Standardized Xin-Ke-Shu Tablets Improves the Disturbances of Lipid, Energy, and Amino Acid Metabolism in a Rabbit Model of Atherosclerosis

Yong Yang, Jing-Bo Peng, Meng Yu, Hong-Mei Jia, Hong-Wu Zhang, Zhong-Mei Zou

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Abstract

Objective: Xin-Ke-Shu (XKS), a patent drug, used to treat coronary artery diseases in China for many years. Previous research indicates that XKS has similar therapeutic effect as atorvastatin (AS) against atherosclerotic in rabbits. However, biochemical assays demonstrate that XKS could have a different therapeutic mechanism from AS. The aim of this study is to explore the mechanism of XKS therapeutic effect, especially those different from AS. Materials and Methods: 1H nuclear magnetic resonance-based metabonomics were applied to profile the low-molecular-weight polar metabolites in the plasma of rabbits fed a high cholesterol diet. Results: Seven of the eleven pathological biomarkers related to atherosclerosis in rabbits were mediated by XKS treatment, namely low-density lipoprotein/very-low-density lipoprotein (LDL/VLDL), lactate, citrate, phosphatidylcholine, glutamine, creatinine, and methionine, as well as two characteristic metabolites of pyruvate and α-glucose. These metabolites involved lipid, energy, and amino acid metabolism, and all could be considered XKS treatment targets. However, AS only affected the metabolic disorders associated with LDL/VLDL and phosphatidylcholine, which is mainly target lipid metabolism. Conclusions: This study indicates that the anti-atherosclerosis effects of AS mainly involve reducing blood–lipid levels, but those of XKS involve a multitargeted activity.

Keywords: 1H nuclear magnetic resonance, atherosclerosis, plasma metabonomics, polar small molecules metabolites, Xin-Ke-Shu

Introduction

Coronary artery disease (CAD), a combination of fatty material, calcium, and plaque buildup inside the coronary arteries, is the most frequent cause of death worldwide.[1] The underlying pathological process includes lipid deposition, oxidation, and modifications that provoke chronic inflammation at susceptible sites in the walls of all major conduit arteries.[2] Hence, an effective therapy should reduce hyperlipidemia and prevent thrombosis. Statins are well known to reduce major cardiovascular events by lowering low-density lipoprotein (LDL) cholesterol levels. However, despite the promising effects of statins on CAD, their adverse effects (including glucose metabolic disorder, myolysis, and hepatotoxicity) during long-term administration are still a concern.[3-5] This has provided worldwide incentive to continue to discover new drugs for CAD prevention and therapy.

Traditional Chinese medicine (TCM) formula are multicomponent medicines that have a holistic therapeutic effect with better therapeutic efficacy and fewer side effects.[6,7] The use of TCM for the treatment of complex, multifactorial diseases, such as CAD, has garnered much interest, especially in the past decades.[8] Xin-Ke-Shu (XKS) tablet, an herbal compound prescription composed of the root of Aucklandia lappa Decne (Mu-Xiang), fruit of Crataegus pinnatifida Bge. (Shan-Zha), root of Panax notoginseng (Burk.) F. H. Chen. (San-Qi), root of Pueraria lobata (Willd.) Ohwi. (Ge-Gen), and root of Salvia miltiorrhiza Bge. (Dan-Shen), is a standardized patent medicine extensively used in the clinical treatment of CAD in China.

Address for correspondence: Prof. Zhong-Mei Zou, No. 151, Malianwa North Road, Haidian District, Beijing 100193, China. E-mail: zmzou@implad.ac.cn

China. It has been manufactured by good manufacturing practice pharmaceutical company and quality-controlled using liquid chromatography (LC)-LTQ-Orbitrap mass spectrometry (MS).\(^9\) Previous research indicates that XKS has similar therapeutic effect as atorvastatin (AS) against atherosclerotic in rabbits. However, biochemical assays demonstrate that XKS could have a different therapeutic mechanism from AS,\(^\text{[10]}\) which the molecular mechanism is still not fully clear.

Evaluation the efficacy and reveal the underlying pathophysiologic mechanism of TCM is a great challenge. The emergence of metabonomics approach provides a powerful method for the study of TCM prescription, which the integrity and systemic features of metabonomics highly coincide with the holistic basis for TCM.\(^{[11]}\) It has been widely used in pharmaceutical research and development, including drug therapy monitoring and drug safety assessment.\(^{[12,13]}\) As the two main analytical spectroscopic approaches in metabonomics, MS and \(^1\)H nuclear magnetic resonance (\(^1\)H NMR) spectroscopy usually offering complementary information, but with different operational performance characteristics.\(^{[14]}\) \(^1\)H NMR-based metabonomics has the advantages of being rapid, unbiased, reproducible, quantitative, nondestructive, and amenable to high-throughput analyses.\(^{[14]}\) It is a powerful approach to selectively profile specific metabolites (e.g. polar small metabolites) in complex biological systems,\(^{[14,15]}\) which these metabolites are difficult to detect by MS spectrometry because of the limitation of the polar small metabolites hard retained on an LC column, not ionizable in MS sources and suffer severe matrix effects during MS analysis.\(^{[16]}\)

Our previous LC-MS-based metabolomics study demonstrates that the action of XKS against AS primarily inhibit the perturbation of small, nonpolar metabolites, which involve lipid-related pathways, including arachidonic acid metabolism, glycerophospholipid metabolism, and fatty acid \(\beta\)-oxidation.\(^{[10]}\) Some small polar metabolites, especially amino acid and energy metabolism-related metabolites, which are hard to detect by LC-MS technique, play vital roles in the formation of AS.\(^{[17-19]}\) However, the regulatory effects of XKS on these specific metabolites are still unclear.

Therefore, in the present study, to unveil a more detailed mechanism of XKS activity, we used \(^1\)H NMR-based metabonomics, to profile the small, polar metabolites in the plasma of rabbits fed a high cholesterol diet (HCD). In addition, multivariate analysis and pattern recognition were used to assess the therapeutic effects of XKS and to identify significantly altered metabolites, and the metabolic pathways involved in XKS or AS treatment.

**Materials and Methods**

**Materials and reagents**

Standard XKS tablets, containing the root of *A. lappa* (Mu-Xiang), the root of *P. notoginseng* (San-Qi), the fruit of *C. pinnatifida* Bge. (Shan-Zha), the root of *P. lobata* (Ge-Gen), and the root of *S. miltiorrhiza* (Dan-Shen) (1:1:15:15:15, w/w), were produced by Wohua Pharmaceutical Co., China (batch No. 090629). Quality control of the XKS tablets was accomplished using an LC-LTQ-Orbitrap approach.\(^{[9]}\) Cholesterol was purchased from Tian Qi Chemical Engineering Co., China. AS was purchased from Jialin Pharmaceutical Co., China. Deuterium oxide (D\(_2\)O, 99.9%) was purchased from Sigma-Aldrich (St. Louis, USA). Ultrapure water (18.2 M\(\text{\Omega}\) cm) was prepared with a Milli-Q® Water Purification System (Millipore, France). All other chemicals were of analytical grade.

**Experimental animals**

Twenty-four male Japanese white rabbits (weighing 2.2 ± 0.2 kg, aged 3 weeks) were purchased from the Laboratory Animal Institute of the Chinese Academy of Medical Science (Beijing, China). All animal experiments were performed under the Control and Approval of the Ethics Committee of the Institute of Medicinal Plant Development, CAMS (Beijing, China). The rabbits were housed individually in cages with food and water freely available. The rabbits were acclimatized to the facilities for a week prior to experimentation. All of the animals were handled humanely throughout the experiment.

**Animal handling and sample collection**

Establishment of the HCD-induced AS rabbit model, drug administration, and plasma sample collection was the same as described previously.\(^{[10]}\) Briefly, after 1 week of acclimatization, the rabbits were randomly divided into four groups (\(n = 6\) per group) according to their body weights: (A) control group, (B) model group, (C) AS group, (D) XKS-fed group. The rabbits in Group A were fed with standard rabbit chow (SC), those in Group B were fed with HCD (standard rabbit chow supplemented with 0.1% thiamazole, 3% cholesterol, 0.7% sodium cholate, w/w/w), those in Group C fed with HCD and AS (4 mg/kg/day), and those in Group D were fed with HCD and XKS tablets (0.34 g/kg/day). The duration of the treatment was 12 weeks.

Blood samples were collected from the ear vein with sodium-heparin tubes. Then, the plasma was separated by centrifugation at 3600 rpm for 10 min at 4°C and stored at -80°C until analysis. At the end of the experiment, all rabbits were euthanatized and autopsied. The aortas were separated from aortic arch to the end piece of the thoracic aorta, then immediately fixed in 10% neutral-buffered formalin (w/v) for histopathological evaluation.

**Plasma biochemistry assays and histopathology**

The degree of atherosclerosis was evaluated by determining the total cholesterol (TC), triglyceride (TG), and low-density lipid-cholesterol (LDL) activities in the plasma using HITACHI 7060 automatic analyzer.

Histopathological changes of aortas were investigated by Sudan IV staining to measuring the atherosclerotic plaques ratio on the aortas. The aortas were scanned using NanoZoomer Digital Pathology image analysis system (Hamamatus, JAP). Image...
Univariate analysis

Experimental values are presented as the mean ± standard deviation. SPSS software package (v 20.0, Chicago, USA) was used for one-way ANOVA. The significance threshold was set at \( P < 0.05 \).

RESULTS

Plasma biochemistry assays and histopathology

As shown in Figure 1, compared with those in the control group, the concentrations of LDL, TC, and TG were markedly elevated \( (P < 0.001) \) in the model group. Compared with the model group, AS and XKS 12-week treatments resulted in a significant decrease of LDL, TC, and TG levels. However, the regulatory effect of AS on LDL, TC, and TG were more pronounced than the effect of XKS, indicating that the anti-AS effects of AS mainly by reduce blood–lipid levels.

As shown in Figure 2a, pathological changes of atherosclerotic plaques in the initial surface of the aortas were apparent in the model group after 12-week feeding with HCD. Treatment with AS and XKS for 12-week induced a significantly reduce in atherosclerotic plaques area ratio compared to the model group [Figure 2b]. In addition, AS and XKS treatment have similar therapeutic effect on atherosclerotic plaques. Biochemistry assays combined with histopathology results indicated that treatment with XKS and with AS may utilize different mechanisms against AS induced by HCD.

Multivariate statistical analysis

The integral metabolite data were assigned and were labeled in the spectra.

Statistical analysis of plasma samples

To evaluate the treatment effects of XKS and AS, unsupervised PCA and supervised PLS-DA were employed. The PCA score plots show that there are no outliers (beyond the 95% confidence interval) in any of the samples [Figure S1]. PLS-DA [Figure 4] score plots show obvious separation between the model and control groups, suggesting that metabolic profiles significant changes induced by HCD. The metabolic profiles of rabbits in both the XKS- and AS-treated groups deviate from that of the model group and are close.
Perturbed metabolic pathways in AS rabbits associated with Xin-Ke-Shu and atorvastatin treatments

Based on the identified biomarkers of pathological changes and drug regulation, the metabolic network was mapped using the KEGG database. Eight metabolic pathways, namely lipid metabolism (P1); glycolysis/glucoseoneogenesis (P2); citrate cycle (P3); glycine, serine, and threonine metabolism (P5); alanine, aspartate, and glutamate metabolism (P6); arginine and proline metabolism (P7); cysteine and methionine metabolism (P8); and tyrosine metabolism (P9) were altered in the plasma of HCD-induced AS rabbits. XKS treatment was able to normalize seven of the altered pathways, namely lipid metabolism (P1); glycolysis/glucoseoneogenesis (P2); citrate cycle (P3); alanine, aspartate, and glutamate metabolism (P6); arginine and proline metabolism (P7); cysteine and methionine metabolism (P8); and pyruvate metabolism (P4). However, AS treatment could only normalize one altered pathway, namely lipid metabolism (P1) [Table 1].

In addition, the most relevant pathways were identified by the metabolic pathway analysis (MetPA) on MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/MetaboAnalyst/). The impact value of pathway analysis with MetPA was applied to evaluate the importance of the pathways on the pathological changes or drug targets [Figure 7 and Table S1]. Four altered metabolic pathways were considered as the most relevant pathways involved in pathological changes or drug targets (Impact >0.1). They were citrate cycle (a); alanine, aspartate, and glutamate metabolism (b); pyruvate metabolism (c); and glycine, serine, and threonine metabolism (d). Citrate (B3), pyruvate (B4), glycine (B7),...
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...glutamine (B8), and betaine (B9) are involved in the four key pathways that may denote the most important drug targets. Among them, citrate cycle (a); alanine, aspartate, and glutamate metabolism (b); and pyruvate metabolism (c) are the targeted pathways related to the therapeutic effects of XKS treatment. However, AS treatment has no effects on these pathways.

**DISCUSSION**

With their multiple components, holistic therapeutic effects, and fewer side effects, TCMs are a promising therapy for CAD. Clinical research has confirmed that XKS can improve arterial elasticity and heart rate variability, and reduce episodes of angina pectoris in CAD. According to our histopathology study, treatment with XKS or AS caused a notable decrease in the atherosclerotic plaque area, suggesting that XKS and AS have similar anti-AS effects. However, AS treatment showed more effect on TC and LDL levels than XKS did in plasma biochemical assays. Therefore, we concluded that treatment with XKS and with AS may utilize different mechanisms against AS.

To understand the mechanism of XKS against AS, a metabonomics approach was used in this study. With the multivariate analysis of ¹H NMR data, the alterations induced by AS were significantly improved after treatment with XKS or AS. Eleven pathological biomarkers were identified to differentiate the metabolic profiles of AS rabbits from those of normal rabbits. XKS treatment mediated the changes of nine metabolites related to pathological changes, as well as two metabolites related to drug regulation. All of these metabolites are considered potential pharmacological biomarkers for XKS treatment targets. However, AS only affected the metabolic disorders of LDL/VLDL and phosphatidylcholine. Pathological combined with metabonomics findings demonstrate that the anti-AS effects of AS mainly target blood-lipid metabolism, but XKS treatment shows multi-targeted effects against AS.

Nine pharmacological biomarkers are relevant to the therapeutic targets of XKS, which primarily involve lipid metabolism (P1); glycolysis/glucoseogenesis (P2); citrate cycle (P3); alanine, aspartate, and glutamate metabolism (P6);...
argine and proline metabolism (P7); cysteine and methionine metabolism (P8); as well as the characteristic pathway of pyruvate metabolism (P4) and the \( \alpha \)-glucose-related glycolysis/gluconeogenesis pathway (P2) [Table 1].

**Lipid metabolism**

Disorders in lipid metabolism, which play a crucial role in the initiation and progression of AS, are considered to cause CAD.\[30\] A marked decrease in lipid metabolism (LDL/VLDL

<table>
<thead>
<tr>
<th>Number</th>
<th>Metabolites</th>
<th>VIP</th>
<th>Chemical shift (ppm)</th>
<th>M/C</th>
<th>A/M</th>
<th>XKS/M</th>
<th>Pathway</th>
</tr>
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<tbody>
<tr>
<td>B1</td>
<td>LDL/VLDL</td>
<td>4.30</td>
<td>0.89 (m), 1.28 (m)</td>
<td>↑**</td>
<td>↓**</td>
<td>↑</td>
<td>Lipid metabolism (P1)</td>
</tr>
<tr>
<td>B2</td>
<td>Lactate</td>
<td>1.93</td>
<td>1.33 (d)</td>
<td>↑*</td>
<td>-</td>
<td>↓*</td>
<td>Glycolysis/gluconeogenesis (P2)</td>
</tr>
<tr>
<td>B3</td>
<td>Citrate</td>
<td>2.73</td>
<td>2.53 (d), 2.69 (d)</td>
<td>↓**</td>
<td>-</td>
<td>↑**</td>
<td>Citrate cycle (P3)</td>
</tr>
<tr>
<td>B4</td>
<td>Pyruvate</td>
<td>-</td>
<td>2.37 (s)</td>
<td>-</td>
<td>-</td>
<td>↑*</td>
<td>Pyruvate metabolism (P4)</td>
</tr>
<tr>
<td>B5</td>
<td>N-acetyl glycoproteins</td>
<td>3.36</td>
<td>2.05 (s)</td>
<td>↑**</td>
<td>-</td>
<td>-</td>
<td>Glycolysis/gluconeogenesis (P2)</td>
</tr>
<tr>
<td>B6</td>
<td>Phosphatidylcholine</td>
<td>2.25</td>
<td>3.23 (s)</td>
<td>↑**</td>
<td>↓**</td>
<td>↑*</td>
<td>Lipid metabolism (P1)</td>
</tr>
<tr>
<td>B7</td>
<td>Glycine</td>
<td>3.95</td>
<td>3.57 (s)</td>
<td>↑*</td>
<td>-</td>
<td>-</td>
<td>Glycine, serine, and threonine metabolism (P5)</td>
</tr>
<tr>
<td>B8</td>
<td>Glutamine</td>
<td>2.51</td>
<td>2.45 (m)</td>
<td>↑**</td>
<td>-</td>
<td>↓**</td>
<td>Alanine, aspartate, and glutamate metabolism (P6)</td>
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<tr>
<td>B9</td>
<td>Betaine</td>
<td>9.13</td>
<td>3.27 (s), 3.90 (s)</td>
<td>↑**</td>
<td>-</td>
<td>-</td>
<td>Glycine, serine, and threonine metabolism (P5)</td>
</tr>
<tr>
<td>B10</td>
<td>Creatinine</td>
<td>1.48</td>
<td>3.05 (s), 4.05 (s)</td>
<td>↓**</td>
<td>-</td>
<td>↑*</td>
<td>Arginine and proline metabolism (P7)</td>
</tr>
<tr>
<td>B11</td>
<td>Methionine</td>
<td>2.59</td>
<td>2.13 (m), 3.78 (m)</td>
<td>↑**</td>
<td>-</td>
<td>↓**</td>
<td>Cysteine and methionine metabolism (P8)</td>
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<tr>
<td>B12</td>
<td>Tyrosine</td>
<td>1.03</td>
<td>6.90 (d), 7.20 (d)</td>
<td>↑*</td>
<td>-</td>
<td>-</td>
<td>Tyrosine metabolism (P9)</td>
</tr>
<tr>
<td>B13</td>
<td>( \alpha )-glucose</td>
<td>-</td>
<td>5.23 (s)</td>
<td>-</td>
<td>-</td>
<td>↑*</td>
<td>Glycolysis/gluconeogenesis (P2)</td>
</tr>
</tbody>
</table>

*\( P<0.05 \), **\( P<0.01 \) compared to model group. s: Singlet, d: Doublet, m: Multiplet, ↑: Increase in signal, ↓: Decrease in signal, -: No statistical change, C: Control group, M: Model group, A: Atorvastatin treatment group, XKS: Xin-Ke-Shu, VIP: Variable importance of projection, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein

Figure 5: Orthogonal partial least squares discriminant analysis score plots (left) and s-plots (right). Model group versus Control group (a: \( R^2_X = 0.609 \), \( R^2_Y = 0.998 \), \( Q^2 = 0.953 \)); Model group versus Atorvastatin treatment group (b: \( R^2_X = 0.384 \), \( R^2_Y = 0.906 \), \( Q^2 = 0.446 \)); Model group versus Xin-Ke-Shu treatment group (c: \( R^2_X = 0.655 \), \( R^2_Y = 0.999 \), \( Q^2 = 0.950 \))
Anti-atherosclerosis effects of Xin-Ke-Shu involve a multitargeted activity

Figure 6: The network of the potential biomarkers changing for atorvastatin, Xin-Ke-Shu, and atorvastatin modulation according to the KEGG pathway database. Column value in histograms is expressed as mean ± standard deviation (n = 6), the blue name means detected potential biomarkers in this work, blue box represents regulated metabolites both by Xin-Ke-Shu and atorvastatin treatment, green box represents regulated metabolites only by Xin-Ke-Shu treatment.

Figure 7: Summary of pathway analysis with MetPA. Each point represents one metabolic pathway; the size of dot and shades of color are positive correlation with the impaction of metabolic pathway (a. tricarboxylic acid cycle; b. Alanine, aspartate and glutamate metabolism; c. Pyruvate metabolism; d. Glycine, serine and threonine metabolism)

B1 and phosphatidylcholine B6) was observed in the XKS and AS groups compared with that in the model group. LDL particles are atherogenic risk factors, especially if oxidized and accumulated in the arterial wall, and are major cholesterol carriers in the circulation. Endothelial cell inflammation and apoptosis could be induced by oxidized LDL overexpression, resulting in vascular endothelial cell expression of adhesion molecules, and leading to enhanced adherence of monocytes to the vascular endothelium. Phosphatidylcholine (B6), a common constituent of platelet-activating factor-like lipids, can lead to higher concentrations of oxidized LDL. The anti-PC IgM has anti-inflammatory properties and can be used as a therapy in atherosclerotic disease. Our metabonomics study demonstrated that XKS and AS can significantly reduce the levels of LDL/VLDL and phosphatidylcholine, but AS treatment showed greater regulatory effects on lipid metabolism disorder compared with that of XKS treatment, which is consisted with clinical chemical analysis results.

Energy metabolism

The tricarboxylic acid (TCA) cycle is a central pathway in the metabolism of sugars, lipids, and amino acids for energy homeostasis and cell metabolism. Accumulating evidence supports that TCA cycle dysfunction plays a key role in the pathogenesis of AS. Citrate (B3) is an intermediate in the TCA cycle. Our previous study demonstrated that citrate had a highly negative association with atherogenic outcomes, and was a better predictor of AS than lipoprotein lipids (e.g., HDL and LDL/VLDL). The decreased citrate in the model group in this study indicates that the formation of ATP is inhibited, which is closely related to the formation of AS. Treatment with XKS corrected the citrate levels, suggesting that XKS ameliorates the dysfunction of the TCA cycle.

Pyruvate (B4) is the end product of glycolysis. Pyruvate is crucial for mitochondrial ATP generation and for several major biosynthetic pathways that feed into the TCA cycle.
Under hypoxic conditions, pyruvate can also be converted to lactate (B2) by lactate dehydrogenase (LDH, EC1.1.1.27) [Figure 8 and Table S2]. The hypoxia induced by the AS state is suggested to be a major stimulator of lactate synthesis from pyruvate. In the present study, the increased levels of lactate suggest the upregulation of glycolysis in atherogenesis, and treatment with XKS reduces the lactate and pyruvate levels in carbohydrate metabolism. In addition, oxidation of long-chain fatty acids in the mitochondria generates the main TCA cycle substrate, acetyl-CoA. Therefore, we conclude that HCD induces lipid accumulation in rabbit plasma, but XKS treatment activates the degradation of fatty acids for energy demand and decreases glycolytic metabolism, which is further supported by the down-regulation of α-glucose (B13) after XKS treatment. The results indicate that the regulatory effects of XKS on glycolysis and TCA cycle intermediates may promote fatty acid degradation and metabolism. As a result, lipid levels are decreased in rabbit plasma by XKS treatment.

**Amino acid metabolism**

The homeostasis of amino acids could be significantly altered by HCD, leading to a metabolic amino acid disorder. Glutamine (B8), a free amino acid, exists in blood and skeletal muscle tissue at high concentrations. It is a physiological inhibitor of nitric oxide (NO) synthesis in intact blood vessels and endothelial cells. Therefore, glutamine accumulation in plasma could inhibit NO generation in endothelial cells, which is responsible for impeding endothelium-dependent vasodilatation. The increased concentration of glutamine in the model group indicates that NO biosynthesis in endothelial cells could have been inhibited, causing endothelial function disorder. Conversely, the decreased concentration of glutamine in the XKS treatment group indicates that XKS may regulate the NO-related pathway to improve endothelial function.

Creatine (B10) is a primary breakdown product of creatine, which is released from the muscle tissue. Studies have reported that creatine supplementation is associated with decreased homocysteine levels in humans. A high level of homocysteine is an independent cardiovascular risk factor associated with ischemic heart attacks and atherosclerotic vascular diseases. In our study, the decreased concentrations of creatinine were detected by NMR in the model group, suggesting that its biosynthesis was inhibited in the development of AS. However, the concentration of creatinine increased in the XKS treatment group, indicating that XKS may regulate homocysteine to improve the AS risk, but AS treatments had no such effect.

Methionine (B11) can be converted to homocysteine through the transmethylation/transsulfuration pathway, which may have atherogenic effects through several mechanisms (including lipid peroxidation). Accumulating evidence suggests that excess dietary methionine can induce the development of AS. Injury/dysfunction of the vascular endothelium is considered the main mechanism of the atherogenic effect induced by methionine. In the present study, the increased concentration of methionine in the model group was reversed by treatment with XKS. However, treatment with AS had no such modulating effect. The results indicate that the therapeutic targets of XKS may inhibit lipid peroxidation to improve endothelial function.
In addition, to identify the metabolite-related genes and proteins, ten representative metabolite biomarkers derived from the plasma-based 1H NMR profiling (i.e., pyruvate, citrate, lactate, phosphatidylcholine, glycine, tyrosine, glutamine, methionine, betaine, and α-glucose) were entered into Metscape, and the compound–reaction–enzyme–gene networks were constructed by the software [Figure 8]. As a result, 78 proteins and 142 genes were found to be involved in AS progression induced by HCD and ameliorated by XKS-related metabolites [Table S2]. Among them, 24 enzymes and 38 genes are involved in the three most relevant pathways (Impact > 0.1 based on MetPA, including citrate cycle; alanine, aspartate, and glutamate metabolism; and pyruvate metabolism) associated with the therapeutic effects of XKS treatment, which confirmed that these enzymes and genes play key roles in the therapeutic targets of XKS.

**Conclusions**

In this study, biochemical assays of the plasma and histological analyses of the aortas revealed that treatment with XKS and with AS may utilize different mechanisms against AS. To understand the mechanism of XKS against AS, we applied 1H NMR-based metabolomics to profile small, polar metabolites in blood. This was combined with multivariate analysis and pattern recognition to identify metabolites with significantly changed levels, and metabolic pathways that were normalized with XKS or AS treatment, to assess the therapeutic effects of XKS. Eleven significantly changed metabolites in plasma were identified, which involved alterations of lipid, energy, and amino acid metabolism, as potential pathological biomarkers related to AS. Further, we applied a metabolomics method to systematically assess the therapeutic effects of XKS and AS against AS. Treatment with XKS could effectively regulate the metabolic alternations of LDL/VLDL (B1), lactate (B2), citrate (B3), phosphatidylcholine (B6), glutamine (B8), creatinine (B10), and methionine (B11), as well as the characteristic metabolites of pyruvate (B4) and α-glucose (B13); these may be the pharmacological targets of XKS against AS. However, AS only regulated the metabolites of LDL/VLDL (B1) and phosphatidylcholine (B6). This study indicates that the anti-AS effects of AS mainly reduce blood-lipid levels, but those of XKS were multi-targeted. Furthermore, the 1H NMR-based metabolomics approach could offer information on small, polar metabolites, which complements information obtained with MS, to explore the therapeutic mechanisms of TCMs. Due to the relatively limited number of XKS doses test and end-points, more doses and end-points are necessary for future studies.

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**Conflicts of interest**

There are no conflicts of interest.

**References**


