Autophagosome activity in macrophage for atherosclerotic plaques in ApoE−/− mice enhanced by Tiaozhi Tongmai Granules

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Abstract  Objective: Atherosclerosis is the fundamental pathophysiologic component of cardiovascular disease, and Tiaozhi Tongmai Granules show great efficiency in the treatment of the disease. However, the mechanism of Tiaozhi Tongmai Granules is still unclear. In this study, we have combined experiments with network pharmacology to explore the anti-atherosclerosis mechanism of Tiaozhi Tongmai Granules.
Method: 120 male ApoE−/− mice were randomly divided into three groups: the model group, Chinese herb group and Atorvastatin group. The model group, Atorvastatin group and Chinese herb group were fed with a high-fat diet, a high-fat diet plus atorvastatin (5.1 mg/kg/d) and a high-fat diet plus Tiaozhi Tongmai Granules (16.5 g/kg/d) for 16 weeks, respectively. Atherogenesis was identified by H&E staining. The colocalization of neutral lipid stain BODIPY and microtubule-associated protein 1 light chain 3 (LC3) and the colocalization of BODIPY and lysosomal-associated membrane protein 1 (LAMP1) within ApoE−/− mice aortic plaques were tested using fluorescence confocal microscopy and the Pearson’s coefficients were calculated. To further explore the anti-atherosclerosis mechanism of Tiaozhi Tongmai Granules, the network pharmacology was used to construct the herb-compound-target network.
Results: The size of the aortic lipid plaque in the Chinese herb group and Atorvastatin group were smaller compared with the model group on the 16th week. Compared with the model group on the 16th week, the BODIPY and LC3 colocalization rate, the BODIPY and LAMP1 colocalization rate of the Chinese herb group and Atorvastatin group all presented significant
increase in the aortic plaque (P < .001), showing that Tiaozhi Tongmai Granules could enhance autophagosome activity in the macrophage. In the herb-compound-target network, 17 active compounds and 27 targets were obtained through literature searching and using LHRI & DAVID Bioinformatics. It was found that 23 targets were correlated with the macrophage. Some of them participated in macrophage inflammatory response, and the other targets could promote/inhibit phagocytosis of the macrophage. It was hypothesized that the active compounds of Tiaozhi Tongmai Granules were acting on these targets and having y the biological effects. Conclusions: In the progression stage of atherosclerosis, Tiaozhi Tongmai Granules can still make the macrophage have higher autophagosome activity, and play a role of anti-atherogenesis.

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Introduction

With the deepening study of atherosclerosis (AS), there has been an increasing number of reports on the function of lipid autophagy in the macrophage. Autophagy, the main metabolic pathway of cell decomposition, is the process of cell delivery of cytoplasmic components to lysosomes for degradation.1 Autophagy can maintain a low basal level in cells, and the level will rapidly up-regulate when cells need adequate nutrition and energy supply.2 It has been demonstrated that the autophagy lysosomal system can directly transport lipid to lysosomes for degradation.3,4 The insufficient degradation and clearance of macrophage cholesterol is caused by the decreased autophagy function, then a large number of lipid droplets accumulate. Increased lipid droplets accelerate the generation of foam cells, leading to the formation of atherosclerotic plaques eventually.5

Mevalonate lactone derivatives, which is called atorvastatin for short, is the first listing of statins and the most commonly used drug to regulate lipid and play a key role against atherosclerosis.6,7 Recent studies have confirmed that atorvastatin can promote the expression of protective autophagy through AMPK pathways.8,9,10 Although atorvastatin is a kind of lipid-lowering drug which promotes autophagy, its mechanism in autophagy regulation has not been elucidated. Long-term use of atorvastatin would cause liver toxicity and myalgia, and some patients are still suffering from plaque progression. So atorvastatin may not be an ideal drug.

Different from Western medicine which concentrates on a specific pathogenic process, traditional Chinese medicine (TCM) has been acknowledged as a popular complementary and alternative medicine in the world. Several Chinese herbs have already been transformed into commercial products from the treatment of AS, such as Xuezhikang Capsule,10 and Zhibituo Capsule.11 Although a lot of clinical trials and basic experimental studies have demonstrated the effectiveness of Chinese herbal products, Chinese medicine still has a predicament in the past decades. Single herb has an intricate composition and more than one active ingredient, not to mention the formula, which hinders deep research on Chinese medicine. Nowadays, the emergence of system pharmacology provides another new perspective for the study of complex Chinese medicine system, for example, the network pharmacology, offers holistic approaches to study the effect of herbal formula.12 Tiaozhi Tongmai Granules is based on the classical prescription—Angelicae Sinensis decoction for supplementing blood (Chinese name—Danggui Buxue Tang) which is comprised of seven herbs capable of treating AS. Our previous study demonstrates that the herbs in Tiaozhi Tongmai Granules produce an anti-atherogenic effect via the hepatocyte ABCA1/macrophage ABCA1 pathway and reduce the level of pro-inflammatory cytokines.13 In this study, we have employed ApoE−/− mice to explore the functional role of Tiaozhi Tongmai Granules in autophagy during atherosclerosis, then the system pharmacology technique has been used to further unveil the mechanism of Tiaozhi Tongmai Granules in the treatment of atherosclerosis.

Materials and methods

Animals and diets

A total of 120 healthy male ApoE−/− mice aged 9—10 weeks with an initial weight of 20 g approximately were purchased from the Beijing HFK Bioscience (Beijing, China) [permit number SCXK(Beijing)2009-0008]. High-fat diet composed of cholesterol 1%, lard 3%, yolk powder 10% and normal diet 86% were obtained from the Beijing HFK Bioscience (Beijing, China). The present study was conducted strictly according to the recommendations set forth in the Chinese Guide for the Care and Use of Laboratory Animals. The Committee on the Ethics of Animal Experiments of the Beijing University of Chinese Medicine approved this study (approval number: 37570).

Drugs

Tiaozhi Tongmai Granules, based on a TCM empirical formula, is comprised of seven ingredients: Milkvetch Root (Astragalus membranaceus (Fisch.) 20 g, Chinese Angelica (Angelica sinensis (Oliv.) Diels) 15 g, Cassia Seed (Sisidacapsularis L.) 30 g, Oriental Waterplantain Rhizome (Alisma orientale (Samuels) Juzep.) 12 g, Hawthorn Fruit (Crataegus maximowiczii C. K. Schneid.) 30 g, Earhtwarm (C. maximowiczii C. K. Schneid.) 15 g, and Sanchi (Panax pseudoginseng Wall. var. notoginseng (Burkill) Hoo et
Seed are prescribed by the Pharmacopoeia method. In the prepared granules, astragaloside IV was detected using previously reported methods. In addition, a component identification test for another main ingredient in the granules, Cassia Seed, was performed according to a previously described method and confirmed that the herb contained chrysophanol and aurantio-obtusin. The two representative markers of Cassia Seed are prescribed by the Pharmacopoeia. The aforementioned components are described in detail in supplemental files (Fig. S1, S2). All herbs were reliably provided by the Beijing University of Chinese Medicine Third Affiliated Hospital.

**Quality control of Tiaozhi Tongmai Granules**

The chief herb or the leading ingredient in Tiaozhi Tongmai Granules is astragaloside IV which acts as one of the main bioactive components, and thus is the marker of quality control for the herb. The content of astragaloside IV was detected using previously reported method. In the prepared granules, astragaloside IV took up 0.046%, which met the requirements (≥0.040%) of the Pharmacopoeia of People’s Republic of China, 2010 Edition. In addition, a component identification test for another main ingredient in the granules, Cassia Seed, was performed according to a previously described method and confirmed that the herb contained chrysophanol and aurantio-obtusin. The two representative markers of Cassia Seed are prescribed by the Pharmacopoeia. The aforementioned components are described in detail in supplemental files (Fig. S1, S2). All herbs were reliably provided by the Beijing University of Chinese Medicine Third Affiliated Hospital.

**Animal groups**

Animal normal diet was given for 1 week, and all ApoE mice were randomly divided into 3 groups (40 in each group). The model group received high-fat diet for 16 weeks before sacrifice. The Atorvastatin group received high-fat diet plus atorvastatin for 16 weeks before sacrifice. The Chinese herb group received high-fat diet plus Tiaozhi Tongmai Granules for 16 weeks before sacrifice.

**Drug intervention**

Atorvastatin clinical equivalent dose was about 5.1 mg/kg/d (equivalent to 40 mg per 70 kg adult per day), and the clinical equivalent dose of Tiaozhi Tongmai Granules was about 16.5 g (crude drug)/kg/d (equivalent to 128 g (crude drug) per 70 kg adult per day) all given via intragastric injection. The two drugs were dissolved in an appropriate amount of distilled water to ensure the drug delivery volume was maintained at 5 mL per mouse. The model group was intragastrically administered with the same volume of distilled water alone without any drugs. Five mice were kept in one cage, and raised in the SPF Animal Laboratory of the Beijing University of Chinese Medicine. They were given adlibitum access to distilled water and high-fat diet.

**Specimen collection**

The mice were fasted for 36 hours before sampling. That is in a nonspecific manner to activate the level of autophagy in vivo by the way of hunger. After sacrificed by overdose of chloral hydrate, the aorta tissue was cut, a part of the aorta was fixed in 10% neutral buffered formalin and embedded in paraffin, waiting for slice and H&E staining. The other part sealed with aluminum foil, was placed in a Frozen Section Machine. The frozen sections were observed by immunofluorescence staining.

**Morphological analysis**

Sections were stained with Harris hematoxylin and eosin (H&E) using Leica ST 5020 Multistainer (Germany). Images were viewed and captured with an Olympus BX53 microscope attached to the Olympus CellSens imaging software platform (Japan). This staining method and other staining methods were basically the same.

**Immunofluorescence staining and confocal laser technology**

Cells were observed through a laser scanning confocal microscope (Olympus, Japan). Rabbit anti-mouse LC3B IgG antibody (L7543, Sigma) and goat anti-rabbit IgG antibody (ab150079, Abcam) were used for the staining of autophagosomes. Rabbit anti-mouse LAMP1 IgG antibody (ab24170, Abcam) and goat anti-rabbit IgG antibody (ab150079, Abcam) were used for the staining of lysosomes. The staining method was similar to other staining methods.

Autophagosomes and lipid droplets were labeled with anti-LC3B antibody and BODIPY, and anti-LAMP1 antibody and BODIPY were labeled with lysosomes and lipid droplets. Each experimental group’s colocalization ratio of LC3B protein and BODIPY, LAMP1 protein and BODIPY was then calculated. And the colocalization of autophagosomes and lipid droplets, lysosomes and lipid droplets were observed with a confocal laser microscope. The two things did not colocalized if the colocalization coefficient was smaller than 0.2. If the colocalization coefficient was larger than 0.3, colocalization formed.

**Statistical analysis**

SPSS (version 18.0) was used for statistical analysis. All data were presented as mean (SD). One-way analysis of variance (ANOVA) was applied to comparing the mean value of single variable measurement data. LSD or S-N-K method was used for multiple comparisons. Game Howell analysis was used if there was heterogeneity in variance. Enumeration data were treated with the chi-square test. P-values less than 0.05 were considered significant.

**Building herb-compound-target network**

All chemical compounds from these 7 herbs were collected from the TCMSP database (http://lsp.nwsuaf.edu.cn/tcmsp.php). Compound molecules screening according to
the compound parameters were provided by the TCMSP database. With the previously developed model from predecessors, the compounds with oral bioavailability (OB) $\geq 30\%$ and drug-likeness index (DL) $\geq 0.18$ as the active molecules were chosen. Meanwhile, the target prediction was performed for the candidate compounds based on the Random Forest (RF) and Support Vector Machine (SVM). Then the herb-compound-target network was built by Cytoscape using the network node of both Degree and Betweenness greater than the mean value as the hub node to do Topology analysis.

Results

The formation of aortic plaque and drug intervention

On the 16th week, the successful rate of the model was 90%. H&E staining results showed that the $\text{ApoE}^{-/-}$ mice aorta had formed a clear plaque, and lipid core could be found. A large volume of lipid plaque was seen in the model group, and lipid plaque occupied most of the lumen. In Figure 1 The H&E staining of $\text{ApoE}^{-/-}$ mice paraffin sections of aorta. (1) A and D for the model group; B and E for the atorvastatin group; C and F for the Chinese herb group. (2) The part of dotted line circle in D, E, F is atherosclerotic plaque. (3) (A, B, C): bars = 100 $\mu$m; (D, E, F) bars = 50 $\mu$m.
Figure 2  Staining of neutral lipids in the frozen sections of aorta (Bars = 10 μm).

Figure 3  Immunofluorescence staining of the frozen sections of aortic LC3B (Bars = 10 μm).
contrast, a smaller volume of lipid plaque in the atorvastatin group and Chinese herb group was seen. Some of their vascular elastic fibers still maintained corrugated continuous arrangement (Fig. 1A–F).

**Lipid droplets distribution and autophagosomes activity of macrophages in atherosclerotic plaques**

On the 16th week, in the ApoE−/− mice aortic plaque tissues, there was uneven wilder distribution of lipid droplets in the shallow and middle of the plaques, while lipid droplets in the deep of the plaques had less distribution. The model group had a large number of lipid droplets in disperse distribution. The atorvastatin group and Chinese herb group had less lipid droplets, and most of them distributed in the shallow layer of the plaques (Fig. 2). On the 16th week, the model group’s aortic plaque LC3B protein expressed a low level. Compared with the model group, the atorvastatin and Chinese herb groups’ aortic plaque LC3B protein expressed a higher level. Autophagosomes was uniformly distributed in plaques (Fig. 3).

**The interaction of autophagosomes and lipid plaques**

The results showed that, on the week 16, lipid droplets and LC3B protein of the model group had no significant colocalization. Lipid droplets still showed a larger dot shape. In contrast, lipid droplets and LC3B protein of the atorvastatin group and Chinese herb group showed a significant colocalization. The number of lipid droplets was not that much and had a small volume. The majority of lipid droplets was dispersed in fine particles (Fig. 4).

The data displayed that the colocalization coefficient of the model group was less than 0.2. Compared with the model group, the colocalization coefficient of the atorvastatin group and Chinese herb group was increased significantly ($F = 128.430$, $P < .001$) to over 0.3. Comparison between the two drug groups, the colocalization coefficient of the atorvastatin group was higher than that in the Chinese herb group, but there was no statistical difference ($P = .060$). (Fig. 5).

**The interaction of lysosomes and lipid plaques**

The results showed that each experimental group’s plaque macrophage LAMP1 protein expression level was nearly the same. However, LAMP1 protein and BODIPY of the model group did not have significant colocalization. In contrast, LAMP1 protein and BODIPY of the atorvastatin group and Chinese herb group had a higher colocalization (Fig. 6).

By the calculation of colocalization ratio of LAMP1 protein and BODIPY, it was found that the colocalization coefficient of the model group was less than 0.2. Compared with the model group, the colocalization coefficient of the atorvastatin group and Chinese herb group was increased significantly ($F = 174.697$, $P < .001$), more than 0.3. The colocalization coefficient of the atorvastatin group was higher than that in the Chinese herb group, but there was no statistically significant difference ($P = .070$). (Fig. 7).
Results of the systematic pharmacological analysis

The corresponding compounds of the 7 herbs and targets information are shown in Table 1. Among them, Milkvetch Root retains most of the compounds, including 25 active compounds and 221 targets. In the herb-compound-target network we have obtained 47 hub nodes, containing 17 active compounds and 27 targets. Through literature searching and using LHRI & DAVID Bioinformatics, we have found that 23 targets were correlated with the macrophage. Those 23 targets are roughly divided into 3 categories, some of them such as Nitric-oxide synthase (NOS), Cyclin A2, and Sodium channel protein presented in the macrophage. Some of the targets participate in macrophage inflammatory response, including Androgen receptor (AR), Prostaglandin G/H synthase 2 (PTGS2), Prostaglandin G/H synthase 1 (PTGS1), Dipeptidyl peptidase IV (DPP4), Thrombin, Trypsin-1, Proto-oncogene serine/threonine-protein kinase Pim-1 (PIM1), Heat shock protein 90 (HSP90), Glucocorticoid receptor (GR), mRNA of Protein-tyrosine phosphatase, non-receptor type 1 (PTPases), Serine/threonine-protein kinase Chk1 (CHEK1) (Fig. 8).

Figure 5  LC3B and BODIPY of the aortic plaque colocalization rate. A, B, C: The colocalization ratio chart: the greater the black dot coverage area is, the higher the degree of colocalization is. D: Pearson colocalization coefficients of each group. *P < .001, compared with the model group.

Discussion

In the present study, the results showed that with the progression of atherosclerotic lesions, the level of autophagy in ApoE/−/− mice aortic tissue was decreased. This results proved that when cells were exposed to lipid stimulation, autophagy levels could be activated and up-regulated. However, when cells loaded with a large number of lipids, the level of autophagy and the degradation ability of autophagy lysosomal system were decreased. Similar as atorvastatin, Tiaozi Tongmai Granules were able to improve the function of autophagy in lipids.
Figure 6  LAMP1 and BODIPY confocal laser (Bars = 10 μm).

Figure 7  LAMP1 and BODIPY of the aortic plaque colocalization rate. A, B, C: The colocalization ratio chart, black dot coverage area is greater, the higher the degree of colocalization. D: Pearson colocalization coefficients of each group. *P < .001, compared with the model group.
Meanwhile, H&E staining results showed the Chinese herb group had a smaller volume of lipid plaque, which also explained the direct relation between autophagy and degradation of lipid droplets.

In TCM theory, the spleen is in charge of transformation and transportation of essence of water and grain, providing the acquired essence for the body, which is also the source of qi and blood. That is to say, the normal transport function of the spleen provides sufficient qi and blood. At the subcellular level, the autophagy lysosomal system, which includes the process of transporting intracellular damaged or denatured proteins and lipids to lysosomal degradation.24 This process is similar to the transport function of the spleen. Intracellular lipid metabolism will be blocked to a certain extent if the cell autophagy capacity is declined.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Herbal compounds and target number.</th>
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<tbody>
<tr>
<td>Herb name</td>
<td>Compounds</td>
</tr>
<tr>
<td>Milkvetch Root</td>
<td>87</td>
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<tr>
<td>Chinese Angelica</td>
<td>125</td>
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<tr>
<td>Cassia Seed</td>
<td>68</td>
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<tr>
<td>Oriental Waterplantain Rhizome</td>
<td>46</td>
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<tr>
<td>Hawthorn Fruit</td>
<td>26</td>
</tr>
<tr>
<td>Earhtwarm</td>
<td>23</td>
</tr>
<tr>
<td>Sanchi</td>
<td>119</td>
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</tbody>
</table>

Figure 8 Herb-compound-target network.
Hindered metabolism contributes to increase volume of lipid droplets, and the quantity also increases. If the lipid droplets exceed the limit beyond tolerance of the cells, the microscopic particles of phlegm will form on the cell. If the cells with particles of phlegm aggregate too much, it will lead to the formation of fatty streaks. On the macroscopic view of the vessel wall tissues, it has already formed a lipid plaque with phlegm. The chief herb in Tiaozhi Tongmai Granules is Milkvetch Root, which is commonly used in TCM to reinforce qi and ascend yang. Chinese Angelica, another herb in Tiaozhi Tongmai Granules, is one of the most important medicine to activate blood circulation. Qi is the commander of blood and blood is the mother of qi. Qi and blood mutually transform. On the other hand, the TCM classic, Bian Zheng Qi Wen (Fantastic Story of Syndrome Differentiation) says excessive phlegm contributes to qi block; normal qi movement promotes phlegm resolving. An adequate amount of qi and blood may guarantee the function of the spleen. Other herbs in Tiaozhi Tongmai Granules include Cassia Seed, Oriental Water plantain Rhizome, Hawthorn Fruit, Sanchi, and Earhtwarm. They work together to improve the function of replenishing qi, activating blood, strengthening the spleen, and resolving phlegm.

In the herb-compound-target network, our data provide a mechanism explaining the link between Tiaozhi Tongmai Granules and autophagy in the macrophage. In these 23 targets, MAPK14 and PPAR-γ are closely related to the macrophage. PPAR-γ in the macrophage is linked to improved macrophage M2 phenotype polarization, exerting their ability to inhibit expression of many inflammatory mediators. Agonists of PPAR-γ can stimulate cholesterol efflux via up-regulating the expression of ABCA1. MAPK14 is an immune modulating signal, which can regulate host response and resist pathogen invasion, stimulate macrophage producing NO, TNF-α and so forth. Another part of targets participates in inflammatory responses. AR and GR from the same family, act as a coordinating hub in anti-inflammatory responses. GR maintained stable due to the function of HSP90. HSP90 is a kind of functionally related proteins, which has a broad interaction with many pro-inflammatory kinase cascades. Recent studies show that HSP90 is responsible for the folding and stabilization of PIM1, which is another target participating in inflammatory response. DPP4 is expressed in inflammatory cells including macrophages, and DPP4 inhibitor exerts anti-atherosclerotic effects and reduces inflammation via inhibition of monocyte activation/chemotaxis. PTGS1 and PTGS2 are responsible for the production of NO and prostaglandin E2 (PGE2), two important inflammatory mediators. PKA-α through PKA-and PKC-dependent pathways down-regulate the expression level of inflammatory factors. In addition, we have also found the relationship between some targets and phagocytosis of the macrophage. Many studies show that after 50 years old, the risk of atherosclerosis in women is gradually increased because of the estrogen level is decreased. However ER promotes phagocytosis of macrophages, especially ox-LDL. GSK-3β is responsible for reduced expression of ABCA1 and SR-B1, ABCA1 facilitates cholesterol export and generation of HDL while SR-B1 promotes bidirectional cholesterol exchange. ABCA1 promoter activity can increase by Etoposide, which is an inhibitor of DNA TOPO II. Consequently, we have speculated that the active compounds of Tiaozhi Tongmai Granules are acting on these targets and have the biological effects.

Conclusion

In this work, our results show that Tiaozhi Tongmai Granules could effectively promote autophagosome activity in the macrophage in the treatment of atherosclerosis, and the herb-compound-target network indicates the active compounds of Tiaozhi Tongmai Granules effecting on 23 potential targets. It is worth to note that these targets could be new breakthrough points of research. Despite these potentially interesting associations, further experimental testing of these hypotheses is necessary.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

SWG conceived the study, QS designed the study. LZ wrote the manuscript. All authors read and approved the final manuscript.

Abbreviations

ABCA1 ATP-binding cassette transporter A1  
AMPK AMP activated protein kinase  
ANOVA one-way analysis of variance  
BODIPY 4,4-disfluoro-3a, 4diaza-s-indacene  
LAMP1 lysosomal-associated membrane protein 1  
LC3 microtubule-associated protein 1 and light chain 3  
SR-B1 scavenger receptor class b1  
TCM Traditional Chinese Medicine

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jtcms.2016.03.002.

References


