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Traditional Mongolian Medicine Qiqirigan-8 Effect on HFD-induced Atherosclerosis in ApoE$^{-/-}$ mice

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Abstract: Atherosclerosis (AS) is a chronic inflammatory disease in which lipid plaques accumulate on the wall of the large and middle arteries, leading to reduction or obstruction of blood flow. It's the basis of variety of cardiovascular diseases. Endothelial cell injury, vascular inflammatory stimulation, abnormal lipid metabolism and coagulation disorders are the main pathological mechanisms of AS. statins are currently the first-line drugs for the treatment of hypercholesterolemia and AS. However, statin therapy has many side effects and cannot completely eliminate the risk of cardiovascular disease. Therefore, the exploration of new drugs is urgently needed.Several studies have shown that Mongolian medicine Qiqirigan8 (MMQ-8) has significant lipid-lowering and anti-inflammatory effects. Therefore, we hypothesized that MMQ-8 might have a pharmacodynamic effect on AS. In this study, we first

systematically investigated the active components, targets and potential pharmacological mechanisms of MMQ-8 against atherosclerosis using network pharmacology. The results showed that the blood components of MMQ-8 and the targets of AS closely related to NF-κB, cAMP, JAK-STAT and other signaling pathways. MMQ-8 can improve lipid metabolism, and reduce plaque area. In conclusion, we have demonstrated that MMQ-8 may exert its anti-atherosclerosis effects through multiple components, targets and pathways.

Key words: Qiqirigan-8, Atherosclerosis, Network pharmacology,

Introduction

AS is a chronic and progressive vascular inflammatory disease of the large and middle arteries. Arterial wall thickening, hardening and plaque formation in AS patients lead to stenosis and obstruction, plaque rupture and bleeding, and induce lesions in organs such as heart, brain and kidney[1, 2]. Epidemiological studies have shown that cardiovascular and cerebrovascular diseases have surpassed infectious diseases and become the leading cause of death and disability worldwide[3, 4]. The detailed pathogenesis of AS is still unclear, but many studies have confirmed that it is closely related to factors such as inflammatory response and lipid metabolism disorders[5-8], vascular endothelial dysfunction[9], genetic factors and intestinal flora disorders[10]. The occurrence and development of each stage of AS have different triggering mechanisms, among which oxidized low density lipoprotein (Ox-LDL) induced by abnormal lipid metabolism is the core of AS. Studies have confirmed that circulating low-density lipoprotein cholesterol (LDL-C) is continuously deposited after vascular endothelial cell injury[11]. Therefore, lipid-lowering statins are widely used in the treatment of AS. However, long-term use of statins does not completely prevent and treat cardiovascular diseases, and may cause side effects such as liver and kidney toxicity, diabetes, cataracts, and frequent muscle damage[12]. Therefore, it is of great significance to explore new anti-AS drugs with low toxicity, high efficiency, economy,

and no or low side effects.

The traditional Mongolian medicine Qiqirigan-8 (MMQ-8) is a compound composed of 8 kinds of plant medicines, such as sea buckthorn, naphthalene, long pepper, sappan, black borneol, broad woody incense, alantane and rhubarb[13]. A number of studies have shown that the plant medicinal ingredients of MMQ-8, such as sea buckthorn, kaonide, sappan and saphena, are rich in terpenoids, flavonoids, alkaloids, phenolic acids, fatty acids and other active substances, and have anti-inflammatory, anti-oxidation, lipid-lowering and protective effects on cardiovascular diseases[14-20]. In addition, MMQ-8 is often used in the treatment of hyperlipidemia, hypertension, coronary heart disease and so on. Experimental studies have also demonstrated a variety of pharmacological effects of MMQ-8, such AS lipid-lowering, anti-inflammatory and anti-oxidation [13]; therefore, we speculate that MMQ-8 has a therapeutic effect on AS. However, whether MMQ-8 has a therapeutic effect on AS has not been reported.

Therefore, in this study, network pharmacology was used to investigate the anti-AS effects of MMQ-8, its potential target proteins in $ApoE^{-/-}$ mouse model of AS induced by High Fat Diet (HFD), and to elucidate the underlying mechanism of MMQ-8 anti-AS. Our results may help to elucidate how MMQ-8 effectively anti-AS and facilitate the development of new anti-AS drugs.

Materials and Methods

Ultra-high performance liquid chromatography-QE-MS

A 400 μL serum sample was collected and 40 μL hydrochloric acid (2 mol/L) was added. The mixture was vortexed for 1 min, left at 4 ° C for 15 min, vortexed with 1.6 mL acetonitrile for 5 min, centrifuged at 12000 rpm for 5 min, and the supernatant was taken with 1800 μL nitrogen and blown to dryness. Then, 150 μL 80% methanol (IS=1000:10) was added to redissolve, vortexed for 5 min, centrifuged at 12000 rpm for 5 min, and 100 μL of the supernatant was taken to the injection bottle for detection. LC-MS/MS analysis was performed using Agilent's 1290 UPLC system with a Waters

UPLC BEH C18 column (1.7μm 2.1*100 mm). The flow rate was 0.4 mL/min, and the injection volume was 3 μL. The mobile phases were 0.1% formic acid aqueous solution (A) and 0.1% formic acid acetonitrile aqueous solution (B). The multi-step linear gradient elution program was 0-3.5 min, 95-85% A; 3.5-6 min, 85%-70%A; 6-6.5, 70-70% a; 6.5-12 min, 70-30%A; 12-12.5 min, 30-30%A; 12.5-18 min, 30-0%A; 18-25 min, 0-0%A; 25-26 min, 0-95% A; 26 -30 min, 95% -95% A.

Using IDA collection method, we used Q Exactive Focus mass spectrometry combined with Xcalibur software to acquire MS and MS/MS data. In all the collection stages, the mass range was 100 to 1500, and then the first three of each stage were selected to allow more in-depth collection of MS/MS information. Sheath gas flow :45 Arb, auxiliary gas flow :15 Arb, capillary temperature :400 ° C, full ms resolution :70000,ms/ms resolution :17500, collision energy :NCE mode 15/30/45, spray voltage :4.0 kV(positive) or -3.6 kV(negative).

Network analysis

According to the detected UHPLC - QE - MS MMQ - 8 into the blood components, from the CHEMBL database (https://www.ebi.ac.uk/chembl/) for the chemical composition of MMQ-8 targets. Card in gene database (https://www.genecards.org) and therapeutic targets (TDD) (https://db.idrblab.net/ttd/), enter the keywords "atherosclerosis" in order to obtain relevant target genes. The names of predicted target genes were then normalized using the UniProtKB search function of the UniProt database (http://www.uniprot.org/). Finally, Venn analysis of MMQ-8 target genes and AS-related genes was performed to obtain common target genes. Of the target genes by metascape network platform GO (http://www.geneontology.org/) and KEGG pathway enrichment analysis (www.kegg.jp/kegg/pathway.html).

Construction of protein-proteininteraction (PPI) network

In order to clarify the interaction between MMQ-8 blood components and atherosis-related targets, the above-mentioned intersection targets of MMQ-8 component targets and AS-related targets were imported into the STRING platform, and then the related functions of Cytoscape 3.8.2 software were used to further analyze

the network. According to the degree value, the core active ingredients, core targets and core pathways of MMQ-8 in the treatment of atherosclerosis were screened.

Animal models and treatments

This study and its experimental procedures were approved by the Medical Ethics Committee of Inner Mongolia Medical University (Approval no: YKD202301165). All animal housing and experiments were conducted in strict accordance with the instituticral wnidsines for care and use of laboratory animals.

SPF ApoE$^{-/-}$ male mice (18-22g, 8-week) and C57BL/6J mice(male) of the same genetic background (Beijing Vitong Lihua Laboratory Animal Technology Co., LTD.SCXK2019-0010), were housed in animal room with a temperature of 22-25℃ and a humidity of 45%-55%. C57BL/6J mice were labeled as blank control after one week of adaptation to normal diet. All other ApoE$^{-/-}$ mice were randomly divided into three groups (n=8 each) : model group, MMQ-8 group and atorvastatin group. The blank group and the model group were given CMC-Na solution daily, the MMQ-8 group was given MMQ-8 (0.38 g/kg) daily, and the atorvastatin group was given atorvastatin calcium tablets (5 mg/kg) daily for 16 weeks.

Experimental Drugs

MMQ-8: prepared according to the dosage in Table 1, powdered and dissolved in CMC-Na. Atorvastatin calcium tablets: Beijing Pfizer Pharmaceutical Industry Co., LTD.,20mg*7 tablets, Chinese drug approval number H20051408.

The method of dose conversion of drugs has been previously elucidated[13].

Serum sampling and biochemical testing

The levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were evaluated using commercially available diagnostic kits (Nanjing Jiancheng Biological Co., LTD., China).

Statistical Analysis

The images were analyzed by Image J and Image-Pro Plus software, and the experimental results were presented as $\bar{x}\pm$SEM. Graphpad Prism software was used to

analyze the data by t-student test, and the differences between groups were tested by one-way ANOVA parametric analysis of variance or non-parametric LSD-t test. $P<0.05$ was considered to indicate statistical difference, and $P<0.01$ was considered to indicate significant difference.

Results

Blood component identification of MMQ-8

To determine the chemical composition of MMQ-8 absorbed into the blood, we performed mass spectrometry analysis of serum samples from rats administered MMQ-8. Serum samples were collected from rats at 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h and from rats without intragastric administration as controls. The chemical composition of MMQ-8 was analyzed by liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS). As shown in Figures 1A and 1B, a total of 916 chemical components were detected, of which 556 were detected in the positive mode and 360 in the negative mode. Further comparison with serum components of non-intragastric administration mice and MMQ-8 components revealed 17 components into the blood, including flavonoids, terpenoids, phenols, alkaloids and phenylpropanoids, among others (FIG. 1C Table 2).

The blood component of MMQ-8 has the characteristics of multi-component and multi-target action

To determine whether MMQ-8 has an anti-AS effect, first we performed network pharmacology analysis of MMQ-8 blood components, using ChEMBL and YaTCM platforms to screen the targets associated with each component. The names of potential targets were then corrected using the Uniprot database and further analysis was performed using the metscape database, which identified a total of 467 drug targets. Cross-analysis was performed with AS targets screened from Genecard database, TTD and OMIM database. A total of 321 intersection targets were identified (FIG 2A). It is suggested that the blood component of MMQ-8 may intervene in the occurrence and development of AS by regulating 321 genes.

Next, the active ingredients were predicted by constructing the drug-compaction-target network diagram. The results showed that liquiritin, eleutheroside, shikonin, tetrahydropiperine, 7, 8-dihydroxyflavone and other components of MMQ-8 into the blood had strong interactions with AS-related targets (FIG. 2B). Cytoscape software was used to draw the protein-protein interaction network, and CytoHubba was used to screen the core targets with high degree value. Key targets were identified, including SRC, AKT1, PIK3CA, ESR1, EGFR, PIK3R1, MAPK, and JAK (FIG.2C). GO functional enrichment is sorted by p-value, The top 10 GO functions were protein kinase activity, positive regulation of phosphorylation, cellular response to nitrogen compounds, positive regulation of programmed cell death, response to organic cyclic compounds, positive regulation of cell migration, circulatory system processes, response to inorganic substances, regulation of protein autophosphorylation, smooth muscle cell proliferation, and so on (FIG. 3A). KEGG pathway enrichment results showed that the key targets of MMQ-8 intervention in AS were significantly enriched in lipid and artery atherosclerosis and inflammation-related regions (FIG. 3B), including NF-κB, PI3K/AKT, cAMP and JAK-STAT signaling pathways. Based on the above results, we preliminarly concluded that MMQ-8 has anti-atherogenic effect and is closely related to inflammatory response.

MMQ-8 can reduce blood lipid levels in ApoE$^{-/-}$ mice induced by High-Fat Diet

ApoE-knockout (ApoE$^{-/-}$) mice are a classic model of atherosclerosis[21, 22].

Cholesterol metabolic disorder is a key causative factor in atherosclerosis, and thus we further examined the effect of MMQ-8 on serum lipid levels in ApoE$^{-/-}$ mice fed an HFD. In this study, atorvastatin, the first choice for the treatment of atherosclerosis, was used as a positive control group. The in vivo study model is shown in FIG 4A. As shown in FIG 4B, MMQ-8 inhibits weight gain induced by high fat diet. The four lipid parameters are indispensable for the study of lipid metabolism. As shown in FIG 4C, the mice treated with MMQ-8 and atorvastatin showed lower plasma levels of TC, TG and LDL-C than the model mice. These results suggested that MMQ-8 has the function of regulating lipid metabolism disorder, and its lipid-lowering effect is similar to atorvastatin.

MMQ-8 ameliorated atherosclerotic lesions and liver lesions in ApoE$^{-/-}$ mice

Plaque caused by excessive lipid deposition in arteries is the most prominent pathological feature of AS[23]. To investigate the effect of MMQ-8 on AS plaques, we performed vascular HE staining assessments. As shown in FIG. 5A, mice treated with MMQ-8 and atorvastatin had significantly less damage than untreated HFD-induced mice. Liver HE staining showed that liver steatosis, cytoplasmic vacuolization, necrosis, and lobular inflammation increased in the model group compared with the control group. In contrast, hepatic steatosis and cytoplasmic vacuolization were significantly reduced in the MMQ-8 group (FIG 5B), indicating that MMQ-8 attenuates HFD-induced hepatic steatosis. Consistent with our previous findings, the absence of hepatotoxicity was further confirmed. These results indicated that MMQ-8 effectively reduced HFD-induced plaque formation and abnormal lipid deposition in AS.

Discussion

AS is the pathological basis of many cardiovascular diseases, and the complexity of its pathogenesis has brought great challenges to the diagnosis and treatment of this disease. Lipid-lowering statins are widely used in the treatment of AS. However, long-term use of statins can not completely prevent and treat cardiovascular disease, and may have a series of side effects. Therefore, more attention has been paid to plant medicine

research. Studies have found that Mongolian medicine Paulier capsule can not only improve the blood lipid levels of ApoE$^{-/-}$ gene knockout mice, but also inhibit the development of atherosclerosis[27]. Traditional medicine QXBW tablets prevent atherosclerosis by reducing serum inflammatory factors, liver function indexes and lipid metabolism disorders[28]. Various phenylpropanoids, flavonoids, terpenoids and alkaloids play a role in the treatment of atherosclerosis by protecting vascular endothelial cells from oxidative stress by activating Nrf2/HO-1[10]. The active ingredients of rhubarb may regulate macrophage polarization by affecting the NF-κB/TLR4/PPARγ pathway, thereby reducing the atherosclerotic effect[29]. HSYA plays a protective role in atherosclerosis by affecting the activation of AKT/mTOR and NF-B pathways in macrophages and reducing inflammatory responses[30] et al.

MMQ-8 is a prescription composed of eight herbs, which is considered to be a commonly used clinical prescription for the treatment of cardiovascular diseases such as hyperlipidemia, hypertension, coronary heart disease, and atherosclerosis in China [13]. However, whether MMQ-8 has an anti-AS effect was not explored. Therefore, in this study, we first identified the blood components of MMQ-8 by HPLC/Q-TOF-MS, and on this basis, we explored the main active components and pharmacological mechanisms of MMQ-8 against AS by network pharmacology study, so as to provide theoretical basis for the subsequent research of MMQ-8.

For a long time, AS has been considered as a cholesterol accumulation lesion, which is caused by the retention of low-density lipoprotein (LDL) containing lipoproteins on the intima of arteries, and then the LDL-induced immune cells absorbed by scavenger receptors continue to infiltrate the atherosclerotic plaque[31]. However, with the hypothesis that atherosclerosis is an inflammatory disease was first proposed in 1999[32, 33], inflammatory response has received more and more attention in the occurrence and development of AS, and many clinical and basic researches have confirmed the importance of inflammation in AS. The process of AS belongs to the inflammatory changes of vascular endothelium (EC), which can be caused by lipid metabolism disorders[24]. Moreover, this process involves a variety of cells[34] and

inflammatory cytokines[35, 36]. The occurrence of inflammatory response is closely related to proinflammatory cytokines, including IL-1β, IL-6[37] and TNF-α[38, 39]. Therefore, based on the previous research basis of MMQ-8 and the characteristics of AS disease, we hypothesized that MMQ-8 has anti-AS effect and its mechanism is related to inhibiting inflammatory response.

In this study, we first identified 17 plasma components of MMQ-8 by HPLC/Q-TOF-MS, including flavonoids, terpenoids and alkaloids. These compounds are widely found in plants, and most of them have pharmacological effects such as anti-inflammatory, anti-oxidative, hepatoprotective, and cardiovascular protection. For example, Homoplantaginin (Hom) is a flavonoid glycoside that is part of the traditional Chinese herb Salvia plebeia R. Br. It has significant anti-inflammatory and antioxidant functions[40]. Recently, some studies have reported that Hom can attenuate endothelial cell inflammation induced by lipid metabolism disorders[41, 42]and LPS-induced liver injury[43]. In addition, some studies have shown that Hom downregulates the release of pro-inflammatory cytokines by inhibiting the NF-κB signaling pathway[44].

Then, the network pharmacology approach was used to further analyze the biological characteristics of MMQ-incorporated components. Cross analysis of target genes associated with AS revealed 321 intersection targets. GO functional enrichment analysis showed that MMQ-8 anti-AS was related to many biological processes, cellular components and molecular functions, including protein kinase activity, positive regulation of phosphorylation, cellular response to nitrogen compounds, cellular response to organic cyclic compounds, positive regulation of cell migration, smooth muscle cell proliferation, and inflammatory response. The key targets of MMQ-8 against AS were mainly enriched in lipid, atherosclerosis and inflammation related regions, including NF-κB, PI3K/AKT, cAMP and JAK-STAT signaling pathways.

Based on the network pharmacology analysis results, we used an HFD-induced $ApoE^{-/-}$ mouse model to establish AS and investigated the effect and underlying mechanism of MMQ-8 on atherosclerosis. The disorder of lipid metabolism is the initial factor of AS. A number of studies have shown [11] that low-density lipoprotein (LDL),

which is rich in cholesterol, has a direct relationship with the development of AS. Most of the cholesterol that accumulates in lesions is derived from plasma LDL. In the early stages of AS, endothelial dysfunction occurs and LDL accumulates in the arterial intima and undergoes oxidative modification to cause AS. In our study, MMQ-8 significantly reduced the HFD-induced levels of TC and LDL-C, indicating that MMQ-8 has the effect of alleviating lipid metabolism disorders.

Modified LDL induces endothelial cells to express adhesion molecules and growth factors and interacts with monocyte receptors to differentiate into macrophages[45]. However, the recruited macrophages express several different polarized phenotypes and play multiple roles in lesion development. Some macrophages may acquire pro-inflammatory M1 type by binding modified LDL to pattern recognition receptors, and secrete pro-inflammatory cytokines, such as I-1β and TNF-α[46]. TNF-α can stimulate endothelial cells, induce platelet aggregation, and promote the expression of matrix metalloproteinases[38], leading to thrombosis and increased plaque instability, thus accelerating the progression of AS. Our study showed that MMQ-8 significantly alleviated HFD-induced aortic plaque formation, and its efficacy was similar to that of atorvastatin.

Conclusion

In summary, this study used network pharmacology and animal experiment preliminary exploring the active compounds, potential targets and mechanisms of MMQ-8 against AS. These results indicate for the first time that MMQ-8 has an anti-atherosclerosis effect . Secondly, MMQ-8 may play a key role in the anti-atherosclerosis process by regulating the protein expression of Nf-κb signaling pathway, cAMP signaling pathway and PI3K-Akt signaling pathway through SRA, AKT1, ESR1 and other targets. However, our study still has some limitations that need to be addressed in future work. In subsequent studies, we will perform in vitro experiments to further elucidate the specific molecular mechanisms of the active components of MMQ-8 in

modulating these biomarkers and potential targets.

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Figure legend

Fig.1 MMQ-8 chemical constituents and metabolites absorbed into blood were identified by UHPLC-QE-MS. (A) Elementary particle flow graph chromatogram in negative ion mode of MMQ-8 analyzed with UHPLC-QE-MS. (B)Elementary particle flow graph chromatogram in positive ion mode of MMQ-8 analyzed with UHPLC-QE-MS. (C) Pie chart illustrating the prototypical blood chemical constituents of MMQ-8.

Fig.2 MMQ-8's chemical constituents absorbed into the blood were analyzed by network pharmacology approach.(A)Venn diagram displayed the overlapping 321 targets of MMQ-8's chemical constituents absorbed into blood and AS related targets. (B) MMQ-8's chemical constituents-targets network module. (C) Protein-protein interaction analysis among proteins encoded by the 321genes. The hub genes were selected from the PPI network using the CytoHubba plugin. The node color was from pale yellow to red, and the corresponding degree gradually larger.

Fig.3 (A) GO enrichment analysis of the 321 potential targets. (B) KEGG enrichment analysis of the 321 potential targets

Fig.4 MMQ-8 reduces blood lipid levels.

(A) The animal experimental scheme. (B)Statistics of body weight in each week and Results of body weight in the 16 weeks. (C) Levels of serum lipids. Serum levels of TC、TG and LDL-C were decreased after MMQ-8 treatment in AS model. Data are presented as means ± SD, n = 6, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$,*p < 0.05, **p < 0.01.

Fig. 5. MMQ-8 alleviated HFD-induced atherosclerotic lesions and liver pathological changes.

(A) Representative image of en face Oil Red O staining of aortas. (B) Quantification of en face lesion area in the aorta (n = 6 per group). Data are presented as means ± SD, n = 6 , $^{\#\#\#}p < 0.001$,* p < 0.05, ** p < 0.01. (C) Representative images of aortic root sections from $ApoE^{-/-}$ mice stained with Haematoxylin and Eosin (H&E) . (D) Quantification of lesion area in the aortic root area with H&E staining (n = 6 per group). Data are presented as means ± SD, n = 6 , $^{\#\#}p < 0.01$,* p < 0.05, ** p < 0.01. (E) Representative H&E staining of liver (n = 6).

Fig.6 MMQ-8 treatment significantly inhibited vascular inflammation.

(A) mRNA expression level of IL-1β、IL-6、TNF-α in vascular tissues. (B) MMQ-8 decreased the number of macrophages. Representative images of artery immunohistochemistry staining for F4/80 (n = 6).(C) Molecule docking of Liquiritin binding to SRC. (D) Molecule docking of Liquiritin binding toAKT1. (E) Molecule docking of trifolirhizin binding to SRC. (F) Molecule docking of trifolirhizin binding to AKT1.