decoction every day. After that, AKR was dried at low temperature, made into powder, and mixed with 0.5% CMC-Na solution to prepare AKR suspension.

Animals and sample collection

The study protocol was approved by the Institutional Animal Care and Use Committee of Chinese People's Liberation Army Academy of Military Medical Sciences (SCXK License No.2012-0004), and conformed to the National Institute of Health guidelines on the ethical use of animals.

Specific pathogen free Male Sprague-Dawley (SD) rats (180-200 g) were purchased from Haidian Medical Laboratory Animal Center (Beijing, China). The animals were housed in standard condition. All rats were assigned to six groups: Control group (C), AKR group (A), TCR-prepared AKR group (T), AKR-TCR 1:1 group (YY), AKR-TCR 1:3 group (YS) and AKR-TCR 3:1 group (SY), randomly. Rats in the Control group were subjected to oral gavage with 0.5% CMC-Na solution, while other groups were administered with corresponding suspension containing AKR of 0.12g/kg body weight (TCR amount was varied but in the referred ratios) for 28 days, respectively. After treatment, all rats were sacrificed after sodium pentobarbital anesthesia (i.p.).

Serum was isolated from blood of the abdominal aorta. The heart was dissected and immediately fixed.

ELISA analysis

The blood of rats was collected after 4 weeks of experiment and centrifuged at 12000 rpm to obtain serum for ELISA. Cardiac Troponin I (cTn-I) and Myoglobin (MB) in the serum was detected by cTn-1 and MB ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Hematoxylin and eosin (HE) staining

Heart tissues were embedded sectioned (5μm) and stained with hematoxylin and eosin (Fuchen Chemical Reagent co., Ltd, Tianjin, China). The sections were observed and photographed using a Leica DM2700M microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 400× magnification.

Transmission electron microscopy

After experiment, the rats were sacrificed and the heart was removed rapidly. The myocardial tissue of about 1 ~ 2 cm^3 was taken from the left ventricular wall of the heart, and pre-fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde (pH =7.4) at 4°C. The buffer was changed for 3 times, with each interval of 10min and then the tissue was stored at 4°C. The tissue was post-fixed with fresh osmate acid and dehydrated with gradient ethanol after being rinsed. Then, the tissue was embedded with propylene oxide and resin and semi-thin sectioned. The slice was stained with toluidine blue and the myocardial tissue at the apex was located. After that, another piece of fixed myocardial tissue was ultra-thin sectioned and the slice was stained with uranyl acetate and lead citrate. The changes of myocardial ultrastructure were observed under H-7650 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

UPLC-MS analysis

Methanol (800 μL) was mixed with the serum (200 μL) to

Table 1: The levels of cTn-I and MB in each group

Groups	cTn-I (ng/L)	MB (ng/L)
C	288.81±44.94	208.39±29.46
T	290.08±22.08$^{\#}$	185.30±55.33$^{\#}$
SY	272.98±58.40$^{\#\#}$	194.91±53.60$^{\#}$
YY	297.39±98.17$^{\#\#}$	201.54±45.31$^{\#}$
YS	371.92±75.07$^{*\#}$	215.91±89.26
A	461.30±59.10*	261.95±89.26*

Note: C=Control group, A=AKR group, T=TCR prepared AKR group, SY=AKR-TCR 3:1 group, YY=AKR-TCR 1:1 group, YS=AKR-TCR 1:3 group. Data were presented as mean ± standard deviation and analyzed by one-way ANOVA and Tukey's test. $^{*}P<0.05$ and $^{**}P<0.01$, compared with the Control group; $^{\#}P<0.05$ and $^{\#\#}P<0.01$, compared with the AKR group.

Table 2: Changing trends of the metabolites of the AKR group compared to the Control group

Biomarkers	AKR group *vs*. Control group
Proline	↑**
Glucose	↓*
Threonine	↓**
Glutamine	↓**
Histidine	↓**
Serine	↓**
Glycine	↓**
Urea	↓**
Citric acid	↓*

Note: ↓ for decrease, ↑ for increase, - for no change. Data were analyzed by chi-square test. $^{*}P<0.05$ and $^{**}P<0.01$, compared with the Control group.

extract the metabolites. After being vortexed for 30s, the mixture was centrifuged at 10000g for 10 min. Then, the supernatant was collected and used for UPLC-MS analysis. The Dionex UltiMate3000 Rapid Resolution Liquid Chromatography and Q Exactive mass spectrum (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used.

Mass spectrometry conditions were as follows: the temperature of positive ion mode scanning electrospray ionization of 350°C, a capillary voltage of 3.5kV, an ion transfer tube temperature of 200°C, the sheath pressure of 42arb, and the auxiliary pressure of 10arb. The acquisition quality range was 50-800m/z.

UPLC chromatographic conditions were as follows. The XBridge BEH Amide chromatographic column (2.1×100mm) (Waters Co., Milford, MA, USA) was used. The flow rate was 0.4mL/min; column temperature was 25°C; the injection volume was 5uL; the mobile phase A was 0.1% formic acid aqueous solution; the mobile phase B was acetonitrile (HPLC/MS; Fisher Co., Ltd., Waltham, MA, USA). The elution gradient was as follows: 0~10min, 10%A; 10~10.1min, 50%A; 10.1~15min, 10%A.

STATISTICAL ANALYSIS

The SPSS 13.0 software (SPSS, Chicago, USA) was used for statistical analysis. Principal components analysis (PCA) and partial least squares-discriminant analysis (OPLS-DA) of SIMCA-P+ 14.0 (Umetrics, AB, Umeå, Sweden) were used to assess the normalized GC-MS spectral data. Variable Influence on Projection (VIP) values of variables were analyzed, with VIP value > 1.0 as significant variables. These variables were used to identify responsible peak(s) of spectrum. Measurement data were presented as mean ± standard deviation. One-Way ANOVA was performed to compare differences among different groups followed by Tukey's test. Count data were analyzed by chi-square test. $P < 0.05$ was considered statistically significant, and $P<0.01$ is considered significant difference.

RESULTS

TCR has protective effect against the cardiac toxicity induced by AKR

To investigate whether TCR has positive effect on AKR induced cardiac toxicity, ELISA, HE staining and TEM were performed. MB and cTn are the biomarkers of myocardial injury and necrosis, which has important clinical significance for the diagnosis and risk stratification of acute myocardial infarction. The increase of MB and cTn-I values may indicate myocardial injury (Li *et al*., 2012). ELISA revealed that the levels of MB and cTn-I in the serum of the AKR group were significantly higher than those in Control group, while TCR treatment could alleviate such increase (table 1). Compared with the other treatment groups, the TCR-prepared AKR group had the best treatment effect.

The histological changes of heart were also examined by HE staining and observed under an optical microscope and TEM. HE staining of the myocardial tissues showed that the rat myocardial cells of the Control group arranged in neat rows with normal shape and uniform color. The myocardial cells of the AKR group arranged in disorder, the striation disappeared, the nuclear swelled, the cytoplasm was slightly stained, the cells shrunk and vacuolar degeneration occurred. The myocardial cells of the TCR-prepared AKR group arranged in neat rows with normal cell morphology and uniform staining. The arrangement of the myocardial cells of the SY group was slightly disordered and the volatile degeneration was not obvious. The YY group showed disordered arrangement of myocardial cells, the striation disappeared, the nuclear swelled, the cytoplasm was slightly stained and no obvious vacuolar degeneration was observed. The YS group showed the arrangement of the myocardial cells was disordered, the nuclear swelled, and vacuolar degeneration occurred (fig. 1A). TEM images showed that the ultra structure of the myocardium in the rats of the Control group was normal. There were dark and horizontal stripes, and the filaments were arranged evenly. The Z lines were parallel to each other, and the

intercalated discs were clearly visible. The shape of mitochondria was oval and the cristae were dense and regular without any abnormal changes. In contrast, AKR group showed obvious myocardial ultrastructure injury, sarcoplasmic reticulum vacuolation, dynamic expansion, mitochondrial swelling, cristae dissolution and the emergence of a large number of myocardial interstitial collagen. In addition, the sarcomere filament was not clear, and no H lines or Z lines or intercalated disc structure was observed (fig. 1B). The other treatment groups had different degrees of myocardial tissue improvement compared with the AKR group and the TCR-prepared AKR group had the best treatment effect. Overall, these results indicate that TCR has a protective effect on the cardiotoxicity induced by AKR.

Metabolic differences in serum after treatment

To identify the metabolic difference after treatment with AKR and AKR+TCR UPLC-MS was performed. Typical total ion chromatogramms on the 28th day were shown in fig. 2. Then, the data was analyzed by PCA and OPLS-DA. fig. 3A shows the PCA score plot. There was well differentiation between AKR group and Control group (R^2=0.742; Q^2=0.488). fig. 3B shows the OPLS-DA score plot. There was clear separation between these two groups (R^2X=0.864; R^2Y=0.934; Q^2=0.884), indicating that AKR induces significant biochemical perturbation. There was an overall goodness of fit of the model. More importantly, TCR administration to rats changed the relationship of AKR group relative to the Control group. As shown in fig. 3A, the TCR-prepared AKR group was almost overlapped with the Control group. These results suggest that TCR could reverse the metabolic change by AKR.

According to the VIP and S-plot (figs. 4A and 5B) and the above-stated threshold, there were 11 metabolites with VIP > 1.0 and 9 biomarkers with probability <0.02 that could significantly differentiate the AKR group from the Control group. Proline was elevated, while glucose, threonine, glutamine, histidine, serine, glycine, urea and citric acid were decreased in AKR group (table 2). Thus, change of these biomarkers may account for the mechanism underlying the toxicity of AKR.

Using pathway topology analysis of Metabo Analyst, 6 pathways involving in the potential biomarkers (Impact > 0.1) were identified. These pathways were Aminoacyl-tRNA biosynthesis, Methane metabolism, Glycine, Histidine metabolism, serine and threonine metabolism, Alanine, Glyoxylate and dicarboxylate metabolism, aspartate and glutamate metabolism (fig. 5). In addition, it was found that these biomarkers are associated with heart failure after the Over Representation Analysis of Metabo Analyst.

DISCUSSION

From the history of traditional Chinese medicine, the detoxification of *Aconitum* can be traced back to more than 200 BC, and its use with TCR had already been mentioned. Chinese herbal medicine must use *Aconitum* together with TCR. TCR contains tannic acids, in which mainly are chebulinic acid, chebulin, terchebin etc. It has the effects of antibacterial, anti-virus, astringent intestinal convergence lung, reduce fire and clearing throat and so on. In practical application, *Aconitum* was often used in combination with a large dose of TCR, or they were used as a drug pair in the form of decoction, or processed into powder pills in order to reconcile the drug, reduce toxicity and exert better curative effect (Yang *et al*., 2013, Yang *et al*., 2011, Gutser *et al*., 1998).

Metabolomics plays an important role in the transmission of biological information between genes, proteins and cells, tissues (Henquet *et al*., 2016, Noga *et al*., 2012) and can be used to evaluate the metabolite profiles of living organisms (Gomez-Casati *et al*., 2013). It has been widely used in clinical medicine, pharmaceutical research, nutrition, food safety, environmental science, toxicology, plant microbiology and other important fields (Nishiumi *et al*., 2010). Here we used UPLC-MS based metabolomics to study the biological differences among normal, AKR, and AKR+TCR treated rats to understand the protective effect of TCR on AKR-caused cardiotoxicity.

Heart is particularly vulnerable to oxygen deficiency caused by blood loss or reduced blood flow. Arrhythmia, as a response to the abnormality of heart caused by pathological changes, can slow the conduction, uneven the repolarization, and then lead to unidirectional conduction block and reentry movement. It will result in changes of electrocardiogram and increased serum myocardial enzymes, myoglobin and troponin. Myocardial cells in hypoxia can cause changes in cardiac endocrine factors and autonomic nerve functions, leading to the occurrence of ventricular arrhythmia (Guo *et al*., 2014).

The UPLC-MS analysis indicated the metabolic changes of rat serum after the treatment with AKR, and increased proline, but decreased glucose, threonine, glutamine, histidine, serine, glycine, urea and citric acid were found. Cardiac physiological activity requires high-energy phosphate to provide continuous energy. Glucose is the main energy source for myocardial phosphorylation. Decreasing glucose content may impede the storage or use of cardiac energy and directly affect systolic and diastolic functions (De *et al*., 2017). Proline and hydroxyproline constitute 25% collagen and the change of myocardial collagen ratio leads to structural matrix change, and finally to myocardial dysfunction such as myocardial fibrosis, hypertrophy or infarction. Glutamine, the most abundant amino acid in plasma, has attracted much attention for its cardioprotective properties.

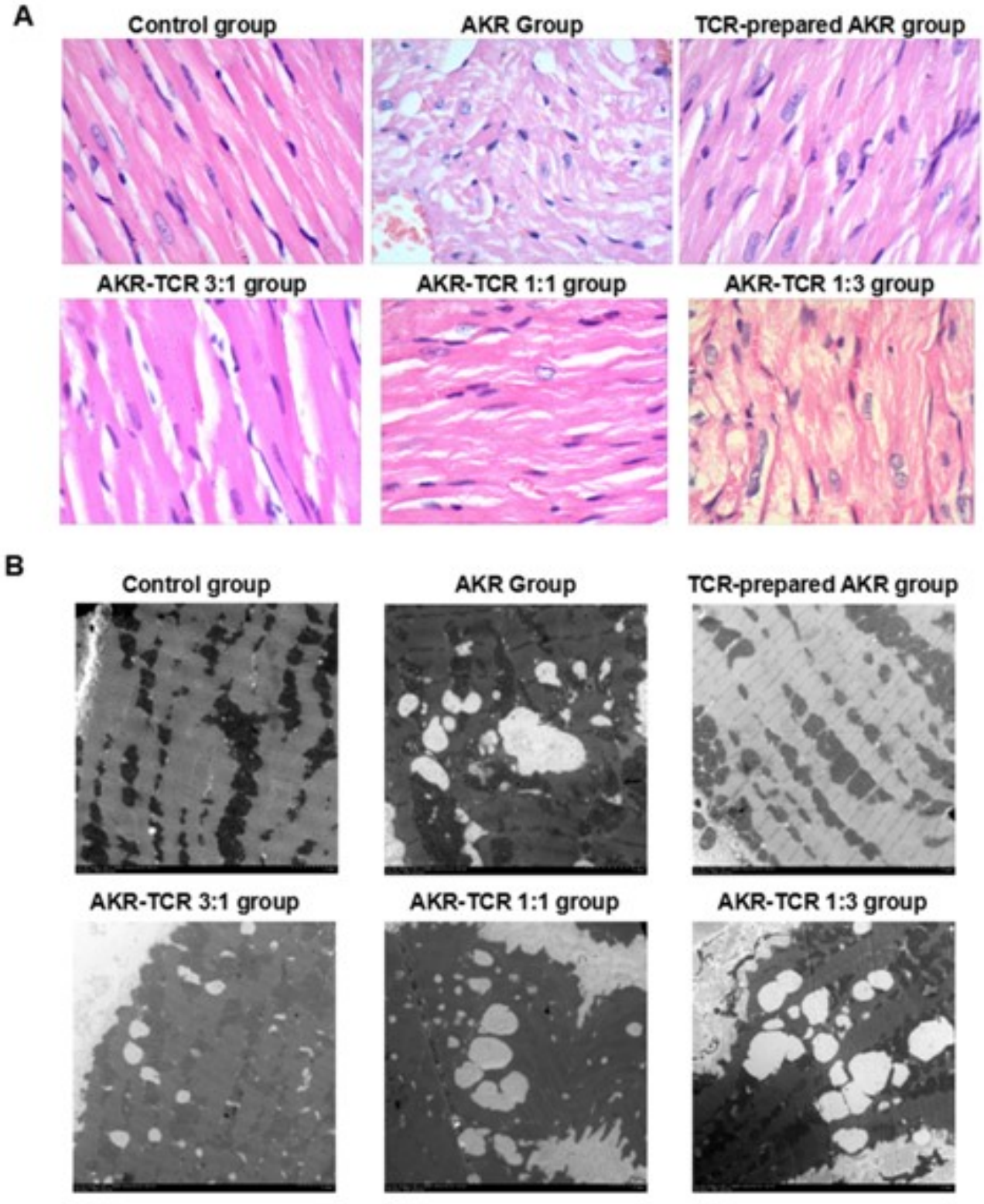

Fig. 1: Histopathology of heart tissues in rats. (A) H&E staining of heart tissues. Magnification: 400×. (B) TEM images of heart tissues. Magnification: 12000×.

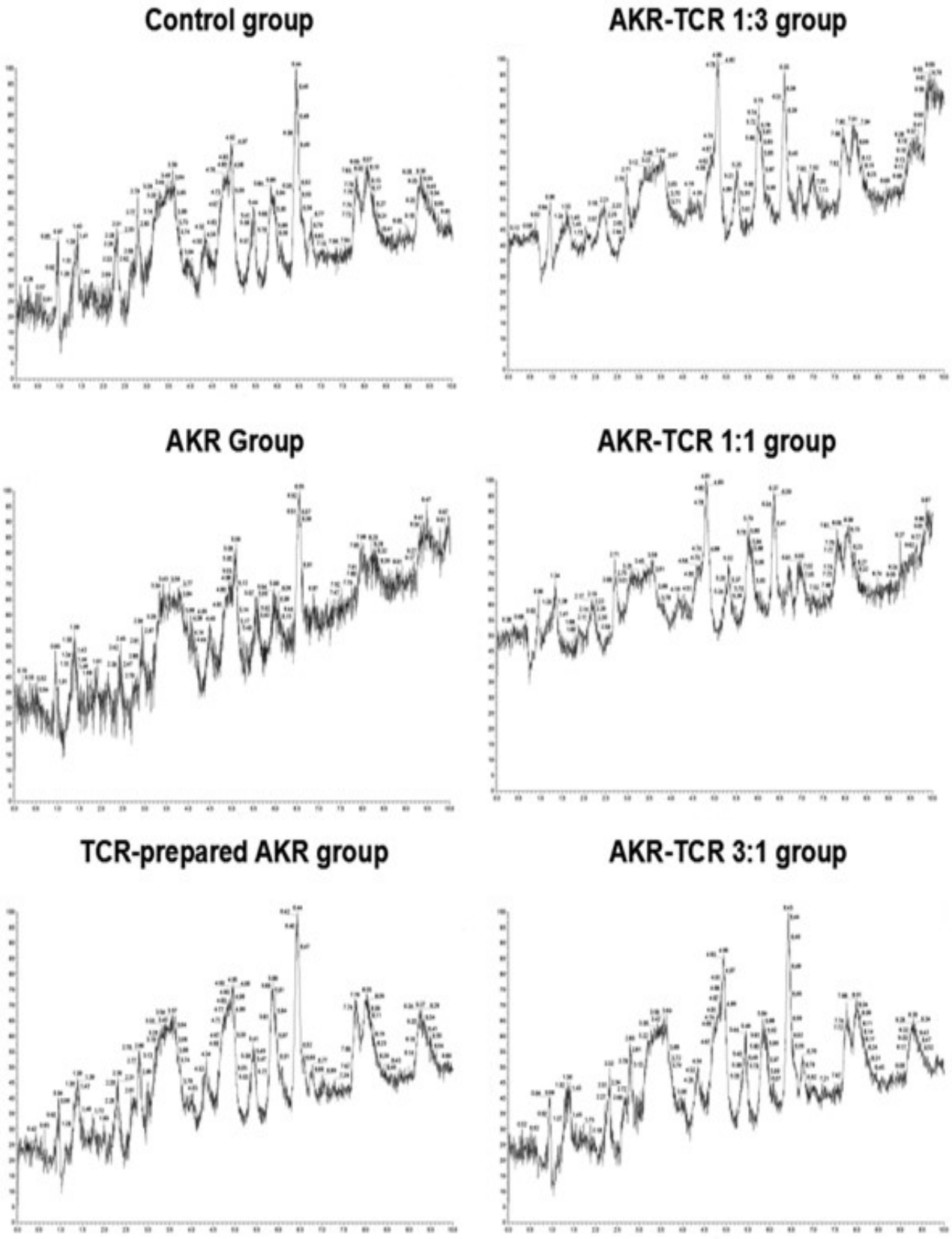

Fig. 2: Representative spectra of total ion flow mass spectrometry in rat serum of the six groups.

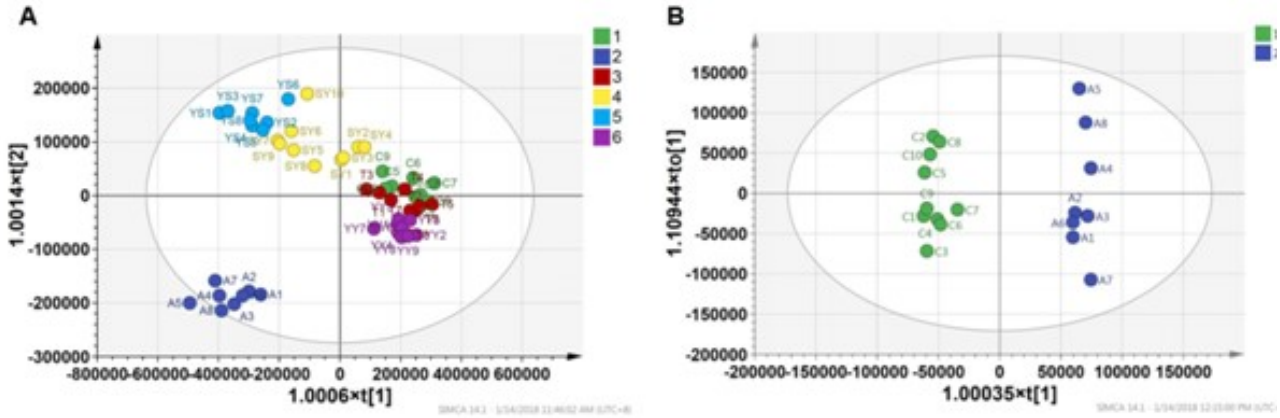

Fig. 3: Separations between the six groups. (A) Score scatter plot of principal components analysis (PCA) of the six groups. (B) Score scatter plot of orthogonal partial least-squares discriminant analysis (OPLS-DA) in the control and AKR groups.

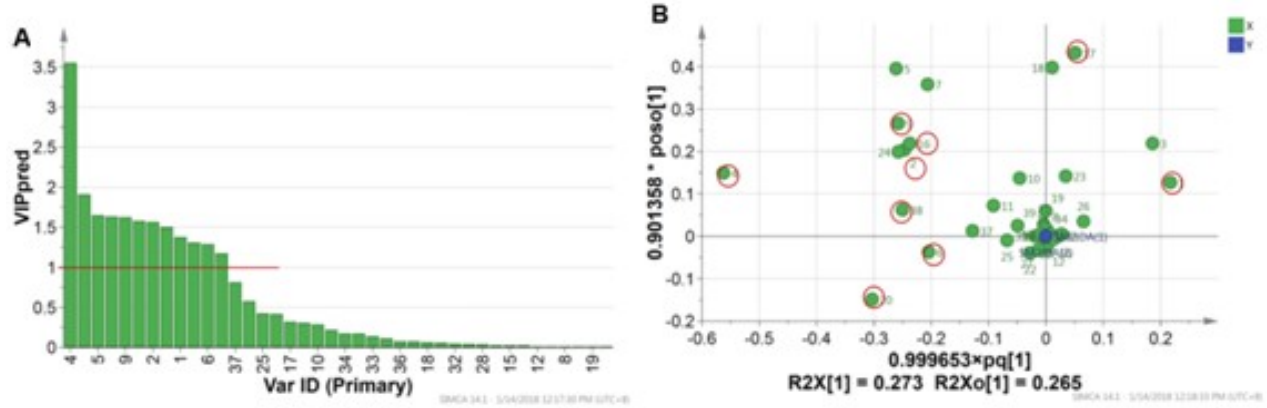

Fig. 4: Multivariate data analysis of metabolites in serum. (A) Variable Influence on Projection (VIP) value of the Control group and AKR group by OPLS-DA analysis. The red line indicates the threshold to separate the 11 biomarkers. (B) S-plot of the Control group and AKR group by OPLS-DA analysis. The 9 biomarkers were indicated by the red circles.

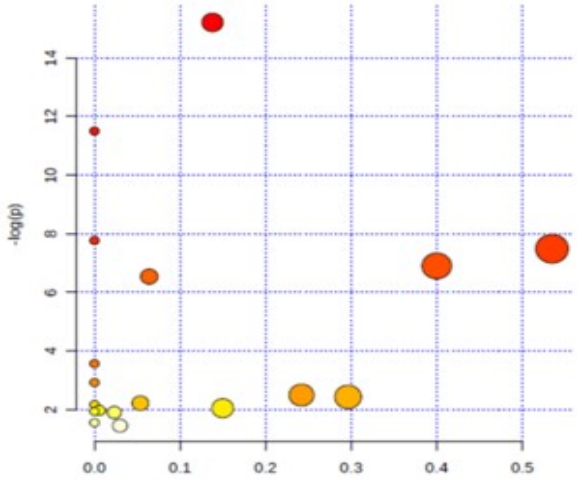

Fig. 5: The result of pathway topology analysis of Metabo Analyst based on the 9 identified markers. The six identified pathways from left to right at $x > 0.1$ are Aminoacyl-t RNA biosynthesis, Glycine, serine and threonine metabolism, Methane metabolism, Histidine metabolism, Glyoxylate and dicarboxylate metabolism, Alanine, aspartate and glutamate metabolism, respectively. The darkness of the circle indicates the correlation between the biomarker and metabolic pathway.

For example, L-glutamine improves cardiac function of patients suffering acute myocardial infarction (Mao *et al*., 2011, Badole *et al*., 2014).

The anaplerotic metabolism of glutamine to citric acid cycle intermediates mediates its primary effect in heart (Lauzier *et al*., 2013). Glycine also exerts anti-inflammatory and antioxidative effects and has been inversely associated with several traditional cardiovascular risk factors, including obesity and hypertension (McCarty and DiNicolantonio, 2014, Oberbach *et al*., 2011). It can scavenge oxygen free radicals to protect myocardial cells as well as improving the energy metabolism of myocardial cells. Decreased glycine content will affect the normal function of myocardial cells (Ding *et al*., 2015). Study in the heart of Dogfish shark (*Squalus acanthias*) found that urea and several urea derivatives could scavenge ROS and prevent post-ischaemia reperfusion damage (Wang *et al*., 1999). Tang *et al*. (Tang *et al*., 2013) found that citric acid and L-malic acid protected against myocardial ischemia/ reperfusion injury in rat model.

This study revealed the protective effect of TCR against the cardiotoxicity to rat heart caused by long-term use of AKR. Nine biomarkers, including proline, glucose, threonine, glutamine, histidine, serine, glycine, urea and citric acid, were identified to be responsible for the differentiation between AKR treated and Control samples. And TCR can reverse the cardiotoxic effect induced by AKR. The results in-depth interpreted the featured application of "TCR-prepared AKR" and provided a new theoretical basis for the safe application of AKR.

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