Tongguan capsule for treating Myocardial Ischemia-Reperfusion Injury: Integrating Network Pharmacology and Mechanism Study

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Research Article

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Abstract

Purpose
Tongguan Capsule (TGC) is widely used in the treatment of coronary atherosclerotic disease and heart failure after myocardial infarction; yet, the exact mechanism of action is still unclear. In this study, network pharmacology and experimental validation were used to examine the mechanism of TGC prescription in the treatment of myocardial ischemia-reperfusion injury (MIRI).

Methods
The components of TGC and candidate targets for MIRI therapy were searched through multiple databases. ClueGO plug-in of Cytoscape was used for enrichment analysis. The binding ability of components to the target was determined by molecular docking. Finally, MIRI model in vivo and hypoxia/reoxygenation (H/R) model in vitro were established for verification.

Results
Molecular docking results suggested that components such as kaempferol, kaempferol, luteolin, tanshinone IIA had a high affinity with autophagy-related proteins in the mTOR pathway. Further in vivo testing indicated that TGC could improve cardiac function and reduce infarct size in a MIRI mouse model. In vitro experiments also suggested that TGC inhibited H/R cell apoptosis by increasing the levels of GSK-3β, LC3, and Beclin1, and by decreasing the expression of mTOR and p62.

Conclusion
To sum up, these data indicated that TGC could protect the heart from MIRI by upregulating autophagy.

1. Introduction
Reperfusion therapy is a milestone achievement in 20th-century cardiology, commonly used in the treatment of acute myocardial infarction (AMI)[1]. Early reperfusion therapy can restore blood flow through the occluded coronary artery immediately on diagnosis, thus protecting the heart function and improving the prognosis of patients with AMI [2]. However, in some patients after coronary interventional treatment, even if the coronary artery stenosis or occlusion is relieved, the distal blood flow TIMI (Thrombolysis in Myocardial Infarction) grade is still 0 to 2, thus suggesting the presence of cardiac microvascular disorders [3–5]. In addition, ischemia-reperfusion (I/R) leads to damage to the structure and function of myocardial microvascular endothelial cells, which is an important mechanism of microvascular disease [4, 6]. Therefore, protecting endothelial cell damage caused by myocardial ischemia-reperfusion injury (MIRI) is essential for treating microvascular disease [7].
Some previous studies have shown that traditional Chinese medicine may have its own unique role in treating MIRI [8, 9]. Tongguan capsule (TGC), a proprietary Chinese medicine composed of *astragalus membranaceus*, *salvia miltiorrhiza*, *borneol*, and *leech*, can promote Qi and blood circulation and remove blood stasis [10, 11]. Based on accurate fingerprint analysis with high-performance liquid chromatography, it has been discovered that TGC contains substantial active compounds, such as tanshinone IIA and salvianolic acid B [12]. Previous studies have shown that TGC can improve myocardial ischemia and cardiac function and inhibit postoperative ventricular remodeling and vascular restenosis in patients with coronary atherosclerotic disease and heart failure [11, 13, 14]. However, the molecular mechanism of TCG is unclear and needs to be preliminarily explored through network pharmacology.

Network pharmacology, based on network database and bioinformation data analysis method, is commonly used to explore the drug's complex mechanism to diseases by searching for components and targets [15–17]. Molecular docking is applied to the study of interactions by simulating the binding between ligands and receptors through computer technology [18]. In this study, compounds and targets of TGC therapy for MIRI were screened through network pharmacological analysis and verified by molecular docking and experiments. The mechanism of TCG in the treatment of MIRI was preliminarily explored to provide a reference for clinical medication.

2. Materials And Methods

The workflow of this study is shown in Fig. 1.

2.1 Active ingredients screen

The TGC active ingredients, with drug-likeness (DL) $\geq 0.18$ and oral bioavailability (OB) $\geq 30\%$, were screened from TCMSP (https://tcmspw.com/tcmsp.php), TCMID (http://119.3.41.228:8000/tcmid/) and BATMAN-TCM (http://bionet.ncpsb.org.cn/batman-tcm/). The targets of the compound were obtained using TCMSP, Swiss TargetPrediction (http://swisstargetprediction.ch/), STITCH (http://stitch.embl.de/). Simultaneously, the UniProt database (https://www.uniprot.org/) was used for gene name standardization.

2.2 Identification of candidate targets

The targets related to "myocardial ischemia-reperfusion injury" were retrieved through GeneCards database (https://www.genecards.org), Online Mendelian Inheritance in Man (OMIM, https://omim.org), and TTD (http://db.idrblab.net/ttd/). The overlapping genes with MIRI and active components were identified as candidate targets.

2.3 PPI Network Construction

The candidate targets were imported into String (https://string-db.org/), and the condition was set as Homo sapiens with a confidence of 0.95 to obtain the relevant information of protein interaction. The
Cytoscape (V3.7.1) was used to construct PPI network through CytoNCA plug-in to calculate topology index, including Betweenness centrality (BC), Closeness centrality (CC), Degree centrality (DC), Eigenvector centrality (EC), Local average connectivity-based method (LAC), and Network Centrality (NC). The core target is the gene larger than the median of each topological index.

2.4 Component-target network

The network between active components and core targets was visualized by Cytoscape, from which the important compounds were obtained according to the degree value.

2.5 Enrichment analysis

With $P$ value < 0.05, the ClueGO plug-in of Cytoscape software was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the core targets in which Homo Sapiens was selected, while $\kappa$ was set to 0.4.

2.6 Molecular docking

The 2D structure of important compounds was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and 3D chemical structures were created through ChemBioDraw software. Protein structures were obtained from the PDB database according to UniProt ID. Water molecules and ligands of protein were removed by Discovery Studio 2016. AutoDock Vina software was used for molecular docking with the binding site of the protein's own ligand as the active pocket.

2.7 Animal model of I/R

Male ApoE$^{-/-}$ mice weighing 18-22g were obtained from Guangdong Medical Experimental Animal Center. All the animals were housed in an SPF environment with a temperature of 22–25 ºC, a relative humidity of 60%, and a light/dark cycle of 12/12 hour; they all had free access to adequate water and food. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Second Affiliated Hospital of Guangzhou University of Chinese Medicine institutional animal care(Ethical number:2016026) and conducted according to the AAALAC and the IACUC guidelines[19].

Mice were randomly divided into 3 groups according to their body weight: Sham group, I/R group, TGC group. All mice were given intragastric administration 5 days before surgery. TGC group was administered with TGC (Lot number:161201, obtained from Guangdong Provincial Hospital of Chinese Medicine) at 1g/kg once a day, while the Sham group and I/R group were administered with the same volume of normal saline. During I/R surgery, the left anterior descending branch was ligated with 8–0 nylon cord. ST-segment elevation and left local ventricular albinism indicated successful operation. The ligation was removed after 45 min of ischemia, and the chest was closed for 24 h reperfusion. Mice in the sham group underwent similar surgery without ligation.

2.8 Echocardiography
The mice were anesthetized with 2% isoflurane (VETEASY, China), and the heart function was assessed using transthoracic echocardiography (Vevo 2100, Canada). After measurement, left ventricular ejection fraction (LVEF) and fractional shortening (FS) were recorded.

2.9 Evans blue-TTC staining

The infarct size of the heart was measured by Evans blue-TTC staining. Briefly, after echocardiography was completed, the LAD was sutured, and Evans blue solution (meilunbio, China) was injected into the coronary artery through the aorta. The heart was quickly removed and snap-frozen at -20°C for 30 minutes. The heart was then cut into 1mm thick slices and placed into TTC solution (Sigma-Aldrich, USA) at 37°C for 15 minutes. The sections were then fixed with 4% paraformaldehyde solution. Infarct area (IA), area at risk (AAR), and left total ventricular area (LV) were evaluated by Image Pro Plus software, and IA/AAR and IA/LV were calculated, respectively.

2.10 Cell model of hypoxic/reoxygenated (H/R)

Human Coronary Artery Endothelial Cells (HCAEC) from BeNa Culture Collection, China, was cultured in DMEM (Gibco, China) supplemented with 10%FBS (HyClone, USA) and 1% Penicillin/Streptomycin in a humidified atmosphere containing 5%CO₂/95% air at 37°C. Before the experiment, the cells were regularly subcultured until reaching an 80% confluence.

Cells were then divided into a control group, H/R group, and TGC group. Cells in the H/R group were cultured in a glucose-free medium with hypoxia (94% N₂, 5% CO₂, 1% O₂) for 3 h, and then transferred to 10% fetal bovine serum (FBS, HyClone, USA) medium with normoxia incubator for 1h. The TGC group was treated with 100µg/mL TGC solution for 7 h before oxygen-glucose deprivation (OGD). Cells in the control group were cultured under normal conditions.

2.11 HOEST33258 staining and immunofluorescence

After the cell medium was discarded, the cells were washed with PBS (HyClone USA) 2 times and rapidly immobilized with 4% paraformaldehyde. The Hoechst 33258 solution (Beyotime, China) was then used to stain the cell for 15 min at room temperature. The images were observed under a fluorescence microscope (Nikon, China).

When preforming immunofluorescence, cell washing and immobilization steps were similar to the HOEST33528 staining. Then, the cells were permeated in 0.5% Triton X-100 for 5 min and sealed in 5%BSA for 2 h. Cells were then incubated with LC3 primary antibody (CST, USA) at 4°C overnight, and then fluorescently labeled secondary antibody (Invitrogen, USA) and DAPI (BOSTER, China) at room temperature. The images were observed under a fluorescence microscope, and the expression level was analyzed based on the fluorescence intensity.

2.12 Western blot
After H/R, cells were dissolved with RIPA buffer containing protease and phosphoprotein inhibitors to obtain protein samples. BCA kit (Thermo Fisher Scientific USA) was used to detect protein content. The protein samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. After being blocked with 5% bovine serum albumin, the PVDF membrane was incubated at 4°C overnight with primary antibody and with secondary antibody (CST, USA) at room temperature for 1 hour. The primary antibody were: GAPDH (CST,USA), GSK-3beta (CST,USA), P-GSK-3β (CST,USA), mTOR (CST,USA), P-mTOR (CST,USA), Beclin1 (CST,USA), p62 (CST,USA). ECL kit (Millipore, USA) was used to visualize the results, and ImageJ software was used to analyze the band intensity.

2.13 Statistical analysis

All data were expressed as mean ± SD and were statistically analyzed by GraphPad Prism 8 software. Statistical significance analysis was performed using one-way ANOVA and multiple comparisons posterior. The difference was considered as statistically significant at $P<0.05$.

3. Results

3.1 Active compounds in TGC

Based on TCMSP, TCMID, and BATMAN, 91 active compounds met screening conditions. 1143 targets of the active compounds were searched from TCMSP, Swiss TargetPrediction, and STITCH.

3.2 Candidate targets

According to GeneCards, OMIM, and TTD, 1302 MIRI-related targets were identified. 393 overlapping genes both in MIRI and TGC were recognized as candidate targets.

3.3 PPI network

By calculating the median of topological indexes, 95 core targets were obtained, including mTOR, GSK-3β, AKT1, PIK3CA, and PIK3R1, etc (Fig. 2).

3.4 Component-target network

In the network (Fig. 3), 90 chemical components in TGC were correlated with the core targets. There are eight active components with degree > 20, which are considered to be important compounds, including quercetin, luteolin, tanshinone IIA, kaempferol, bifendate, etc.

3.5 Enrichment analysis

GO enrichment analysis (Fig. 4A) indicate that the targets were mainly enriched in positive regulation of phosphorylation, positive regulation of kinase activity, angiogenesis, positive regulation of protein transport, negative regulation of apoptotic signaling pathway. The KEGG results (Fig. 4B) further
suggested that TGC in MIRI treatment might be related to the PI3K–Akt signaling pathway, mTOR signaling pathway, which has a key role in autophagy and apoptosis.

### 3.6 Docking

KEGG analysis suggested that TCG might improve MIRI by regulating autophagy-related proteins in the mTOR pathway. To further verify the interaction of TCG with targets of this pathway (Fig. 5), we performed the docking of important compounds with the following proteins: GSK-3β (PDB: 1H8F), mTOR (PDB: 4JSV), Beclin1 (PDB: 6HOJ), and LC3 (PDB: 7ELG). The value of affinity reflects the strength of docking. The lower the value is, the stronger the ligand binds to the receptor. As shown in Table 1, the affinity values of the important components with GSK-3β, mTOR, Beclin-1, and LC3 were all <-5 kcal/mol, suggesting that TGC may interact with autophagy related proteins in the mTOR pathway to improve MIRI.

Table 1  
Docking score of important compounds with GSK-3β, mTOR, Beclin1, and LC3

<table>
<thead>
<tr>
<th>Molecular</th>
<th>GSK-3β (kcal/mol)</th>
<th>mTOR (kcal/mol)</th>
<th>Beclin1 (kcal/mol)</th>
<th>LC3 (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>quercetin</td>
<td>-9</td>
<td>-7.8</td>
<td>-8.6</td>
<td>-6.5</td>
</tr>
<tr>
<td>tanshinone IIA</td>
<td>-9.5</td>
<td>-9</td>
<td>-9.7</td>
<td>-6.9</td>
</tr>
<tr>
<td>kaempferol</td>
<td>-9.1</td>
<td>-7.9</td>
<td>-8.6</td>
<td>-6.5</td>
</tr>
<tr>
<td>luteolin</td>
<td>-8.8</td>
<td>-8.1</td>
<td>-8.6</td>
<td>-6.4</td>
</tr>
<tr>
<td>bifendate</td>
<td>-7.6</td>
<td>-6.4</td>
<td>-8.2</td>
<td>-5.5</td>
</tr>
<tr>
<td>przewalskin b</td>
<td>-9.3</td>
<td>-8</td>
<td>-9.1</td>
<td>-6.1</td>
</tr>
<tr>
<td>mono-O-methylwightin</td>
<td>-8.6</td>
<td>-7.6</td>
<td>-7.8</td>
<td>-5.2</td>
</tr>
<tr>
<td>2-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxypropyl)-7-methoxy-3-benzofurancarboxaldehyde</td>
<td>-8</td>
<td>-8</td>
<td>-8.4</td>
<td>-5.6</td>
</tr>
</tbody>
</table>

### 3.7 TGC capsule improves MIRI in mice

Echocardiography showed abnormal ventricular wall motion in mice after surgery (Fig. 6A). Compared with Sham group, LVEF and FS in I/R group decreased significantly \((P < 0.05)\). After TGC treatment, these two parameters were significantly increased \((P < 0.05)\), suggesting that TCG can improve cardiac function after MIRI (Fig. 6B).

Evans Blue /TTC staining was then used to assess the myocardial infarction area (Fig. 6C). IA/LV and IA/ARR in the TGC group were significantly lower than in the I/R group \((P < 0.05)\), indicating that the TGC group can reduce the proportion of myocardial infarction area (Fig. 6D).
3.8 TGC can reduce the apoptosis of H/R cells

In Hoechst 33258 staining, the nucleus was bright blue, indicating apoptosis. The ratio of bright blue nucleus in the TGC group was significantly lower than that in the H/R group, indicating that the apoptosis rate of cells in the TGC group decreased (Fig. 7). These results suggested that TGC could inhibit the apoptosis of H/R coronary endothelial cells.

3.9 TGC up regulates the LC3 expression in H/R cells

To clarify the expression of autophagic proteins, we performed immunofluorescence detection of LC3. As shown in Fig. 8, the level of LC3 protein (red fluorescence) in the TGC group was significantly increased compared with the H/R group, suggesting that the inhibition of TGC on H/R induced apoptosis may be related to enhanced autophagy.

3.10 TGC inhibits apoptosis by regulating autophagy-related proteins in the mTOR pathway

As shown in Fig. 9, after TGC treatment, the level of p-GSK-3β and Beclin1 increased, while p-mTOR and p62 were decreased compared with the H/R group ($P<0.05$). It was further suggested that TGC inhibited apoptosis of coronary endothelial cells by regulating autophagy-related proteins in the mTOR pathway.

4. Discussion

Network pharmacology analysis suggested that the important active ingredients in TGC, including quercetin, luteolin, tanshinone IIA, and kaempferol, can decrease MIRI through mTOR and autophagy pathways. Experimental data has further shown that TGC could reduce the area of myocardial infarction and increase the left ventricular ejection fraction by regulating cell apoptosis and the expression of autophagy proteins such as LC3, Beclin1, and p62.

Previous studies have shown that the level of autophagy affects the repair of ischemia-reperfusion injury [20]. Autophagy enhances the survival of cardiomyocytes after ischemia-reperfusion injury by removing damaged mitochondria and other cellular contents [21]. In the case of hypoxia or lack of nutrient substrates, autophagy can provide energy for the body by degrading damaged intracellular organelles, thereby exerting a cytoprotective effect [22–24]. Numerous cardiovascular diseases are associated with endothelial dysfunction, and regulating endothelial autophagy in endothelial cells may be a new frontier in treating these conditions [25–28].

The autophagy initiation is regulated by multiple signal pathways [29, 30]. mTOR is a relatively conserved serine/threonine-protein kinase that regulates various biological functions, including cell division, differentiation, apoptosis, and autophagy [31–33]. Under oxidative stress, mTOR can promote tissue repair by regulating the process of autophagy protein, such as beclin1, p62, and LC3 [34–36]. A previous study suggested that the mTOR signaling pathway is inhibited during the ischemia-reperfusion phase,
and the expression of Beclin1 is increased [37]. In addition, Dai et al found that Beclin1 knockout inhibits the formation of autophagosomes and promotes the occurrence of apoptosis [37].

GSK-3β is a conserved serine/threonine kinase that can exert biological effects by controlling mTOR [38]. By regulating the expression of GSK-3β and mTOR, autophagy signals can be induced to reduce myocardial infarction size and promote cardiac function recovery in MIRI mouse models [39].

The present study found that the important active components of TGC have a role in protecting the heart by regulating autophagy. When cells are under stress, quercetin can alleviate oxidative cell stress-induced damage and restore mitochondrial functions through enhancing autophagy [40]. Moreover, luteolin can improve cardiac function by up-regulating autophagy of cardiac myocytes, inhibiting inflammation and apoptosis [41, 42]. Also, tanshinone IIA induces autophagy by regulating the mTOR signaling pathway to reduce myocardial cell apoptosis [43]. Kamanol up regulates GSK-3β to inhibit the mTOR pathway and alleviates ox-LDL-induced apoptosis by increasing LC3-II and Beclin 1 [44, 45].

Molecular docking results showed that the important compound of TGC had a high affinity with the target protein, suggesting that TGC may protect the heart through autophagy proteins associated with the mTOR pathway. Based on autophagy, exploring the mechanism of TGC in the treatment of MIRI from the molecular perspective can further clarify the drug target, which is conducive to the research and development of new medicine for coronary heart disease.

In summary, based on network pharmacology and experiments, we found that when myocardial ischemia-reperfusion injury occurs, TGC treatment can improve cardiac function, the mechanism of which may be by activating GSK-3β to inhibit the downstream protein mTOR, increasing the level of Beclin1, thereby promoting autophagy to reduce apoptosis of cells (Fig. 10). The present study has some limitations. Relevant research is needed to further verify this data in order to clarify the mechanism of TGC in the treatment of MIRI and provide a reference for clinical treatment.

Declarations

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Author Contributions JL and LC jointly designed and conducted this study. HC assists with experiments such as echocardiography detection. HC and SZ performed experimental data analysis and interpretation of results. HY and FL revised and polished the manuscript. QC and LW conducted technical guidance and supervision for the study.
**Conflicts of interest** All authors declared that there are no conflicts of interest.

**Availability of data and material** The raw data of this study are available upon request.

**Code availability** Not applicable.

**Ethics approval** All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Second Affiliated Hospital of Guangzhou University of Chinese Medicine institutional animal care (Ethical number: 2016026) and conducted according to the AAALAC and the IACUC guidelines.

**Consent to participate** All procedures in this study do not involve human experiments.

**Consent for publication** All authors agreed to publish.

### References


Figures
Figure 1

Workflow for TGC in the treatment of MIRI
Figure 2

Acquisition of core targets. (A) MIRI-related targets identified based on GeneCards, OMIM, and TTD. (B) The overlapping genes both in TGC and MIRI are candidate targets. (C) 95 core targets were obtained according to BC, CC, DC, EC, LAC, and NC.
Figure 3
Component-target network. The pink hexagon nodes refer to the target, and the circular nodes of different colors represent the compound of various traditional Chinese medicines. The size of circular nodes reflects the degree of the component.
Figure 4

GO and KEGG enrichment analysis
Figure 5

The important components docking with GSK-3β, mTOR, Beclin1, and LC3. (A) In the active pocket of GSK-3β, kaempferol formed hydrogen bonds with GLY A:79 and GLU A:80, and there was an alkyl interaction with VAL A:82. (B) Quercetin interacts with MET B:2345, TRP B:2239, and LEU B:2185 in mTOR to form π-sulfur, π-π stacking, and π-alkyl, respectively. (C) Docking with Beclin 1, Tanshinone IIA forms π-cation, π-σ and π-alkyl interactions with ARG C:65, TYR A:61, and PHE B:62, respectively. (D) In addition to
hydrogen bonding with ASP A:52, Luteolin also forms π-cation interaction with ARG A:14 and π-alkyl interaction with VAL A:50 in the binding of LC3.

Figure 6

TGC improves MIRI in mice. (A) Representative images of M-mode echocardiography after MIRI. (B) LVEF and FS measured according to echocardiography (n=6). (C) Representative images of Evans blue/TTC
staining. (D) The area of myocardial infarction was measured according to Evans blue/TTC staining (n=3). *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7

TGC reduces H/R cells apoptosis
Figure 8

TGC up regulates LC3 protein expression in H/R cells. Blue fluorescence represents the nucleus, and red fluorescence represents LC3.
Figure 9

TGC inhibits apoptosis by regulating autophagy-related proteins in the mTOR pathway. (A) TGC decreased the level of P-mTOR and increased P-GSK-3β, which was assessed by Western blot. (B) TGC reduced the level of p62 and increased Beclin 1, which was assessed by Western blot. n=3. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 10

The interaction of autophagy-related proteins in the mTOR signaling pathway in MIRI. Green lines represent activation, while red lines refer to inhibition. (Reference: KEGG database).